INVESTIGATION OF THE EFFECT OF A PROBIOTIC-SUPPLEMENTED DIET ON THE HAEMOCYTE PROTEOME OF THE ABALONE *HALIOTIS MIDAE*

By

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"*Never lose faith ... all you need is time to understand, sensibility to find a better way to solve the issue and courage to go ahead.*" Pope Francis

"*For as God knew all things, before they have been created, so now He still sees all of them, after the creation.*" Eclessiastes 23:20

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ABSTRACT

Haliotis midae is an economically important South African abalone species which has been cultured since the 1990s. *H. midae* farming constitutes approximately 93% of the country's total marine aquaculture industry. However, the slow growth rate of this animal and the potential for disease outbreaks remain a concern for farmers. Therefore, government and private institutions have joined efforts to investigate ways of enhancing abalone production, to increase abalone growth rates and improve disease resistance. Several studies have shown that probiotic microorganisms can significantly increase the growth rate and disease resistance of *H. midae*. To date, no comprehensive studies have been conducted to characterise these physiological improvements at the molecular level. Thus, the aim of this study was to evaluate the effect of a probiotic-supplemented diet on the haemocyte proteome in *H. midae*.

Two probiotic-strains, *Vibrio midae* SY9 and *Debaryomuces hansenii* AY1, were introduced into *H. midae* via a kelp-based feed. Changes in the haemocyte proteome were analysed using isobaric tag for relative and absolute quantification (iTRAQ) coupled with LC-MS/MS. A total of 128 haemocyte proteins were identified. Proteins that were found to vary significantly in their expression levels in haemocytes sampled from abalone fed the probiotic-supplemented diet were identified as COP9 signalosome subunit 4, phosphorylase, T-complex protein 1

subunit gamma, V-type proton ATPase subunit B, Rab 1 and Ra-related protein Rab 1A. Differential expression of COP9 signalosome subunit 4 (up-regulated) and Ras-related protein Rab 1A (down-regulated) was confirmed by western blot analysis.

Bioinformatics analysis revealed proteins with immune class GO terms that functioned in metabolism, apoptosis, cell adhesion, immune response, stress response, and response to endogenous and external stimulus. Hierarchical clustering analyses showed that proteins with similar expression patterns mostly belonged to the same immune classes. Analysis of protein interaction networks indicated that all the differentially expressed proteins may indirectly interact with each other. It was also found that the neurotrophic tyrosine kinase receptor was the central molecule within the interaction network, suggesting that this protein may play a crucial role within the protein interaction network that contains all the differentially expressed proteins. Biochemical pathway analysis indicated that phagosomal maturation was the most significant canonical pathway identified, in which V-type proton ATPase and Ras-related protein Rab have fundamental importance.

Changes in Ras-related protein Rab 1A expression were further investigated in the cytosolic and membrane fractions of haemocyte cells using western blot analysis and cellular immunochemistry. The expression of this protein was found to be down-regulated both in cytosolic and membrane fractions from haemocytes sampled from *H. midae* fed a probiotic-supplemented diet. Although an association between Ras-related protein Rab 1A and F-actin (cell cytoskeleton) was not detected, confocal microscopy confirmed Ras-related protein Rab 1A down-regulation. Thus, results from this study suggest that Ras-related protein Rab 1A may play a key role in *H. midae* immune response, when this species of abalone is fed with a probiotic-supplemented diet.

This is the first time that a large-scale proteomics approach has been used to investigate proteome changes in haemocytes sampled from *H. midae* fed a probiotic-supplemented diet. The findings of this study, regarding the protein profile, interaction networks, molecular pathways and a putative molecular indicator of *H. midae* immune response, provide a foundation from which future studies can be conducted in order to increase our understanding of how probiotics affect the abalone immune system.

LIST OF ABBREVIATIONS

1D	One-dimensional
2D	Two-dimensional
β	Beta
γ	Gamma
^{0}C	Degrees celsius
%	Percentage
&	And
ABC	Ammonium bicarbonate
ACN	Acetonitrile
ANOVA	Analysis of variance
ATP	Adenosine triphosphate
BCA	Bicinchoninic acid
BLAST	Basic local alignment search tool
BSA	Bovine serum albumin
cfu	Colony forming units
DTT	Dithiothreitol
EDTA	Ethylenediaminetetraacetic acid
ESI	Electrospray ionization
F-actin	Filamentous actin
FAO	Food and Agriculture Organization
FASP	Filter-assisted sample preparation procedure
FDR	False discovery rate
FITC	Fluorescein isothiocyanate
g	Gram(s)
GO	Gene Ontology
GTP	Guanine triphosphate
h	Hour(s)
HCl	Hydrochloric acid
HPLC	High pressure liquid chromatography
IAA	Iodoacetamide
IEF	Isoelectrofocusing

IPG	Immobilised pH gradient
iTRAQ	Isobaric tag for relative and absolute quantitation
KEGG	Kyoto encyclopaedia for genes and genomes
l	Litre(s)
LC-MS/MS	Liquid chromatography tandem mass spectrometry
Ltd	Limited
M	Molar(s)
MAPK	Mitogen-activated protein kinase
mg	Milligram(s)
MHBSS	Modified hank's balance salt solution
ml	Millilitres(s)
mM	Millimolar(s)
MMTS	Methyl methanethiosulfonate
MS	Mass spectrometry
MS/MS	Tandem mass spectrometry
m/z	Mass to charge ratio
nl	Nanolitre(s)
nm	Nanometre(s)
nM	Nanomolar(s)
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate-buffered saline
pH	Potential of hydrogen
p*I*	Isoelectric points
PTM	Post-translational modification
Rab	Ras-related protein Rab
rpm	Revolutions per minute
SAB	Sample application buffer
SDS	Sodium dodecyl sulphate
SEM	Standard error of means
sp.	Species
SSS	Sterile sea salts
TBS	Tris-buffered saline
TBST	Ttris-buffered saline with Tween 20

TCEP	Tris (2-carboxyethyl) phosphine
TE	Tris-EDTA
TEAB	Triethylammonium bicarbonate
TEMED	N, N, N', N'-Tetramethylethylenediamine
TFA	Trifluoroacetic acid
TM	Trademark
Tris	Tris(hydroxymethyl)aminomethane
TSA	Tryptone soy agar
TSB	Tryptone soy broth
µg	Microgram(s)
µl	Microlitre(s)
ULB	Urea lysis buffer
v	Volume
V	Volt(s)
v/v	Volume per volume
w/v	Weight per volume
YPD	Yeast peptome D-glucose

CHAPTER 1 – LITERATURE REVIEW

1.1. INTRODUCTION

The demand for seafood has increased globally in recent years, making it one of the most traded food categories (Naylor et al., 2000). According to the FAO (2014), capture fisheries and aquaculture production increased from around 70 million tonnes in 1970 to approximately 160 million tonnes in 2012. While non-food uses of total fish products decreased from 23.4 to 21.7 million tonnes between 2007 and 2012, human consumption of these products increased from 117.2 to 136.2 million tonnes during the same period. This rapidly rising demand is motivated by the human population growth, increasing family profits, and urbanization. The high consumption of fish products is due to the increase of fish production and an more effective supply channels (FAO, 2014; Naylor et al., 2000; Rodgers et al., 2015).

Aquaculture, which refers to fish and shellfish farming, has become a fast-growing food producing sector, and currently accounts for half of the total fishery products consumed by humans (Cook and Gordon, 2010). In 2012, the live weight equivalent of global aquaculture production was estimated to be around 90.4 million tonnes, which was evaluated to be worth US$ 144.4 billion (FAO, 2014). Marine aquaculture, in particular, has been recognized as the sector that is expanding rapidly (Halley and Semoli, 2010).

Among marine aquaculture production, abalone farming has shown rapid growth. Indeed, abalone meat is considered a delicacy, and a valuable source of protein. It is one of the highest-priced seafood varieties globally (Cook, 2014), and its consumption is considered a luxury. South African abalone, in particular, has an excellent reputation and is regarded as one of the best in the world (Sales and Britz, 2001). While the proportion of abalone production (in tonnage) is marginal in comparison to total aquaculture production in South Africa, trading of this expensive delicacy generates enough economic profit to warrant the attention of authorities. South African governance structures have taken measures to ensure that the production of abalone achieves its potential, as they trust that abalone farms will aid local economies, reduce poverty, empower communities and provide employment (Halley and Semoli, 2010). To this end, several studies have been conducted by both private and public South African institutions with the aim of finding ways to improve abalone farming. Currently, one of the ways in which researchers have been trying to improve abalone production is through the understanding of abalone immune system.

1.2. ABALONE

Abalone, also known as sea snails, ear-shells or sea ears, are marine mollusc organisms that belong to the class Gastropoda, within the Haliotidae family (Fallu, 1991). The genus *Haliotis* contains 100 species distributed throughout the world. Large specimens of abalone are mostly found in temperate zones, whereas smaller specimens are found in tropical zones. The majority of abalone populations are found in the cold waters of New Zealand, South Africa, Australia, western North America, and Japan. Abalone reside in the rocky surfaces of subtidal zones and prefer shallow and turbulent water with high levels of dissolved oxygen. They avoid being exposed to light. During the day they are usually found hidden in crevices on rocky reefs and under rocky overhangs where they attach sturdily to avoid predation or other hazards, such as being washed off rocks by waves (Fallu, 1991).

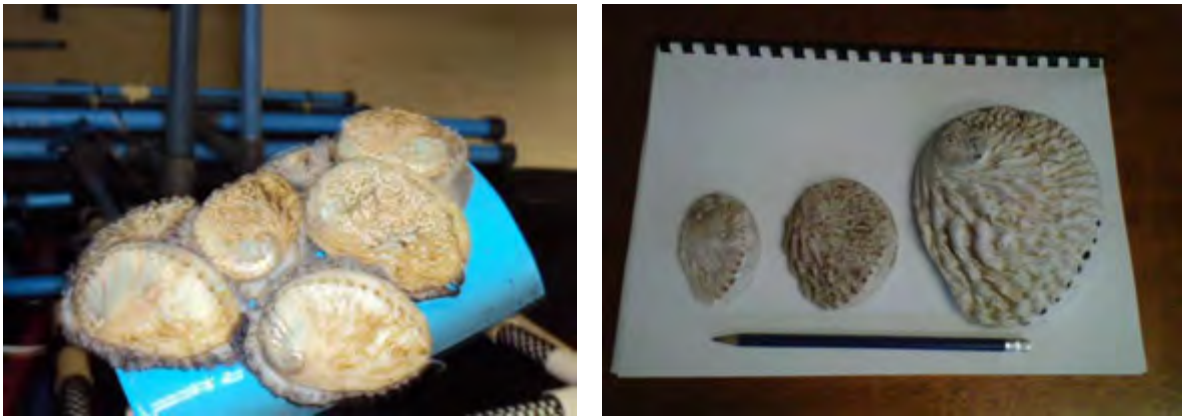
Six species of *Haliotis* can be found in the waters of the Southern African region, namely: *H. midae, H. spadicea, H. parva, H. speciosa, H. queketti* and *H. pustule*. These species, excluding *H. pustule,* are considered endemic to South Africa. Among them, *H. midae*, also known as South African abalone or 'perlemoen' abalone, is commercially valuable and is the only cultivated abalone species (Sales and Britz, 2001).

1.2.1. Biological description of *Haliotis midae*

H. midae is found in the southern regions of South Africa's Western Cape Province (Halley and Semoli, 2010; van der Merwe et al., 2011). The body of *H. midae* is large, fleshy and has a muscular foot used to crawl and attach to hard surfaces (Fallu, 1991; Jarayabhand and Paphavasit, 1996). The top of its body is mostly covered by a shell that is single, oval, spiral, rough and flat (Figure 1.1). Along the edges of the shell is a row of small pores that allow water to reach the gills for oxygen absorption and move waste away from the body (Fallu, 1991).

Abalone are dioecious organisms – the spawning period generally occurs in summer, with multiple events occurring in one season. Abalone fertilization occurs externally when eggs and sperm are released from the pores of male and female species. The fertilized eggs hatch into planktonic larvae, which metamorphose to juveniles after about one week. The juveniles lose their capacity to swim shortly after the process of metamorphosing, and only survive if they find rocks or hard surfaces on which to settle. Abalone start to crawl and graze when they

achieve between 10 to 20 mm. They can grow up to 200 mm and achieve maturity within approximately four to six years (Troell et al., 2006).



(a) (b)

Figure 1.1. *H. midae* at the Marine and Coastal Management Research Aquarium in Sea Point (Western Cape) are kept in baskets inside a tank with aerated and circulated sea water. (a) Living abalone attached to a polyethylene substrate, and (b) shells of different sized abalone found at Sea Point beach.

In terms of nutrition, the larval phase of the abalone life cycle is a non-feeding phase, relying on the yolk sack and the absortion of nutriets through the skin. Once settled, the larvae develop into miniature abalone and begin feeding on benthic diatioms (Sales and Britz, 2001). Adult abalone are generalist and opportunistic herbivores (Troell et al., 2006). They eat brown algae (e.g. *Ecklonia. maxima* and *Laminaria pallida*), red algae (e.g. *Gracilaria spp, Gelidiu, spp*) and green algae (e.g. *Ulva sp*). However, the brown algae which are comprised of polysaccharides such as alginate and cellulose, are generally the preferred diet of adult abalone (Iehata et al., 2010). Alternatively, the farmed abalone may eat formulated feed (Troell et al., 2006).

1.2.2. Production of abalone

Abalone is cultured in several countries such as Chile, Japan, South Africa, Australia, Taiwan, New Zealand, Mexico, Thailand, and Philippines. In the aforementioned countries, cultured abalone activity is likely to increase in the future. Among these countries, South Africa is the biggest producer outside of Asia (Britz et al., 2009); wherein *H. midae* has been a commercially

grown species for more than 17 years (Mouton and Gummow, 2011). In South Africa, the commercial aquaculture of this species was initially to compensate for a reduction in wild stock due to overexploitation (Dang et al., 2012; Sales and Britz, 2001; Troell et al., 2006).

The sustainability of *H. midae* farming relays partially on prolific gamete production, the requirement of a minimal amount of broodstock adults, the planktonic and non-feeding larval stages, and abalone tolerance to high stocking densities (Sales and Britz, 2001). The majority of abalone farms in South Africa are located in the Western Cape, where some farms have both hatcheries and on-growing facilities while others have only on-growing facilities. Abalone are farmed on-share in a flow-through raceway system, in which the water is pumped from the sea into the tanks (Sales and Britz, 2001; Troell et al., 2006).

Nowadays, more than 22 licensed farms are cultivating and commercialising *H. midae*. The profits from abalone production contribute significantly to the economy of the country. In 2011 it generated approximately R 379 million, which was 93% of marine aquaculture production (FAO, 2014).

1.2.3. Description of the abalone immune system

Research in marine invertebrate immunology is important to the aquaculture sector mainly for the purpose of monitoring infection and disease outbreaks (Bachère et al., 1995). The immune system, in a broad sense, is a mechanism that allows a living organism to discriminate between "self" and "non-self." When an organism encounters alien particles, the immune system reacts immediately to kill or remove these from the host (Magnadottir, 2010; McComb et al., 2013), and it is a vital process to ensure the host's survival (Ellis et al., 2011). Abalone, as other marine organisms, can be challenged by generalised and specialised pathogens that naturally exist in their environment; and these can be harmful, especially when the host is in stress. For its defense mechanism, the abalone's soft body is protected by a ciliated epithelium that produces mucus, which acts as an initial physical trap and barrier to colonisation by pathogenic organisms (Cheng et al., 2004).

Unlike vertebrates that present both innate (non-specific and natural) and adaptive (specific and induced) immune systems as mechanisms of defense (Linde et al., 2009; Magnadottir, 2010), invertebrates only possess an innate immune system (Linde et al., 2009). The innate immune system is composed mainly of humoral and cellular defense mechanisms that work to maintain the host's homeostasis (Hooper et al., 2011; Zong et al., 2008). On one hand, humoral defenses are reactions carried out by active molecules stored within and released from haemocytes; the active immune molecules include reactive oxygen species (ROS), lectins such as fibrinogen-related proteins (FREPs), lysozymes, agglutinins, antimicrobial peptides (AMPs), aminopeptidases, acid phosphatase, alkaline phosphatase and peroxidase (Khimmakthong et al., 2013; Loker, 2010; Marmaras and Lampropoulou, 2009). On the other hand, cellular defenses are all processes performed by haemocytes which are the principal cellular effector mediating molluscan immune responses (Humphries and Yoshino, 2003). Additionally, haemocytes play a role in nutrient digestion, metabolite transport, and wound or shell repair. However, their main role is in internal defense (Hooper et al., 2007; Travers et al., 2009), making them responsible for processes such as infiltration, aggregation, encapsulation, cytotoxic reaction, and phagocytosis (Feldhaar and Gross, 2008; Gestal et al., 2008). In all these defense mechanisms, phagocytosis is critically important to fight against invading pathogens (Pearson et al., 2003; Stuart and Ezekowitz, 2008; Zong et al., 2008).

Phagocytosis removes microorganisms, pollution particles, and damaged or apoptotic cells of the host, being an essential element of the immune defense system (Pearson et al., 2003; Roth and Kurtz, 2009). Phagocytosis consists of a number of stages that lead to the elimination of unwanted particles that includes binding of particles to the cell surface, engulfment of the particle by pseudopod extension, and fission and fusion reactions to form phagolysosomes (Gestal et al., 2008; Rupper and Cardelli, 2001). The unwanted particles inside the phagosome are destroyed by lower pH levels, hydrolysis and radical attacks (Henneke and Golenbock, 2004). Among the different molecules that participate in the phagocytic process, the family of small GTPases Ras-related proteins such as Ras, Rho, Rab, and Arf have been frequently reported to be associated with the phagocytic process. For instance, Rab and Arf play a crucial role in regulating membrane and protein trafficking. The formation of a phagocyte cup has been proposed to involve the recruitment of Rab 11, Arf 6 and members of SNARE family proteins (Rupper and Cardelli, 2001). Rab, specifically, plays an important role in the formation

and maturation of the phagosome, which is crucial during host defense responses (Zong et al., 2008).

Our understanding of molluscan immunity is still in its infancy (Humphries and Yoshino, 2003). Prior studies on molluscan immunobiology focused on activities associated with circulating haemocytes and humoral factors to neutralise invading organisms. In this process, invaders are detected via humoral and haemocyte-bound recognition factors, triggering the production of cytokines that mediate the recruitment of additional haemocytes, activation of phagocytosis and the production and/or release of a wide range of antimicrobial compounds (Allam and Raftos, 2015). Recent discoveries to date imply that molluscan immune-mediated signal transduction is well developed and most likely present molecular pathways that are common with vertebrates and the model invertebrate, *Drosophila* (Humphries and Yoshino, 2003). However, no precise mechanism for "immune memory" has been established in molluscan or other invertebrates. Indeed, molluscs do not produce antibodies and they cannot be protected against infection through vaccination (Brokordt et al., 2015). Therefore, alternative mechanisms must be used to help these organisms maintain a healthy environment and increase their performance in aquaculture systems.

1.3. PROBIOTICS

The Russian scientist and Nobel laureate, Eli Metchnikiff, was the first to demonstrate, at the beginning of the 20th century, that certain species of microorganisms can modify the flora of the host gut and replace harmful microbes. Furthermore, in 1953, Kollath introduced the term 'probiotic' in reference to microorganisms and their metabolic products that contribute to the equilibrium of microorganism populations inside a host's intestine (Zhou et al., 2009). Over the years, several other definitions of probiotics have been developed, for instance by Parker (1974) and Fuller (1989); these authors focused on the role of microbial feed supplements that benefit the host by improving its intestinal balance (Verschuere et al., 2000).

Indeed, to be considered a probiotic, the microorganism must contain a set of properties, namely: (i) be non-harmful to the host for which it is desired; (ii) reach the location of where the effect is required to take place; (iii) be accepted by the host and possess the potential for

colonization and replication within the host; and (iv) contain no virulence-resistant or antibiotic-resistant genes (Denev et al., 2009; Kumar et al., 2008). Inside the host, probiotics may produce inhibitory compounds, compete with other microorganisms for nutrients and adhesion to the gastrointestinal tract, produce essential nutrients such as vitamins, fatty acids, and enzymatic molecules that contribute to digestion (Defoirdt et al., 2007).

As examples, the production of certain inhibitory compounds can prevent the occurrence of disease in the probiotics' host by acting antagonistically against the pathogenic microbes and preventing their proliferation within the host (Mohapatra et al., 2012). The host's immune system can be activated by the interaction of the probiotic with the host's epithelial cells, and by modulating the secretion of anti-inflammatory cytokines, lead to a decrease in inflammatory response. Some bacteria seem to modulate the host's innate humoral responses and, consequently, facilitate the exclusion of potential pathogens (Denev et al., 2009). Probiotics influence non-specific immune responses such as phagocytic cell activity, natural killer cell activity and lysozyme levels (van Hai and Fotedar, 2010), hence their use is encouraged as a preventative measure rather than further antibiotic treatment (Gatesoupe, 1999).

The overuse of antibiotics for disease treatment has resulted in the development of antibiotic-resistant bacteria, leading to the reduction of antibiotic efficiency and, subsequently, failure to recover from sickness. Additionally, the release of antibiotic-resistant bacteria into the environment can transfer antibiotic-resistant genes to other microorganisms (Kesarcodi-Watson et al., 2008) and may lead to an accumulation of antibiotics in the environment, which can be absorbed by other organisms (Zhou et al., 2009). It is on this basis that several countries introduced policies with strong restrictions on the use of antibiotics for the treatment of diseases (Kesarcodi-Watson et al., 2008).

1.3.1. Probiotics in aquaculture

In their natural environment, aquatic organisms are mostly surrounded by pathogens that exist independently of their host animal in comparison to terrestrial animals (Verschuere et al., 2000). In aquaculture systems, pathogens are generally opportunistic organisms that can reach a high density depending on the environmental conditions (Matsuzaki and Chin, 2000).

Therefore, one of the major challenges for the aquaculture sector is to achieve a system that minimises untreated effluents, habitat destruction, and the spread of pathogens and alien species (Sales and Britz, 2001). The discharge of effluents without treatment not only leads to ecosystem pollution, but also damage to the ecosystem around the aquatic farms, causing pathogenic microorganisms to proliferate and spread rapidly (Kumar et al., 2008). As previously mentioned, treatment with antibiotics is avoided as their use may result in the development of antibiotic-resistant bacteria, damage or harassment of normal microflora in the aquaculture environment (Gibson et al., 1998; Matsuzaki and Chin, 2000; Zhou et al., 2009).

On the other hand, the use of probiotics within the aquaculture sector has been encouraged and is gaining acceptance (Gomez-Gil et al., 2000). Probiotics seem to be ideal for sustainable aquaculture production. Thus, the intention is to have beneficial microorganisms with no toxic side effects, effectively improving environmental conditions and raising the immunity of cultured organisms (Kesarcodi-Watson et al., 2012; Matsuzaki and Chin, 2000; Mohapatra et al., 2013; Zhou et al., 2009).

One of the first publications to discuss probiotics in aquaculture was by Kozasa in 1986, in which probiotics in aquaculture was defined as “live microbial adjunct which has a beneficial effect on the host by modifying the host-associated or ambient microbial community, by enhancing its nutritional value, increasing the host response towards disease, or improving the quality of the environment” (Kesarcodi-Watson et al., 2008).

1.3.1.1. Mode of application of probiotics in aquaculture

The expansion of using probiotics in aquaculture is a complex process that requires a deep investigation, a full-scale trial and an economic assessment (Denev et al., 2009). Indeed, the effective use of probiotics involves appropriate application in terms of time period and dosages, as well as suitable methods of administration (Matsuzaki and Chin, 2000).

There are several ways to administer probiotics in an aquaculture system. However, two main ways are frequently implemented: 1) through feed supplements (gut probiotics) which can be blended with feed and administered orally to enhance the useful microbial flora of the gut and,

2) through the immersion of probiotics in the aquatic medium so that they can proliferate and exclude the pathogenic bacteria by consuming all available nutrients (Denev et al., 2009). Administration through feed supplement, specifically, is considered the most practical and effective (Nayak, 2010). In fact, a brief period of feeding probiotic supplements followed by the feeding of a basal diet enhances the host immune response, particularly with regard to disease resistance (Matsuzaki and Chin, 2000).

During probiotic administration it is important to know the concentration that must be supplied to the host in order for the probiotic to reach the beneficial levels required by the host. In aquaculture, the dose of probiotics varies from 10^{6-10} cfu.g^{-1} feed, depending on the host and immune parameters (Nayak 2010). Macey and Coyne (2005) used the probiotics *Vibrio midae SY9, Debaryomyces hansenii AY1* and *Cryptococcus sp*. SS1 to stimulate the immune system of *H. midae*. In this study it was found that the use of probiotics at a concentration of 10^7 cfu.g^{-1} of feed stimulated the host's immune system after infection with pathogenic bacteria *Vibrio. anguillarum*, and increased the growth rate of the abalone. Similar results were reported in additional studies focusing on *H. midae* as well as other species of abalone. The probiotic strain *Pseudoalteromonas sp.* C4 was used by Ten Doeschate and Coyne (2008), at a concentration of $2.4x10^8$ cfu.g^{-1} of kelp cake. The authors showed that this concentration of probiotic increased the resistance of *H. midae* to pathogenic infection. Silva-Aciares et al. (2011) mixed three strains of probiotics (*Vibrio sp.* strain F15 UMA, *Vibrio sp*. strain C2-UMA, and *Angarivorans albus* strain F1-UMA) at a concentration of $2\text{-}5x10^6$ cfu.cm^{-2} on macro algal surfaces to stimulate the immune system of *H. refescens*. Their results showed an increase in survival and growth of the animal. In research done by Iehata et al. (2010), the probiotic *Pediococcus sp.* was prepared at a concentration 10^9 cfu.g^{-1} of dried feed so as to improve the gut environment of *H. gigantea*.

1.3.1.2. Benefits of probiotics for aquaculture

In aquatic systems, organisms have a direct and permanent interaction with water, which means that there is direct contact between potential pathogens and both internal and external surfaces of the host (Ringø and Birkbeck, 1999). The use of probiotics thus provides the host protection against pathogens by overcoming the adverse consequences of antibiotics and

chemotherapeutic substances. In aquaculture, probiotics have been shown to improve water quality and feed conversion rates; they also enhance the gut environment and modulate the host's immune system (van Hai and Fotedar, 2010; Mohapatra et al., 2013). Immune modulation by probiotics supplied to the host is possible through anti-pathogenic activities that are achieved due to singular or combined production of anti-microbial compounds, siderophores, lysozymes, proteases and hydrogen peroxides, as well as the alteration of pH within the gut. Probiotics have also been reported to compete with pathogenic microorganisms for nutrients, adhesion, and energy; they supply macro and micro nutrients as well as digestive enzymes to the host (van Hai and Fotedar, 2010; Matsuzaki and Chin, 2000). Martinez Cruz et al. (2012) present an extensive report on the use of probiotics in aquaculture in which they mention that probiotics increase stress tolerance and have beneficial effects on the reproduction of aquatic species.

In the aquatic environment, probiotics also improve the decomposition of organic matter (faeces, wastes, remnants or leftover feed) and reduce nitrogen and phosphor concentrations. They also control ammonia nitrite and hydrogen sulphite concentrations, thus creating a friendly environment within the system (Matsuzaki and Chin, 2000; Zhou et al., 2009). Several studies confirm the benefits of using probiotics in aquaculture. For instance, in farmed shrimp, probiotics bolstered the host's defense against viral infection (Zhao et al., 2011). A bacteria isolated from abalone *H. discus hanni* was shown to degrade alginate (Matsuzaki and Chin, 2000). In another study, Gibson et al. (1998) reported that *Bifidobacterium sp.* and LAB (lactic acid bacteria) protect the host against potential pathogens by competitive exclusion and producing antibacterial agents (bacteriocins).

1.3.1.3. Species of probiotics used in aquaculture

Generally, probiotics can be bacteria, cyanobacteria, microalgae, brown and red algae, terrestrial fungi and marine yeast (Kesarcodi-Watson et al., 2008; Zhou et al., 2009). Commercially available species of probiotics include pure strains, defined mixtures of specific strains, and associations of strains and undefined mixtures (Denev et al., 2009; Matsuzaki and Chin, 2000; Newaj-Fyzul et al., 2013).

Probiotics currently used in the aquaculture industry include a wide range of bacteria such as *Lactobacillus, Bifidobacterium, Pediococcus, Streptococcus, Carnobacterium, Bacillus, Flavobacterium, Cytophaga, Pseudomonas, Alteromonas, Aeromonas, Enterococcus, Nitrosomonas, Nitrobacter* and *Vibrio*; as well as two main yeast strains, *Saccharomyces* and *Debaryomyces* (Denev et al., 2009). However, the most commonly used species of probiotics belong to *Lactobacillus sp., Bacillus sp., Bifidobacterium sp., Vibrio sp., Enterococcus sp. and Saccharomyces sp*. (Kumar et al., 2008).

Most research on probiotics for molluscan aquaculture has been performed in bivalves, such as oysters, scallops, and clams (Kumar et al., 2008). For example, four potential probiotic strains, *Alteromonas macleodii, Neptunomonas sp., Phaeobacter gallaeciensis,* and *Pseudoaltermonas sp.* have been revealed to protect the larvae of the aforementioned molluscan species (Pacific oyster, flat oyster, and great scallop) against pathogens (Kesarcodi-Watson et al., 2012). Experiments with Pacific oyster larvae, *Crassostrea gigas*, showed that the non-pathogenic probiotic *Aeromonas* could be used to prevent the death of larvae infected with *Vibrio tubiashii* (Gibson et al., 1998).

Probiotics studies have been conducted in gastropods such as abalone. For instance, *H. refescens* had improved growth and survival when fed a natural diet of macroalgae *Macrocystis integrifolia* supplemented with probiotics *Vibrio sp* C21-UMA, *Agarivorans albus* F1-UMA and *Vibrio sp.* F15-UMA (Silva-Aciares et al., 2011). *H. gigantea* had an improved gut microflora and nutrition status when fed the probiotic *Pedioccus sp* strain Ab1 supplemented in a commercial diet (Iehata et al., 2010). Three putative probiotic strains *V. midae* SY9, *D. hansenii* AY1 and *Cryptococcus* sp. SS1 (one bacterium and two yeasts) improved the survival of abalone *H. midae* after infection by the pathogen *V. anguillarum* (Macey and Coyne, 2005). For these reasons, the use of probiotics should be a focus when seeking to improve the health management of aquaculture systems.

The probiotics used in this study were the marine microorganisms *Vibrio midae* SY9 and *Debaryomyces hansenii* AY1. *V. midae* SY9 colonizes and adheres to the mucous lining of the abalone gut. There is a close association between the bacterium, its extracellular protease and ingested feed particles that suggest that *V. midae* SY9 increases *in situ* digestive enzyme levels and consequently improves feed digestion in farmed abalone (Huddy and Coyne, 2014). Macey

and Coyne (2006) demonstrated that both *V. midae* SY9 and *D. hansenii* AY1 colonize the digestive tract of *H. midae*, and that there is a positive correlation between the presence of these probiotics and increased enzyme activity. They suggested that the probiotics *V. midae* SY9 and *D. hansenii* AY1 could be successfully cultivated to high cell densities for incorporation into commercial abalone feed formulations.

1.4. PROTEOMICS AND BIOINFORMATICS

Proteins participate in almost all cellular processes. Information regarding protein descriptions, activity, and interaction is central to understanding a biological system. Therefore, proteome characterisation has received significant attention in various biological studies during the last few years (Cristea et al., 2004; Lane, 2005; Walther and Mann, 2010). The first studies on protein sought to map proteins from *Escherichia coli* (O'Farrell, 1975), mouse (Klose, 1975), and guinea pig (Scheele, 1975). However, the term 'proteomics' was first coined only in 1990 to refer to a large-scale characterisation of the entire protein complement within a biological system (Anderson and Anderson, 1996; Wilkins et al., 1996). At the present time, proteomics is a discipline that merges protein biochemistry, genome biology, and bioinformatics to determine the structural, functional, spatial and temporal expression of proteins in cells, tissues or whole organisms (Karr, 2008; Lane, 2005; Lin et al., 2003; Scherp et al., 2011).

In most biological studies, the main goal of proteomics is to find those proteins that exhibit changes due to a treatment or condition. Once this is established, it is then possible to identify, quantify, and characterise the maximum number of differentially expressed proteins (Graham et al., 2005; Jones and Cooper, 2011). Investigation into how protein levels change between a control and a specific perturbation, and to determine the relative abundance of proteins between these two states, are crucial variables to consider in molecular biology research (López, 2005; Nzoughet et al., 2009; Walther and Mann, 2010).

In addition to focussing on proteome characterisation, much research has looked into functional proteomics, which is based on the hypothesis that interaction between proteins suggests their common involvement in a biological function. Employing a functional proteomics approach allows the identification of protein interaction networks, molecular pathways, and activities (Aebersold and Cravatt, 2002; Köcher and Superti-Furga, 2007; Kolch and Pitt, 2010). The investigation of proteomic functions has provided new insights into research areas such as biomarker discovery, cancer prevention, drug treatment and disease screening (Hustoft et al., 2012; Monti et al., 2005). This is achieved mostly due to the improvement of mass spectrometry techniques (Smith and Figeys, 2006).

1.4.1. Overview of the mass spectrometry-based proteomics approach

Mass spectrometry is now universally recognized as the main tool for large-scale proteomics studies (Andersen and Mann, 2000). This tool relies almost exclusively on its ability to measure peptide masses accurately, which the mass spectrometry performs with remarkable precision thanks to the new generation of instruments (Zimmer et al., 2006).

There are two processes by which proteins are analysed in mass spectrometry: the top-down process and the bottom-up process. The top-down process starts with intact proteins that are cleaved in the gas phase rather than in solution. Basically, the protein is fragmented inside the mass spectrometer to create a ladder of ions indicative of the protein sequence. On the other side, the bottom-up process (or shotgun proteomics), identifies proteins by the tandem mass spectrometry analysis of peptides derived from enzymatic digestion of intact proteins. The resulting peptide mixture is chromatographically fractionated and introduced into a tandem mass spectrometer spectrometry (Evans et al., 2012; Lin et al., 2003; Steen and Mann, 2004). Shotgun proteomics has recently emerged as a powerful approach for characterising the proteomes of biological samples, with the goal being to identify the form and quantity of each protein in a high-throughput manner. This is done by coupling liquid chromatography with tandem mass spectrometry (Demirev et al., 2005; Evans et al., 2012; Graham et al., 2011; Lin et al., 2003; Steen and Mann, 2004).

Two-dimensional (2D) electrophoresis and mass spectrometry are the main approaches used in proteomics studies (López, 2005). Previous proteomics studies were based on 2D electrophoresis, a sensitive technique for analysing protein mixtures, as other approaches had not yet been discovered. In 2D electrophoresis, proteins are essentially separated in the first dimension by a charge using isoelectric focusing (IEF); the charge-focused proteins are then separated in the second dimension by size, using sodium dodecyl sulphate (SDS) and polyacrylamide gel, which generates protein spots (Aldred et al., 2004; Jungblut et al., 1999). These spots are then visualised by staining the gel with hydrophobic dyes such as Coomassie, silver, zinc or fluorescent stains (Lin et al., 2003). Protein spots that differ between treatments are selected, excised and in-gel digested to peptides (Moresco et al., 2008). Trypsin is the most commonly used protease, since it has a well-defined specificity and hydrolyses the peptide bonds only when the carbonyl group is followed by an arginine (Arg) or lysine (Lys) residue, with the exception being when Lys and Arg are N-linked to Aspartic acid (Asp) (Hustoft et al., 2012). After protein digestion, the resulting peptides are then purified and analysed by mass spectrometry (Moresco et al., 2008).

The applications of 2D electrophoresis are mostly in the context of detecting and quantifying modifications in protein expression in response to different conditions (Aldred et al., 2004; Jungblut et al., 1999). Although this gel-based approach was for long considered the workhorse of proteomics, recent studies have shown that this approach suffers several drawbacks, such as the number of samples that can be analysed per gel (which is only one sample), the poor detection of low abundant proteins, and the number of identified proteins per sample (Gygi et al., 2000; Righetti et al., 2004). Advances in mass spectrometry approaches have transformed proteomics studies (Cravatt et al., 2007; Smith and Figeys, 2006; Walther and Mann, 2010), shifting these from a gel-based approach to a direct mass spectrometric analysis (Tyers and Mann, 2003). Mass spectrometry has also made noteworthy contributions to the discovery of fundamental biological processes, and the detection and characterisation of low abundant proteins, apart from measuring hundreds to thousands of proteins within a biological system (Bantscheff et al., 2012; Cristea et al., 2004). These approaches have been applied across a wide diversity of taxa such as bacteria (Dworzanski et al., 2006), plants (Baginsky and Gruissem, 2006), invertebrates (Walker et al., 2006) and mammals (Pilch and Mann, 2006).

The most commonly used techniques for mass spectrometry analysis are liquid chromatography electrospray ionization tandem mass spectrometry (LC-ESI-MS/MS, or ESI), and liquid chromatography matrix-assisted laser desorption ionization tandem mass spectrometry (LC-MALDI-MS/MS, or MALDI) (McCormack et al., 1997; Medzihradszky et al., 2000; Zhen et al., 2004). Both ESI and MALDI are soft ionisation processes in which ions are generated with low internal energies and, as a result, undergo minimal fragmentation. ESI is carried out in conjunction with nano-spray high-performance liquid chromatography (HPLC), in which the samples are dissolved in a solvent and pumped through a thin capillary or needle that is raised to a high potential, producing a Taylor cone upon exiting the needle tip. On the other hand, MALDI uses irradiation by laser to desorb intact molecules that are co-crystallized in a photoactive matrix solution. Detailed descriptions of both techniques can be found in several reviews (Domon and Aebersold, 2006; Gingras et al., 2005; Guerrera and Kleiner, 2005; Jungblut et al., 1999)

Two strategies are used to provide quantifiable expression analysis in proteomics. The first is the label-free quantitative method that uses spectral counting, in which the number of MS/MS spectra assigned to a particular protein have been shown to correlate directly with the abundance of that protein (Bantscheff et al., 2007). The second strategy, the isotope-labelling method, is one of the most successful mass spectrometry achievements in proteomics research and is used to facilitate the quantitation and differential comparison of proteins, either at different times or under different conditions (Ahmad and Lamond, 2013; Graham et al., 2011).

There are three main methods of introducing stable isotopes to proteins: metabolic labelling, enzymatic labelling, and chemical labelling. Metabolic labelling involves cell culture, whereby the incorporation of isotopic labels occurs during the processes of cellular metabolism and protein synthesis (Amanchy et al., 2005; Ong et al., 2002). On the other hand, enzymatic labelling involves the incorporation of oxygen-18 during proteolysis or cleavage of the protein (Reynolds et al., 2002; Yao et al., 2001). Lastly, chemical labelling uses chemical reagents to label digested proteins in terminals of specific amino acids (Aggarwal et al., 2006; Sun et al., 2012; Zieske, 2006). Of these three methods, isotope labelling performed by chemical reagents has shown to fulfil several aspects that were previously considered as gaps in proteomics studies.

Chemical labelling can be performed in proteins by the use of isotope-coded affinity tags (ICAT), or alternatively, in peptides using isobaric tagging for relative and absolute quantitation (iTRAQ) (Graham et al., 2011). The iTRAQ approach was pioneered by Ross et al. (2004) and since then the protocol has been developed further. iTRAQ is based on isobaric tags containing a balance group and a reporter group of masses (*m/z*) 113, 114, 115, 116, 117, 118, 119 and 121 (8-plex kit). In the iTRAQ protocol, proteins from up to eight states are digested with trypsin, resulting in a peptide mixture; each cleaved peptide has a free amine group. Each sample is labelled with one specific reagent by attachment of the label through the amine-specific reactive group. The labelled samples are mixed, separated by liquid chromatography and introduced into the mass spectrometer. During tandem mass spectrometry, the reporter group is released from the labelled peptides, and the peak areas of the resultant ions allow the abundance of that particular peptide to be estimated (Aggarwal et al., 2006; Lengqvist et al., 2007; Ow et al., 2009; Schwacke et al., 2009; Zieske, 2006). The iTRAQ reagents are efficient at labelling nearly all peptides, providing high protein identification sequence coverage, and allowing a sensitive quantification of low-abundant proteins (Jain et al., 2009). The main advantage of iTRAQ over ICAT and other isotope-labelling methods is that it allows the simultaneous analysis of up to eight samples, thus decreasing the overall time needed for mass spectrometry analysis. Other methods can only analyse up to two samples at the same time. Another advantage of iTRAQ is that “b” and “y” ions derived from peptides labelled with its tags are indistinguishable, resulting in higher MS/MS intensity and, therefore, more reliable peptide identification (Shadforth et al., 2005). A simple representation of iTRAQ analysis is shown in Figure 1.2.

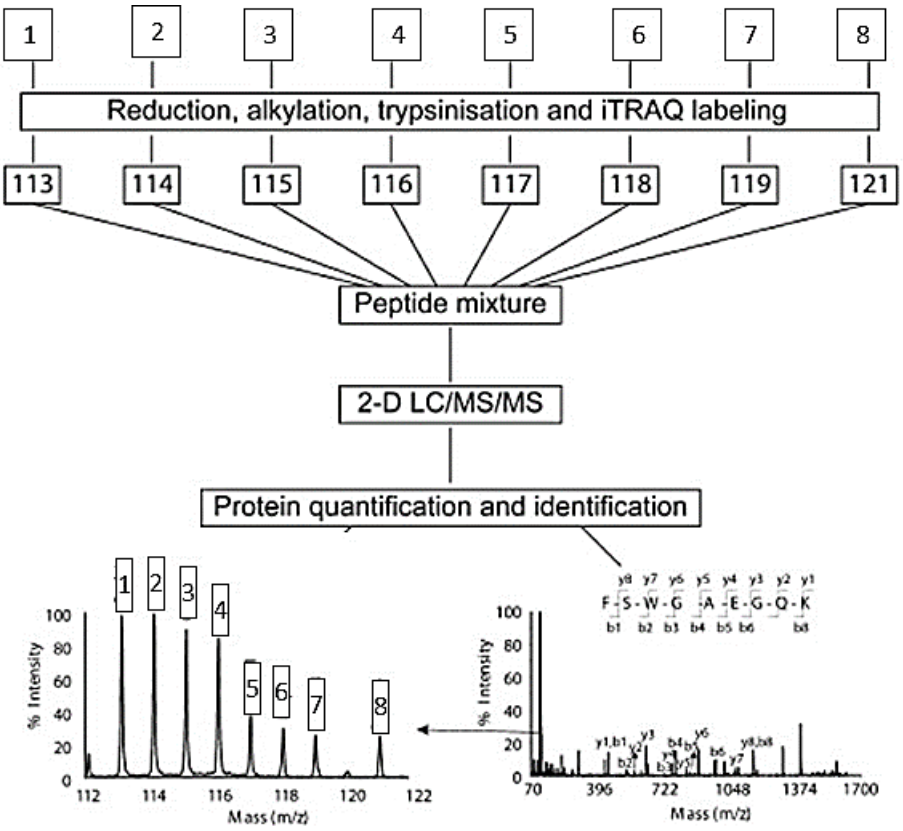


Figure 1.2. Schematic representation of iTRAQ's workflow. Up to eight protein samples are reduced, alkylated, and tryptically digested. The resulting peptides are labelled by iTRAQ tags *m/z*113, 114, 115, 116, 117, 118, 119, and 121. The labelled peptides are combined and analysed using liquid chromatography coupled with tandem mass spectrometry (2D LC/MS/MS). Database searches and bioinformatics procedures allow for protein identification and quantification. Adapted from Tyler et al. (2011).

1.4.2. Bioinformatics for mass spectrometry-based proteomics data analysis

Proteomics have witnessed major advances as a result of improvements in mass spectrometry techniques. The field is now a data-intensive discipline that requires extensive analytical and data-mining support (Cox and Mann, 2007); consequently, bioinformatics has become a pivotal tool for proteomics studies. Bioinformatics is fundamental to reducing the overall analysis time and to provide accurate results regarding the analysis of protein interaction networks, signalling pathways, amino acid sequences and protein structure (Cristoni and Mazzuca, 2011; Lisacek et al., 2006; Palagi et al., 2006).

New software packages and algorithms are continuously being developed to process mass spectrometry data efficiently and bring new insights to the understanding of proteomics results (Graham et al., 2011; Karr, 2008). Such packages have been developed to translate mass spectrometry data into a list of identified proteins and peptides, by comparing the *in-silico* peptide data of an entire database to the data acquired by the mass spectrometer analysis (Smith and Figeys, 2006). The most commonly used packages are SEQUEST, MASCOT, and MS-Tag (Cottrell and London, 1999; Eng et al., 1994; Mann and Wilm, 1994; Nesvizhskii et al., 2007; Ohlund et al., 2011; Romero-Rodriguez et al., 2014).

There are two main strategies used to assign fragment ion spectra to a particular peptide: (1) database searching – the fragment ion spectra are searched against a known database in which all of the proteins have been *in-silico* digested to produce a theoretical library of potential matches, from which the best match is then reported; (2) *de novo* sequencing – the amino acid sequence of the peptide is derived exclusively from the interpretation of the fragment ion spectra. This method is beneficial if there is no sequence information on the protein of interest in the database against which the search is being performed, or if there are unexpected modifications or polymorphisms in the protein. Generally, *de novo* sequencing is carried out following a database search strategy and is applied only to the unidentified spectra (Graham et al., 2011). A number of databases are available for protein identification, such as NCBI, EMBL, SWISS-PROT, and Uniprot (Bessarabova et al., 2012).

There is a possibility of including false peptide assignments during generation of the best matching peptides from the database. False positive identifications can occur, for instance, due to low-quality spectra, the presence of contaminants and the fragmentation of more than one peptide at a time. Therefore, it is good practice to ascertain a False Discovery Rate (FDR) for protein output lists from the onset. The most widely used method to establish a FDR is using a target-decoy database; most protein lists are reported with 1% FDR rates, indicating that there is more than 99% probability that the proteins reported in the list are true identifications (Graham et al., 2011; Van Nierop and Loos, 2011).

Common software applications such as Peaks, Scaffold, and ProteoIQ are used to identify proteins from raw peptides data. These software packages give report as list of accession numbers and gene names that contains marginal biological annotation, and experimental mass

spectrometry details instead of the biological significance of the data (McAfee et al., 2006). Therefore, establishment of the biological meaning of the experiment with regard to gene ontology analysis, protein interaction networks, and molecular pathways is performed using additional bioinformatics programs (Graham et al., 2011), such as KEGG, STRING, Cytoscape, and Ingenuity. Additional information regarding bioinformatics software in relation to mass spectrometry-based proteomics analysis can be found in reports such as those by Aebersold (2003), Cristoni and Mazzuca (2011), Deutsch et al. (2008) and Vitek (2009).

1.4.3. Proteomics in abalone research

Proteomics has been applied to the investigation of several areas within the aquaculture sector, including nutrition, identification of molecular biomarkers, seafood safety, and animal health (Piñeiro et al., 2003). In marine organisms, proteomics analyses are increasingly being implemented to monitor responses to challenges such as environmental changes (stress response and adaptation) and diseases (Bolton et al., 2013). Although a number of proteomics studies have been documented concerning marine invertebrates such as sponges, clams, mussels, oysters, scallops, shrimps and crabs, little work has been performed involving abalone. A review of these studies is available from Slattery et al. (2012).

Previous proteomics studies of abalone included an investigation of the potential molecular mechanisms of heterosis in hybrids of *H. gigantea* and *H. discus hannai* (Di et al., 2015a); characterisation of proteins involved in fertilization processes in *H. rufescens* (Palmer et al., 2013); identification of novel shell matrix proteins in *H. tuberculata* and *H. asinine* (Bédouet et al., 2012; Marie et al., 2011); and investigation of the mechanism of chemical toxicity in *H. diversicolor supertexta* (Liu et al., 2011; Zhou et al., 2010). As yet, there have been no proteomics studies that focus on the effect of probiotics on the abalone immune system, despite these immune stimulants being shown to have an important effect on the host's defense response in species such as *H. rufescens*, *H. discus hannai*, *H. gigantea*, *H. iris*, and *H. midae* (Hadi et al., 2014; Jiang et al., 2013; Macey and Coyne, 2005; Silva-Aciares et al., 2011).

1.5. RATIONALE AND AIMS OF THIS STUDY

The global demand for seafood has contributed to the development of the aquaculture sector (Qin et al., 2005), including the culture of abalone, which is a marine shellfish with one of the highest commercial value globally (Oakes and Ponte, 1996). However, there are several factors that negatively impact on the production of abalone; one of the major concerns is susceptibility to a possible disease outbreak during the long grow-out phase required to reach market size (Di et al., 2015b; Hooper et al., 2007; Mouton and Gummow, 2011; Zhuang et al., 2010). In South Africa, where *H. midae* is the most economically important seafood species cultivated, the farms are clustered along the Western Cape coastline, and some farms share boundaries, making the spread of diseases between farms inevitable (Mouton and Gummow, 2011; Sales and Britz, 2001). The use of antibiotics and other chemicals to control pathogenic organisms is not encouraged due to the possible development of antibiotic-resistant bacteria. Such bacteria can damage the normal microflora, cause microdybiosis, and result in environmental accumulation of antibiotics (Kumar et al., 2008). On the other hand, abalone, as an invertebrate, lack an adaptive immune response and depends solely on its innate immune system to fight against pathogenic infection (Di et al., 2015b; Xue et al., 2008).

Enhancement of the abalone immune system during grow-out phase could have a significant positive effect on production. Strategies employed by abalone farmers to accomplish immune stimulation include avoiding stress through the reduction of handling, grading, and transportation. Furthermore, restricted access to facilities, water quality control, and nutrient supplementation to help maintain a healthy environment. The use of dietary supplements to improve immune functions and resistance to infection has been shown to be suitable for abalone production (Chen and Tan, 2005; Dang et al., 2011). Probiotics, in particular, are feed supplements that offer protection by modifying the host environment through the production of inhibitory compounds, by competing with pathogenic-bacteria for essential nutrients and adhesion sites, or by modulating the immune response (Balcázar et al., 2006a, 2006b). In several species of abalone, experimental trials have shown that a probiotic-supplemented diet increased the growth rate and survival of the animals when challenged with a pathogenic bacterium. Therefore, probiotic-supplemented diets have been an important research focus area for the improvement of abalone production (Hadi et al., 2014; Jiang et al., 2013; Macey and Coyne, 2005). Our current understanding of the abalone immune response, when probiotic-

supplements are administered, is based mostly on host physiological responses (Cordero et al., 2014). The molecular mechanisms of the host immune response to probiotics remain poorly understood. New insights into the abalone molecular response to a probiotic-supplemented diet may provide a better understanding of the nature of the host defense response, such as protein biological function, protein interaction networks and protein biochemical pathways related to the immune system. This knowledge may help the development of molecular biomarkers that will allow the health status of farmed abalone to be monitored. The prediction of possible disease outbreaks may help abalone farmers to mitigate against the spread of an infectious disease at an early stage.

The recent development of high-throughput mass spectrometry techniques that can identify, quantify and compare the expression of several proteins from different conditions in one set, overcomes the shortfalls of 2D electrophoresis in proteome studies. Mass spectrometry coupled with increasingly more sophisticated bioinformatics tools has expanded our understanding of molecular mechanisms within biological systems (Aggarwal and Lee, 2003; Fenselau, 2007; McAfee et al., 2006). The review by Rodrigues et al. (2012) emphasises the power of proteomics to address various aquaculture-related questions such as those relating to animal welfare, health, quality, safety and nutrition.

To the best of our knowledge, there are no comprehensive proteomic studies of the effect of diet-supplements on the *H. midae* immune system. Thus, the objective of this study is to use a high-throughput proteomics approach to characterise changes in the *H. midae* haemocyte proteome elicited by a probiotic-supplemented diet, since previous studies showed that this treatment enhances host survival during pathogenic infection (Macey and Coyne, 2005). Thus, the three main aims of this study were to:

i. Identify and quantify proteins expressed in haemocytes from *H. midae* fed a probiotic-supplemented diet using iTRAQ, a mass spectrometry-based proteomics approach that is capable of comparing up to eight different samples. This analysis was performed in order to determine whether the probiotic-diet affected haemocyte protein expression. The effectiveness of iTRAQ LC-MS/MS and the Uniprot database for the identification of identification proteins from a non-model organism

such as *H. midae* would be checked by validating the results with the use of an alternate technique.

ii. Describe the biological functions, interaction networks, and molecular pathways modulated by haemocyte proteins that were identified, giving special attention to proteins that were differentially expressed in probiotic-fed abalone, in order to identify which protein, immune functions and biochemical processes best describe the effect of a probiotic-diet on *H. midae*. These analyses would be performed using bioinformatics packages such as IPA and STRING that are capable of predicting and mapping protein interactions and finding canonical pathways that include the proteins comprising the dataset.

iii. Characterise the expression of Ras-related protein Rab 1A, a protein that may be associated with immune stimulation of *H. midae* fed a probiotic-supplemented diet. This would include a comparison of the expression of this protein in the membrane and cytosolic fractions of haemocytes sampled over the course of the experiment, since members of Ras-related proteins are thought to translocate between these two cellular locations when the cell is stimulated. The results of this investigation may advance our understanding of Ras-related protein expression in haemocytes in response to probiotic induced stimulation of the abalone immune system.

CHAPTER 2 – ANALYSIS AND VALIDATION OF HAEMOCYTE PROTEIN EXPRESSION IN *HALIOTIS MIDAE* FED A PROBIOTIC-SUPPLEMENTED DIET

2.1. INTRODUCTION

Probiotics are live microorganism supplements that, in aquaculture, have been shown to improve water quality, the growth rate and immune response of the host, and consequently reduce the use of chemicals as well as antibiotics to control pathogenic infections (Matsuzaki and Chin, 2000; Mohapatra et al., 2013; Nayak, 2010). Several studies reported the benefits of probiotics for both vertebrates and invertebrates produced by aquaculture (Hadi et al., 2014; Kesarcodi-Watson et al., 2012; Lauzon et al., 2010; Tovar-Ramirez et al., 2010). Most of these studies focussed on the effect of probiotic supplements on the physiology of the host, looking specifically at growth rate and disease resistance. However, a better understanding of the effect of these supplements can only be obtained through the investigation of biological functions that are influenced by the host immune response.

Almost all biological functions are essentially mediated by proteins (Ooi et al., 2010; Suter et al., 2015). Therefore, protein characterisation during probiotic stimulation of host may provide new insights into the immune response and possibly contribute to new strategies for probiotic application in an aquaculture system. A number of studies have used proteomic approaches to investigate the effect of pathogenic bacteria and environmental conditions on the host immune system (Fields et al., 2012; Liu et al., 2013; Malécot et al., 2011; Somboonwiwat et al., 2010; Tomanek et al., 2011). On the other hand, only a few studies have investigated the effect of probiotic supplements on the host proteome, and these were conducted on vertebrates such as the rainbow trout *Oncorhynchus mykiss* (Brunt et al., 2008) and Atlantic cod *Gadus morhua* (Sveinsdóttir et al., 2009).

To the best of our knowledge, there are no proteomic studies that have investigated the effect of probiotic supplements on the immune response of invertebrates. Therefore, the effect of a probiotic-supplemented diet on the *Haliotis midae* haemocyte proteome was investigated in this study – *H. midae* is a species of abalone that has the highest commercial value in South Africa (Sales and Britz, 2001; Troell et al., 2006). It was hypothesised in this study that a probiotic-supplemented diet would affect the expression of proteins involved in *H. midae* immune activity. In general, the physiological activities of all organisms, including abalone, experience acute phase protein response when exposed to an external or internal stimulus.

Specifically, many defense-related molecules change in concentration (up- or down-regulation) in response to a stimulus in order to restore homeostasis (Bayne and Gerwick, 2001; Gerwick et al., 2002).

Studies on the effect of a probiotic-supplemented diet on the *H. midae* immune system have been performed (Macey and Coyne, 2005, 2006; Ten Doeschate and Coyne, 2008). These studies reported that a probiotic-supplemented diet enhanced the abalone growth rate and disease resistance. The molecular mechanisms by which the abalone immune system responds to a probiotic-supplemented diet remains unknown. Characterisation of the proteins that may participate in abalone immune activity may potentially lead to the identification of partner and effector proteins, protein interaction networks and molecular pathways related to the *H. midae* immune response. This investigation might contribute to the large body of work that aims to develop strategies to assist *H. midae* farmers to reduce stock loss. In this regard, the findings of this study might help to identify molecular indicators that are important in evaluating the abalone's immune state. This may allow abalone farmers to implement strategies to avoid potential infection and disease outbreaks.

Isobaric tag for relative and absolute quantification (iTRAQ) is one of the several techniques that can be used to evaluate protein expression (Ernoult et al., 2008; van Nierop and Loos, 2011; Unwin et al., 2010). It has multiple advantages over other isotope labelled-based proteomic techniques, specifically the metabolic and enzymatic isotopes. The iTRAQ-based proteomic approach can analyse up to eight samples from different conditions in a single run. This iTRAQ feature allows for flexible experimental design since several replicates can be analysed in the same mass spectrometry run (Fuller and Morris, 2012; Shadforth et al., 2005; Zieske, 2006). The ability to analyse multiple replicates in the same mass spectrometry run, reduces potential experimental variation. Therefore, iTRAQ-coupled with LC-MS/MS was employed in this study to investigate the immune response of abalone stimulated by a probiotic-supplemented diet.

This study had two main aims. The first aim was to characterise the proteome response in haemocytes of *H. midae* fed a probiotic-supplemented diet. To this end, we employed iTRAQ coupled with LC-MS/MS to analyse the proteome of *H. midae* haemocytes. The second aim of this study was to validate the iTRAQ-identified proteins that were found to be differentially

expressed in the haemocytes of *H. midae* fed a probiotic-supplemented diet. This was accomplished by using western blot analysis to confirm the identification and quantification of specific differentially expressed *H. midae* haemocyte proteins.

2.2. MATERIALS AND METHODS

The preparation of all media and solutions used in this study is described in Appendix A.

2.2.1. Routine housing and maintenance of *H. midae* stocks

The abalone used in this study were kindly donated by the I&J abalone farm in Gansbaai, South Africa. Upon collection by the researcher, the animals, which were approximately 70 mm in length, were stored in a plastic container that was kept in an ice box and transported to the Department of Agriculture, Forestry and Fisheries Research Aquarium in Sea Point, South Africa, where experiments were conducted.

The abalone were routinely maintained in 98 l polyethylene tanks. The polyethylene tanks were supplied with aerated, flow-through (330 $l.h^{-1}$) seawater at 15 – 18 ºC. Each tank was stocked with 40 abalone. The animals were fed once a week with fresh kelp (*Ecklonia maxima*). The tanks were cleaned on a weekly basis.

2.2.2. Preparation of kelp cakes supplemented with probiotic strains

Two probiotic strains were used for this study: *Vibrio midae* SY9 and *Debaryomyces hansenii* AY1. These species were previously isolated and proven to function as probiotics when provided to *H. midae* via the feed fed to the abalone (Macey and Coyne, 2005). Both species of probiotics were available in our laboratory. *V. midae* SY9 was routinely cultured in tryptone soya broth (TSB) supplemented with 2.5% NaCl (w/v) (Appendix A.1.1). *D. hansenii* AY1 was routinely cultured in yeast peptone D-glucose (YPD) broth (Appendix A.1.2).

For the preparation of kelp cakes, each probiotic strain was cultured in 4 l of broth until the stationary phase was reached. The cultures were concentrated by continuous flow centrifugation (Centrifuge Stratos, Thermo Scientific) at 16 000 rpm and 4 ºC. The probiotic-supplemented diet (Figure 2.1) was prepared as kelp cakes (Appendix B.1.1), which were a

mixture of dried seaweed (*E. maxima*), agar, probiotics and sea salt solution (SSS) (Appendix A.2.1). The kelp cakes were stored at 4 ^{o}C until required, and the duration of storage never exceeded a seven days period.

In order to determine the viability of each strain under storage conditions, the titre of the probiotics in the diet was determined in the freshly prepared kelp cakes and this was repeated after seven days of storage at 4 ^{o}C. One gram of kelp cake was homogenised in 1 ml of SSS and mixed. Serial dilutions of 100 µl were plated onto both TSB and YPD agar plates. Colony forming units (cfu) were counted and the final titre was calculated. This was performed in duplicate.

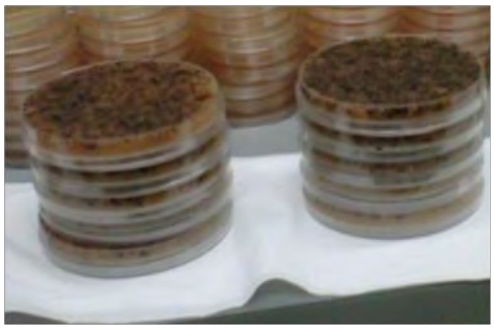


Figure 2.1. Petri dishes containing kelp cakes supplemented with probiotic strains. This feed was used to study the effect of a probiotic-supplemented diet on the haemocyte proteome of cultured *H. midae*. The kelp cakes were prepared using dried seaweed (*E. maxima*), agar, probiotics and SSS.

2.2.3. Experimental set up of the feeding trial using a probiotic-supplemented diet

For this experiment, 80 randomly-selected abalone were divided equally between two polyethylene tanks (Figure 2.2) and each tank was taken to represent a biological replicate. Two independent experiments were performed in this study; therefore, a total of four biological repeats were performed.

Before the probiotic feeding experiment, abalone were acclimatised for a period of two weeks. During this acclimation period, the animals were fed probiotic-free kelp cakes three times weekly. Any remaining feed in the tanks was removed prior to supplying fresh feed.

The abalone were starved for three days prior to the commencement of the experiment to ensure that the animals would rapidly consume the feed supplemented with the probiotic strains, as recommended by Macey and Coyne (2005).

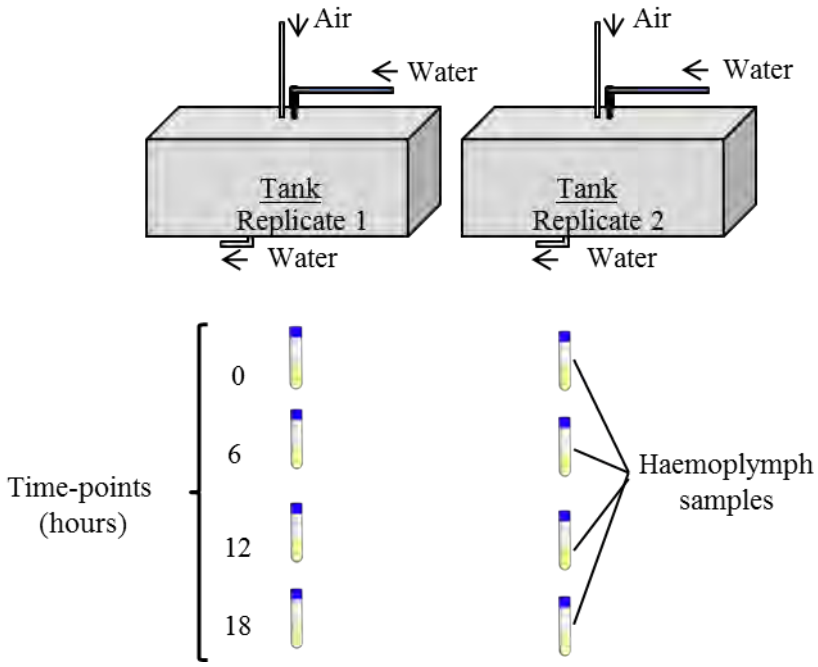


Figure 2.2. Schematic representation of the experimental design of the probiotic-supplemented diet feeding trial. Two polyethylene tanks of 98 l were supplied with circulating seawater per experiment and each tank was stocked with 40 abalone. Each tank represents a biological repeat. The experiment was performed in duplicate to allow for a total of four biological repeats.

Over the course of the experiment, haemolymph samples were collected from abalone at four different sampling time-points. The sampling time-points were selected based on previous studies performed in the laboratory in which abalone fed similar probiotics exhibited a stimulated immune response within the first hour of experiment. Therefore, haemocyte samples were collected at 0 hours (before adding the probiotic-supplemented diet to the tanks), as well as at 12, 18, and 36 hours after the introduction of the probiotic-supplemented diet. At each sampling time-point, approximately 0.4 – 0.7 ml of haemolymph was extracted from the pedal

sinus of between 4 to 7 abalone using a 2 ml syringe and 26G x ½ inch needle. After the extraction of haemolymph, the abalone were removed from the experimental tanks and placed in recovery tanks to safeguard against resampling. All haemolymph samples obtained from the same tank per sampling time-point were pooled in glass test tubes to a final volume of 4 ml, and samples were kept on ice. An amount of 3 ml sample was then split into two 1.5 ml aliquots in separate tubes. The Eppendorf tubes were centrifuged for 10 minutes at 8 500 rpm and 15 ^{o}C. The supernatant was discarded and the haemocyte pellets were stored at -80 ^{o}C until required. The remaining 1 ml of haemolymph was used for haemocyte cell counts and phagocytosis assay.

2.2.4. Determination of haemocyte concentration

The total number of circulating haemocytes in the haemolymph was determined using a haemocytometer and a compound light microscope at 40x magnification. In order to improve statistical accuracy, the haemocyte count was performed in triplicate. The concentration of the haemocytes was adjusted to 10^6 cells.ml^{-1} with Modified Hank's Balance Salt Solution (MHBSS) (Appendix A.2.2), to ensure that a consistent haemocyte concentration was used across all samples for the phagocytosis assay.

2.2.5. Determination of phagocytic activity of haemocytes

In order to assess whether the *H. midae* immune system was stimulated by the probiotic-supplemented diet, a phagocytosis assay was performed according to the method described in Macey and Coyne (2005). Per sampling time-point, an amount of 100 µl of haemolymph sample containing haemocytes, at a concentration of 10^6 cells.ml^{-1} in MHBSS, was placed inside separate squares (1.5 cm^2) prepared with Vaseline gel on a coverslip, which was previously washed in 50 % acetic acid (Appendix A.2.3). The coverslip was placed in a moist and dark incubation chamber for 30 minutes so as to allow the haemocytes to adhere to the coverslip. The unfixed haemocytes were removed by gently washing each coverslip with MHBSS. An aliquot of 100 µl FITC-labelled *Vibrio anguillarum* (Appendix B.2.1), at a

concentration of 10^8 ml^{-1} of phosphate buffered saline (PBS) (Appendix A.2.6), was then placed inside the gel square containing the haemocytes, to give a bacterium:haemocyte ratio of 100:1. The coverslips were then returned to the moist incubation chamber. Following incubation for 20 minutes in the dark, the coverslip was gently washed with MHBSS. A volume of 100 µl methanol was placed on the coverslip to allow fixation of the cells to the substrate. The coverslip was then gently rinsed with MHBSS.

In order to stain the genomic DNA of the haemocytes, 100 µl of ethidium bromide (Appendix A.2.7), at a concentration of 100 $\mu g.ml^{-1}$ in PBS, was added to each coverslip. The ethidium bromide solution was removed after 1 minute by rinsing with MHBSS; the remaining liquid was removed using a pipette. This assay was performed in triplicate at each sampling time-point.

The haemocytes were viewed using an inverse fluorescent microscope (Nikon Inverted Microscope DIAPHOT-TMD containing a Nikon EDI-Fluorescent attachment TMD-EF) at 400x magnification using a 510 nm excitation filter. Images were captured using an AxioCam (Zeiss) camera with its corresponding software, AxioVision AC version 4.4. The percentage of phagocytosis was determined by counting a total of 100 haemocytes using the following equation: % phagocytosis = (phagocytic haemocytes/total haemocytes counted) x 100. The mean and standard error of phagocytic activity was calculated for each sampling time-point (three replicates).

2.2.6. Characterisation of the proteome profile of haemocytes sampled from *H. midae* fed a probiotic-supplemented diet

A schematic overview of the experimental plan is illustrated in Figure 2.3. A description of how this was performed is included below.

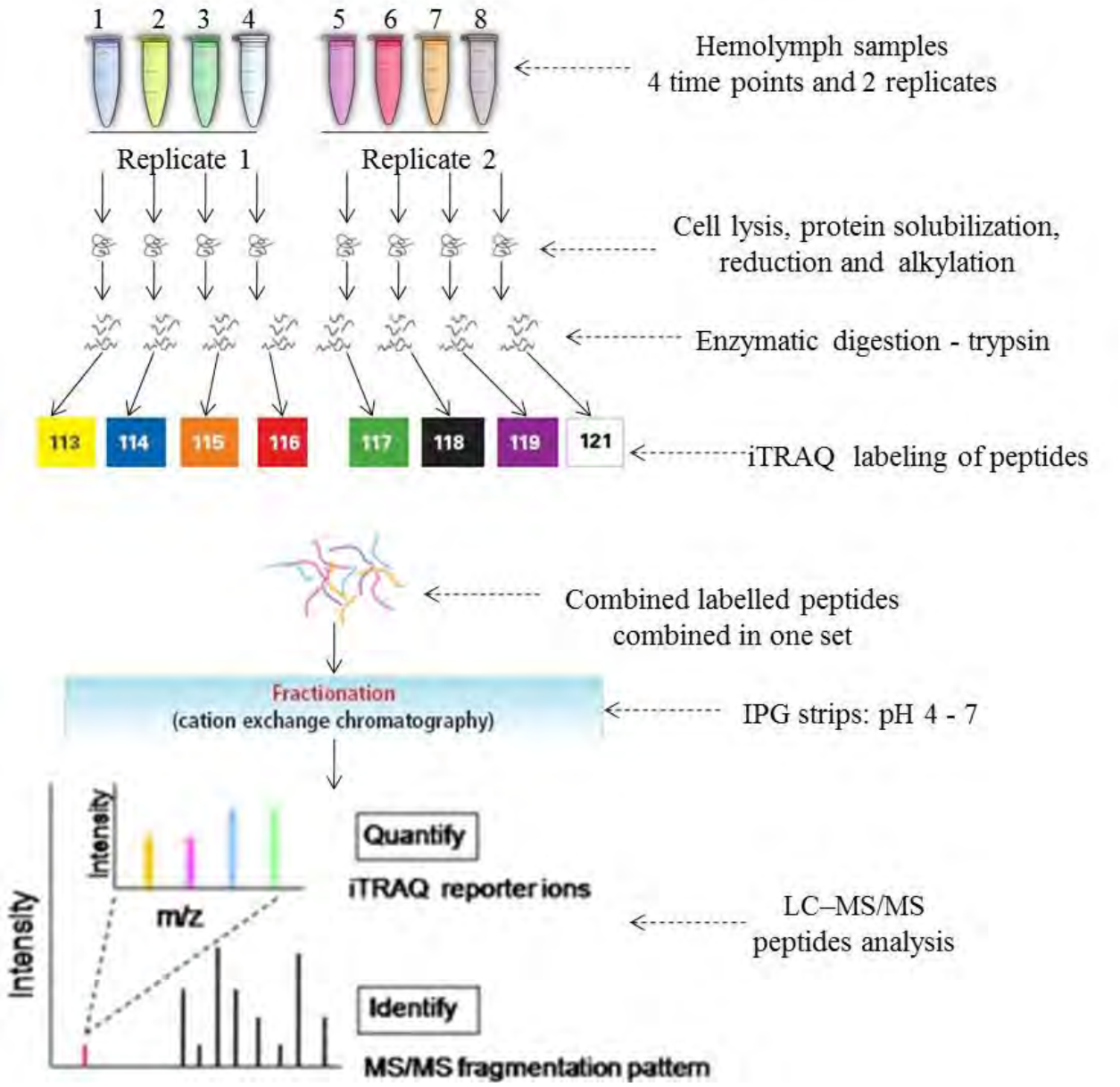


Figure 2.3. A brief schematic representation of the iTRAQ analysis design workflow that consists of two replicates; each replicate is composed of four sampling time-points (0, 12, 18, and 36 hours). At each sampling time-point, proteins were isolated, tryptic digested and peptides labelled with 8 plex iTRAQ tags. Subsequently, the labelled peptides were combined, Off-gel fractionated and analysed as one set in LC-MS/MS. The output data was processed for protein identification and quantification using bioinformatics software. The entire procedure was repeated twice. Thus, there was a total of four biological replicates in this study. Illustration adapted from Pastor et al. (2013).

2.2.6.1. Protein isolation from abalone haemocytes

Haemocyte pellets that had been stored at -80 °C were thawed at room temperature. The pellets were re-suspended in 50 µl of lysis buffer (Appendix A.2.9), and the samples mixed by pipetting. The samples were then vortexed for 10 minutes at room temperature, or until the pellets were dissolved. The samples were centrifuged for 30 minutes at 10 000 rpm and 4 °C. The supernatant from each sample was transferred to low-bind Eppendorf tubes.

2.2.6.2. Protein quantification

The protein content of each sample was quantified using the Thermo Scientific Pierce Bicinchoninic acid (BCA) Protein Assay kit according to the manufacturer's instructions. Next, each sample was separated into aliquots containing 300 µg of protein. The aliquots were stored in Eppendorf tubes at -80 °C, until required.

2.2.6.3. Filter-aided sample preparation

A filter-aided sample preparation (FASP) (Wisniewski 2009) protocol was used to reduce the concentration of detergents and salts in the protein samples before enzymatic digestion. This step was necessary because the haemocytes were lysed in a high concentration of SDS, which is not compatible with mass spectrometry analysis. The 300 µg samples stored at -80 °C were thawed at room temperature and incubated for 10 minutes in a water bath at 95 °C in order to denature the proteins. The samples were allowed to cool to room temperature prior to being centrifuged briefly. The proteins were then reduced by adding 0.5 M of tris(2-carboxyethyl)phosphine (TCEP) (Appendix A.2.10) at a ratio of 10:1 (v/v) sample:TCEP.

The samples were briefly vortexed and centrifuged, and then incubated for 1 hour in a 60 °C water bath. Each protein sample was placed in a 30 kDa molecular weight cut-off (MWCO) centrifuge filter, placed in a collector Eppendorf tube and centrifuged for 20 minutes at 9 000 rpm at 20 °C. The samples on the filters were alkylated by adding 100 µl of the buffer Triethylammonium bicarbonate in urea (UT) (Appendix A.2.12) containing 0.15M of methyl-methanethiosulphonate (MMTS) (Appendix A.2.11). The samples were briefly vortexed,

centrifuged, and incubated for 15 minutes at room temperature. In order to remove SDS from the samples, 300 µl of UT buffer (Appendix A.2.13) was added to each sample before centrifugation at 20 ºC for 20 minutes at 9 000 rpm. The flow-through in the collector tube was discarded. This washing step was repeated three times and the samples were mixed by briefly vortexing between each wash. The pH of the samples was adjusted to pH 8 using 1M TEAB (approximately 10 µl was added *per* sample). Protein reduction was performed by adding 100 µl of 0.5 M TEAB to each sample. The samples were briefly vortexed and centrifuged for 20 minutes at 9 000 rpm at 20 ºC. The flow-through in the bottom of the collector tube was discarded. The reducing step was performed twice, with brief vortexing between each step. Typically, 30 µl of the sample remained in the filter and was digested with trypsin.

2.2.6.4. Trypsin digestion

The samples from section 2.3.6.5 were transferred to low-bind Eppendorf tubes. An amount of 5 µg of proteomics-grade modified trypsin (Appendix A.14) was added, and the samples were briefly centrifuged. Optimal trypsin digestion occurs at an alkaline pH. Therefore, the pH of the samples was measured using pH strips and adjusted to approximately pH 9, where necessary, using 0.5 M TEAB. Each Eppendorf tube was sealed with parafilm, and tryptic digestion proceeded for 18 hours at 37 ºC. The parafilm was removed from the tubes and these were centrifuged for 10 minutes at 9 500 rpm at 15 ºC. An amount of 100 µl of 0.5 M TEAB was added to each Eppendorf tube; the samples were briefly vortexed and centrifuged for 10 minutes at 9 500 rpm at 20 ºC.

2.2.6.5. Labelling of peptides with iTRAQ tags

An 8plex iTRAQ kit was obtained from ABSciex South Africa Pty (Ltd) and was routinely stored at -20 ºC. The kit contained eight labels (113, 114, 115, 116, 117, 118, 119 and 121). The iTRAQ kit was thawed to room temperature and used according to the manufacturer's instructions, albeit with the following modification: a volume of 70 µl of isopropanol (Sigma-Aldrich) was added to each tube containing the labels, vortexed and centrifuged briefly. The content of each iTRAQ label tube was transferred to Eppendorf tubes containing tryptic

peptides. There were a total of eight samples (two replicates with four sampling time-points each), and each of them received different iTRAQ reagents for labelling (Table 2.1).

The samples were incubated at room temperature for 2 hours for labelling by the iTRAQ reagents. The eight samples were subsequently pooled into the same Eppendorf tube and mixed by brief vortex. Approximately 500 µl of the sample was used for further analysis.

In order to remove excess iTRAQ reagents, each sample was purified using a PepClean C-18 centrifuge column kit (Thermo Scientific) according to the manufacturer's instructions. The samples were then dried using a Savant SC110 Speed–vac (Thermo Scientific) for approximately 1 hour.

Table 2.1. Two experiments were performed to evaluate the effect of a probiotic-supplemented diet on the proteome of *H. midae* haemocytes. Each experiment contained two replicates and each replicate consisted of four sampling time-points (as described in section 2.2.3.). A total of two 8 plex iTRAQ kits were used, one for each experiment, as represented by colours green and blue.

	Experiment 1								Experiment 2							
	Replicate 1				Replicate 2				Replicate 3				Replicate 4			
Time-point (hours)	0	12	18	36	0	12	18	36	0	12	18	36	0	12	18	36
iTRAQ labels	113	114	115	116	117	118	119	121	113	114	115	116	117	118	119	121

2.2.6.6. Peptide off-gel fractionation

Dried peptides were re-suspended in 360 µl of millipore water and 1.440 ml of off-gel peptide stock solution (10% (v/v) of glycerol and 1% (v/v) ampholytes). The peptide sample was placed in a 12 well frame containing a 13 cm immobilize IPG gel strip (GE Healthcare) 3 – 10 linear pH range. Thereafter, the peptides were separated according to their isoelectric point (p*I*) using off-gel fractionation (Agilent 3100 OFFGEL Fractionator), according to the method used by Michel *et al*. (2003). Electro-focusing was performed up to a total of 20 kVh. The 12 fractions were recovered and kept in 12 separate Eppendorf tubes. C-18 centrifuge columns were used

to remove interfering contaminants, and the peptides were dried and stored at -80 ºC for ESI-MS/MS analysis.

2.2.6.7. Mass spectrometry analysis

To remove contaminants from the OFFGEL fractionation solutions and prevent clogging of the trapping column, each of the 12 cleaned peptide fractions were desalted prior to LC-MS/MS using Pierce C18 Spin columns. The fractions were then analysed on an Agilent 1200 series HPLC-Chip/MS system interfaced to an Agilent 6530 Accurate-Mass Q-TOF LC/MS. The HPLC–chip configuration consisted of a 160 nl enrichment column and a 150 mm x 75 µm analytical column. Mobile phases employed were: A) 0.1% (v/v) formic acid in water and B) 90% (v/v) acetonitrile with 0.1% (v/v) formic acid. An 84 minute long gradient method was used for the LC separation. Sample loading onto the enrichment column was done at 1% B. The gradient used for the analytical column began at 1% B, was raised to 8% B at 1 minute, maintained at 8 % B until 4 minutes, raised to 16% B at 18 minutes, 28% B at 50 minutes, 42% B at 70 minutes, 70% B at 80 minutes, maintained at 70% B until 82 minutes and then brought back to 1% B at 84 minutes. Samples were loaded at 2 µL.minute^{-1} flow rate and eluted at 0.4 µL.minute^{-1} flow rate. An Agilent 6530 Accurate-Mass Q-TOF LC/MS operating in high resolution (4GHz) positive ion mode was used for all experiments. The MS source conditions were: Source temperature: 350 ^{0}C; Capillary voltage: 1955 V; Fragmentor voltage: 175 V and; Drying gas flow rate: 4.3 µL.minute^{-1}. The mass spectrometer was set to perform data acquisition with a selected mass range of 200-1700 m/z and an acquisition rate of 6 spectra.second^{-1} in MS mode and m/z region of 90-1700 and an acquisition rate of 3 spectra.second^{-1} in MS/MS mode. Isolation width for MS/MS mode was set to 4 amu. Ramped collision energy slope was 3.9 and offset at 4.2.

2.2.7. Database searching and protein identification

The raw spectral data files obtained from the mass spectrometer (.d format) were first converted to .mzML format using the free tool MSConvert ProteoWizard, version 3.0.3662 (Kessner et

al., 2008); the data were then uploaded into Peaks Studio (Bioinformatics Solutions Inc. version 6.0).

In order to achieve a complete fragmentation pattern and low noise, the data were firstly refined with the following parameters: parent ion m/z tolerance at 0.1, retention time tolerance window of 30 seconds, and precursor charge correction. Secondly, *de novo* sequencing was performed using the following parameters: precursor mass tolerance of 20 ppm using monoisotopic mass, fragmentation ion of 0.1 Da, and trypsin for the cleave enzyme, so as to aid the identification of novel peptides, particularly because the study was performed on a non-model organism. Thereafter, database searches were conducted with the following parameters: parent mass error tolerance of 20.0 ppm using monoisotopic mass, fragment mass error tolerance of 0.1 Da, trypsin as the cleave enzyme, 3 maximum missed cleavages per peptide, one non-specific cleavage, fixed modifications- iTRAQ 8plex (K, N-term) and beta-methylthiolation, variable modifications- iTRAQ 8plex (Y) and oxidation (M), maximum variable PTM *per* peptide of 3. The protein identity search was conducted against the UniProtKB/Swiss database that included all mollusc protein sequences (downloaded in July 2013). The false discovery rate was automatically generated by Peaks Studio using a concatenated decoy database. The Peaks quantification outputs were filtered as 1% of false discovery rate, which set the -10 logP score at a threshold below which peptides would not be considered in the analysis. Data that satisfied the above-mentioned criteria were then auto-normalized by Peaks Studio to correct for total channel intensity differences, and then filtered to select only proteins with two or more unique peptides, which was considered a positive identification (Muñoz-Gómez et al., 2014). The data were exported as .csv (MS-DOS) format.

Using Microsoft Excel 2013, the protein data were manually edited to remove proteins with less than two unique peptides and zero intensity values, as well as those with the same accession numbers. The software package CLC Main Workbench (version 6.0) was used to $\log_2$ transform and quantile normalise the data. Protein expression values were then normalised to the control sampling time-point (0 hours).

2.2.8. Validation of differentially expressed proteins identified using iTRAQ analysis

It is common practice to validate protein expression data obtained by mass spectrometry using an alternate technique (Johnson and Gaskell, 2006; Slattery et al., 2012). Therefore, western blot analysis was used to validate the expression of proteins that had been identified to have undergone statistically significant changes in expression levels over the course of the experiment by iTRAQ analysis. Since there are no commercially available antibodies that are species-specific for *H. midae*, selection of the polyclonal antibodies used in this study was based on the highest sequence similarity between the immunogen and the target protein identified by iTRAQ. Thus, only three of the statistically differentially expressed proteins could be validated by western blot analysis, namely: COP9 signalosome subunit 4 (COP9 S4), Ras-related protein Rab 1A (Rab 1A), and V^+ATPase subunit B (V-ATPase B).

The primary antibodies, anti-COP9 subunit 4 (HPA036894), anti-Rab 1A (SAB2700768), and anti-V^+ATPase subunit B (HPA008147) were purchased from Sigma-Aldrich (South Africa) and were all polyclonal raised in rabbits. The secondary antibody (peroxidase conjugated) was raised in goats (anti-rabbit) and was purchased from Sigma-Aldrich (South Africa).

2.2.8.1. Experimental design and protein isolation

The preparation of the probiotic-diet used in this study is described in section 2.2.2., and the experiment, consisting of three biological replicates, was set up as described in section 2.2.3. For haemolymph extraction, three to four animals were sampled per sampling time-point, at 0 hours (control), 6, 12 and 18 hours after feeding the probiotic-supplemented diet to abalone. To ensure that the probiotic-supplemented feed stimulated a physiological response, the total number of circulating haemocytes and haemocyte phagocytic rate were assessed over the course of the experiment as described in sections 2.2.4 and 2.2.5. Subsequently, haemocyte total soluble protein was isolated and the total protein concentration was determined, as described in sections 2.2.6.1 and 2.2.6.2. Samples were then divided into 30 µg aliquots and stored at -80 ^{o}C for western blot analysis.

Before performing western blot analysis, cross-reactivity between each antibody and the target protein was tested, and the concentration of protein required to visualise a signal on the nitrocellulose membrane was determined. The minimal concentrations of both primary and secondary antibodies, which showed a robust immunoblot signal, are shown in Table 2.2. Some of the protein bands were not detected at the expected molecular size; therefore two-dimensional sodium dodecyl sulphate – polyacrylamide gel electrophoresis (2D SDS-PAGE) was performed to address whether the bands detected by western blot analysis were indeed the proteins of interest. Thereafter, the spots on the 2D SDS-PAGE were excised and subsequently analysed by LC-MS/MS for protein identification. The process of identification is described below.

Table 2.2. The sequence homology (%) between abalone protein and protein orthologues to which an antibody exists (based on Uniprot database), and the concentration of the proteins and antibodies (diluted in blocking buffer) used in western blot analysis.

Protein name	Antibody code (Sigma)	Sequence homology (%)	Protein (µg)	Antibody (v/v)	
				Primary	Secondary
COP9 signalosome complex subunit 4	HPA036894(1)	77.1	20	1:500	1:10 000
Ras-related protein Rab A1	SAB2700768(2)	67.8	20	1:250	1:10 000
V-type proton ATPase subunit B	HPA008147(3)	80.3	20	1:500	1:20 000

(1) 0.5 $\mu g.\mu l^{-1}$ in PBS, pH 7.2, containing 40% glycerol and 0.02% sodium azide.
(2) 0.5-1.0 $\mu g.\mu l^{-1}$ in PBS, pH 7.2, containing 40% glycerol and 0.02% sodium azide.
(3) 0.5 $\mu g.\mu l^{-1}$ in PBS, pH 7.2, containing 40% glycerol and 0.02% sodium azide.

2.2.8.2. Western blot analysis

In order to analyse changes in protein relative expression levels in haemocytes sampled from abalone at different sampling time-points over the course of the probiotic-supplemented feed experiment, one-dimensional sodium dodecyl sulphate – polyacrylamide gel electrophoresis (1D SDS-PAGE) analysis was performed and followed by immunoblot analysis.

2.2.8.2.1. Protein separation by 1D SDS-PAGE

Protein samples (20 µg) were first diluted in 5x Sample Application Buffer (SAB) (Appendix A.2.15) to a final concentration of 5 $\mu g.\mu l^{-1}$. The samples protein:SAB master mix were vortexed for 10 minutes at room temperature. Subsequently, the samples were briefly centrifuged and incubated for 10 minutes in a 75 ^{o}C water bath to denature the proteins. The samples were briefly vortexed and centrifuged for 30 seconds, pipetted into the wells of a 4% v/v stacking gel (Appendix A.2.16) and separated electrophoretically through a one-dimensional 12% polyacrylamide separating gel (Appendix A.2.17). One well was loaded with a molecular mass marker (Page Ruler^{TM} prestained protein ladder 10 – 170 kDa, Fermentas) for estimation of the protein size. The gels were electrophoresed in a Mini-Protean gel electrophoresis tank (Bio-Rad, South Africa) at a constant voltage of 100 V in 1x SDS electrophoresis buffer (Appendix A.2.18) at 4 ^{o}C until the bromophenol blue front of the sample application buffer had reached the bottom of the gel. The gels were then removed from the electrophoresis tank and prepared for western blot analysis.

2.2.8.2.2. Protein transfer to nitrocellulose membrane and immunoblotting

Following 1D SDS-PAGE, the gels were incubated in a cold Towbin buffer (Appendix A.2.19) for 5 minutes, so as to remove residual SDS. Additionally, a nitrocellulose membrane (Whatman Protran^{TM} 0.2 µM pore size) previously cut to fit the gel was also placed in cool Towbin buffer to equilibrate. Subsequently, the membrane and gel were placed between two pieces of pre-soaked Whatmann 3 mm filter paper and sandwiched together in a transfer cassette. This was then loaded vertically into a transfer tank (Bio-Rad mini Trans-Blot cell, South Africa), which was then filled with Towbin buffer. The protein gel was electroblotted onto the nitrocellulose membrane for an hour at 100 V 4 ^{o}C.

The nitrocellulose membrane was removed from the cassette and protein transfer confirmed using Ponceau S reversible total protein stain (Appendix A.2.20). Images were captured using the Molecular Imager ChemiDoc XRS+ system (Bio-Rad) with ‘colorimetric’ settings optimised for faint bands.

The nitrocellulose membranes were washed in Tris Buffered Saline (TBS) (Appendix A.2.21) to remove excess Ponceau S stain. Afterwards, the nitrocellulose membranes were blocked by immersing them in a container with blocking buffer (Appendix A.2.22) for one hour at room temperature with shaking. The nitrocellulose membranes were then incubated in the primary antibody (Appendix A.2.23) for 18 hours at 4 ^{o}C with shaking. Following this, the nitrocellulose membranes were washed three times in Tris Buffered Saline Tween (TBST) (Appendix A.2.24) and once in TBS for 15 minutes at room temperature with shaking. The nitrocellulose membranes were then incubated in the secondary antibody (Appendix A.2.25) for 2 hours at room temperature with shaking. The nitrocellulose membranes were washed three times in TBST and once in TBS for 15 minutes at room temperature with shaking.

2.2.8.2.3. Densitometric analysis of western blot

The visualization of proteins was performed using the WesternBright ECL HRP chemiluminescent detection kit (Advansta) as the substrate, according to the manufacturer's instructions. The chemiluminescent signal was acquired using the Molecular Imager ChemiDoc XRS+ system (Bio-Rad), where the immunoblot was exposed for 100 seconds, capturing an image every 10 seconds. The images with the fewest saturated pixels were selected for analysis.

The density (intensity.mm^{-2}) of each positive signal was calculated using the Image Lab Software (version 2.0.1, Bio-Rad). An area lacking signal was defined for background subtraction. The size of the bands detected by the antibody was determined using a molecular mass marker (Page Ruler^{TM} prestained protein ladder 10 – 170 kDa, Fermentas) and the molecular mass analysis tool provided in Bio-Rad's ImageLab software. The relative density of the bands was normalised to the respective β-actin signals. The intensity of the bands at 6, 12 and 18 hours was calibrated in relation to the control (0 hours), meaning that protein expression was presented as either an increase or a decrease in fold change of the target protein at each sampling time-point relative to the control.

2.2.8.3. Protein spot identification by mass spectrometry analysis

In order to confirm that the antibody used to detect COP9 signalosome subunit 4 was binding the target protein (since the 1D SDS-PAGE western blot showed an unexpected protein size), the putative protein spot was excised from the 2D SDS-PAGE and sequenced by mass spectrometry. Thus, two gels of 2D SDS-PAGEs were prepared: one was used for western blot (visualization of the target spot) and the other for excision of the protein spot for LC-MS/MS analysis.

For each SDS-PAGE, a volume containing 250 µg of soluble protein from the control haemocyte sample was placed in a microfuge tube containing an equal volume of 80% acetone (Appendix A.2.26); the sample was briefly mixed by vortexing and incubated for 30 minutes at -20 ºC. The sample was then vortexed for 10 seconds and centrifuged for 20 minutes at 8 500 rpm at 15 ºC. The supernatant was discarded, and the protein pellet allowed to air dry at room temperature for 10 minutes. The pellet was then re-suspended in 100 µl of urea lysis buffer (ULB) (Appendix A.2.27) because this buffer is compatible with solutions used in 2D SDS-PAGE analysis.

Protein separation in the first dimension was carried out on an immobilised pH gradient (IPG) according to the manufacturer's instructions (Bio-Rad). Briefly, rehydration solution (Appendix A.2.28) containing the total protein sample was loaded onto a tray with an IPG strip (7 cm, pH 4-7, Bio-Rad). The IPG strip was allowed to rehydrate for 16 hours at room temperature. A focusing tray for the Bio-Rad PROTEAN^{TM} Isoelectric focusing (IEF) system for 7 cm strips was prepared by covering the electrode filaments with Whatman's filter paper wicks, and the rehydrated strips containing the protein sample were placed onto the focusing tray (Bio-Rad). The IPG strips were covered with mineral oil, and the focusing tray was placed into the PROTEAN^{TM} Isoelectro focusing (IEF) Cell (Bio-Rad). The proteins were focussed in the first dimension under the following conditions: 250 V for 20 minutes, 4000 V for 2 hours, 4000 V for 20000 Volt-hours, 500 V for 24 hours (holding step).

Once the IEF was complete, the IPG strips were incubated in equilibration buffer 1 (Appendix A.2.29) for chemical reduction, and then in equilibration buffer 2 (Appendix A.2.30) for chemical alkylation, for 10 minutes each. The strips were removed from the equilibration buffer, dried with a brief application of tissue paper and loaded onto a pre-made 12%

polyacrylamide separating gel. The IPG strips were held in place by the addition of 1 ml 0.5% agarose (Appendix A.2.31). A molecular mass marker (Page Ruler^{TM} prestained protein ladder 10 – 170 kDa, Fermentas) was included for estimation of the protein size. The samples were placed in a Mini-Protean gel electrophoresis tank (Bio-Rad, South Africa) containing 1x SDS running buffer; they were then electrophoresed at 100 V at 4 ^{o}C until the bromophenol blue front of the sample application buffer had reached the bottom of the gel.

One of the 2D SDS-PAGEs was stained for 6 hours with Coomassie blue R250 solution (Appendix A.2.32) and subsequently incubated for approximately 12 hours in a destaining solution (Appendix A.2.33). Both staining and destaining procedures were performed at room temperature on a shaker. The gel was visualised using a Molecular Imager ChemiDoc XRS+ system (Bio-Rad). Images were captured using a Silver Fast colour scanner, and JPEG file formats were saved for a visual representation of the gel images.

To aid the identification of the target protein spot on the gels during spot excision, the second 2D SDS-PAGE was subjected to western blot analysis as described in sections 2.2.8.2 and 2.2.8.4. The nitrocellulose membrane was immersed in Ponceau S reversible total protein stain for 1 minute. It was then carefully washed in water, and the image captured using the Molecular Imager ChemiDoc XRS+ system (Bio-Rad), with 'colorimetric' settings optimised for faint bands, in order to compare the position of the spot between images of the gel and immune blot.

In the 2D SDS-PAGE, the protein spots of interest (exhibiting the size and isoelectric point of the target proteins) were selected for further identification using LC-MS/MS. The spots were manually excised from the Coomassie-stained 2D SDS-PAGE using a sterile scalpel. Each gel spot was placed in a microfuge tube and completely dehydrated using a Savant SC110 Speed-vac (Thermo Scientific) for approximately 30 minutes, followed by storage at -20 ^{o}C until required. Prior to mass spectrometry analysis, the spots were digested using the Protein in Gel Tryptic Digestion method (Appendix B.2.2). The extracted peptides were dehydrated until no liquid remained; this was done using Speed-vac (Thermo Scientific) for approximately 45 minutes. The spots were stored at -80 ^{o}C until required, and then sent for mass spectrometry sequencing (LC-MS/MS) at the Mass Spectrometry Unit of the Department of Molecular and Cell Biology, University of Cape Town.

The protocol followed for densitometric analysis of the western blots (section 2.2.8.2.3) was performed twice so as to ensure the accuracy of the results. The mass spectrometry analysis was performed as described in section 2.2.6.7, and data were processed as described in section 2.2.7.

2.2.9. Statistical analysis

All statistical analyses were conducted using SPSS 20 software (SPSS Inc.). For the descriptive statistics, means and standard errors of means (± SEM) of protein expression values were presented per sampling time-point. One-way ANOVA analyses were performed to determine whether there was a statistical difference ($p < 0.05$) in protein expression over the course of the experiment. One-way ANOVA results were used only when the Levene test of homogeneity of variances was not statistically different ($p > 0.05$). Post hoc multiple comparison tests (Tukey test) were performed when the differences between means were statistically significant.

2.3. RESULTS

2.3.1. Stability of probiotics in kelp cakes

The viability of the probiotics *V. midae* SY9 and *D. hansenii* AY1 following incorporation in the kelp cakes, and after seven days of storage, was tested to ensure that the animals were fed a sufficient amount of live probiotics per gram of feed. On the day of preparation, the concentration of both strains, SY9 and AY1, in the kelp cakes was greater than 10^8 cfu.g^{-1} of feed; although the viable concentration of both strains decreased (during both experiment 1 and 2) after seven days of storage at 4 ºC, it remained above 10^6 cfu.g^{-1} of feed (Figure 2.4).

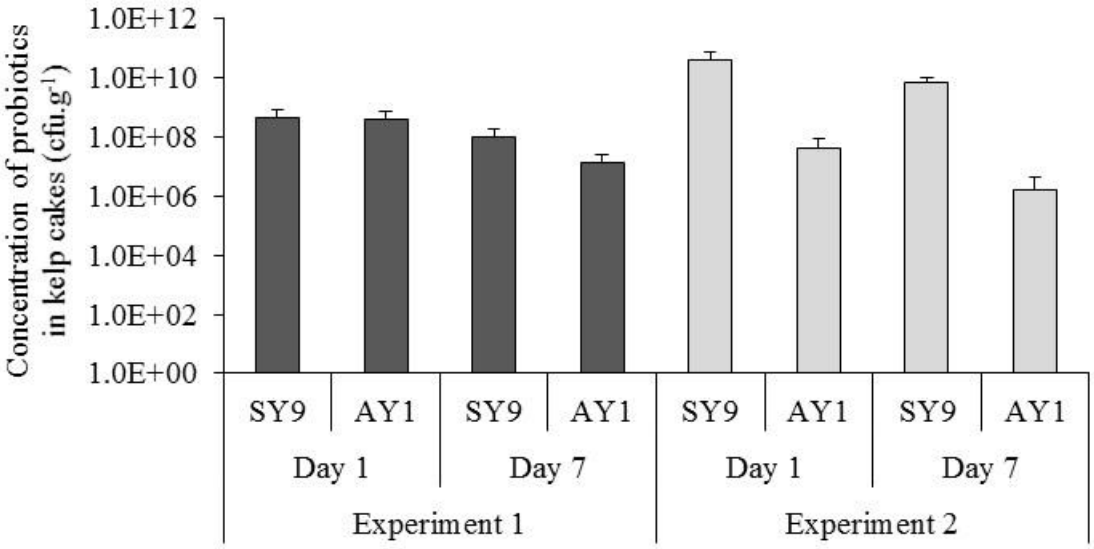


Figure 2.4. Viable concentration of the probiotic strains SY9 and AY1 immediately after preparation of kelp cakes and following seven days storage at 4 ºC. The data are presented as mean (n = 4) ± SEM of colony forming units per gram of feed.

2.3.2. Concentration of circulating haemocytes in haemolymph of *H. midae*

In order to assess whether the probiotic-supplemented diet influenced the total number of circulating haemocytes in *H. midae*, the concentration of cells was analysed during the course of the feeding experiment. During the course of experiment 1, there was an increase in the mean number of haemocytes in haemolymph. It rose from $3.0x10^6$ cell.ml^{-1} at 0 hours (control) to approximately $4.0x10^6$, $4.7x10^6$ and $3.6x10^6$ cell.ml^{-1} at 12, 18 and 36 hours, respectively (Figure 2.5). Statistical analysis performed using one-way ANOVA showed that there was a significant increase in the number of haemocytes at 12 and 18 hours in comparison to the control sample ($F = 8.02$ df 3, $p < 0.05$). During the second experiment, the mean number of haemocytes increased from $3.7x10^6$ cell.ml^{-1} at 0 hours to approximately $5.3x10^6$, $4.6x10^6$, and $4.8x10^6$ cell.ml^{-1} at 12, 18 and 36 hours, respectively (Figure 2.5). One-way ANOVA revealed that all these changes were statistically significant ($F = 7.57$, df 3, $p < 0.05$).

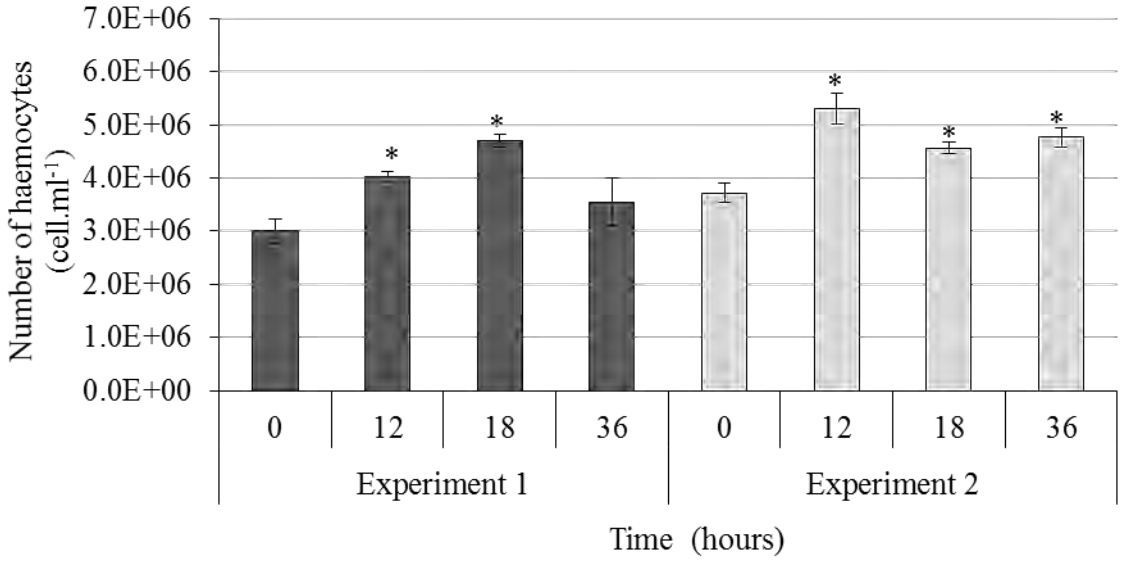


Figure 2.5. The total amount of haemocytes per ml of haemolymph sampled from *H. midae* fed with a probiotic-supplemented diet. The data are presented as mean (n = 4) ± SEM. An asterisk (*) indicates a significant difference ($p < 0.05$) in haemocyte numbers at a particular sampling time-point compared to the control (0 hours).

2.3.3. Phagocytic activity of *H. midae* haemocytes

The phagocytic capability of circulating haemocytes was evaluated over the course of the experiment to determine the effect of the probiotic-supplemented diet on *H. midae*. The results obtained in both experiments showed that there was an increase in the number of haemocytes phagocytosing bacteria. In experiment 1, the percentage of phagocytic activity in the circulating haemocytes was below 20% at 0 hours; however, it increased at all subsequent sampling time-points. The increase was statistically significant at 18 and 36 hours ($F = 82.22$ df 3, $p < 0.05$), reaching approximately 35% and 50 %, respectively.

In experiment 2, the percentage of phagocytic activity in circulating haemocytes increased from approximately 17% at 0 hours, to approximately 29% and 42% at 12 and 18 hours, respectively. At the end of the experiment (at 36 hours), the percentage of phagocytic activity was approximately 45% (Figure 2.6). Further analysis performed using one-way ANOVA revealed that these changes were statistically different ($F = 34.77$ df 3, $p < 0.05$).

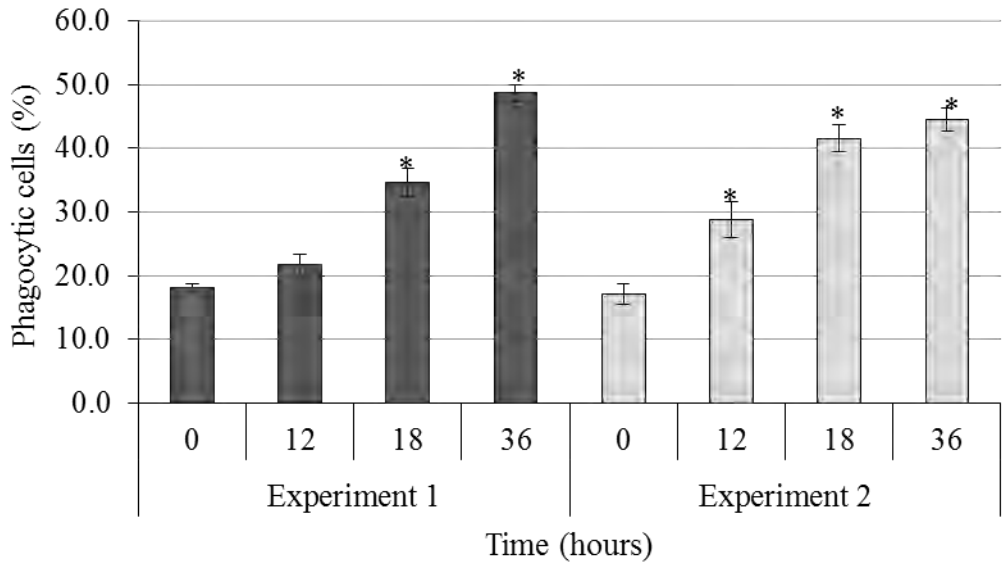


Figure 2.6. Percentage of phagocytic haemocytes in haemolymph sampled from *H. midae* fed with a probiotic-supplemented diet. The data are presented as mean (n = 4) ± SEM. An asterisk (*) indicates a significant difference ($p < 0.05$) of the percentage of phagocytic cells at a particular sampling time-point compared to the control (0 hours).

2.3.4. Identification of the haemocyte proteome of *H. midae* fed with a probiotic-supplemented diet

The identification and quantification of proteins expressed in abalone fed probiotics were investigated using iTRAQ and LC-MS/MS analysis. The two iTRAQ experiments (each including two replicates) identified a total of 317 proteins (iTRAQ experiment 1) and 338 proteins (iTRAQ experiment 2), respectively. A total of 128 proteins, common to both iTRAQ experiments 1 and 2 (Figure 2.7), were used to further investigate the effect of probiotics on the haemocytes proteome of *H. midae*. This group was composed of several proteins, of which 40S ribosomal proteins, actin, calmodulin, heat shock protein 70, haemocyanin, Ras-related proteins, T-complex protein 1, tropomyosin and ubiquitin were presented in different subunits. The total number of peptides and the number of peptides identified in each protein, as well as the mean expression values of the proteins at each sampling time-point (0, 12, 18 and 36 hours) over the course of the experiment, are presented in Table 2.3.

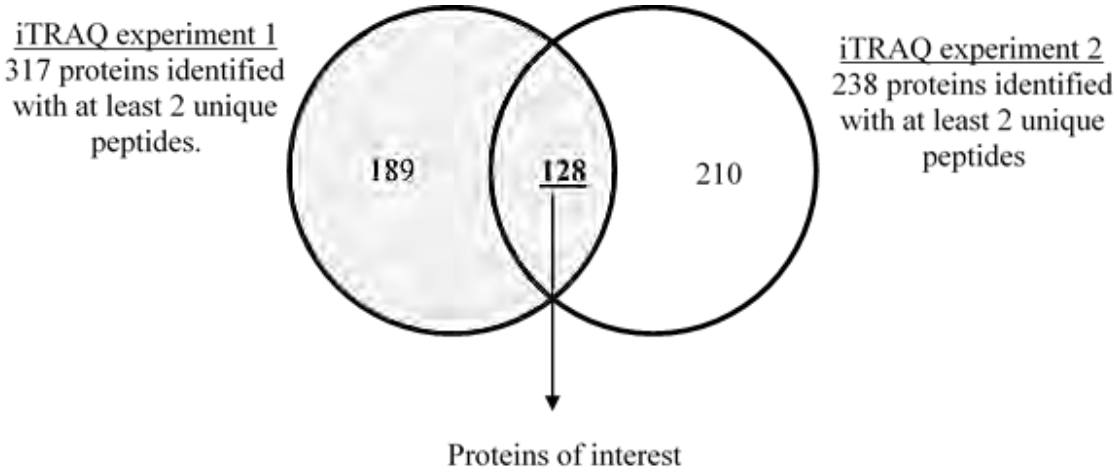


Figure 2.7. Number of proteins identified from two independent experiments. Each circle represents an iTRAQ experiment. The intercept includes the number of proteins common to both experiments. Proteins outside the intercept are unique to that experiment; 189 and 210 for experiment 1 and 2, respectively. The common proteins (n = 128) were used to investigate the effect of a probiotic-supplemented diet on the *H. midae* haemocyte proteome.

Table 2.3. Proteins identified in haemocytes from *H. midae* fed with a probiotic-supplemented diet. Accession numbers and descriptions were obtained from the UniProt database. The scores and percentage of coverages are referring to the peptides identified, and molecular masses are referring to the proteins. Expression values are presented as mean fold-change from four replicates, and the data, previously log_2 transformed, quantile normalised and normalised to the control, were collected at four different sampling time-points (hours).

			Coverage	Number of	Unique	Mass	Expression values			
Accession number	**Description**	**-10lgP**	**(%)**	**peptides**	**peptides**	**(kDa)**	**0**	**12**	**18**	**36**
K1QWC3|K1QWC3_CRAGI	40S ribosomal protein S3	70.2	11	4	2	25.9	0	-0.078	0.011	-0.078
B3TK56|B3TK56_HALDV	40S ribosomal protein S3a (Fragment)	95.8	33	7	6	19.5	0	-0.006	0.127	0.089
K1QCB0|K1QCB0_CRAGI	40S ribosomal protein S5	66.8	19	2	2	14.2	0	0.011	-0.154	0.061
B3TK66|B3TK66_HALDV	40S ribosomal protein S8	118.9	30	6	5	24.0	0	-0.010	0.038	0.060
K1R6S5|K1R6S5_CRAGI	40S ribosomal protein S9	66.2	13	10	4	64.3	0	-0.017	0.033	-0.215
B6RB17|B6RB17_HALDI	40S ribosomal protein SA	116.8	26	7	5	35.2	0	0.166	-0.035	0.127
K1Q273|K1Q273_CRAGI	60S ribosomal protein L14	82.3	26	5	4	18.2	0	-0.105	-0.115	-0.102
K1QNG9|K1QNG9_CRAGI	60S ribosomal protein L23a	98.9	42	8	7	19.0	0	0.098	0.006	0.039
K1RH70|K1RH70_CRAGI	6-phosphogluconate dehydrogenase_ decarboxylating	131.1	29	22	15	53.1	0	-0.001	-0.064	-0.144
Q6SQL9|Q6SQL9_9GAST	Actin (Fragment)	254.6	61	25	2	22.9	0	-0.045	-0.320	0.021
B3SND4|B3SND4_HALDV	Actin depolymerisation factor/cofilin	71.7	25	6	6	18.1	0	0.036	-0.067	0.199
K1QG58|K1QG58_CRAGI	Actin	337.0	75	55	3	41.8	0	-0.050	-0.331	0.008
K1R1E2|K1R1E2_CRAGI	Actin-related protein 2/3 complex subunit 2	52.7	7	3	2	32.8	0	0.210	-0.123	0.071
K1QLZ1|K1QLZ1_CRAGI	Actin-related protein 3	157.8	32	13	12	47.5	0	0.155	0.185	0.220
K1RW85|K1RW85_CRAGI	Adenosylhomocysteinase	63.9	15	9	7	47.5	0	0.047	0.020	-0.364
D7EZG9|D7EZG9_CRAGI	Adenylyl cyclase-associated protein	53.9	6	5	3	67.3	0	-0.348	-0.222	-0.371
B3TK64|B3TK64_HALDV	ADP_ATP carrier protein (Fragment)	37.3	13	2	2	16.7	0	-0.242	-0.158	-0.256
K1R0Y9|K1R0Y9_CRAGI	ADP_ATP carrier protein	87.3	14	4	4	33.3	0	-0.064	-0.081	-0.022
B6RB72|B6RB72_HALDI	ADP-ribosylation factor 2	169.8	53	10	6	20.9	0	0.010	-0.031	-0.098
K1PTH4|K1PTH4_CRAGI	ADP-ribosylation factor	143.9	11	14	8	124.2	0	0.065	0.002	0.208
Q8ITH0|Q8ITH0_BIOGL	Alpha-actinin (Fragment)	205.5	26	26	6	89.5	0	-0.145	0.036	-0.226

Table 2.3 (Continued)

Accession number	Description	-10lgP	Coverage (%)	Number of peptides	Unique peptides	Mass (kDa)	Expression values			
							0	12	18	36
K1PYW8|K1PYW8_CRAGI	Annexin	49.5	5	9	3	125.1	0	0.177	0.153	0.249
sp|P51544|KARG_HALMK	Arginine kinase	108.7	18	9	6	39.8	0	-0.026	0.354	0.087
K1R6Z7|K1R6Z7_CRAGI	ATP synthase subunit alpha	126.1	23	15	12	59.9	0	0.078	0.071	-0.097
B3TK26|B3TK26_HALDV	Calmodulin 2	118.7	28	10	5	21.9	0	0.383	0.302	0.555
sp|P02595|CALM_PATSP	Calmodulin	161.8	43	9	2	16.8	0	-0.304	-0.012	-0.028
B3TK60|B3TK60_HALDV	Calmodulin-dependent protein kinase (Fragment)	142.8	33	8	8	23.6	0	-0.248	-0.051	0.424
J9U877|J9U877_9BIVA	Calreticulin	71.4	6	4	4	49.0	0	0.171	0.194	0.176
Q86DH9|Q86DH9_APLCA	Cdc42	72.4	26	5	4	21.4	0	-0.195	0.013	-0.051
H9AWU2|H9AWU2_HALDH	Chaperonin containing T-complex polypeptide subunit zeta	130.2	21	14	8	58.5	0	-0.031	0.239	0.255
B6RB18|B6RB18_HALDI	Chaperonin containing tcp1	144.9	27	14	12	59.3	0	-0.037	-0.104	-0.008
K1PNR3|K1PNR3_CRAGI	Clathrin heavy chain 1	144.4	20	46	32	191.9	0	-0.076	-0.067	-0.114
K1QLP5|K1QLP5_CRAGI	Coatomer subunit delta	96.9	7	7	5	91.7	0	-0.189	-0.318	-0.123
K1R3V8|K1R3V8_CRAGI	COP9 signalosome complex subunit 4	44.6	6	3	2	46.5	0	0.505	0.461	0.598
K1PK85|K1PK85_CRAGI	Cullin-associated NEDD8-dissociated protein 1	98.5	11	15	10	138.8	0	-0.001	-0.057	-0.269
K1QHK9|K1QHK9_CRAGI	Dynein heavy chain_ cytoplasmic	130.2	5	32	12	561.6	0	0.000	-0.045	0.076
Q45Y88|Q45Y88_HALRU	Elongation factor 1-alpha (Fragment)	173.5	33	18	3	45.1	0	0.062	-0.017	-0.121
B3TK20|B3TK20_HALDV	Fructose-bisphosphate aldolase (Fragment)	110.2	18	4	3	24.1	0	0.120	0.042	-0.015
B6RB97|B6RB97_HALDI	Gelsolin OS	143.4	35	9	8	23.4	0	-0.118	-0.197	0.000
K1PM29|K1PM29_CRAGI	Glucose-6-phosphate 1-dehydrogenase	88.9	25	25	11	47.8	0	0.239	0.316	0.106
E4W3F6|E4W3F6_HALDV	Glutathione-S-transferase	93.5	19	4	4	31.0	0	0.098	0.034	0.073
B6RB30|B6RB30_HALDI	Glyceraldehyde-3-phosphate dehydrogenase	234.8	56	22	16	31.5	0	0.060	-0.074	-0.047
Q8I0U4|Q8I0U4_HALTU	H2 protein (Fragment)	345.4	25	104	14	380.5	0	0.012	0.070	0.096
A2TF45|A2TF45_9BIVA	Heat shock protein 70	220.8	33	28	2	71.3	0	0.158	0.298	0.378
B4E3Z5|B4E3Z5_9BIVA	Heat shock protein 70kDa A (Fragment)	88.0	42	5	2	12.3	0	0.126	0.276	0.345
Q9GP18|Q9GP18_HALTU	Hemocyanin (Fragment)	365.9	28	130	10	392.9	0	-0.021	0.297	0.192

Table 2.3 (Continued)

Accession number	Description	-10lgP	Coverage (%)	Number of peptides	Unique peptides	Mass (kDa)	Expression values 0	12	18	36
Q53IP9|Q53IP9_MEGCR	Hemocyanin 1	157.4	13	57	28	391.5	0	-0.055	-0.216	-0.143
Q1MVA1|Q1MVA1_MEGCR	Hemocyanin 2	223.7	15	65	26	391.5	0	-0.104	0.230	0.182
Q5XLV2|Q5XLV2_ENTDO	Hemocyanin A-type (Fragment)	95.8	7	31	7	332.0	0	-0.128	0.030	-0.164
B5RHQ6|B5RHQ6_9MOLL	Hemocyanin fgh (Fragment)	102.1	11	15	5	92.9	0	-0.122	0.010	-0.130
Q9BJ58|Q9BJ58_ENTDO	Hemocyanin G-type (Fragment)	108.4	8	32	7	334.9	0	-0.112	0.049	-0.133
sp|O61363|HCYG_ENTDO	Hemocyanin G-type_ units Oda to Odg	108.4	7	31	7	331.9	0	-0.108	0.056	-0.147
C7FEG7|C7FEG7_HALDV	Hemocyanin isoform 1 (Fragment)	282.5	22	107	33	381.8	0	-0.161	0.028	-0.053
Q27Q57|Q27Q57_SEPOF	Hemocyanin subunit 1	132.3	7	33	5	383.2	0	0.109	0.179	0.430
A2CI32|A2CI32_9BIVA	Histone H2A	88.6	45	5	4	13.4	0	0.065	0.002	0.063
I1SKJ3|I1SKJ3_APLCA	Histone H4	174.1	58	10	8	11.3	0	-0.023	-0.018	-0.053
I1VYX2|I1VYX2_MYTTR	Isocitrate dehydrogenase [NADP]	87.3	15	9	4	50.5	0	0.099	0.047	-0.044
Q6KC56|Q6KC56_MEGCR	Keyhole limpet hemocyanin1	153.5	13	52	26	358.9	0	-0.049	-0.217	-0.139
Q6KC55|Q6KC55_MEGCR	Keyhole limpet hemocyanin2	223.7	15	65	26	391.5	0	-0.104	0.250	0.180
K1PV35|K1PV35_CRAGI	Kyphoscoliosis peptidase	91.0	8	8	4	77.8	0	0.013	-0.069	0.242
Q9U9B6|Q9U9B6_MYTED	Major vault protein (Fragment)	82.1	16	4	3	31.8	0	-0.270	-0.308	0.104
K1QQR1|K1QQR1_CRAGI	Major vault protein	102.1	10	9	5	96.3	0	-0.174	-0.014	-0.151
B6RB90|B6RB90_HALDI	Malate dehydrogenase (Fragment)	81.4	18	6	3	25.8	0	-0.063	0.051	-0.107
K1R3N2|K1R3N2_CRAGI	Methylmalonic aciduria type A protein_ mitochondrial	34.8	9	5	2	35.4	0	-0.036	-0.036	0.131
A6MD73|A6MD73_HALDI	Mx	45.4	7	5	2	57.6	0	-0.005	-0.079	-0.255
K1Q122|K1Q122_CRAGI	Myosin regulatory light chain sqh	138.9	38	8	7	19.6	0	-0.137	-0.146	-0.035
Q45R40|Q45R40_APLCA	Nonmuscle myosin II (Fragment)	278.9	29	76	22	220.2	0	0.006	0.020	-0.027
B6RAZ8|B6RAZ8_HALDI	Omega class glutathione-s-transferase 1	94.3	14	4	4	27.4	0	-0.030	0.002	0.052
D3K380|D3K380_PINFU	Peroxiredoxin	92.2	30	9	5	22.3	0	-0.060	0.090	-0.047
K1Q615|K1Q615_CRAGI	Peroxiredoxin-1	102.6	19	7	2	28.9	0	0.380	0.106	0.201

Table 2.3 (Continued)

Accession number	Description	-10lgP	Coverage (%)	Number of peptides	Unique peptides	Mass (kDa)	Expression values			
							0	12	18	36
K1QCC1|K1QCC1_CRAGI	Phosphoglycerate kinase	99.7	10	4	2	43.0	0	-0.113	-0.158	-0.008
K1Q948|K1Q948_CRAGI	Phosphorylase	94.1	17	21	12	103.9	0	0.301	0.537	0.278
K7ZQ52|K7ZQ52_PTEPN	Pif	43.2	4	6	3	86.6	0	0.068	-0.129	-0.037
C7EAA2|C7EAA2_HALAI	PL10-like protein	164.2	26	27	15	86.0	0	0.125	0.042	0.005
K4INQ5|K4INQ5_9BIVA	Proteasome 26S subunit (Fragment)	61.5	18	5	4	27.4	0	-0.017	-0.255	0.167
K1R6F1|K1R6F1_CRAGI	Proteasome subunit alpha type	60.0	20	4	3	28.0	0	0.197	0.031	-0.055
B6RB63|B6RB63_HALDI	Protein disulfide isomerase	250.8	38	31	14	55.2	0	-0.077	-0.091	0.148
K1PWR0|K1PWR0_CRAGI	Protein SET	98.9	28	9	6	28.1	0	-0.402	-0.081	-0.022
G9K380|G9K380_HALDV	Putative 60S ribosomal protein L3	97.8	18	8	6	46.1	0	-0.029	-0.127	-0.107
B3TK70|B3TK70_HALDV	Putative RNA-binding protein	87.4	21	3	3	19.0	0	-0.164	-0.231	-0.190
K1PJ46|K1PJ46_CRAGI	Pyruvate kinase	64.6	11	8	3	53.0	0	-0.279	-0.086	-0.302
B8XW76|B8XW76_HALDV	QM-like protein	99.8	26	10	3	25.1	0	0.130	0.169	-0.134
K1S151|K1S151_CRAGI	Rab GDP dissociation inhibitor beta	48.9	8	5	3	50.0	0	0.036	0.229	0.068
B6RB76|B6RB76_HALDI	RAB protein	106.5	34	6	4	22.5	0	0.059	-0.267	-0.249
A7L3I9|A7L3I9_HALCR	RAB1 (Fragment)	130.8	61	10	4	16.0	0	-0.240	-0.326	-0.431
K1PUJ1|K1PUJ1_CRAGI	Radixin	126.2	13	9	3	69.5	0	0.125	0.153	0.149
B6RB29|B6RB29_HALDI	Ran-1-prov protein	92.9	25	7	2	25.2	0	-0.081	-0.250	0.003
K1R4S7|K1R4S7_CRAGI	Ras-related protein Rab-10	111.6	27	8	3	24.2	0	0.079	0.038	-0.339
K1QD28|K1QD28_CRAGI	Ras-related protein Rab-11A	94.8	12	3	2	23.4	0	-0.098	-0.173	-0.252
K1QX44|K1QX44_CRAGI	Ras-related protein Rab-11B	113.5	28	5	4	19.3	0	-0.093	-0.193	-0.275
K1QC78|K1QC78_CRAGI	Ras-related protein Rab-14	64.6	19	4	2	23.9	0	-0.168	-0.585	-0.529
B6RB23|B6RB23_HALDI	Ras-related protein Rab-1A	170.6	58	12	4	22.8	0	-0.125	-0.296	-0.304
K1PZ08|K1PZ08_CRAGI	Ras-related protein Rab-7a	126.2	41	9	3	23.1	0	-0.051	-0.050	-0.300

Table 2.3 (Continued)

Accession number	Description	-10lgP	Coverage (%)	Number of peptides	Unique peptides	Mass (kDa)	Expression values 0	12	18	36
K1QQ39|K1QQ39_CRAGI	Ras-related protein RHA1	51.1	11	6	6	54.8	0	-0.100	-0.131	-0.313
K1PR25|K1PR25_CRAGI	Regulator of differentiation 1	82.2	8	6	4	82.9	0	0.126	0.017	0.088
B6RB96|B6RB96_HALDI	Ribosomal protein l5 (Fragment)	97.8	19	6	4	28.8	0	-0.029	-0.065	-0.138
Q70MM6|Q70MM6_CRAGI	Ribosomal protein S3 (Fragment)	95.4	17	4	2	20.7	0	-0.024	0.003	-0.063
Q4H451|Q4H451_CRAGI	Ribosomal protein S5	66.8	14	3	2	23.0	0	0.014	-0.159	0.074
A9LMJ6|A9LMJ6_HALDI	Ribosomal protein S9	71.8	34	8	4	22.3	0	0.022	-0.070	-0.186
K1PY30|K1PY30_CRAGI	Septin-2	64.2	5	2	2	72.4	0	0.322	0.214	0.070
K1S6V7|K1S6V7_CRAGI	Serine/threonine-protein phosphatase 2A 65 kDa regulatory subunit A alpha isoform	90.9	10	14	12	117.5	0	0.208	0.079	-0.077
K1QB83|K1QB83_CRAGI	Serine/threonine-protein phosphatase	80.7	9	3	2	37.2	0	-0.667	-0.677	-0.193
K7R2X9|K7R2X9_9BIVA	SET nuclear oncoprotein (Fragment)	82.5	9	3	2	25.7	0	-0.402	-0.072	-0.020
E6Y2Z7|E6Y2Z7_HALDV	Splicing factor arginine/serine-rich 4	100.1	31	5	4	18.4	0	0.083	-0.134	0.130
K1RAJ1|K1RAJ1_CRAGI	T-complex protein 1 subunit alpha	82.8	5	5	2	75.3	0	0.151	0.462	0.048
K1S4Q2|K1S4Q2_CRAGI	T-complex protein 1 subunit delta (Fragment)	89.9	16	8	5	54.3	0	-0.436	-0.433	-0.191
K1RLC5|K1RLC5_CRAGI	T-complex protein 1 subunit epsilon	78.8	8	7	2	75.0	0	0.097	-0.430	-0.233
K1R466|K1R466_CRAGI	T-complex protein 1 subunit gamma	108.7	13	10	6	63.9	0	0.377	0.033	0.069
K1QX02|K1QX02_CRAGI	Tenascin-X	86.0	4	84	39	1722.8	0	-0.303	-0.585	-0.039
B1N693|B1N693_HALDI	Thioredoxin peroxidase 1	71.3	18	5	3	28.0	0	-0.005	0.142	0.272
E1B300|E1B300_9BIVA	Thioredoxin peroxidase	92.2	16	5	2	21.9	0	-0.072	0.080	-0.054
K1QVK0|K1QVK0_CRAGI	Transaldolase	69.1	7	6	4	62.9	0	0.077	-0.030	-0.119
B6RB35|B6RB35_HALDI	Transgelin	96.6	10	3	3	20.8	0	0.106	-0.007	0.120
K1PVA1|K1PVA1_CRAGI	Transitional endoplasmic reticulum ATPase	92.8	12	13	6	88.7	0	0.112	0.128	0.066
H9LJ34|H9LJ34_CRAAR	Transketolase-like protein 2 (Fragment)	63.9	22	7	4	22.4	0	0.002	0.053	0.039
K1RBC9|K1RBC9_CRAGI	Transketolase-like protein 2	88.7	18	18	11	74.9	0	0.001	-0.012	0.083
Q45Y86|Q45Y86_HALRU	Triosephosphate isomerase (Fragment)	128.3	31	6	4	22.6	0	0.187	0.085	0.267

Table 2.3 (Continued)

Accession number	Description	-10lgP	Coverage (%)	Number of peptides	Unique peptides	Mass (kDa)	Expression values			
							0	12	18	36
Q7YZR4|Q7YZR4_HALAI	Tropomyosin 1	150.2	43	19	5	32.9	0	-0.058	-0.081	0.045
Q7YZR3|Q7YZR3_HALAI	Tropomyosin 2	136.1	42	19	5	32.9	0	-0.059	-0.121	0.038
B7XC62|B7XC62_HALDI	Tropomyosin	150.2	43	19	5	32.9	0	-0.036	-0.072	0.036
B0B039|B0B039_MYTED	Ubiquitin (Fragment)	119.1	13	4	3	25.1	0	-0.038	-0.075	-0.004
D2XEB0|D2XEB0_CRAHO	Ubiquitin	127.9	38	7	6	14.7	0	-0.034	-0.004	-0.043
Q8T6A1|Q8T6A1_APLCA	Ubiquitin/ribosomal L40 fusion protein (Fragment)	127.0	36	6	5	14.1	0	0.019	0.034	-0.021
K1P339|K1P339_CRAGI	Vinculin	138.1	22	33	21	125.2	0	0.002	-0.205	-0.078
B0RZD4|B0RZD4_PECMA	Vitellogenin	48.9	5	20	6	265.1	0	-0.158	-0.012	-0.017
K1Q9V3|K1Q9V3_CRAGI	V-type proton ATPase catalytic subunit A	64.0	8	8	3	70.9	0	0.040	-0.074	-0.170
K1QI28|K1QI28_CRAGI	V-type proton ATPase subunit B	104.4	19	9	7	58.0	0	0.372	-0.046	0.127
K1RQC1|K1RQC1_CRAGI	Xanthine dehydrogenase/oxidase	57.6	6	11	5	148.5	0	0.110	0.107	0.038

2.3.5. Identification of differentially expressed in haemocyte proteins isolated from *H. midae* fed a probiotic-supplemented diet

In order to ascertain whether a diet containing probiotics affected abalone haemocyte protein expression, the 128 proteins that were common to both experiments were subjected to statistical analysis. One-way ANOVA revealed that seven of the proteins were differentially expressed with statistical significance ($p < 0.05$). Annexin, COP9 signalosome complex subunit 4, phosphorylase, T-complex protein 1 subunit gamma and V-type proton ATPase subunit B, were the proteins identified as up-regulated during the experiment; of these five proteins, COP9 signalosome complex subunit 4 had the largest fold change increase. On the other hand, Rab 1 (Fragment) and Ras-related protein Rab 1A were down-regulated over the course of the experiment (Table 2.4).

Several other proteins in the dataset showed down- or up-regulation at almost all sampling time-points in comparison to the control; however, the differences were not statistically significant ($p > 0.05$). The top 10 proteins that showed the largest fold change are presented in Table 2.5 and include calmodulin 2, peroxiredoxin-1, septin-2, and glucose-6-phosphate 1-dehydrogenase, which were up-regulated; and Serine/threonine protein phosphatase, T-complex protein 1 subunit delta, Protein SET, and Adenylyl cyclase-associated protein, which were down-regulated.

Table 2.4. Differentially expressed haemocyte proteins from *H. midae* fed with a probiotic-supplemented diet. Expression values are presented as mean fold-change (n = 4, except n = 3 where indicated by an asterisk, *) of the protein intensities, which were previously log_2 transformed, quantile normalised and normalised to the control. One-way ANOVA showed that there was statistically significant difference ($p < 0.05$) in at least one sampling time-point compared to the control.

		Expression				
Accession number	**Description = source**	**0**	**12**	**18**	**36**	**p-value**
K1PYW8|K1PYW8_CRAGI	Annexin OS=*Crassostrea gigas*	0	0.320	0.276	0.335	0.020*
K1R3V8|K1R3V8_CRAGI	COP9 signalosome complex subunit 4 OS=*Crassostrea gigas*	0	0.505	0.461	0.598	0.011
K1Q948|K1Q948_CRAGI	Phosphorylase OS=*Crassostrea gigas*	0	0.301	0.537	0.278	0.027
A7L3I9|A7L3I9_HALCR	RAB1 (Fragment) OS=*Haliotis cracherodii*	0	-0.240	-0.326	-0.431	0.007
B6RB23|B6RB23_HALDI	Ras-related protein Rab-1A OS=*Haliotis discus discus*	0	-0.182	-0.387	-0.474	0.035*
K1R466|K1R466_CRAGI	T-complex protein 1 subunit gamma OS=*Crassostrea gigas*	0	0.377	0.033	0.069	0.039
K1QI28|K1QI28_CRAGI	V-type proton ATPase subunit B OS=*Crassostrea gigas*	0	0.372	-0.046	0.127	0.017

Table 2.5. The top 10 proteins with the largest fold change (no statistically significant difference; $p > 0.05$) identified in haemocytes from *H. midae* fed with a probiotic-supplemented diet. Data are from four sampling time-points (hours), presented as mean fold-change (n = 4) of the protein intensities, which had been log_2 transformed, quantile normalised and normalised to the control.

		Expression values			
Accession number	**Description**	**0**	**12**	**18**	**36**
Up-regulated					
B3TK26|B3TK26_HALDV	Calmodulin 2 OS=*Haliotis diversicolor*	0	0.383	0.302	0.555
K1Q615|K1Q615_CRAGI	Peroxiredoxin-1 OS=*Crassostrea gigas*	0	0.380	0.106	0.201
K1PR25|K1PR25_CRAGI	Regulator of differentiation 1 OS=*Crassostrea gigas*	0	0.126	0.017	0.088
K1PY30|K1PY30_CRAGI	Septin-2 OS=Crassostrea *gigas*	0	0.322	0.214	0.070
K1PM29|K1PM29_CRAGI	Glucose-6-phosphate 1-dehydrogenase OS=Crassostrea gigas	0	0.239	0.316	0.106
Q45Y86|Q45Y86_HALRU	Triosephosphate isomerase *(Fragment)* OS=*Haliotis rufescens*	0	0.187	0.085	0.267
J9U877|J9U877_9BIVA	Calreticulin OS=Hyriopsis cumingii	0	0.171	0.194	0.176
A2TF45|A2TF45_9BIVA	Heat shock protein 70 OS=Laternula elliptica	0	0.158	0.298	0.378
K1QLZ1|K1QLZ1_CRAGI	Actin-related protein 3 OS=Crassostrea gigas	0	0.155	0.185	0.220
K1RAJ1|K1RAJ1_CRAGI	T-complex protein 1 subunit alpha *OS=Crassostrea* gigas	0	0.151	0.462	0.048
Down-regulated					
K1QB83|K1QB83_CRAGI	Serine/threonine-protein phosphatase OS=*Crassostrea gigas*	0	-0.667	-0.677	-0.192
K1S4Q2|K1S4Q2_CRAGI	T-complex protein 1 subunit delta (Fragment) OS=*Crassostrea gigas*	0	-0.435	-0.433	-0.191
K1PWR0|K1PWR0_CRAGI	Protein SET OS=*Crassostrea gigas*	0	-0.401	-0.081	-0.022
D7EZG9|D7EZG9_CRAGI	Adenylyl cyclase-associated protein OS=*Crassostrea gigas*	0	-0.347	-0.221	-0.371
sp|P02595|CALM_PATSP	Calmodulin OS=*Patinopecten sp.*	0	-0.304	-0.012	-0.028
K1QX02|K1QX02_CRAGI	Tenascin-X OS=*Crassostrea gigas*	0	-0.303	-0.585	-0.039
K1PJ46|K1PJ46_CRAGI	Pyruvate kinase OS=*Crassostrea gigas*	0	-0.279	-0.086	-0.302
B3TK64|B3TK64_HALDV	ADP_ATP carrier protein (Fragment) OS=*Haliotis diversicolor*	0	-0.242	-0.158	-0.256
K1QLP5|K1QLP5_CRAGI	Coatomer subunit delta OS=*Crassostrea gigas*	0	-0.189	-0.317	-0.123
K1QC78|K1QC78_CRAGI	Ras-related protein Rab-14 OS=*Crassostrea gigas*	0	-0.167	-0.585	-0.529

2.3.6. Validation of iTRAQ results by western blot analysis

A complementary molecular technique was employed to validate the expression profile of haemocyte proteins that showed statistically significant changes when *H. midae* was fed a probiotic-supplemented diet. Only three of the seven significantly regulated proteins could be validated by western blot since compatible, commercially polyclonal antibodies were only available for COP9 signalosome subunit 4, Ras-related protein 1A and V-type proton ATPase subunit B.

Although a single band was observed on the western blot probed with anti-COP9 S4, the protein size was 37 kDa instead of the predicted 46 kDa (Figure 2.8a). Further analysis performed using 2D-SDS PAGE for spot extraction (Figure 2.8b) and identification by LC-MS/MS successfully confirmed that the antibody specifically detected COP9 S4 (Appendix C.3). The expression of COP9 S4 increased over the course of the experiment by 1.3 fold in comparison to the control at 0 hours (Figure 2.8c). Statistical analysis performed using one-way ANOVA showed that there was a significant difference in the expression of this protein at 12 and 18 hours ($F=14{,}67$, df 3, $p < 0.05$).

The Ras-related protein Rab-1A western blot showed two bands: one band at 30 kDa, which is an unexpected band, and another band at 23 kDa, which is similar to the predicted size of the target protein (Figure 2.9a). The expression of the first protein increased over the course of the experiment. The expression of the second protein decreased at all sampling time-points, being down-regulated by approximately 5 – 10 fold at 6, 12 and 18 hours in comparison to the control (Figure 2.9b). Statistical analysis performed using one-way ANOVA revealed that the change was significantly different ($F=165$, df 3, $p < 0.05$).

The western blot probed with anti-V-type proton ATPase subunit B exhibited multiple bands, including a band at the predicted protein size of 58 kDa (Figure 2.10a). Expression of this protein was higher than the control over the duration of the experiment (Figure 2.10b). However, one-way ANOVA showed that there was no statistically significant difference between the expression of this protein at the experimental and control sampling time-points ($F=0.81$, df 3, $p > 0.05$).

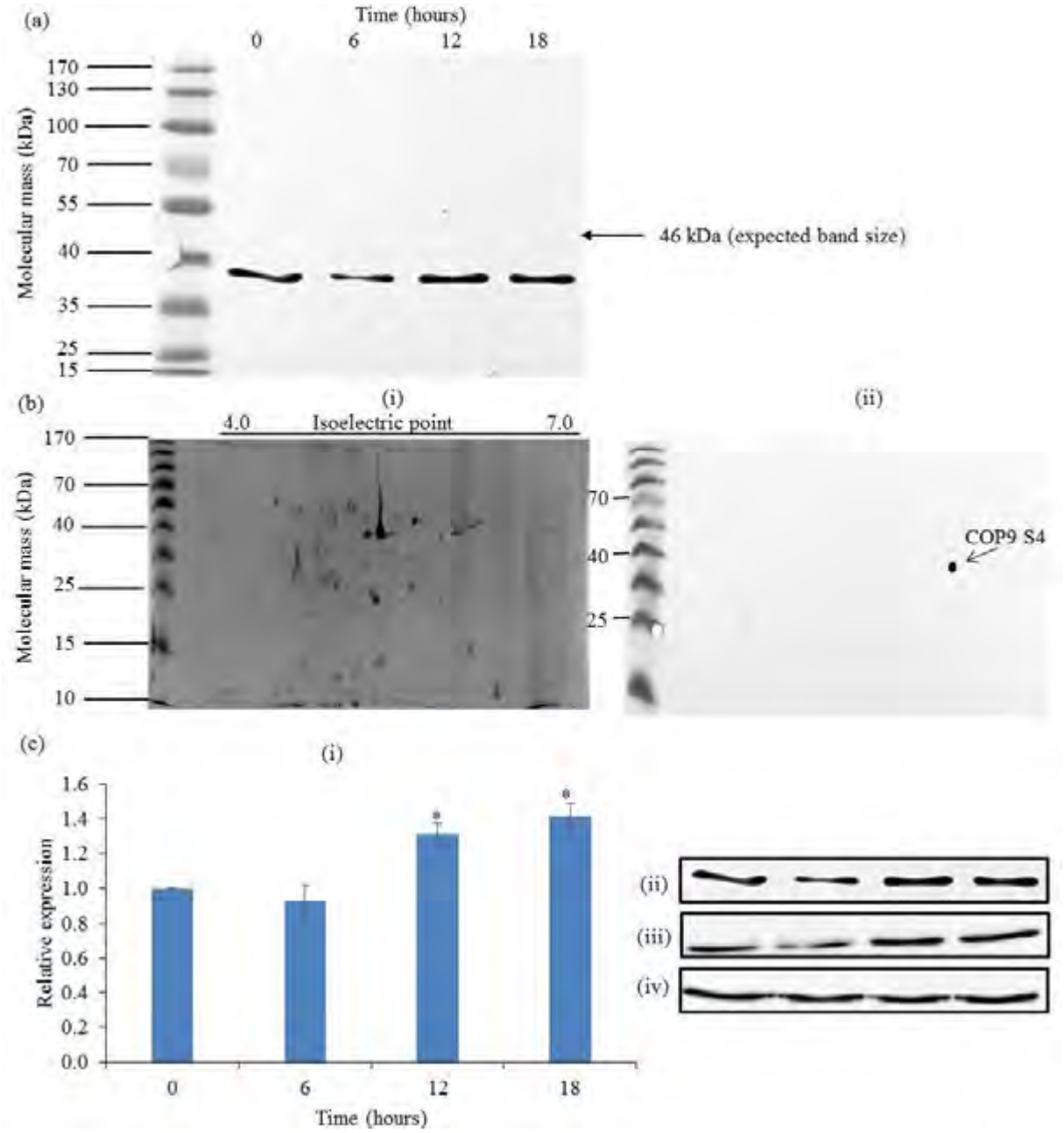


Figure 2.8. Validation of COP9 S4 expression in haemocytes from *H. midae* fed with a probiotic-supplemented diet. (a) Western blot signal detecting COP9 S4 at 37 kDa. (b.i) Coomassie-stained 2D SDS-PAGE of the haemocyte protein prior to spot picking and identification by tandem mass spectrometry, and (b.ii) position of the protein spot following western blot analysis. (c.i) The bar graph shows the mean (n = 3) ± SEM of relative expression of the protein volume intensity relative to the control (0 hours). An asterisk (*) indicates a statistically significant difference ($p < 0.05$) at this sampling time-point in comparison to the control, and (c.ii – iv) inlaid images are the chemiluminescent western blot signals obtained from different biological replicates.

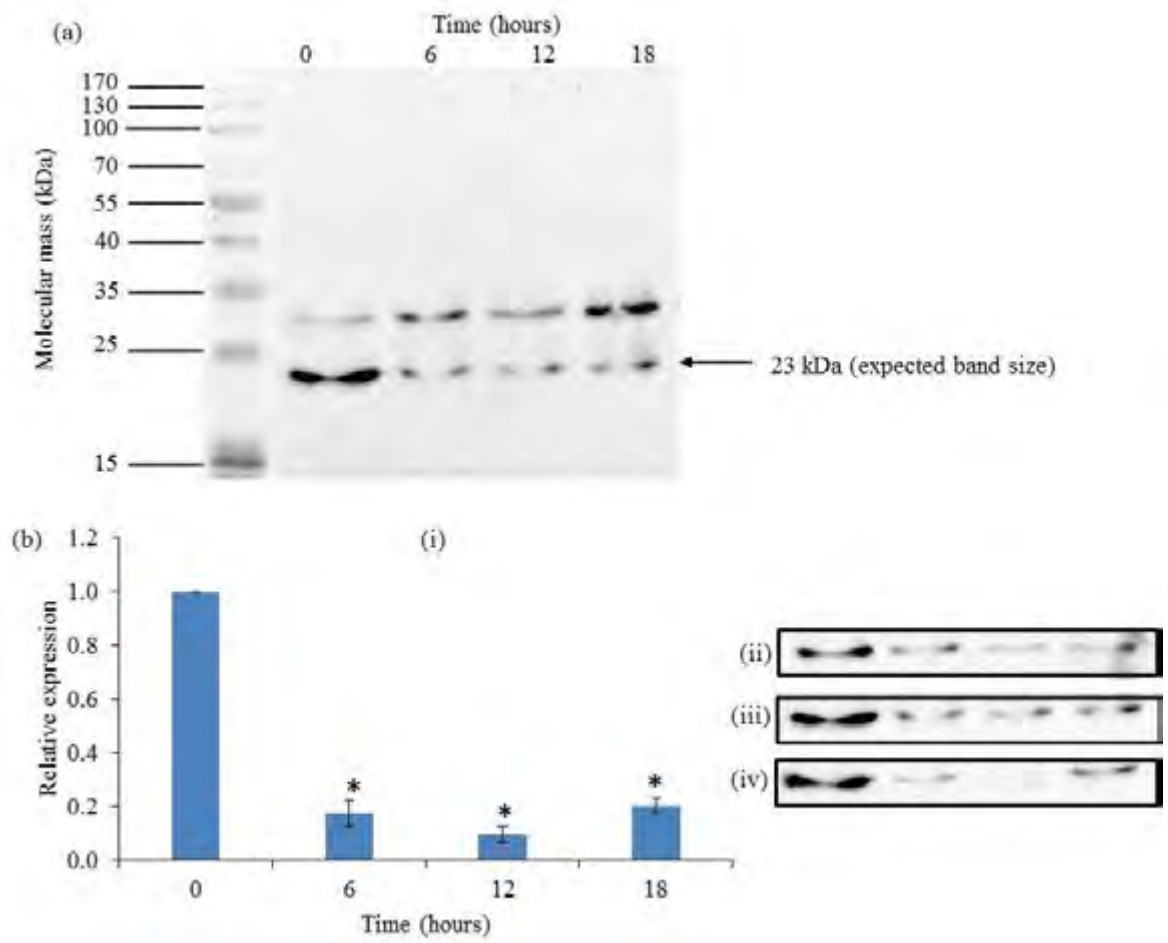


Figure 2.9. Validation of Rab 1A expression in haemocytes from *H. midae* fed with a probiotic-supplemented diet. (a) Western blot signal detecting Rab 1A at 23 kDa. (b.i) The bar graph shows the mean (n = 3) ± SEM of relative expression of the protein volume intensity relative to the control (0 hours); an asterisk (*) indicates a statistically significant difference ($p < 0.05$) at this sampling time-point in comparison to the control. (bii – iv) Inlaid images are the chemiluminescent western blot signals obtained from the three biological replicates.

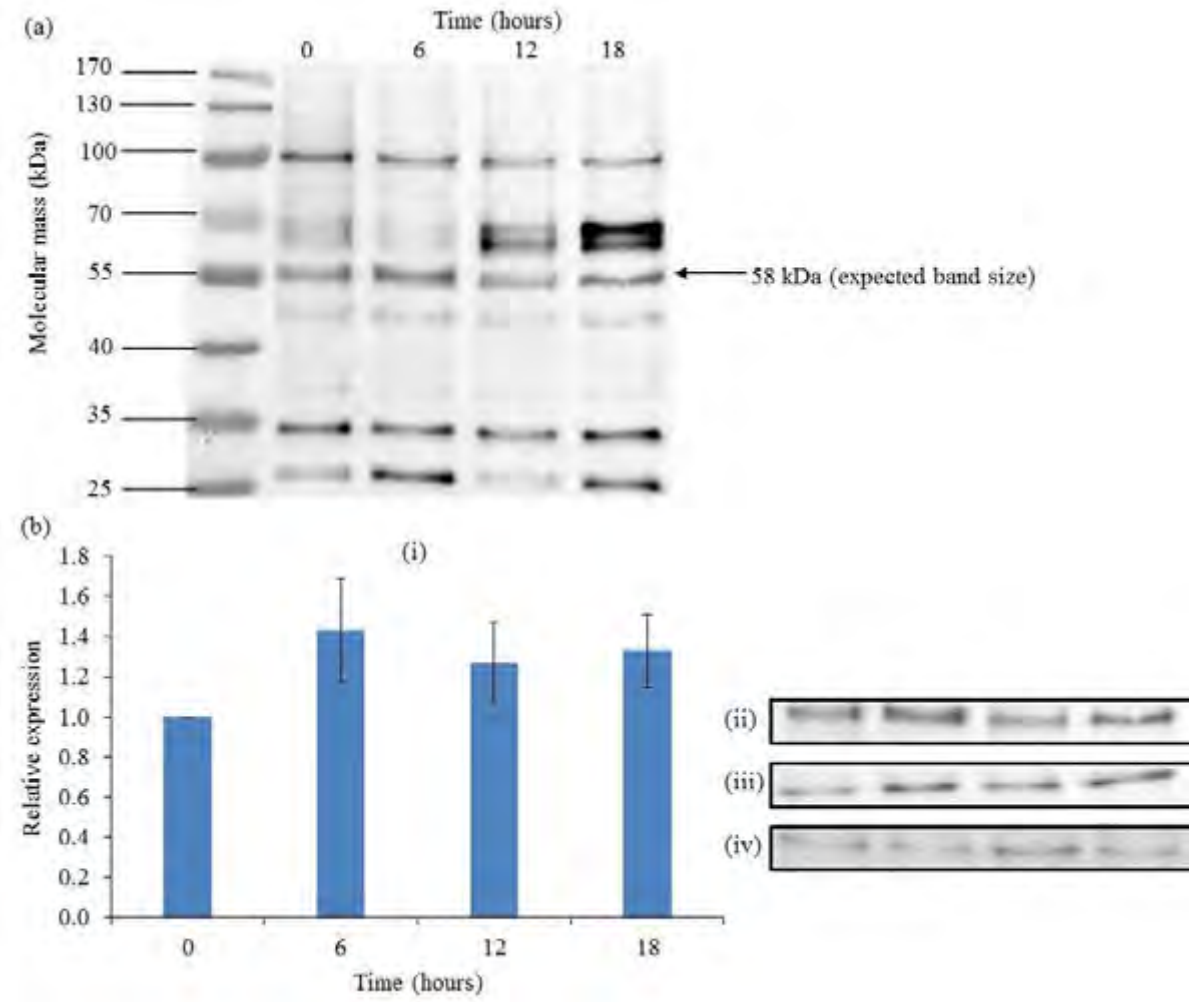


Figure 2.10. V-ATPase S4 expression in haemocytes from *H. midae* fed with a probiotic-supplemented diet. (a) Western blot signal detecting at 58 kDa. (b.i) The bar graph shows the mean (n = 3) ± SEM of relative expression of the protein volume intensity relative to the control (0 hours). Statistical analysis showed that there was no evidence of a significant difference ($p > 0.05$) between the sampling time-point in comparison to the control.(b.ii – iv) Inlaid images are the chemiluminescent western blot signals obtained from different biological replicates.

2.4 DISCUSSION

The tendency to use probiotics in aquaculture is growing due in part to the demand for environmentally friendly production, which prohibits the application of antibiotics in order to prevent the development of antibiotic-resistant strains (Gatesoupe, 1999). Previous studies on the use of probiotic-supplements fed to cultured *H. midae* have shown that these enhance the host's growth and immune system (Macey and Coyne, 2005, 2006; Ten Doeschate and Coyne, 2008). Since proteins mediate the function of almost the entire biological system (Cristea et al., 2004; Paz et al., 2011; Smith and Figeys, 2006; Walther and Mann, 2010), we hypothesised that proteins would likely be involved in immune activity when abalone are immune stimulated by a probiotic-supplemented diet. In this study, a proteomics approach was employed to investigate the effect of a probiotic-supplemented diet on the *H. midae* immune system in an attempt to obtain new insights into their effect at the molecular level.

Although there are several methods for analysing protein expression (Karr, 2008; Kurien and Scofield, 2009; Smith and Figeys, 2006; Taylor and Wrana, 2012), recent studies in molecular biology have used mass spectrometry-based proteomic methods (Fernández-Boo et al., 2014; Wang et al., 2015a, 2013a; Yang et al., 2015). These methods have been valuable for analysing the effect of different stimuli at a proteome level (Beck et al., 2011). Mass spectrometry-based methods are useful for prompt identification of proteins that are differentially expressed between experimental and control groups, and may aid the understanding of the biochemical pathways involving the target proteins (Fuller and Morris, 2012; Sveinsdóttir et al., 2009). In light of this, we used the iTRAQ label mass spectrometry-based method to analyse abalone haemocyte proteins. This investigation allowed the identification of several proteins, some of which were differentially expressed. Western blot analysis was subsequently employed to validate the expression of some of these key proteins.

Initially, we assessed the number of circulating haemocytes and their phagocytic activity, as these parameters are considered to be acceptable indicators of an immune response in abalone (Chen and Tan, 2005; Dang et al., 2011; Hooper et al., 2011). The number of circulating haemocytes and their phagocytic activity was significantly increased in *H. midae* fed the probiotic-supplemented diet. This result was consistent with other studies which showed that a probiotic-supplemented diet stimulates the abalone immune system (Dang et al., 2011; Hadi

et al., 2014; Jiang et al., 2013; Macey and Coyne, 2005; Ten Doeschate and Coyne, 2008). Consequently, we sought to ascertain whether haemocyte protein expression was affected when the abalone were fed a probiotic-supplemented diet.

2.4.1. Description of the *H. midae* haemocyte proteome

In this study, we identified a total of 527 proteins from two independent experiments using iTRAQ labelling and mass spectrometry. The 128 proteins found to be common to both experiments were considered sufficient for obtaining an overview of the abalone haemocyte proteome and its response to a probiotic-supplemented diet. iTRAQ LC-MS/MS has previously been used to analyse protein samples from several aquacultured species, resulting in the identification of a similar number of protein identifications to this study (Lü et al., 2014; Marancik et al., 2013; Meng et al., 2014; Valdenegro-Vega et al., 2014). Special consideration was given to proteins that were over-represented within the 128 protein dataset, such as ribosomal protein, hemocyanin, calmodulin, Ras-related protein, and t-complex subunit 1.

Ribosomal protein is the principal component of ribosomes, a complex organelle responsible for protein synthesis from transcribed mRNA. In addition, this protein carries extra-ribosomal functions related to a variety of cellular mechanims (Doudna and Rath, 2002; Lindström, 2009; Makkapan et al., 2014). For instance, ribosomal S3, which was also identified in this study, was reported to be the link between DNA repair pathways and apoptosis (Jang et al., 2004). Although ribosomal proteins were not differentially expressed in *H. midae* fed with a probiotic-diet, these proteins have been reported to be involved in the immune response of the zebrafish *Danio rerio* (Lü et al., 2014), the Chinese mitten crab *Eriocheir sinensis* (Meng et al., 2014), and the abalone *H. diversicolor* (Wang et al., 2008).

Another protein that was noted to be over-represented in this study was hemocyanin; however, this is not surprising because hemocyanin is a copper-containing protein present in the haemolymph of arthropods and molluscs, representing up to 95% of the total proteins (Sellos et al., 1997; Xu et al., 2015). While hemocyanin is mainly known as a respiratory protein, it is involved in several physiological processes such as protein storage, osmoregulation, molt cycle, and exoskeleton formation (Adachi et al., 2005; Engel et al., 2001). Furthermore,

hemocyanin has been reported to play a role in non-specific innate immune defense, and its immunological functions include phenoxidase activity (Decker and Rimke, 1998; Lee et al., 2004), antimicrobial properties (Lee et al., 2003) and antiviral properties (Lei et al., 2008). Using cell culture, for instance, it was found that haemocyanin isolated from the shrimp *Penaeus monodon* possesses antiviral properties (Zhang et al., 2004). Additionally, hemocyanin was amongst the haemocyte proteins that were found to be up-regulated and directly involved in the host defense response of the same species of shrimp (Somboonwiwat et al., 2010). Although we identified several hemocyanins, none showed differential expression in haemocytes, suggesting that probiotic-supplementation of feed may not influence haemocyanin expression in abalone.

Calmodulin was also abundant within the identified haemocyte protein dataset. It is a versatile calcium-binding protein that can activate several enzymes as well as ion pumps and channels (Karabinos and Bhattacharya, 2000; Liu et al., 2015). Calmodulin is reported to function as an important signalling molecule in host-pathogen interactions when retrovirus was investigated in mammalian cells (Chattopadhyay et al., 2013). In vertebrates, calmodulin is shown to regulate a range of other proteins involved in immune functions, such as programmed cell death, autophagy, inflammation and immune response (Berchtold and Villalobo, 2014). Li et al. (2014) reported that calmodulin was up-regulated when the Chinese mitten crab (*Eriocheir sinensis*) was stimulated with pathogenic bacteria and suggested that the change in the expression of this protein was related to stress and immune response. In this study, calmodulin 2 was up-regulated and showed one of the highest fold changes at all sampling time-points. However, the differences in fold changes across the sampling time-points were not statistically significant. These results may indicate that the expression of calmodulin 2 in haemocytes of *H. midae* was possibly regulated by the use of a probiotic-supplemented diet. However, this hypothesis needs to be tested in further studies.

Ras-related protein Rab and t-complex subunit 1 were also over-represented in this study; these proteins will be discussed in section 2.4.2. Some family members of these proteins were differentially expressed when *H. midae* was fed the diet supplemented with probiotics, and statistical analysis showed that the differences were statistically significant.

2.4.2. Identification of proteins with significant differential expression in haemocytes from *H. midae* fed with a probiotic-supplemented diet

The discovery and validation of proteins that respond to the use of a probiotic-diet provide important steps to increase our understanding of the physiological processes that occur during a host's response to this stimulus. In this study, the expression of annexin, COP9 signalosome complex subunit 4, phosphorylase, T-complex protein 1 subunit gamma, V-type proton ATPase subunit B, Rab 1 and Ras-related protein Rab-1A showed statistically significant changes in haemocytes sampled from abalone fed a probiotic-supplemented diet. The relevance of these proteins in this regard is discussed below.

Annexin was up-regulated at all sampling time-points in relation to the control, which suggests that the use of a probiotic-supplemented diet likely resulted in an alteration in the expression of annexin in the haemocytes of *H. midae*. Annexin is a member of calcium and phospholipid binding proteins (Enrich et al., 2011; Lizarbe et al., 2013). It is implicated in several processes, including the interaction between membranes and actin-based cytoskeleton (Enrich et al., 2011). It is reported to have anti-inflammatory properties against bacteria during the host's immune defense (Lim and Pervaiz, 2007; Perretti and D'Acquisto, 2009). Similar findings were reported by Yeh and Klesius (2010), who found that six members of the annexin family were up-regulated in the gills of the channel catfish *Ictalurus punctatus* infected with the pathogenic bacteria *Edwardsiella ictaluri*, concluding that annexin has an important role to play during the immune response of channel catfish.

In another study, annexin a13 was found to induce a protective immune response against infection of the rockfish *Sebastes schlegeli* by the ectoparasite *Microcotyle sebastis* (Choi et al., 2009). Annexin a3 was also one of the up-regulated proteins during the immune response against the pathogenic bacterium *Aeromonas hydrophila* infecting the gill of the zebrafish *Danio rerio* (Lü et al., 2014). Furthermore, annexin a1 was found to be up-regulated in gill mucus from Atlantic salmon, *Salmo solar*, infected with ameboic gill disease (Valdenegro-Vega et al., 2014). These results collectively indicate that annexin may be a suitable candidate for further investigation of molecular pathways involving the effect of probiotics on the abalone immune system.

COP9 signalosome complex subunit 4 was found to be up-regulated at all sampling time-points in comparison to the control. This protein is a member of a multiprotein complex (COP9 signalosome or CSN) that consists of eight subunits (CSN1-CSN8) (Echalier et al., 2013; Lee et al., 2011; Serino et al., 1999; Wei and Deng, 2003; Wei et al., 1998, 2008). Each of the subunits was identified as a binding surface of several regulatory proteins, but the specificity and biological relevance of these interactions still needs to be investigated (Hannß and Dubiel, 2011).

The COP9 protein has been reported to play an important role in the control of the expression of other proteins (Echalier et al., 2013), as well as being involved in several cellular and developmental processes (Stotland et al., 2012; Wei and Deng, 2003), such as DNA repair, cellular homeostasis, cell cycle regulation, MAPK signalling, hormone signalling, axonal guidance, and embryogenesis (Fang et al., 2008; Oron et al., 2007). Therefore, the hypothesis that COP9 S4 might be associated with an immune stimulus that was probably mediated by the probiotic-supplemented diet lacks supporting data.

Although several investigations have been performed on COP9 proteins, most of them are related to plant physiology (Busch et al., 2003; Hind et al., 2011; Karniol and Chamovitz, 2000; Schwechheimer and Deng, 2001; Serino et al., 1999); only a few are specific to immune systems (Fukumoto et al., 2006; Pearce et al., 2009), with scarce reports relating COP9 signalosome subunit 4 to immune function. Nevertheless, to the best of our knowledge, the current study is the first to report the differential expression of COP9 signalosome subunit 4 in abalone fed with a diet enriched with probiotics. A more insightful investigation would support these findings and increase our understanding of the role of COP9 signalosome subunit 4 in abalone.

V-type proton ATPase subunit B, or V^+ATPase B, showed rapid up-regulation at 12 hours, after which its expression at the subsequent sampling time-points returned to a level similar to that of the control. V^+ATPase B protein belongs to the vacuolar ATPase family, an ATP-driven proton pump. V^+ATPases are the main proteins responsible for acidification of intracellular compartments of secretory vesicles, endosomes, lysosomes, Golgi vesicles and plasma membranes (Cipriano et al., 2008; Forgac, 2007). Additionally, Beyenbach and Wieczorek (2006) stated that V^+ATPase is required for normal functioning of the Golgi complex,

endoplasmic reticulum, and vacuoles. Therefore, the fast response of this abalone haemocyte protein may reflect its role in regulating cellular homeostasis. Indeed, pH homeostasis in cellular organelles is crucial for several biological functions (Beyenbach and Wieczorek, 2006; Forgac, 2007; Hinton et al., 2009).

V^+ATPases also play a role in processes such as protein sorting, endocytic trafficking, protein degradation, signalling, fusion of secretory vesicles with plasma membranes, and in endo and exocytosis (Forgac, 2007; Pérez-Sayáns et al., 2012). Thus, it is highly relevant that V^+ATPase B showed differential expression in haemocytes, which may be influenced by immune stimulation mediated by a probiotic-supplemented diet. These results are supported by Coyne (2011) who stated that haemocytes require a regular supply of energy to maintain cellular functions, especially during immune-related processes.

Similar to the results of this study, several other studies reported the differential expression of V^+ATPase upon host immune stimulation, and some examples are given here: in a study by Candas et al. (2003) investigating the effect of Cry toxin – which is an insecticide produced by a soil bacterium – in larvae of the insect, *Plodia interpunctella*, the authors found that the level of V^+ATPase was high in larvae that were resistant to the toxin. This result was inferred to be associated with an elevated cellular energy that may be necessary to combat toxic stress and maintain homeostasis. Yan et al. (2009) reported that there is a functional connection between V^+ATPase and endosomal trafficking in *Drosophila*, suggesting that the acidification of intracellular compartments in cells is crucial for notch signalling. Singh et al. (2014) observed an increased expression of V^+ATPase B in the neutrophils of adult zebrafish, *D. rerio*, upon chemically induced inflammation. It can therefore be concluded that the use of a probiotic-supplemented diet likely influenced the differential expression of V^+ATPase B in *H. midae* haemocytes as the abalone may be immune stimulated. Thus, this protein may be a suitable candidate for consideration in further studies in order to further our understanding of the abalone immune response.

Phosphorylase catalyses the addition of a phosphate group from an inorganic phosphate to an acceptor substrate. This process has a central role in the regulation of several cellular processes (Bollen and Stalmans, 1992; Hofer, 1996; Pereira et al., 2011; Rauch et al., 2011; Tafesse and Eguzozie, 2010), such as cell–cell and cell–substrate interactions, cell cycle progression,

differentiation, channel and transporter activities, and gene transcription, including during immune response (Almo et al., 2007). For instance, when a cell receives stimulation, messengers are generated through the signal network to perform a physiological response. This response is conducted through the modification of proteins' phosphorylation status, either phosphorylation or dephosphorylation (Sopory and Munshi, 1998). The reversible phosphorylation route modulates protein functions in various ways, such as through the alteration of protein localization and protein stability. Therefore, it is not surprising that protein phosphorylase was found to be differentially expressed in this study. However, since only a limited number of studies have shown a link between phosphorylase and immune stimulation in invertebrates, further investigation must be performed to aid the understanding of phosphorylase activity in *H. midae* haemocytes.

T-complex protein 1 subunit gamma, or TCP 1 γ, was also up-regulated in haemocytes from abalone fed a probiotic-supplemented diet, and the changes in expression of this protein were statistically significant. This protein belongs to the TCP 1 (CCT or TriC) protein family, which consist of eight subunits, namely α, β, γ, δ, ε, ζ, η and θ (Souès et al., 2003). TCP protein is a chaperone that facilitates the folding of many structural proteins; it may also be involved in the regulation of cellular processes such as signalling (Llorca et al., 2000; Yam et al., 2008).

There is little evidence suggesting a link between TCP and the invertebrate immune system (Cyrne et al., 1996; Leroux and Candido, 1997); however, there are some reports from plant studies that indicate the involvement of this protein in defense response (Kim et al., 2014; Koyama et al., 2007; Viola et al., 2012). For instance, TCP 1 was suggested to play a crucial role in developmental regulation and defense response, enabling *Arabidopsis thaliana* to deal with abiotic stress (Li, 2015). Despite the aforementioned, Brackley and Grantham (2010) reported that TCP 1 is required for the activity of the cytoskeletal proteins actin and tubulin in order to achieve their native state. Llorca et al. (2000) reported that ATPase and GTPase binding sites also play a crucial role in actin and tubulin correct folding. The link between actin and these three proteins, all of which (TCP, ATPase, and GTPase) were differentially expressed in haemocytes sampled from abalone fed a probiotic-supplemented diet, may increase our understanding of the molecular pathways that are altered during immune stimulation by a probiotic-supplemented diet. However, it is necessary to conduct further studies that focus on the activity of these proteins in immune-stimulated abalone.

Ras-related protein Rab-1A and Rab 1 were the only proteins that showed down-regulation with statistical significance. These two proteins belong to the GTPase family that is well-known for its function in translocating proteins through membranes or operate in protein biosynthesis, signal transduction, and transport of vesicles within the cell (Caetano-Anollés et al., 2012). In addition, GTPases have been identified in a range of cellular processes in all eukaryotic organisms, including biosynthesis, vesicle transporting, cell proliferation, cytoskeleton organization, and response to stimulus (Vernoud et al., 2003; Wang et al., 2013b). Due to the importance of these proteins in immune activity, the expression of a member of this family was investigated in Chapter 4.

2.4.3. Validation of iTRAQ results using an orthogonal approach

After completion of mass spectrometry-based proteomics analysis, it is common practice to validate the expression of some of the target proteins through alternative methods to ensure the accuracy of the initial results (Johnson and Gaskell, 2006; Rodriguez-Suárez and Whetton, 2013; Slattery et al., 2012; Sun et al., 2012). Therefore, in this study, western blot analysis was used to validate the expression of three proteins, namely COP9 signalosome subunit 4, Ras-related protein Rab 1A, and V^+ATPase subunit B (V^+ATPase B), that were shown to be differentially expressed in haemocytes from *H. midae* fed a probiotic-supplemented diet.

Although COP9 signalosome subunit 4 was detected with a molecular weight of 37 kDa, instead of the predicted 46 kDa, two-dimensional SDS-PAGE followed by spot sequencing using mass spectrometry confirmed that the antibody was indeed binding to the correct antigen. According to Ghosh et al. (2014), the reasons for a protein having an unexpected molecular mass include post-translational modification, post-translation cleavage, and relative charge. In the present study, the position of the protein on the gel remained the same over the time course of the experiment. On this basis, we speculate that a change in the relative charge of the protein may explain the variation in the observed size of COP9 signalosome subunit 4.

Nevertheless, the western blot results confirmed the results from iTRAQ analysis, which showed that the expression of COP9 signalosome subunit 4 was up-regulated when *H. midae* were fed the probiotic-supplemented diet. To the best of our knowledge, this is the first time

that COP9 signalosome subunit 4 has been reported in abalone haemocytes and shown to respond to probiotic-supplemented feed.

Ras-related protein Rab 1A was the second protein to be validated by western blot. The antibody used to detect this protein reacted with a protein band at 23 kDa, which is the expected size for Ras-related protein Rab 1A. Similarly, western blot analysis showed that this protein is significantly down-regulated in agreement with the findings obtained using iTRAQ analysis. Thus, it is suggested that Ras-related protein Rab 1A warrants further investigation with regard to its effect on the haemocytes of *H. midae* fed a probiotic-supplemented diet.

Apart from the predicted band, an unexpected 30 kDa band was also detected on the western blot performed to identify Ras-related protein Rab-1A. Contrary to Rab-1A (23 kDa), the 30 kDa band seemed to increase its expression over the experiment. This result may indicate that Rab-1A undergoes a PTM, such as glycosylation. According to Chauhan et al. (2013), glycoproteins have been implicated in several cellular processes such as immunogenicity, antigenicity and pathogenicity. Most importantly, glycoproteins have been implicated in cell surface properties that are crucial for host-pathogen interactions and pathogen resistance to host complement killing. However, this hypothesis needs to be confirmed through appropriate experiments.

The expression of V^+ATPase B was the last protein to be validated. Although a band was detected at the predicted size of 58 kDa, other bands were also detected at different molecular masses, such as at 28, 34 and 50 kDa. This may be an indication that the antibody was detecting other V^+ATPase subunits that have a range of molecular weights (Arata et al., 2002; Beyenbach and Wieczorek, 2006; Jefferies et al., 2008; Pérez-Sayáns et al., 2012). A study by Kodippili et al. (2014) used western blot to detect dystrophin protein which is 427 kDa. The nitrocellulose membrane showed various smaller bands, suggesting that the multiple cross-reactive bands may either be protolithic fragments of the full-length protein, isoforms, or other proteins. Ghosh et al. (2014) reported that antibodies that recognize nonspecific proteins can still be utilized for western blot if the nonspecific interactions occur at a molecular mass that is sufficiently different from the target protein, to allow accurate quantification of the target protein.

We assessed the expression of the 58 kDa band and found that it was up-regulated at all sampling time-points, though the increase was not statistically significant. These findings differed slightly from those obtained using iTRAQ analysis, in which the expression of V^+ATPase B was up-regulated at 12 hours and then returned to levels similar to those recorded for the control at the 18 and 36 hour time-points. Since the antibody detected additional bands besides the expected 58 kDa protein, and expression of the 58 kDa protein was not consistent with the data obtained by iTRAQ, the function of this protein was not investigated further in this study. An alternative approach is needed to validate the expression of V^+ATPase B shown by iTRAQ analysis.

In summary, iTRAQ labelling proved to be an effective tool for investigating the effect of a probiotic-supplemented diet on the *H. midae* haemocyte proteome. We identified and quantified a total of 128 proteins common to both experiments conducted in this study. Although the expression of several proteins were found to change over the duration of the experiment, only seven showed statistically significant differential expression. These were annexin, COP9 signalosome complex subunit 4, phosphorylase, T-complex protein 1 subunit gamma, V-type proton ATPase subunit B, Rab 1, and Ras-related protein Rab-1A. Further analysis using western blots allowed us to validate the expression of three of these proteins: COP9 signalosome complex subunit 4, V-type proton ATPase subunit B, and Ras-related protein Rab 1A. Of these, Ras-related protein 1A proved to be the most suitable candidate warranting further research aimed at elucidating the molecular changes that occur in abalone haemocytes in response to immune stimulation. The importance of this protein with regard to immune system activity is supported by studies conducted in other cultured species such as the crab *E. sinensis* (Wang et al., 2013b); the shrimps *L. vannamei* and *P. japonicus* (Wang et al., 2015a; Wu and Zhang, 2007), and the fish *S. ocellatus* (Hu et al., 2011b). Consequently, expression of Ras-related protein Rab 1 in *H. midae* haemocytes was chosen for further investigation (Chapter 4).

CHAPTER 3 – FUNCTIONAL ANALYSIS OF PROTEINS IDENTIFIED IN HAEMOCYTES FROM *HALIOTIS MIDAE* FED A PROBIOTIC-SUPPLEMENTED DIET

3.1. INTRODUCTION

High-throughput proteomics generates data that can be interrogated using databases containing the characteristics of each of the identified molecules, such as functional and biological proprieties, and their interactions with other proteins (Szklarczyk et al., 2014). However, in order to gain insight into the biological context and the interpretation of results of this proteomic study, appropriate bioinformatics analysis is required (Oveland et al., 2015).

Several bioinformatics programs have been designed to investigate protein functionality. The most recently used programs are described by Malik et al. (2010) and Lehne and Schlitt (2009); some of these programs include online resources that assist in providing known and predicted information about a variety of proteins (Jensen et al., 2009; Szklarczyk et al., 2014). Gene ontology (GO) annotation is, generally, one of the first steps in bioinformatics analysis of proteomics data. This method of analysis can determine whether proteins show enrichment for a particular function and serves as a meta-tool to search for integrated information regarding molecular pathways, domains and other important biological attributes (Malik et al., 2010). Bioinformatics tools that are available for the identification of molecular pathways include Reactome, KEGG (Kyoto Encyclopedia of Genes and Genomes), PANTHER (Protein Analysis Through Evolutionary Relationships), and STKE (Signal Transduction Knowledge Environment); while those that can be used to identify protein interaction networks include MINT (Molecular interactions), BIND (Biomolecular Interaction Network Database), DIP (Database of Interacting Proteins), and STRING (Search Tool for the Retrieval of Interacting Genes/Proteins) (Malik et al., 2010). Other open source tools include Cytoscape, which is a valuable and popular open-source program that allows visualization, analysis and interpretation of protein or gene networks (Enright et al., 2005; Su et al., 2014). IPA (Ingenuity Pathways Analysis) is another noteworthy program that is applicable for analyses of, example, molecular pathways, protein-protein interaction network(s) and the visualization of result outputs (Orchard, 2012).

The aim of this chapter was to use bioinformatics software to provide biological meaning to the proteins identified in Chapter 2, wherein iTRAQ, a quantitative mass spectrometry-based proteomic analysis, was employed to explore the proteome of haemocytes from *H. midae* fed a probiotic-supplemented diet. The approach focused on the use of protein interaction network

analysis to explore whether the proteins identified as differentially expressed are associated with host immune functions.

To the best of our knowledge, this report is the first to address the functional analysis of the haemocyte proteome from *H. midae* stimulated by the dietary inclusion of probiotics. Understanding the biological function, the interaction network and the molecular pathways involving the proteins identified in *H. midae*, especially those proteins that were differentially expressed putatively as a result of a probiotic-supplemented diet, will bring new insight into the effect of probiotics on the host immune system. Additionally, the results of this study may help to demonstrate the importance of a computerized-based approach in omics research, particularly when studying non-model organisms such as abalone.

3.2. MATERIALS AND METHODS

In this chapter, a total of 128 proteins identified and quantified in Chapter 2 were analysed using bioinformatics. A list of protein descriptions used in this chapter, including accession numbers and expression values from four different time-points, can be found in table 2.3.

3.3.1. Immune functional analysis of protein identified in haemocytes from *H. midae* fed a probiotic-supplemented diet

In order to elucidate whether the proteins identified in haemocytes of *H. midae* were involved in immune activities, protein analysis was done using immune classification. Firstly, the list containing accession numbers of all proteins was retrieved into UniProt, www.uniprot.org, to obtain *fasta* files of the protein identities. The obtained *fasta* files were uploaded into Blast2GO 3.1.0.0 software for GO term assignment and annotation. Blast2GO is a tool that allows the functional annotation of protein data in order to reveal the role of protein in a biological system (Götz et al., 2008).

To identify immune-related biological processes associated with GO terms, the GO terms generated in Blast2GO were uploaded as *txt* files into CateGOrizer, a GO classification tool (Na et al., 2014). The classification was performed based on "accumulative all occurrences counts" through "Immune System Gene Classes". REViGO (Reduce and Visualize Gene Ontology) was employed to summarize and visualize immune GO terms in an interactive graph (Supek et al., 2011).

The interactive graph generated by REViGO was downloaded as a *xgmml* file for further analysis in Cytoscape 3.3.0 software (Shannon et al., 2003). Cytoscape is a software program that allows the exploration of functional interactions within the data and the presentation thereof in a flexible manner. Immune classes that were identified using Cytoscape were presented as an interactive network map.

In order to group the identified protein according to their expression pattern, hierarchical clustering analysis was performed. In addition, hierarchical clustering analysis facilitated

further identification of the predominant biological functions in each group. The list of proteins and their corresponding expression values were uploaded into Cluster 3.0 (de Hoon et al., 2004). Cluster 3.0 is a program that uses protein or gene expression values as input and, thereafter, scans the data set to identify any similarities in expression patterns. The program is able to generate a dendrogram to highlight protein or gene expression relationships between samples. As parameters, the data were ordinated based on Euclidean distance performed in complete linkage to provide a reasonable solution. The *cdt* file generated by Cluster 3.0 was uploaded into Java TreeView 1.1 software in order to view the results of the hierarchical clustering analysis as a heat-map and perform comparative analysis (Saldanha, 2004). Clusters were derived from the stem of the dendrogram; a correlation of at least 0.95 was used to identify sub-cluster groups of proteins with similar patterns in expression values. Each of these sub-clusters was further analysed to determine whether they were involved in any immune function, as previously described in this section. Thus, the sub-clusters that showed diverse immune classes were subjected to interaction network analysis using STRING (Szklarczyk et al., 2011), in order to establish relationships between the proteins and ascertain which other proteins interacted with the target proteins.

3.3.2. Analysis of protein interaction network and biological pathways involving proteins identified in haemocytes from *H. midae* fed a probiotic-supplemented diet

In order to investigate the relationship between proteins identified in haemocyte samples of *H. midae*, protein accession numbers were used to blast putative human protein orthologues in the UniProt database (Appendix C.5). This was performed as there is no information about protein interaction in *H. midae*.

Both proteins encompassed in clusters with immune-related functions (in up-regulated and down-regulated groups) and those differentially expressed were used to evaluate the possibility of association in terms of protein functionality. The below programs were used to explore protein interactions:

(1) STRING) was used to generate a protein-protein interaction network within each clusters' data. This software identifies known and predicted protein-protein interactions based on information from curated databases such as BioGRID, MINT, DIP and BIND (Malik et al., 2010). The search was set for multiple names using the *Homo sapiens* database.

(2) IPA was employed to generate protein-protein interactions among differentially expressed proteins. This program was used because, in addition to the analysis of protein interaction, it considers the expression values of the proteins, thus showing if there is up-regulation or down-regulation in protein expression. The program also performs canonical pathway analysis to identify other proteins that participate in closed biological networks in which the target proteins are involved. Thus, putative human orthologues of the six target proteins (statistically differentially expressed) and the associated expression values from four different time-points – 0 hours (control), 12 hours, 18 hours and 36 hours – were uploaded into IPA to map casual networks and find canonical pathways involving the target proteins. The parameters used for these analyses included: flexible format; Ingenuity and UniProt identifiers; and "not specified arrays" (for the upload data). The cut-off was set as default at 1.3; the threshold value was 0.05; and the scoring method was Fisher's Exact Test p-value.

3.4. RESULTS

3.4.1. Identification of the immune classes of proteins identified in haemocytes from *H. midae* that was fed a probiotic-supplemented diet

Based on CateGOrizer *Immune System Gene Classes*, 332 GO terms were mapped to 108 immune class ancestor terms. A total of 185 unique terms from the data set were found to belong to at least one of the 13 immune class GO terms identified, and 137 odd terms in this data set did not belong to any immune class GO term. GO terms related to metabolism were the most abundant, comprising more than 50% of the total count. This was followed by the terms associated with stress response and immune response (Figure 3.1).

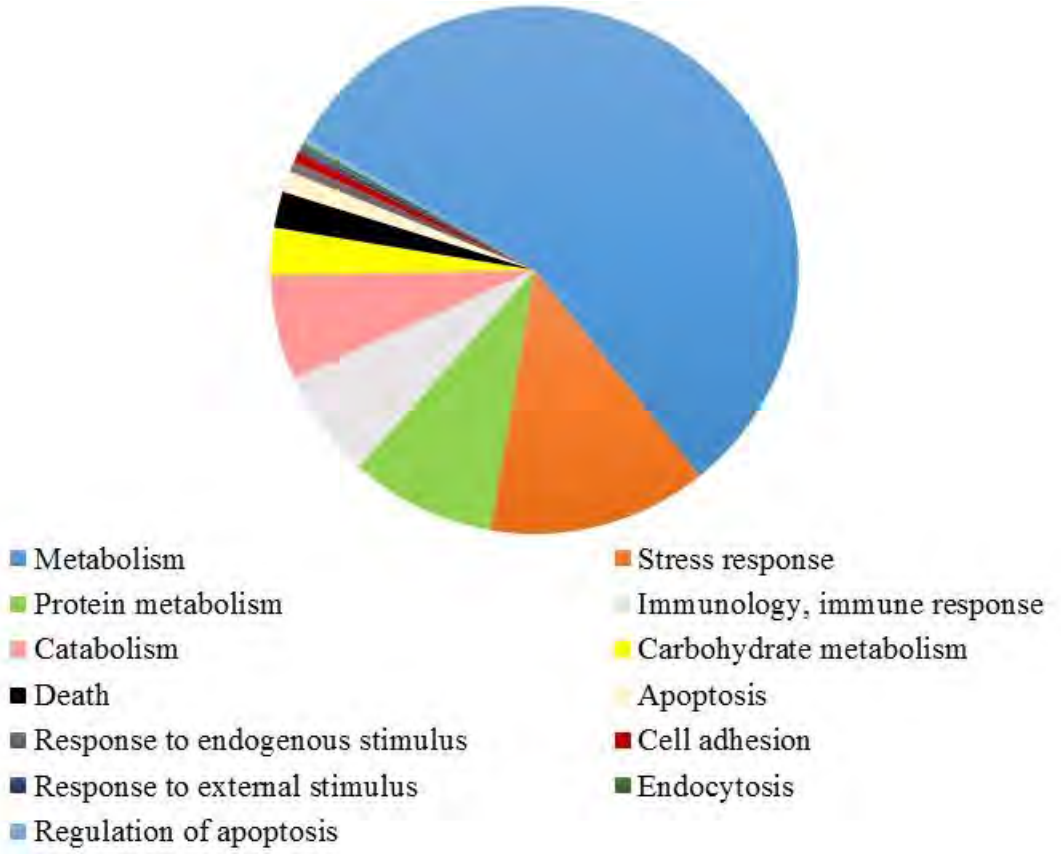


Figure 3.1. Representation of the overall biological functions of GO terms of proteins identified in haemocytes from *H. midae* fed a probiotic-supplemented diet. The pie-chart was generated using CateGOrizer and was based on immune classifications.

Based on the GO terms mentioned above, Cytoscape generated a functional interactive map in which nodes represented immune-related categories, and edges represented interactions. Several nodes were identified; for reasonable visual presentation the classes related to metabolism and catabolism were omitted, but can be found in Appendix C.6. Thus, two main groups were identified; the first group was composed of immune-related categories such as cellular response to stress, response to external stimulus, defense response, and immune response-regulating signalling pathways. The second group comprised of immune-related categories such as the regulation of biosynthesis, regulation of gene expression, regulation of protein modification process and translation. Other independent immune-related classes were identified in isolated nodes, such as endocytosis, apoptotic process, death and phosphorylation (Figure 3.2).

A heat-map dendrogram cluster analysis was used to classify the proteins according to their pattern of protein expression at 0 hours (or control), 12 hours, 18 hours and 36 hours (Figure 3.3). Two main clusters of proteins were identified: one group containing proteins down-regulated at most time-points (Figure 3.3a) and a second group containing proteins up-regulated at most time-points (Figure 3.3b). A correlation index of 0.95 was used to find sub-clusters of proteins displaying a high similarity within the main clusters (“a” and “b”). Consequently, a total of 12 sub-clusters were identified; these were numbered 1 – 12 (Figure 3.3). Sub-clusters 1 – 3 are mostly composed of down-regulated proteins, which are indicated in green. Sub-clusters 10 – 11 are mostly composed of up-regulated proteins, which are indicated in red. The remaining sub-clusters were composed of proteins whose expression values showed slight variation during the experiment.

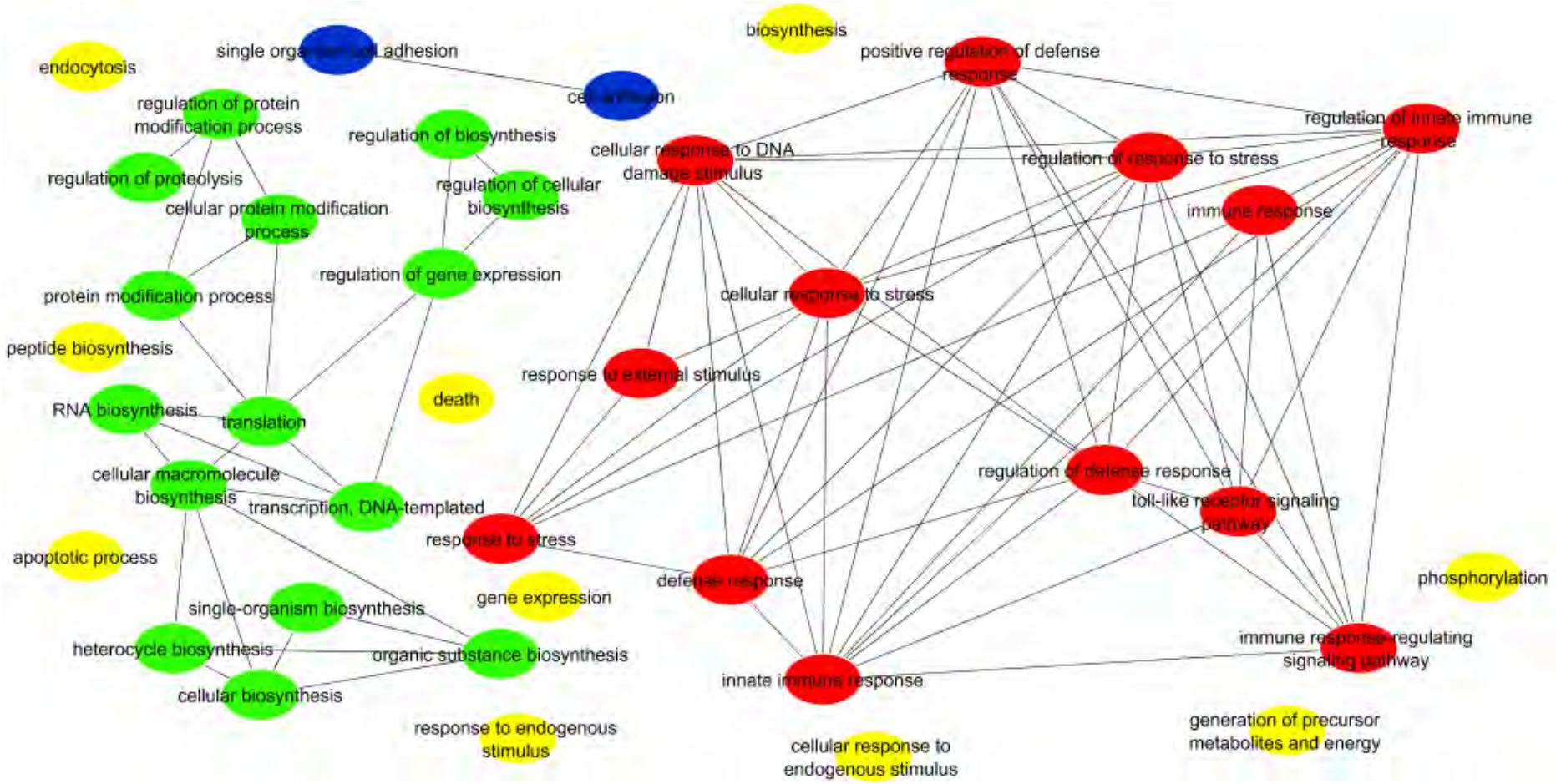


Figure 3.2. Functional interaction networks between immune classes identified in proteins from haemocytes samples of *H. midae* fed a diet supplemented with probiotic. The graph was generated using Cytoscape software. Nodes indicate immune classes and edges indicate relationships between the classes. Nodes with the same colour are connected, except the yellow nodes, as they are not functionally connected to other nodes in the dataset.

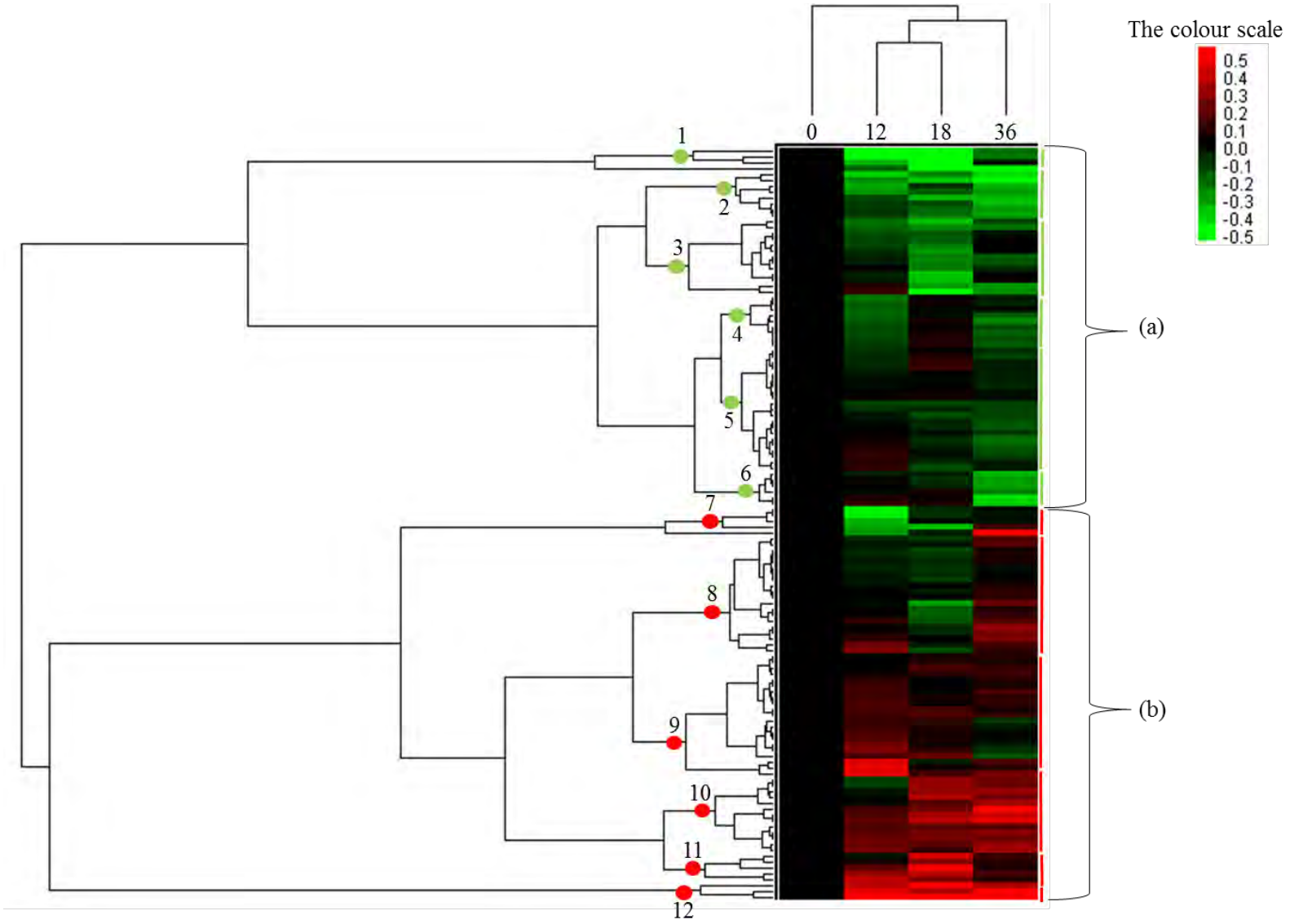


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Figure 3.3. Heat-map dendrogram showing the hierarchical clustering of 128 common proteins identified during an experiment in which *H. midae* were supplemented with probiotic through their diet. Columns indicate sampling time-points in hours and rows reflect individual proteins. Each square represents the mean (n = 4) protein intensity. The expression value for each protein was log2 transformed, quantile normalized and normalized to the control before analysis. Green indicates a decrease and red indicates an increase in protein expressions in comparison to 0 hours, which is indicated by colour black. The heat-map dendrogram is composed of two main clusters: down-regulated (a) and up-regulated (b). Each of the groups features sub-clusters (1 – 12) that were selected based on a correlation index of at least 0.95. The list of proteins and their expression values used to generate this dendrogram are presented in Table 2.3 (Chapter 2).

3.4.1.1. Immune classification and identification of protein-protein interactions within the sub-cluster

Immune classification performed on each of the 12 sub-clusters (see Figure 3.3 above) revealed that most of these were composed of classes related to metabolic processes. Within the main cluster containing proteins that were down-regulated at most of the time-points (see Figure 3.3a above): more diverse immune classes were identified in sub-clusters 2 and 5, such as response to stress, response to stimulus, defense response to bacteria, cell adhesion, cytokine production, endocytosis, apoptosis, and death (Figure 3.4a). Within the second main cluster, which contained proteins that were up-regulated at most of the time-points (see Figure 3.3b above), sub-clusters 8 and 9 presented more diverse immune classes, including those related to response to stress, cell adhesion, apoptosis, and death (Figure 3.4b). The other sub-clusters (1, 3, 4, 6, 7, 10, 11, and 12) included immune functions that were mostly related to metabolic processes. A detailed list of all immune classes identified in each sub-cluster (1 – 12) can be found in Appendix C.7.1 – 2.

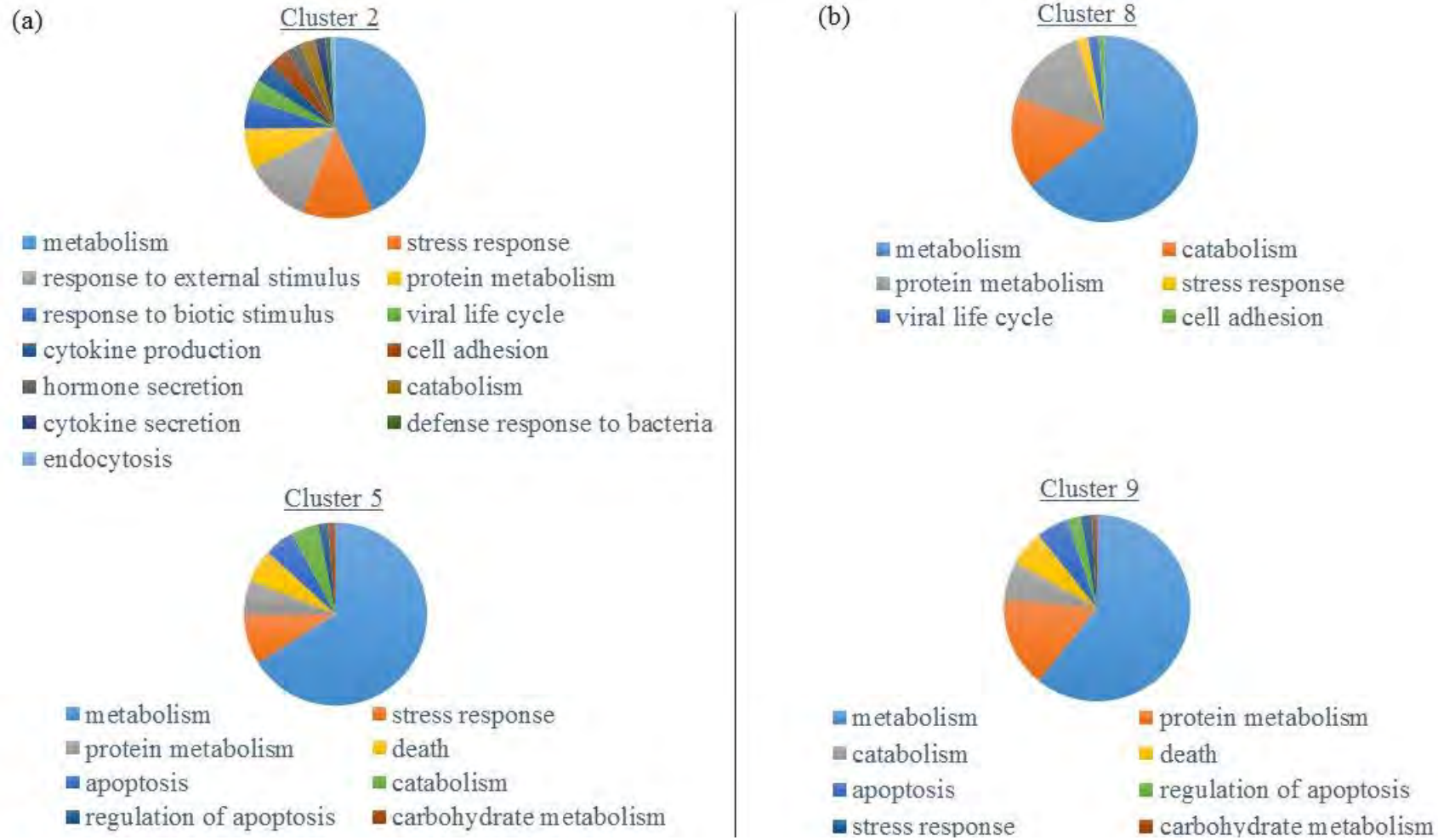

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Figure 3.4. Representation of the biological function of GO terms of proteins expressed with a similar partner. (a) Sub-cluster 2 and 5 belong to the group of mostly down-regulated proteins. (b) Sub-cluster 8 and 9 belong to the group of mostly up-regulated proteins. These sub-clusters presented diverse immune classes, while the remaining sub-clusters were composed of immune classes related to metabolism. The proteins were identified in haemocytes samples from *H. midae* that were fed a probiotic-supplemented diet. The pie charts were generated in CateGOrizer and are based on immune classifications.

Protein interactive network analysis based on protein data from sub-clusters containing diverse immune classes (sub-clusters 2, 5, 8 and 9) revealed that Rab 1A, Rab 11A and Rab 11B (sub-cluster 2) interacts extensively with its predicted functional partners – MYO5B (myosin) and UBC (ubiquitin C) – within the network (Figure 3.5a). Furthermore, it was identified that UBC was part of sub-cluster 5, in which this protein showed interaction with several other predicted functional partners – such as RPL (ribosomal protein L), UBA52 (ubiquitin A 52) and EEFIA2 (eukaryotic translation elongation factor 1 alpha 2) (Figure 3.5b). In sub-cluster 8, UBB (ubiquitin B) and RPSA (ribosome protein SA) showed more interaction with other proteins, within the network (Figure 3.6a). In sub-cluster 9, RP (ribosome proteins) seemed to be the most interactive protein within the sub-cluster (Figure 3.6b).

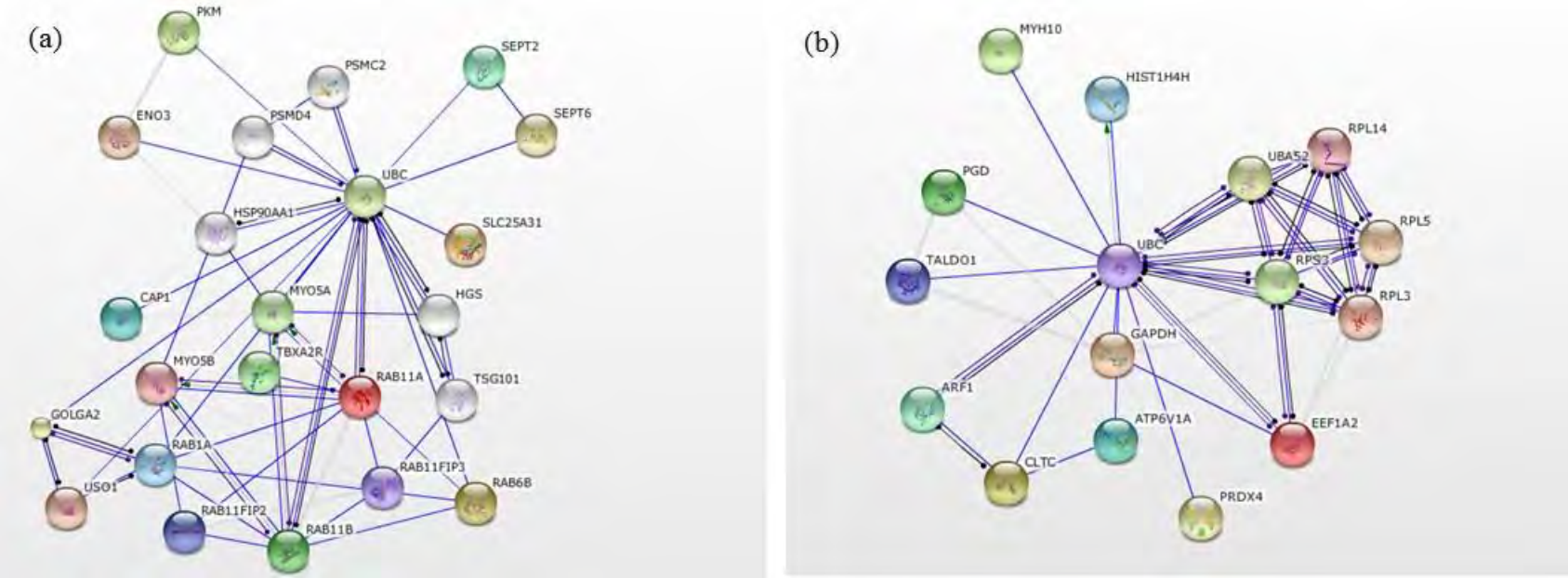

Figure 3.5. Protein interaction networks based on data from sub-clusters of proteins which showed diverse immune classes: (a) sub-cluster 2, (b) sub-cluster 5. Nodes with different colours indicate that the protein presents a different function. Coloured edges indicate direct interaction, and grey edges indicate indirect interaction. Graphs were generated by STRING. Nodes indicate proteins and edges indicate a functional relationship between them. **Protein abbreviations:** RAB11A (Ras-related protein Rab 11A), SLC25A31 (solute carrier family 25), RAB6B (Ras-related protein Rab 6B), PKM (pyruvate kinase), RAB6B (Ras-related protein Rab 11B), SEPT2 (septin 2), CAP1 (adenylate cyclase-associated protein 1), RAB1A (Ras-related protein Rab 1A), RAB11FIP2 (Ras-related protein Rab 11 family interacting protein 2), RAB11FIP3 (Ras-related protein Rab 11 family interacting protein 3), MYO5B (myosin VB), USO1 (vesicle docking protein homolog), ENO3 (enolase 3), SEPT6 (septin 6), GOLGA2 (golgin A2), UBC (ubiquitin C), MYO5A (myosin VA), TBXA2R (thromboxane). EEF1A2 (eukaryotic translation elongation factor 1 alpha 2), GAPDH (glyceraldehyde-3-phosphate dehydrogenase), CLTC (clathrin), MYH10 (myosin), PGD (phosphogluconate dehydrogenase), ARF1 (ADP-ribosylation factor 1), ATP6V1A (ATPase, H^+ transport), HIST1H4H (histone cluster 1), RPL14 (ribosomal protein 14), RPL3 (ribosomal protein 3), RPL5 (ribosomal protein 5), PRDX4 (peroxiredoxin 4), UBA52 (ubiquitin A-52), and RPS3 (ribosomal protein S3).

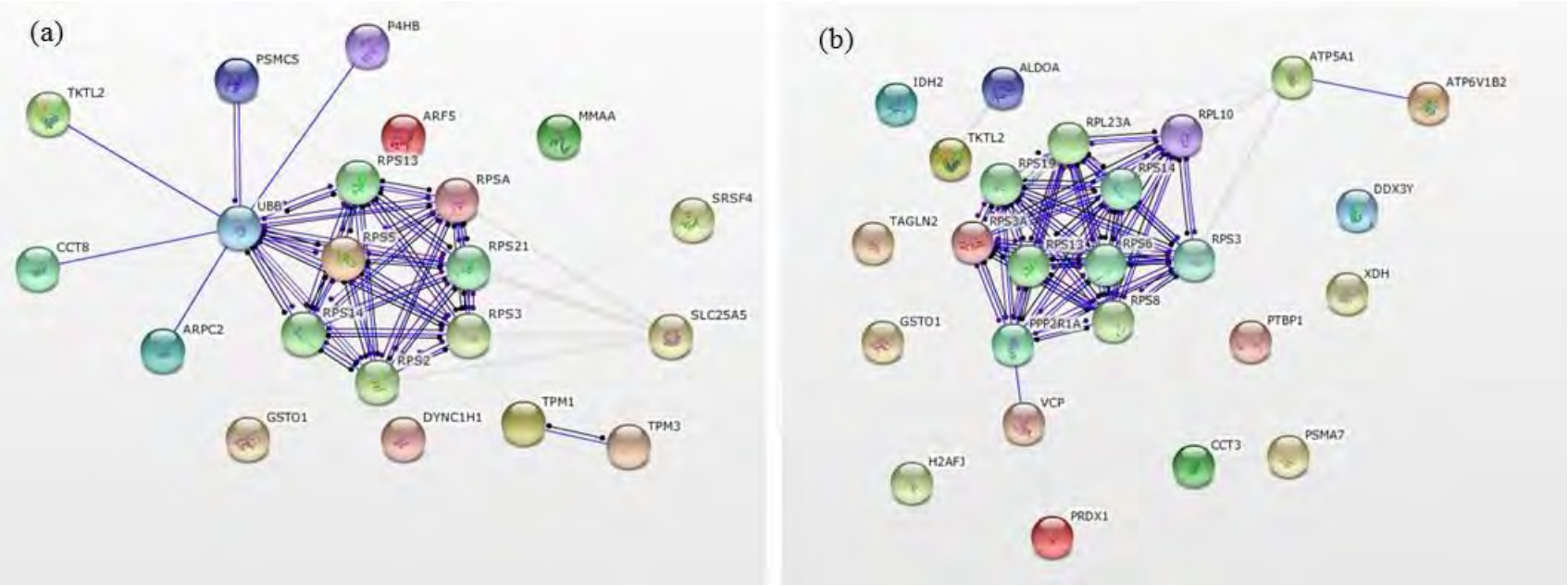

Figure 3.6. Protein interaction networks based on data from sub-clusters of proteins that showed diverse immune classes: (a) sub-cluster 8, (b) sub-cluster 9. Nodes with different colours indicate that the protein presents a different function. Coloured edges indicate direct interaction and grey edges indicate indirect interaction. Graphs were generated by STRING. Nodes indicate proteins and edges indicate a functional relationship between them. **Protein abbreviations:** MMAA (methylmalonic aciduria A), CCT8 (chaperonin containing TCP1, subunit 8), ARPC2 (actin related protein2/3 complex, subunit 2), UBB (ubiquitin B), PSMCS (proteasome, prosome macropain, 26S), P4HP (proly4-hydroxylase, beta polypeptide), RPSA (ribosomal protein SA), DYNC1H1 (dynein, cytoplasmic 1, heavy chain 1), TPM3 (tropomyosin 3), GSTO1 (glutathione S-transferase omega 1), SLC25AS (solute carrier family 25, adenine 5), SRSF4 (serine/arginine-rich splicing factor 4), PTBP1 (polyprimidine tract binding protein 1), VCP (valosin containing protein), TAGLN2 (transgelin 2), PSMA7 (proteasome, prosome macropain, subunit alpha type 7), XDH (xanthine dehydrogenase), H2AFJ (H2A histone family member J), RPL24A (ribosomal protein L23A), RPS2 (ribosomal protein S2), RPS3 (ribosomal protein S3), RPS6 (ribosomal protein S6), RPS8 (ribosomal protein S8), RPS13 (ribosomal protein S13), RPS14 (ribosomal protein S14), and RPS19 (ribosomal protein S19).

3.4.2. Profile of the interaction network and biological pathways involving proteins differentially expressed in haemocytes from *H. midae* fed a probiotic-supplemented diet

Annexin A6, COP9 signalosome subunit 4, phosphorylase, Ras-related proteins Rab 1A, TCP complex subunit gamma, and V-type proton ATPase subunit B are the proteins that showed differential expression in *H. midae* fed a probiotic-supplemented diet. The interaction network generated using Ingenuity software and a list of putative human orthologues of these proteins revealed that there were a number of proteins that indirectly connected the target proteins into the interactive network. Among these proteins, neurotrophic tyrosine kinase, receptor 1 (NTRK1) showed to be particularly important in linking the six differentially expressed proteins (Figure 3.7). Phosphorylase (PYG) was the only protein that did not show a direct interaction with NTRK1 and participated indirectly in the glycogen synthase pathway.

Additionally, Ingenuity software mapped five canonical pathways wherein some of the target proteins are involved; phagosome maturation showed to be the most relevant pathway identified ($p = 3.42E-02$). The phagosome maturation pathway showed that members of Ras-related proteins and V-type proton ATPase play an important role in phagosome maturation. Both proteins act at the membrane level of phagosomes and endosomes throughout the process of phagosome maturation (Figure 3.8).

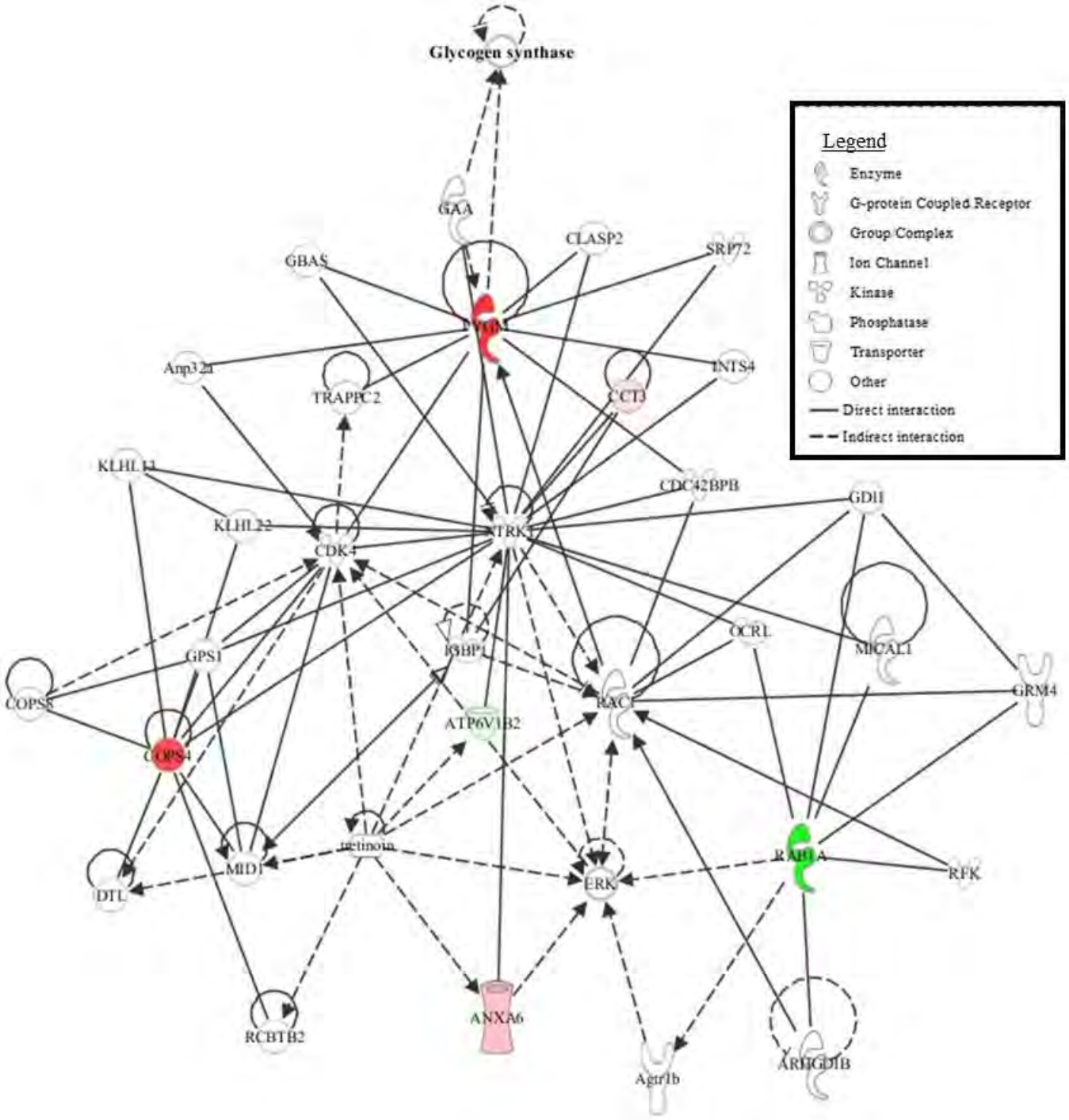

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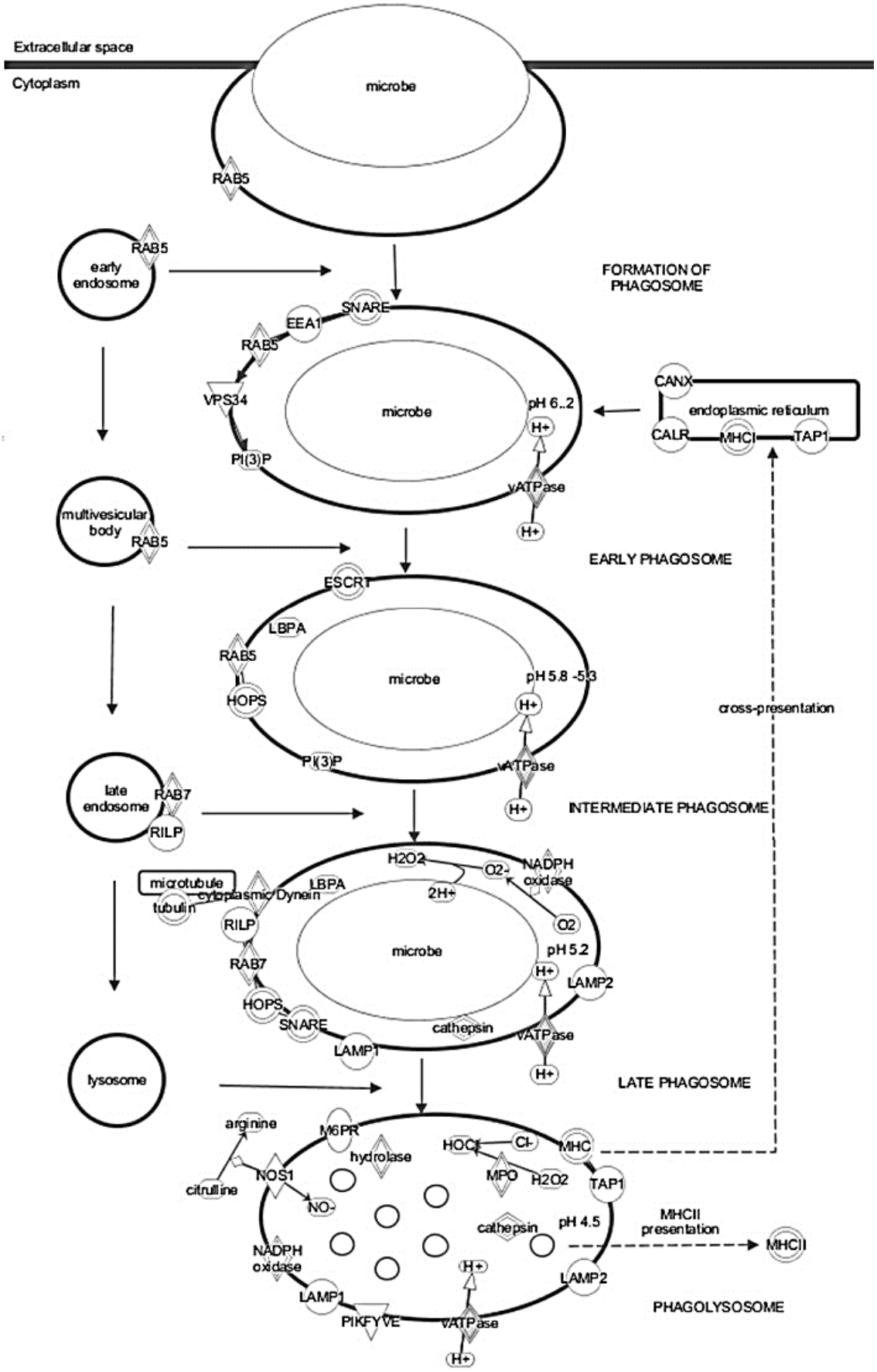
Figure 3.7 Interactive network of differentially expressed proteins identified in haemocytes from *H. midae* fed a probiotic-supplemented diet. Coloured symbols refer to proteins differentially expressed in the experiment, and non-coloured symbols refer to predicted partner proteins. Solid and dotted edges represent direct and indirect interactions. Arrow-head edges and simple edges indicate action on and binding only. The molecular network was generated using IPA. **Protein abbreviation:** PYGM (phosphorylase), CCT3 (TCP complex subunit gamma), COPS4 (COP9 signalosome subunit 4), ATP6V1B2 (V^{+}ATPase subunit B), RAB1A (ras-related protein 1A), ANXA6 (annexin A6), GAA (glucosidase alpha, acid), CLASP2 (Cytoplasmic Linker Associated Protein 2), SRP72 (Signal-recognition-particle), Anp32a (acidic nuclear phosphoprotein 32 family member A), TRAPPC2 (trafficking protein particlecComplex 2), INTS4 (integrator complex subunit 4), KLHL13 (kelch like family member 13), KLHL22 (kelch-like family member 22), CDK4 (cyclin-dependent kinase 4), NTRK1 (neurotrophic tyrosine kinase, receptor 1), CDC42BPB (CDC42 binding protein kinase beta, DMPK-like), CDI (contact-dependent growth-inhibition), COPS8 (COP9 Signalosome Subunit 8), GPSI (G protein pathway suppressor 1), IBP (ice-binding protein), RAC (Ras-related protein Rac), OCRL (inositol polyphosphate 5-phosphatase), MICAL1 (microtubule associated monooxygenase, calponin and LIM domain containing 1), GRM4 (glutamate receptor, metabotropic 4) DTL (denticleless protein homolog), MID1 (midline-1), RCBTB2 (RCC1 and BTB domain-containing protein 2), ERK (extracellular-signal-regulated kinase), and ARHGDIB (Rho GDP dissociation inhibitor beta).



(see next page for caption)

Figure 3.8. Canonical pathway of phagosome maturation generated by Ingenuity. This is the most significant canonical pathway identified based on the differentially expressed proteins from haemocytes of *H. midae* fed a probiotic-supplemented diet. The differentially expressed proteins, Rab and V^{+}ATPase, play a role along the entire phagosome pathway, meaning that this pathway was most likely affected by the probiotic-supplemented diet.

3.5. DISCUSSION

The current study used bioinformatics to investigate the biological functions of a set of proteins identified in haemocytes from *H. midae* that were fed a probiotic-supplemented diet (Chapter 2). As probiotics were added to the abalone's diet to stimulate its immune system, our analyses then focused on the immune classification of the overall proteins as well as the identification of protein interaction networks and molecular pathways involving the differentially expressed proteins.

Based on Categorizer analysis, some biological processes, including response to stress, response to external and internal stimulus, cell adhesion, apoptosis, death, catabolism, and metabolism, were over-represented in haemocytes of *H. midae.* Over-representation of metabolic processes, which comprised more than half of the GO terms, is not surprising, as proteins are generally connected to several metabolic activities. Indeed, metabolism drives all biological processes performed by cells, including immune processes; the goal then is to provide immune cells with sufficient energy and metabolic intermediates in order to perform their roles more effectively (Ganeshan and Chawla, 2014).

One of our hypotheses was that proteins with similar functions may exhibit similar expression patterns. Pereira-Leal et al. (2004) reported that proteins with associated functions are maintained and regulated simultaneously. This was confirmed in our study by the identification of four clusters that followed a similar expression pattern. In fact, apart from the metabolic function that was identified in all sub-clusters, only four of the 12 sub-clusters contained proteins that showed specific immune functions. These results may indicate that *H. midae* haemocyte proteins with similar functions followed the same expression pattern when the immune system of the abalone was stimulated by a probiotic-supplemented diet.

Within the four sub-clusters, cell adhesion, response to stress, apoptosis, and death were the common immune-related functions. The immune system relies on apoptosis and death processes to maintain and remove damaged cells for both normal and pathologic cells (Chen et al., 2015; Opferman, 2008); and the dysregulation of apoptosis and cell death are related to disease (Kennedy et al., 2014). The identification of proteins related to cell adhesion in the same cluster containing proteins related to apoptosis may indicate that these proteins work

together to regulate the immune system. Cell adhesion plays a critical role in immune response and inflammation, regulating contact between cells and the cell-matrix (Elangbam et al., 1997; Zhang et al., 2015). In fact, proteins related to cell adhesion, such as integrin, are important for signal transduction. Their effects include the activation of members of the GTPase family – leading to changes in cytoskeletal organization, activation of mitogen-activated protein (MAP) kinase pathways and activation of an array of protein and lipid kinases. All these processes influence cell-cycle progress and cell survival. Indeed, cells are unlikely to proliferate and survive except when they interact with a substrate (Hynes, 1999).

The other highlighted process in our results was the response to stress. This process is complex and comprises a wide range of activities mediated by a range of proteins involved in the transport, fold, and assembly of newly synthesized proteins. Eventually, and under adverse conditions, the synthesis of these proteins changes to perform reparation and protection of cellular proteins (Solé et al., 2000). Generally, all organisms possess stress proteins and are conserved among the species. When the stress tolerance is exceeded, the cells activate the apoptotic process to ensure the survival of healthy cells and removal of damaged cells during stressful environmental conditions (Kültz, 2005). Our view in this regard is that the identification of all these processes in relation to immune activity in some clusters within our data set may indeed underscore our hypothesis that protein expression in haemocytes from *H. midae* probably followed the same pattern during the experiment in which probiotics were supplemented into the abalone's diet. However, further research is required to investigate the regulation of key proteins involved in these pathways in a similar study.

Another interesting finding in this study was the identification of functions related to response to stimulus in only one sub-cluster (cluster 2). In general, cells respond to several environmental stimuli by adjusting their molecular interaction through mechanisms such as differentiation, modification and degradation (Samir et al., 2015). As we used a probiotic-diet to stimulate the abalone's immune system, our results highlighted the group of proteins related to immune function. Therefore, we used a protein interaction network approach to predict partner proteins associated with those proteins whose immune function is associated with response to stimulus.

We represented the interaction network by using STRING, based on our four clusters of interest, to understand the protein relationship among the clusters and other partner proteins. Indeed, it is well known that proteins do not act in isolation. Instead, proteins rely on interaction with other proteins and molecules to realize their function (Lehne and Schlitt, 2009; Wang et al., 2014; Wetie et al., 2014). Essentially, protein interactions govern all biological systems and cellular activity (Ooi et al., 2010; Suter et al., 2015). In our results, we found that cluster 2, which was composed of proteins down-regulated during the experiment, presented more interactions among each other in comparison to the up-regulated protein clusters. Another remarkable finding was the presence of UBC (ubiquitin C) as the main partner protein in cluster 2. Furthermore, we recognize that UBC was one of the proteins to constitute cluster 5 – which was one of the four clusters containing immune proteins – and interacts with several other proteins within the clusters. The advantage of using interaction network analysis relies on the fact that it shows direct and indirect connections between proteins that share a substrate in a metabolic pathway, regulate each other transcriptionally, or participate in multi-protein assemblies. The interaction network analysis is based on the premise that associated proteins may share functional activities (von Mering et al., 2003; Pereira-Leal et al., 2004).

In proteomic studies, the identification and prediction of binding pattern molecules lead to better understanding of the importance of the target protein in a biological system (Cannataro et al., 2010; Kenley and Cho, 2011). According to Laukens et al. (2015), the number of interactions connecting a protein may indicate its importance within the network. Based on the protein interaction network generated in this study, we identified UBC as the focal protein in our network. Ubiquitin belongs to a family of structurally conserved proteins known to fulfil diverse processes in eukaryotic cells. It functions through covalent attachment to other cellular proteins, thereby changing the stability, localization, or activity of the target protein (Pickart and Eddins, 2004). Transcription, cell cycle, antigen processing, signalling and cellular defense are some of the biological processes in which ubiquitin plays a role (Ciechanover and Iwai, 2004). A study by Booth et al. (2002) demonstrated the role of ubiquitylation in the endocytic pathway. Indeed, ubiquitin regulates signalling receptors of the immune system and functions as a signal for internalization of plasma membrane proteins in the endo-lysosomal pathway.

The relationship between ubiquitin and the immune system – particularly endocytosis – has been mentioned in several studies (Mosesson et al., 2003; Nakatsu et al., 2000; Schnell and

Hicke, 2003; Strous and Govers, 1999). Fu et al. (2011) found that ubiquitin was down-regulated in *Crassostrea hongkonenis* that had been challenged with pathogenic bacteria; and showed that this protein plays an important role in immune defense mechanisms of this invertebrate. In a review, Hicke (2001) mentioned that ubiquitin fulfils a crucial role in membrane trafficking and ubiquitination functions at multiple subcellular locations in order to effect down-regulation of membrane proteins. Additionally, Hicke and Dunn (2003) reported that ubiquitin regulates the transport of proteins among membrane compartments. These statements support our results which show that UBC was associated with down-regulated proteins such as Ras-related proteins. Ras-related proteins are members of the Ras superfamily of small GTPases. They are known to play a crucial role in membrane trafficking (Hu et al., 2011a; Stenmark et al., 2001). These results highlight our interest in using Ras-related protein Rab 1A to investigate the effect of a probiotic-diet in *H. midae*, prompting us to analyse the function of one Ras-related protein member (which was identified as differentially expressed in abalone fed a probiotic-supplemented diet); this is reported in Chapter 4.

According to Krämer et al. (2013), the use of casual network analysis is important to detect novel regulators that operate through the target's molecules, especially when little information about the gene or protein of interest is available. The Ingenuity interaction analysis showed that our six differentially expressed proteins mapped to a single interaction network in which the NTRK1, a family of nerve growth factor receptors, was a central molecule. This suggests that NTRK1 may play an important role in the network. However, it is important to note that our interaction network analysis was based on human orthologue proteins.

Neurotrophins were initially identified in vertebrates, wherein they play an essential role in the development and maintenance of the vertebrate's nervous system, regulating its neural survival, axonal growth, guidance, synaptic plasticity, and long-term potentiation events (Benito-Gutiérrez et al., 2006; Braunger et al., 2014). Cancer studies in humans have shown that this protein plays a role in neuronal development, cell survival, apoptosis and cell proliferation (Huang and Reichardt, 2003; Terry et al., 2011; Wang et al., 2015b). On the other hand, McKay et al. (1999) reported in their review that homologues of neurotrophic factor receptors have been identified in several invertebrate species; they described neurotrophins as being highly conserved across diverse phyla. However, this protein was reported to be absent in invertebrates such as *Drosophila melanogasteror* and *Caenorhabditis elegans* (Adams et al.,

2000; Ruvkun and Hobert, 1998). Nevertheless, a study performed in molluscan *Aphysia sp.* suggested that neurotrophin receptors exist in this species and may play an important role in neural plasticity (Gruenbaum and Carew, 1999).

Based on potential functional protein homology at the level of three-dimensional structures, it is suggested that neurotrophic factors also exist in the snail pond *Lymnaea stagnails* (Montgomery et al., 2002). Indeed, the isolation of tyrosine receptor kinase from the central nervous system of this species of mollusc revolutionized the neurotrophic field, as it presented many of the features that characterise the vertebrate tyrosine receptor kinase (Benito-Gutiérrez et al. 2006). Evidence exists for both unique invertebrate and neurotrophic factors and invertebrate homologues of vertebrate neurotrophic factors (Montgomery et al., 2002); and according to Benito-Gutiérrez et al. (2006), the rudimentary neurotrophic system reported in some invertebrates possibly indicates the acquisition of complex nervous systems at the invertebrate/vertebrate transition. Thus, we hypothesise that a human NTRK1 orthologue may also occur in *H. midae* haemocytes, and due to predicted functional interaction with the differentially expressed proteins identified during the experiment, this neurotrophin protein may have an important role in preserving the abalone immune system. This theory remains to be confirmed in further studies.

Lastly, we found that phagosome maturation was one of the significant canonical pathways in which the differentially expressed abalone haemocyte proteins play a role. This pathway is a component of the pathogen-influenced signalling pathway. During the process of phagosome maturation, particles such as bacteria and apoptotic cells are trafficked into a series of acidified membrane-bound structures, ultimately leading to particle degradation. Phagosome maturation is crucial to the host response to bacterial pathogens and for returning the immune system to normal homeostasis (Kinchen and Ravichandran, 2008; Scott et al., 2003). Several proteins are found in the phagosome membrane, including proton transporter ATPase, other transporters and ion channels, heterotrimeric G proteins, monomeric GTPases of the Rab and Rho families, actin and microtubule binding proteins, COP proteins of vesicle coats, and a spectrum of signalling proteins such as protein kinase C and phospholipase (Griffiths and Mayorga, 2007). In our analysis, we identified V-ATPase and Rab as the proteins from our dataset that participate in the process of phagosome maturation.

A number of studies have reported the importance of these two proteins for the normal activity of the phagosome (it will be discussed later). Rab proteins are key molecules responsible for normal phagosome activity, and multiple Rab proteins have been identified from the early stage of phagosome formation to the regulation of phagosome maturation (Egami, 2016). Brumell and Scidmore (2007) reported that 18 Rab proteins were associated with phagosome formation, and 12 of these proteins were present on the phagosome at a given stage of maturation; this suggests that a complex network of Rab GTPases regulate phagosome maturation. Rab 4, Rab 5, Rab 7, Rab 11, Rab 22 and Rab 25 are some of the Rab proteins reportedly associated with endocytic compartments (Bucci et al., 1992; Van Der Sluijs et al., 1991; Wang et al., 2000; Zhang et al., 2009). Rab 7 is one of the Rab proteins that has been studied extensively – it is known as a key regulator in the process of phagosome maturation (Yu et al., 2008). Mutation or dysfunction of Rab 7 was suggested to cause human disease due to trafficking disorders (Zhang et al., 2009). Both Rab 5 and Rab 7 are present on both bacteria- and apoptotic cell-containing phagosomes (Kinchen and Ravichandran, 2008). On the other hand, V-ATPase is an additional key protein in phagosome activity, and several investigations have been conducted to describe the role of V-ATPase in this regard (Sturgill-Koszycki et al., 1994). V-ATPase must be delivered to the phagosome for membrane acidification. The reason is that cathepsins, which are important proteins present in phagosomes and required for degradation of phagocytosed particles, are only activated at low pH levels (Lennon-Duménil et al., 2002).

This study only provides preliminary information concerning the biological functions associated with proteins identified in haemocytes of *H. midae,* the predicted interaction network, and the canonical pathway involving some target proteins. This is because the bioinformatics analysis was limited by the non-model organism status of abalone. It is for this reason that human orthologue proteins were used for the analysis instead. Another limitation is that some proteins lacked interaction data, which most likely prevented their identification in the interaction network. However, several of our target proteins seemed to be conserved among eukaryotic species. According to Bell et al. (2009), there are several human homologue proteins in invertebrates that are highly relevant for use as novel molecules.

In summary, the use of bioinformatics to investigate the biological function of abalone haemocyte proteins identified by mass spectrometry appears to be reasonable. As the proteins are associated with an annotation that characterises the molecules, this information can be

accessed through different servers both free and commercially available. We also confirmed the hypothesis that those proteins that present similar expression patterns were more likely to present a related biological function. Additionally, we bring new insight regarding protein interaction networks and the canonical pathways associated with the proteins differentially expressed in *H. midae* fed a probiotic-supplemented diet. Therefore, for future work, a deeper investigation of the activity of tyrosine kinase, ubiquitin C and the phagosome pathway may be important to increase our understanding of the effect of probiotics on host immune responses.

CHAPTER 4 - INVESTIGATION OF THE EFFECT OF A PROBIOTIC-SUPPLEMENTED DIET ON THE EXPRESSION OF A PUTATIVE RAS-RELATED PROTEIN RAB 1A, IN *HALIOTIS MIDAE* HAEMOCYTES

4.1. INTRODUCTION

Rab protein belongs to the superfamily of small GTPase Ras-related proteins, a group composed of five members: Rab, Ras, Rho, Ran, and Arf (Hu et al., 2011b; Jiang et al., 2006; Kahn et al., 1992; Stenmark et al., 2001). GTPases are molecular switches that translocate proteins through membranes, operate in protein biosynthesis, signal transduction and transport of vesicles within a cell (Caetano-Anollés et al., 2012; Diekmann et al., 2011). Members of this superfamily fulfil functions in a variety of biological processes, such as differentiation, morphogenesis, cell division, cytokinesis, and molecular trafficking (Vernoud et al., 2003; Wang et al., 2013b). Rab is particularly characterised by its crucial function in the regulation of membrane and vesicle trafficking, a conserved mechanism from yeast to higher eukaryotes, for the transportation of proteins and other macromolecules inside and outside cells (Cheung and de Vries, 2008; Pavlos and Jahn, 2011; Zerial and McBride, 2001).

Rab has also been reported to participate in immune-related activities (Brighouse et al., 2010) in diverse groups of organisms such as plants (Kwon et al., 2009), crabs (Wang et al. 2013), shrimps (Wang et al., 2015c; Wu and Zhang, 2007), fish (Hu et al., 2011b) and mammals (Krzewski and Cullinane, 2013; Sakamoto et al., 2011). Indeed, members of the Rab family are described as highly conserved and function similarly in different organisms (Brighouse et al., 2010; Vernoud et al., 2003). Despite this, and to the best of our knowledge, there is no report linking the effect of probiotics to the expression of Rab in *H. midae*.

An experiment performed in *H. midae* to investigate the host immune response to a probiotic-supplemented diet revealed that the expression of Rab 1A was differentially expressed in haemocytes of this species (Chapter 2). As a follow-up study, we sought to evaluate the relative expression of Rab between membrane and cytosolic fractions of *H. midae* haemocytes when the host immune system is stimulated by a probiotic-supplemented diet. Based on the statement that there are two forms of Rab in the cell – the inactive form, located in the cytoplasm, and the active form, located in membranes (Liu and Storrie, 2012; Seabra et al., 2002) – and that Rab is activated during cellular stimulation (Krzewski and Cullinane, 2013; Seabra et al., 2002), we hypothesized that the expression of Rab 1A would be up-regulated in the membrane fraction (active form) from the haemocytes since it was down-regulated in samples of soluble

proteins from the haemocytes of abalone that were immune stimulated with a probiotic-supplemented diet (Chapter 2).

To the best of our knowledge, this study is the first to investigate the expression variation of Ras-related protein Rab 1A from abalone whose immune systems were stimulated by the dietary inclusion of probiotics. Characterisation of the functionality of Ras-related protein Rab between cytosol and membrane fraction of the abalone haemocytes will help us understand the mechanism of activation of this protein that has been documented to switch the localization between these two fractions in order to mediate the translocation of effector proteins between different membrane compartments inside the cell.

4. 2. MATERIALS AND METHODS

The preparation of all media and solutions used in this study is described in Appendix A.

The routine housing of abalone and the process of feed preparation referred to in this chapter are described in Chapter 2.

4.2.1. Abalone probiotic-feeding experiment and sample collection

Sixty randomly selected abalone were divided equally between four polyethylene tanks of 98 l capacity. The tanks were aerated and supplied with flow-through (330 $l.h^{-1}$) seawater at 15 – 18 ^{o}C (Figure 4.1). The animals were allowed to acclimatise to these conditions for a period of two weeks, during which they were fed probiotic-free kelp cakes three times weekly. Any remaining feed in the tanks was removed prior to the supply of fresh feed. After the acclimation period, the feed was withheld from abalone for a period of three days in order to ensure the rapid consumption of feed supplemented with a probiotic strain (Macey & Coyne 2005).

During the experiment, samples were collected at four time-points that had been pre-determined based on a similar experiment (Chapter 2). Approximately 0.4 – 0.8 ml of haemolymph samples were extracted from the pedal sinus of between 3 to 4 abalone (to obtain a total volume of 3 ml) using a 2 ml syringe and 26 G x ½ inch needle. The first sampling time-point was prior to placing the probiotic-supplemented feed, kelp cakes (Appendix B.1.1), into the experimental tanks (zero (0) hours, or control). Thereafter, the probiotic-supplemented feed was fed to the abalone, and abalone haemolymph samples were extracted at 6, 12 and 18 hours post the introduction. After the extraction of haemolymph at each time-point, the bled abalone were removed from the experimental tanks and placed in recovery tanks; this was performed to ensure that the abalone were not used again during the experiment.

All haemolymph samples obtained from the same tank per time-point were pooled into the same glass test tube to a final volume of 4 ml. The test tubes were kept on ice; however, this was only for short-term storage. Of the total haemolymph sample, 1 ml was used for total

haemocyte counting and for the phagocytosis assay. The remainder of the sample was used for western blot analysis of membrane and cytosolic assessment of Rab 1A expression, as well as immunofluorescence analyses of cellular expression of Rab 1A.

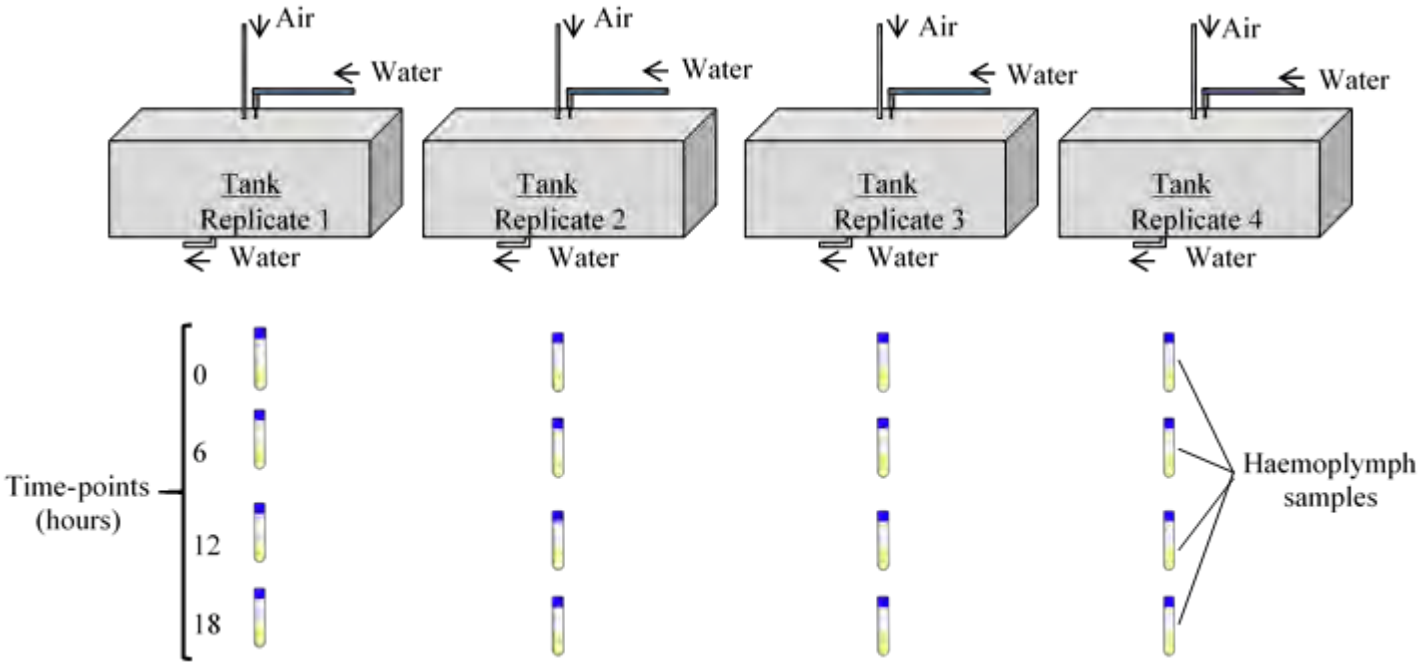


Figure 4.1. Schematic representation of the *H. midae* probiotics feeding experiment. Four polyethylene tanks of 98 l were used in this experiment; each tank was stocked with 15 animals. The time-points selected (0, 6, 12 and 18) represent the hours at which the haemolymph was sampled based on the feed supplementation time, where samples taken at 0 hours – before the addition of the feed – were used as control samples and were taken before the addition of the feed. After the addition of feed, the remaining samples were taken at 6 hourly intervals.

4.2.2. Determination of total haemocyte concentration and phagocytic activity

To ensure that the probiotic-supplemented feed stimulated the abalone's physiology, the total number of circulating haemocytes and their phagocytic rates were assessed during the experiment. This was done using approximately 1 ml of haemolymph sample per time-point. The methodology for these analyses is described in sections 2.3.4 and 2.3.5.

4.2.3. Extraction of cytosolic and membrane proteins from *H. midae* haemocytes to assess the expression of Ras-related protein Rab 1A

It has been reported that the Ras-related protein Rab switches its form between membrane and cytosol in cases of its activation and de-activation due to stimulation (Krzewski and Cullinane, 2013; Seabra et al., 2002). Therefore, to evaluate the expression of this protein in membrane and cytosol fractions from abalone haemocytes sampled during the experiment, a commercially available membrane protein extraction kit, Mem-Per^{TM} Plus (Thermo Scientific Pierce), was used according to the manufacturer's instructions, with minor modifications.

Briefly, approximately 5x10^6 haemocytes were centrifuged at 1 800 rpm for 5 minutes at room temperature. The supernatant was discarded and the pellets resuspended in 1.5 ml of Cell Wash Solution, briefly vortexed and centrifuged at 1 800 rpm for 5 minutes at room temperature. The supernatants were subsequently discarded. This procedure was repeated for a total of three times. The protein pellets were resuspended in 0.75 ml of permeabilization buffer, briefly vortexed to homogenise the suspension and then incubated for 10 minutes at 4 ^oC on an orbital shaker (100 rpm). The samples were subsequently centrifuged at 13 000 rpm for 15 minutes at room temperature. The supernatants containing cytosolic proteins were transferred to new microfuge tubes and kept in a container with ice (short-term storage) until required. The pellets, which contained the membrane proteins, were resuspended in 0.5 ml of solubilization buffer by pipetting. The samples were incubated for 30 minutes at 4 ^oC on a shaker. Thereafter, the samples were centrifuged at 13 000 rpm for 30 minutes at room temperature. The supernatant containing membrane-associated proteins was transferred to a new microfuge tube and kept in a container with ice (short-term storage) until required. Both cytosolic and membrane protein fractions were quantified using the Thermo Scientific Pierce Bicinchoninic acid (BCA) Protein Assay kit according to the manufacturer's instructions.

Due to the interference of the buffer (from Mem-Per^{TM} Plus) with downstream applications (western blot analysis), both cytosolic and membrane proteins were precipitated in 80% acetone; followed by resuspension in ULB.

Briefly, the extracted protein samples were placed in a microfuge tube containing an equal volume of 80% acetone (Appendix A.2.26), which was briefly mixed by vortexing and incubated for 30 minutes at -20 ^{o}C. The samples were then vortexed for 10 seconds and centrifuged for 20 minutes at 8 500 rpm at 15 ^{o}C. The supernatants were discarded and the protein pellets allowed to air dry at room temperature for 10 minutes. The protein pellets were then resuspended in 100 µl of ULB (urea lysis buffer) (Appendix A.27).

4.2.4. Western blot analysis of Ras-related protein Rab 1A expressed in cytosolic and membrane haemocyte protein fractions

To quantify and compare the expression of Rab 1A in the cytosolic and membrane haemocyte protein fractions, the proteins were first separated according to size by one-dimensional SDS-PAGE followed by immunoblot analysis.

4.2.4.1. Protein separation by one-dimensional SDS-PAGE

For each sample, 20 µg of protein was first diluted in 5x Sample Application Buffer (Appendix A.2.15) to a final concentration of 5 $\mu g.\mu l^{-1}$; this was then vortexed for 10 minutes at room temperature. Thereafter, each sample was briefly centrifuged and incubated for 10 minutes in a 75 ^{o}C water bath to denature the proteins. Samples were then briefly vortexed, centrifuged for 30 seconds and pipetted into pre-made gel wells of 4% (v/v) stacking gel (Appendix A.2.16) and 12% (v/v) polyacrylamide separating gel (Appendix A.2.17). One well was loaded with a molecular mass marker (Page Ruler^{TM} prestained protein ladder 10 – 170 kDa, Fermentas) for the estimation of protein sizes. Protein samples were electrophoresed in a Mini-Protean gel electrophoresis tank (Bio-Rad, South Africa) at a constant voltage of 100 V in 1x SDS electrophoresis buffer (Appendix A.2.18); this was done at 4 ^{o}C until the bromophenol blue front of the sample's application buffer reached the bottom of the gel. The gels were then removed from the electrophoresis tank and prepared for western blot analysis.

4.2.4.2. Western blotting to the nitrocellulose membrane

Following one-dimensional SDS-PAGE, the gels were incubated in a cold Towbin buffer (Appendix A.2.19) for 5 minutes, so as to remove residual SDS. Additionally, a nitrocellulose membrane (Whatman Protran^{TM} 0.2 μm pore size) previously cut to fit the gels was also placed in a cool Towbin buffer to equilibrate. Subsequently, the membranes and gels were placed between two pieces of pre-soaked Whatmann 3 mm filter paper and sandwiched together in a transfer cassette. The cassette was loaded vertically into the transfer tank (Bio-Rad mini Trans-Blot cell), which was then filled with a Towbin buffer, according to the manufacturer's instructions. Proteins in the gels were electro-blotted onto the nitrocellulose membranes for 1 hour at 100 V 4 ^{o}C. The nitrocellulose membranes were removed from the cassette, and transfer of the proteins to the membranes was confirmed using Ponceau S reversible total protein stain (Appendix A.2.20). Images were captured using the Molecular Imager ChemiDoc XRS+ system (Bio-Rad) with 'colorimetric' settings optimised for faint bands.

The nitrocellulose membranes were washed in Tris Buffered Saline (TBS) (Appendix A.2.21) to remove excess Ponceau S stain. Afterwards, the nitrocellulose membranes were blocked by immersing them in a container with blocking buffer (Appendix A.2.22) for 1 hour at room temperature with shaking. The nitrocellulose membranes were then incubated in the primary antibody (Appendix A.2.23) for 18 hours at 4 ^{o}C with shaking. Following this, the nitrocellulose membranes were washed three times with Tris Buffered Saline Tween (TBST) (Appendix A.2.24) and once in TBS for 15 minutes at room temperature with shaking. The nitrocellulose membranes were then incubated with the secondary antibody (Appendix A.2.25) for 2 hours at room temperature with shaking. The nitrocellulose membranes were washed three times with TBST and once in TBS for 15 minutes at room temperature on a shaker.

4.2.4.3. Densitometric analysis

The detection of proteins was performed using the WesternBright ECL HRP chemiluminescent detection kit (Advansta) according to the manufacturer's instructions. The chemiluminescent signal was acquired using the Molecular Imager ChemiDoc XRS+ system (Bio-Rad) – where

the immunoblot was exposed for 100 seconds, capturing an image every 10 seconds. The images with the lowest saturated pixels were selected for analyses.

The density (intensity.mm^{-2}) of each positive signal was calculated using Image Lab Software (version 2.0.1, Bio-Rad). An area lacking signal was defined for background subtraction. The relative density of the bands was normalised to the respective β-actin, which band displayed stable (with no change), as previosly mentioned in a study by Zong et al. (2008). The size of the bands detected by the antibody was determined using a molecular mass marker (Page Ruler^{TM} prestained protein ladder 10 – 170 kDa, Fermentas) and the molecular mass analysis tool provided in Bio-Rad's ImageLab software. The intensity of the bands at 6, 12 and 18 hours was calibrated in relation to the control (0 hours), meaning that protein expression was presented as either increase or decrease in fold change of the target protein at each time-point relative to the control.

4.2.5. Immunochemical analysis of Ras-related protein Rab 1A cellular expression in *H. midae* haemocytes

Haemolymph samples collected from abalone at 0 hours (control) and after 12 hours of feeding a probiotic-supplemented diet (see section 4.3.1) were subjected to immunochemical analysis to evaluate Rab 1A expression. Three slides were prepared per time-point.

4.2.5.1. Haemocyte preparation for confocal imaging

Samples containing haemocyte cells were diluted to a concentration of 10^5 cells.ml^{-1} using PBS (Appendix A.2.6). A volume of 100 µl of the diluted sample was plated onto the coverslips that were previously acid-washed, labelled, and kept inside a one-well tissue culture plate. The coverslips were incubated at room temperature for 30 minutes, inside a dark and humid chamber, to allow the cells to attach to the surface of the coverslips. Ice-cold PBS (± 2 ml) was used to remove those cells that did not attach to the coverslip. The cells that attached to the surface of the coverslips were fixed by adding approximately 2 ml of 2% paraformaldehyde

(Appendix A.2.34) onto the coverslips for 10 minutes at room temperature. The paraformaldehyde was then removed using a pipette, and the coverslips were washed by immersing them in PBS (± 2 ml) for 10 minutes. The cells were then permeabilized with 2 ml of 1% Triton X-100 (Appendix A.2.35) for 10 minutes at 4 °C. To reduce non-specific staining, the coverslip cells were washed twice with 2 ml of PBS. Thereafter, the coverslips were incubated for 1 hour, at room temperature, in 2 ml of 1% Bovine serum albumin and Triton X-100 (BSA-Triton X-100) blocking solution (Appendix A.2.36). Following this, the coverslips were incubated in 200 µl of primary antibody (1:100 in blocking solution) to detect Rab 1A (Appendix A.2.23). The coverslips were incubated in a dark and humid chamber for approximately 18 hours at 4 ºC, after which the cells were gently washed with 2 ml of PBS for a total of three times.

The coverslips were then incubated in 200 µl of secondary antibody conjugated to fluorophores (1:500 in blocking solution) anti-rabbit ALEXA 488 (Appendix A.2.37) for 1 hour at room temperature. The coverslips were gently washed with 2 ml of PBS; this was repeated three times. The cells were counterstained in 2 ml of Hoechst Nuclear stain (Appendix A.2.38) and incubated in a dark, humidified chamber for 10 minutes at room temperature. Next, the coverslips were incubated in 2 ml of PBS for 10 minutes at room temperature. The coverslips were incubated in 2 ml of 100 nM Rhodamine Phalloidin (Appendix A.2.39), in a dark, humidified chamber for 30 minutes, to stain F-actin (actin cytoskeleton that interacts with cell membrane) of haemocyte cells. The coverslips were then gently washed and incubated in 2 ml PBS for 10 minutes at room temperature. Coverslips containing the cells were mounted onto glass slides with 50 µl of Mowiol (Appendix A.2.40). This was followed by incubation for 1 – 2 hours inside a dark chamber at room temperature, to air dry, before being stored at 4 ºC in the dark until visualization. In order to establish background interference levels and detect if there were any non-specific binding sites, two control coverslips were prepared: cells stained without any antibodies, and cells only exposed to the primary antibody.

4.2.5.2. Confocal microscopic imaging of haemocytes

For visualization and comparison of protein expression, the prepared slides were mounted onto a Zeiss Axiovert 200M LSM 150 Meta NLO Confocal Microscope, using a 40x oil immersion

objective lens and a multitrack configuration using 488 nm (Argon laser) for Alexa 488; 561 nm (Solid state laser) for Rhodamine Phalloidin; and 750 nm (MaiTai two photon laser) for Hoechst nuclear. Stain excitation lines were employed to minimise bleeding between fluorophores. Emission filters used were 500-550 nm band pass (BP), 575-630 nm BP and 390-465 nm BP for Alexa 488, Rhodamine Phalloidin and Hoechst nuclear stain, respectively. The photomultiplier gain and offset were adjusted to exclude any background fluorescence emitted by the cells and fluorophores. At least five fields of view for two experiments were imaged. The images were analysed using ZEN imaging software to measure the intensities of the proteins and compare the results from the experimental (12 hours) and control (0 hours) haemocyte cells. Imaging parameters were kept the same for all data acquisitions in order to not affect pixel intensity for co-localization analysis. The single label control samples were used to set appropriate cross-hair threshold values which were kept constant for the analysis of all double-label experimental samples.

A total of three slides (one per replicate tank) were prepared per time-point (0 hours and 12 hours), in which four fields of observation were analysed per slide. The selected field of observation was based on the presence of 3 – 6 cells for better visualization. A total of 10 observations were used to calculate the mean intensity of Rab 1A in haemocyte cells and for comparison of the time-points.

4.2.6. Statistical analysis

All statistical analyses were conducted using SPSS 20 software (SPSS Inc.). For the descriptive statistics, the mean and standard error of means (± SEM) of protein expression were presented per time-point. One-way ANOVA analysis was performed to determine whether there was a statistical difference ($p < 0.05$) in protein expression over time. One-way ANOVA results were considered valid only when the Levene test of homogeneity of variances was not statistically different ($p > 0.05$). Post hoc multiple comparison tests (Tukey test) were performed when the differences between the means were statistically significant. Independent T-test analyses were performed to compare the means of the immunofluorescence of protein intensities between two groups of samples, one from the 0 hours and another from the 12 hour sample.

4.3. RESULTS

4.3.1. Comparison of the relative expression of Ras-related protein Rab 1A protein in the cytosolic and membrane fractions

A Mem-Per^{TM} Plus kit (Thermo Scientific Pierce) was used to separate proteins present in haemocyte samples in two fractions – membrane proteins and cytosolic proteins. The separation was done to evaluate whether there was any change in cytosolic and membrane expression of Rab 1A in haemocytes sampled from *H. midae* fed a probiotic-supplemented diet.

The relative expression of Rab 1A in the cytosol decreased over the course of the experiment in comparison to the control. Rab 1A was down-regulated approximately 3.3 fold at 6, 12 and 18 hours in relation to the control (Figure 4.2. a.). Statistical analysis performed using one-way ANOVA showed that these differences were statistically significant ($F = 121.20$, df 3, $p < 0.05$).

Similarly, the relative expression of Rab 1A in the membrane fraction also decreased over the course of the experiment in comparison to the control (Figure 4.2. b.). Membrane associated Rab 1A was down-regulated 1.7 fold after 6 hours and 2.5 fold after 12 and 18 hours. Statistical analysis using one-way ANOVA showed that these differences were statistically significant ($F = 16.24$, df 3, $p < 0.05$).

Additionally, the relative expression of Rab 1A was compared between membrane and cytosol fractions at each experimental sample point. Control data were not tested since the experimental time-points were normalised according to control levels. The results showed that the expression of Rab 1A at each sampling time-point was always higher in the membrane fraction in comparison to the analagous cytosolic sample. However, statistical analysis performed using an independent T-test showed that the difference in Rab 1A expression between the two haemocyte fractions was statistically significant only at 6 hours ($t = -2.91$, df 6, $p < 0.05$) and 18 hours ($t = -2.95$, df 6, $p < 0.05$), but not at 12 hours ($t = -1.99$, df 6, $p > 0.05$).

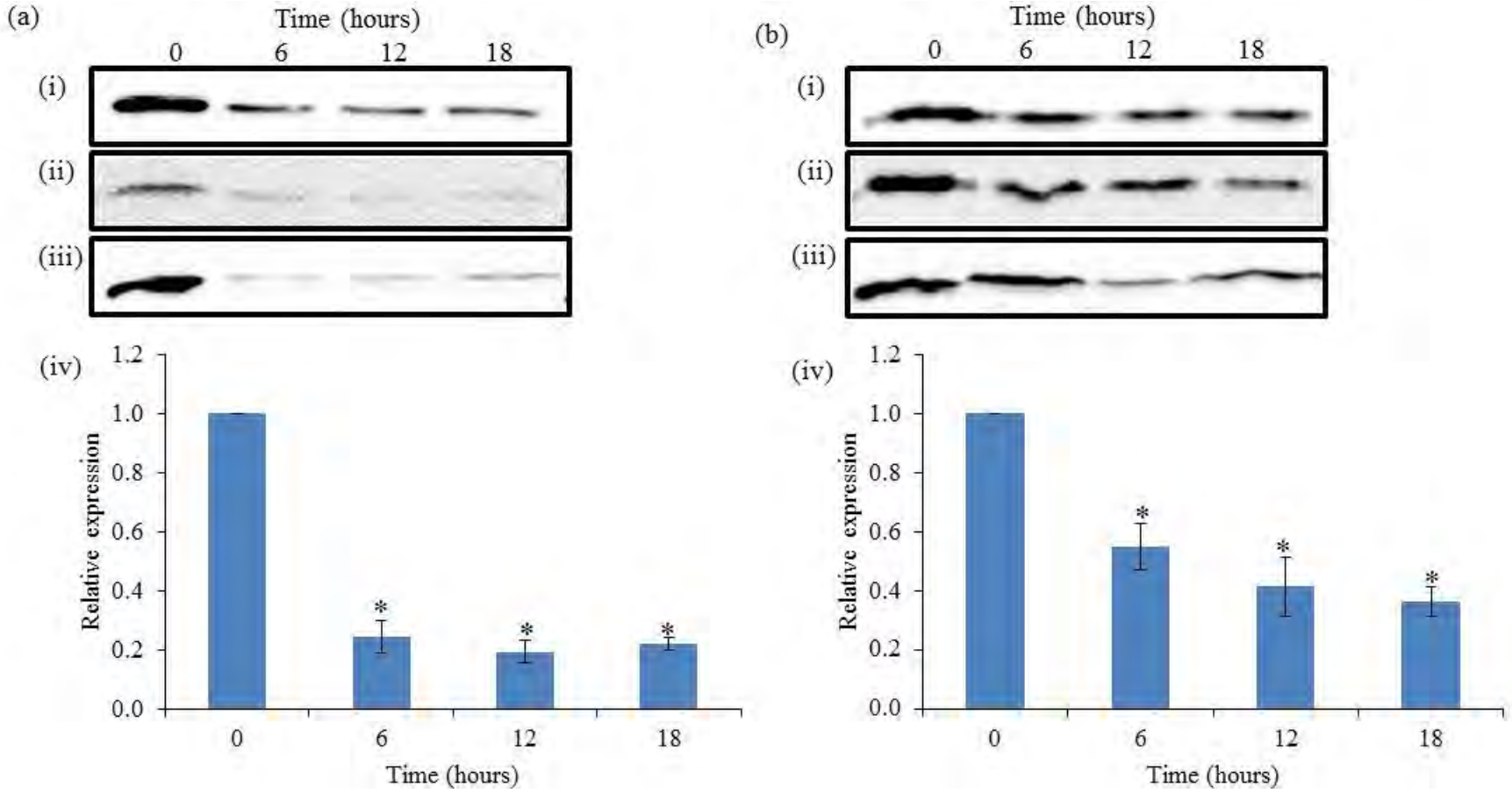


Figure 4.2. Western blot analysis of Rab 1A expression in haemocytes from *H. midae* fed a probiotic-supplemented diet. (a) Proteins from the cytosol fraction and (b) proteins from the membrane fraction. (i – iii): inlaid images show the chemiluminescent western blot signals obtained from different biological replicates. (iv) The bar graphs show the mean (n = 4) ± SEM of relative expression of the protein volume intensity relative to the control (0 hours). An asterisk (*) indicates a statistically significant difference ($p < 0.05$) at this sampling time-point in comparison to the control.

4.3.2. *In situ* examination of Ras-related protein Rab 1A expression in *H. midae* haemocytes

Immunochemistry was performed to assess Rab 1A *in situ* expression in haemocytes. The relative expression of Rab 1A decreased 1.8 fold in haemocytes sampled from abalone fed the probiotic-supplemented diet for 12 hours in comparison to the control (Figure 4.3). Independent T-test analysis showed that the difference in Rab 1A expression between these two groups was statistically significant ($t = 5.28$, $df\ 18$, $p < 0.05$). Indeed, Rab 1A could not be detected in association with the haemocyte membrane at 12 hours. Yellow colour, generated by merging the Rab 1A (green) and cell cytoskeleton (red) signals, was not visible in the 12 hour samples as Rab 1A expression had decreased at this time-point (Figure 4.4).

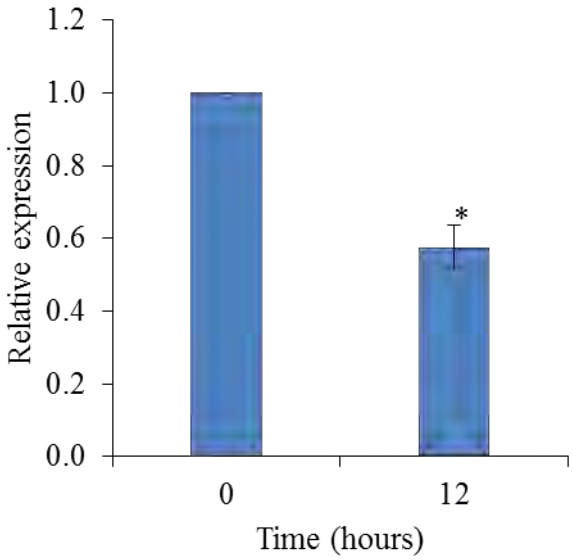
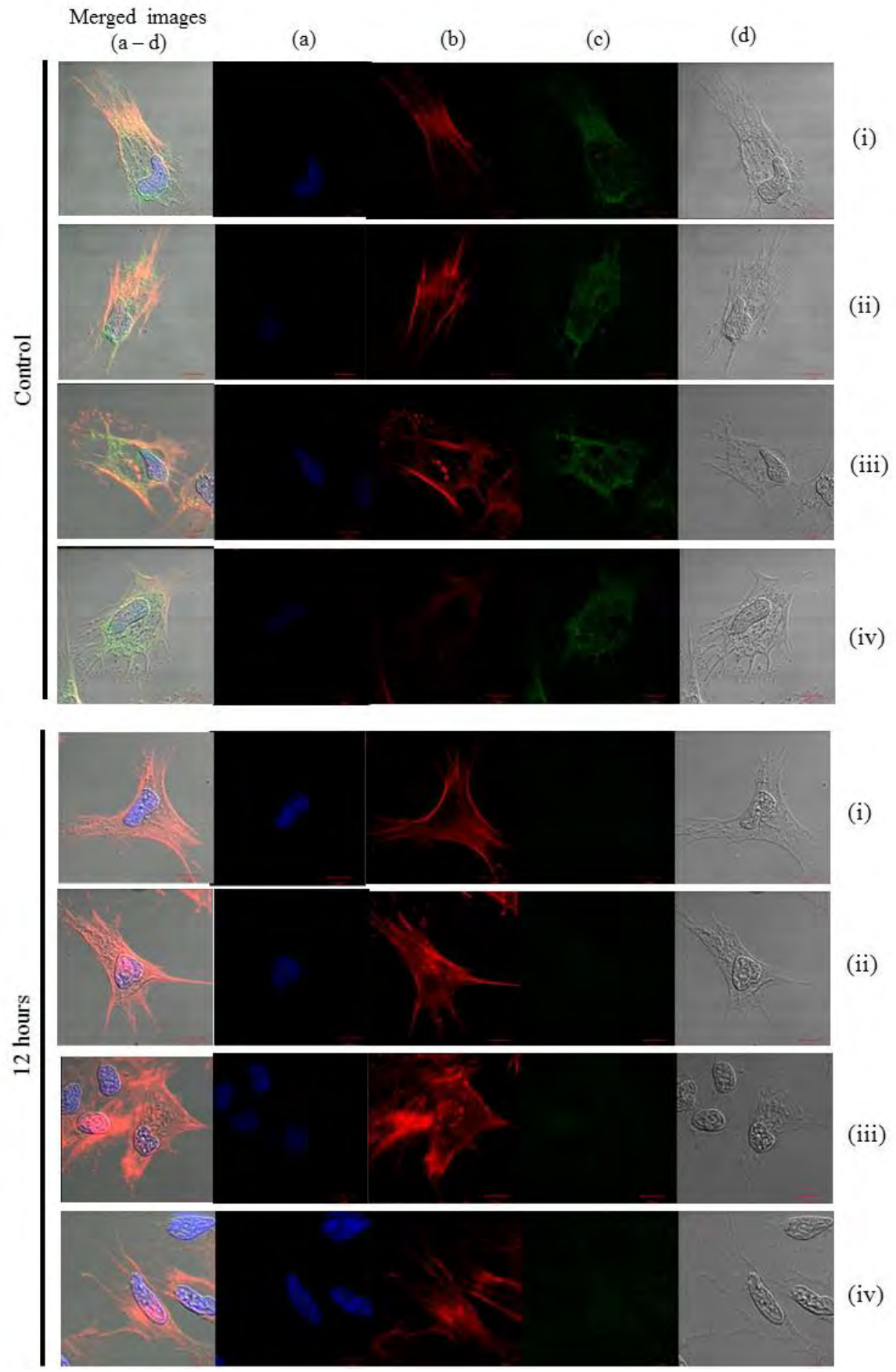


Figure 4.3. Relative expression of Rab 1A in haemocytes sampled from *H. midae* fed a probiotic-supplemented diet. The values are means (n = 10) ± SEM from different fields of view observed in two replicates. An asterisk (*) indicates a statistically significant difference ($p < 0.05$) between the two sampling time-points.



(See next page for caption)

Figure 4.4. Immunofluorescent imaging of haemocyte cells from *H. midae* fed a probiotic-supplemented diet. (i – iv) Representative replicates images of haemocytes at the 0 (control) and 12 hour (experimental) sampling time-points. The images were taken using different channels: (a) blue for nuclei stained with Hoechst, (b) red for membrane cytoskeleton stained with Rhodamine Phalloidin, and (c) green for Rab 1A stained with ALEXA 488. (d) Light microscopy images of the haemocytes show the format of the cell. Scale bar = 5 µm.

4.4. DISCUSSION

Ras-related Rab GTPases, or simply Rabs, are well known for their role in membrane trafficking and their involvement in almost every step of vesicle transportation in eukaryotic cells (Liu and Storrie, 2012; Pfeffer, 2001; Stenmark, 2009). They switch between a cytosolic (inactive) and a membrane (active) form to interact with a variety of effector proteins in order to deliver proteins (effectors) to specific intracellular compartments (Fukuda, 2008; Langford et al., 2002; Saxena and Kaur, 2006).

Rab 1A was differentially expressed in haemocytes from *H. midae* fed a probiotic-supplemented diet. Rab 1A levels in both the membrane and cytosolic haemocyte fractions decreased significantly over the course of the experiment, suggesting that the use of probiotics as an abalone feed supplement mediated the change in Rab 1A expression. Indeed, similar results were obtained in our previous study in which only soluble proteins were assessed (Chapter 2). However, the finding that this protein was also down-regulated in the membrane fraction is a novel result and is discussed in greater detail later in this section.

Similar to our finding, the differential expression of Rab proteins has been reported in several other studies in which the host was challenged with pathogenic bacteria. For instance, in the Chinese mitten crab, *Eriocheir sinensis,* challenged with the pathogenic bacterium *Vibrio angullarium* (added to the water), the levels of Rab 1 and Rab 3 in haemocytes were significantly up-regulated (Wang et al., 2013b). In shrimp, *Penaeus japonicas*, the level of Rab was up-regulated in specimens resistant to WSSV (white spot syndrome virus), suggesting that this protein is involved in anti-viral immunity (Wu and Zhang, 2007). In another study also conducted in *P. japonicas*, Rab was seen to play a role in the phagocytic activity of the haemocytes (Zong et al., 2008). In Red drum, *Sciaenops ocellatus*, over-expression of Rab increased the host's resistance to intracellular invasion by *Edwardsiella tarda*, leading to the authors' suggestion that the protein plays a crucial role in the host's immune defense against infection by pathogenic bacteria (Hu et al., 2011b). According to Hu et al. (2011b), differential expression of Rab proteins has been found in several human diseases, including cardiomyopathy, lung, prostate and pancreatic cancer. Therefore, it is not surprising that Rab was differentially expressed in *H. midae* fed a probiotic-supplemented diet.

However, a major difference between the above-mentioned studies and the present one is that expression of Rab increased in the former, but decreased in the latter. This may be because our investigation focused on the effect of probiotic bacteria while the others challenged the host organism with pathogenic bacteria. While host immune stimulation by pathogenic bacteria may mediate an increase in Rab expression Hu et al. (2011b), we propose that a probiotic-supplemented diet may lead to a reduction in Rab expression in *H. midae* haemocytes.

A question that may arise from a comparison of the results of our study and those mentioned above is how the expression of Rab protein can be up-regulated in other studies (stimulated with pathogenic bacteria) and not here (stimulated with probiotics). A possible explanation of our results may be supported by Pei et al. (2012), who reported that there are two pools of Rab proteins in the cell: one that is newly synthesised, and the other that recycles between the cytosol and target membranes. The authors hypothesised that these two pools of Rab proteins may have different roles in a temporal context: a rapid one that responds to the instant requirements of a specific pathway, and an extended one that sustains active transport. However, the molecular regulatory mechanism that controls the internal pool versus newly synthesised Rab is not well understood.

Another potential explanation of our results, which revealed decreased levels of Rab protein in abalone fed a probiotic-supplemented diet, may relate to the characteristics of this protein. Similar to other GTPases, Rab proteins act as molecular switches, cycling between an inactive form in the cytosol to an active form in the membrane (Figure 4.5). During the activation process, pre-existing cytosolic Rab-GDP (guanine diphosphate)-GDI (GDP displacement inhibitor) complex is dissociated, and the GDI is removed to allow Rab-GDP to attach to the cell membrane. In the membrane, Rab is activated by exchanging the GDP with GTP (guanine triphosphate) that is catalysed by guanine nucleotide exchange factors (GEFs). Thus, Rab-GTP forms a reversible complex with other proteins, meaning Rab-GTP-effector (Rojas et al., 2012). After Rab-GTP-effector has performed its function, Rab is recycled to the cytosol. In the membrane, Rab-GTP is deactivated to Rab-GDP by GTPase activating proteins (GAPs) that hydrolyse the GTP. Cytosolic GDI recovers the Rab protein to form Rab-GDP-GDI complex, which is the inactive form (Barr and Lambright, 2010; Itzen and Goody, 2011; Rojas et al., 2012; Zerial and McBride, 2001). A more detailed explanation of how Rab proteins are

activated and deactivated in cells can be found in several reports (Grosshans et al., 2006; Langford et al., 2002; Peter et al., 1994; Saxena and Kaur, 2006).

Considering the current knowledge regarding Rab activation/deactivation and our results, we speculate that the *H. midae* immune system is stimulated in response to the probiotic-supplemented diet, resulting in the activation of Rab 1A. If the active form of Rab (Rab-GTP-effector) was not detected in our samples by the antibody, it would appear that Rab was down-regulated over the course of the experiment. In other words, it may be that the antibody used in our analysis only recognised Rab 1A in its inactive form (before binding to other molecules or effectors). This explanation is supported by Stein et al. (2003), who reported that the interaction between Rab and its effector proteins may result in post-translational modifications while Vernoud et al. (2003) found that Rab association with membranes is promoted by post-translational lipid modification. Indeed, the reversible localization of Rab is dependent on the post-translational modification of a cysteine motif at the carboxyl terminus of the protein (Stenmark et al., 2001). There is also a growing indication that the transcriptional rate of Rab gene expression changes during immune activation, wherein the cells require higher levels of this protein (Pei et al., 2012). Therefore, we postulate that Rab 1A in *H. midae* haemocytes undergoes structural changes that cause the protein not to be recognised by the antibody. However, this needs to be confirmed in future work in which Rab and its effector proteins are investigated in haemocytes of *H. midae* in a similar experiment.

Although researchers state that the inactive form of Rab is localized in the cytosol and that Rab becomes activated and localized mostly in membranes following a specific stimulus (Barr, 2013; Fukuda, 2008; Szatmári and Sass, 2014), our results show that Rab protein levels were similar in both the cytosolic and membrane fractions of *H. midae* haemocytes at time 0 (control samples). Our results may be supported by Chavrier et al. (1990), who mentioned that each membrane compartment in the cytoplasm is likely to be associated with distinct Rab proteins. Indeed, according to Pfeffer (2012), Rab proteins in the cytosolic pool represent half of the total Rab proteins. Therefore, Rab will probably always occur in membrane samples. Although inactive Rab protein can occur in the membrane as Rab-GDP (Figure 4.5), it is not capable of interacting with effector proteins unless it converts to the active form (Rab-GTP). According to Scheffzek and Ahmadian (2005), the active and inactive forms of Rab differ in the presence or absence of γ-phosphate in one of the structural terminals; and this terminal is involved in the

interaction between the protein and its effectors. Theoretically, Rab proteins can only interact productively with downstream effectors when bound to GTP (Donovan et al., 2002).

Different effectors determine the function of the Rab complex, such as vesicle motility, tethering and fusion (Rojas et al. 2012), that acts in the cytoplasmic leaflet of the membrane (Seabra et al., 2002; Yaneva and Niehaus, 2005; Zerial and McBride, 2001); these effectors are diverse kind of proteins, such as coiled-coil proteins involved in membrane tethering or docking, while others are enzymes or cytoskeleton-associated proteins. Gillingham et al. (2014) and Hutagalung and Novick (2011) describe some of the effectors and diseases in which Rab protein plays a crucial role. Therefore, Rab is capable of regulating several molecular events (Jain et al., 2012).

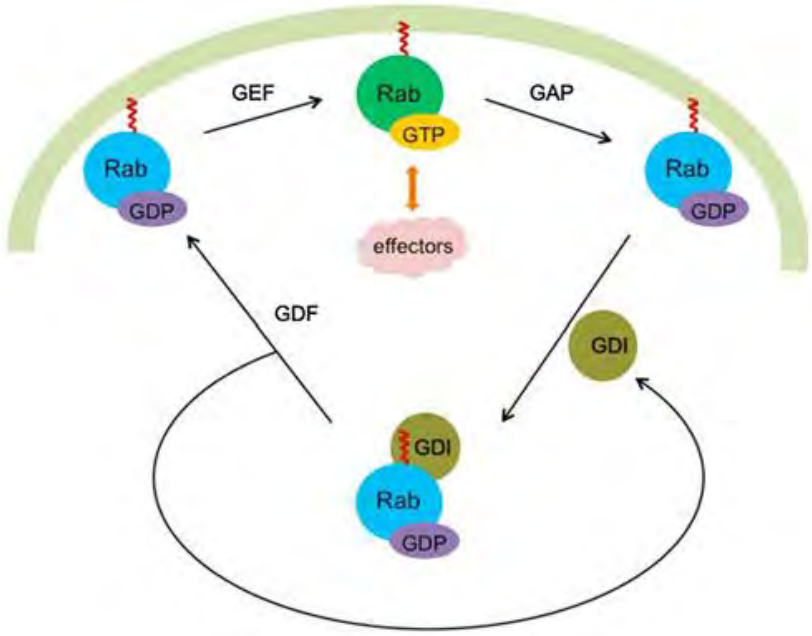


Figure 4.5. The inactive form of Rab is located in the cytosol (Rab-GDP-GDI), and the active form is located in membrane (Rab-GTP-effector). GEF and GAP mediate Rab switching between the two forms. Image adapted from Liu and Storrie (2012).

There are some indications that Rab proteins may be directly linked to the membrane cytoskeleton (Jordens et al., 2005). Indeed, Rab plays a crucial role in membrane trafficking (Barr, 2013; Stenmark, 2009). Therefore, we used immunochemistry to investigate Rab 1A *in situ*. We found no specific evidence of co-localization between the F-actin (actin cytoskeleton

that interacts with the cell membrane) of *H. midae* haemocytes and Rab 1A; however, expression of this protein was reduced in haemocyte cells sampled from abalone fed a probiotic-supplemented diet. This result supports our previous finding using western blot and iTRAQ analysis (Chapter 2).

Since we could not find any evident association between Rab 1A and F-actin during the experiment (12 hours), we suggest that future work investigates specific molecular effectors of Rab 1A protein to establish whether there is any possible translocation of Rab within the cell. According to Zerial and Stenmark (1993) and Tisdale et al. (1992), Rab 1A transports newly synthesised proteins from the endoplasmic reticulum to the Golgi complex. The vesicle docking protein p115, Golgi matrix protein (GM130), giantin, Golgi reassembly and stacking protein 65 (GRASP65), golgin-84, component of oligomeric Golgi complex 6 (COG6), Oculocerebrorenal syndrome of lowe (OCRL1), microtubule associated monooxygenase, calponin and LIM domain Containing 1 (MICAL-1), inositol polyphosphate-5-phosphatase B (INPP5B) are known effectors of Rab 1 protein (Liu and Storrie, 2012). Among the proteins mentioned above, p115, GM130 and GRASP65 have been reported to function between the endoplasmic reticulum and Golgi complex (Allan et al., 2000; Alvarez et al., 2001; Sannerud et al., 2006; Zerial and McBride, 2001). For instance, Rab 1 recruits the tethering factor p115 onto coat protein (COP) II vesicles budding from the endoplasmic reticulum (donor compartment) to the Golgi complex (receptor compartment); apart from this, Rab possibly performs other functions during the transition between cytosolic and membrane location (Mizuno-Yamasaki et al., 2012; Moyer et al., 2001).

Understanding the activity of cellular vesicles in this type of study is important, as it may provide new insight into the effect of probiotics on Rab activity in *H. midae* haemocytes. According to Shandala and Brooks (2012), Rab proteins function to control vesicular compartments and transport target vesicles and their internal cargo to specific cellular destinations, including the plasma membrane. All membranes of endocytic and exocytic vesicles present different Rab proteins, which serve to distinguish the different types of vesicles (Csépányi-Kömi et al., 2012). Since endocytic trafficking is central to normal cellular physiology, Rab and associated proteins are a critical regulator of endocytosis, a fundamental cellular process required for the uptake and intracellular transport of macromolecules (Chen et al., 2009; Stein et al., 2003). The role of Rab in exocytic, endocytic and intracellular trafficking

explains their importance in the immune system since it counteracts pathogenic bacteria and viral infection (Corbeel and Freson, 2008). Rab proteins are also the central node of the machinery that regulates trafficking of organelles, including phagosomes. The dynamic and complex process of phagosome maturation is the result of multiple interactions between the phagosome and various intracellular compartments (Griffiths and Mayorga, 2007; Gutierrez, 2013). The importance of Rab protein in phagosome activity was discussed in Chapter 3; wherein phagosome maturation was identified as the most relevant canonical pathway within the protein data set.

In summary, we found that Rab 1A was highly expressed both in membrane and cytosolic fractions from haemocytes sampled from *H. midae* fed a probiotic-supplemented diet. Rab 1A was down-regulated in the membrane and cytosolic haemocyte fractions obtained at 6, 12 and 18 hours after abalone had been provided with the probiotic-supplemented diet. We postulate that the probiotic-supplemented diet mediated activation of Rab 1A (from the Rab-GDP form to the Rab-GTP form), facilitating the interaction of this protein with effector proteins. Alternatively, the probiotic bacteria may have induced a post-translational modification of Rab 1A that prevented detection of the protein by the antibody in the western blot assay, leading to the erroneous conclusion that Rab 1A was down-regulated. Our hypothesis that Rab 1A was co-localized in the haemocyte membrane-cytoskeleton was not confirmed by immunochemistry. Further studies into Rab 1A and its effector proteins may elucidate the mechanism of action of this protein in *H. midae* haemocytes. Additionally, investigation of transport processes from the endoplasmic reticulum to the Golgi complex, phagosome maturation and vesicle activity may provide greater understanding of Rab 1A activity and function in *H. midae* fed a probiotic-supplemented diet.

CHAPTER 5 – GENERAL DISCUSSION AND FUTURE WORK

5.1. SYNTHESIS OF THE STUDY

Farming of the abalone, *H. midae*, is the largest marine aquaculture subsector in South Africa (Halley and Semoli, 2010). Due to the ecominic importance of this industry to the country, much research has been conducted in an attempt to understand the key requirements for optimal abalone production (Bolton et al., 2013). Obstacles encountered by those involved in this sector range from slow growth rates of abalone to poor water quality and disease outbreaks (van der Merwe et al., 2011). In recent years, disease outbreaks and high mortality have been the major concerns for abalone farmers. As such, much effort is being put into understanding the abalone immune system in order to develop improved farming techniques. In this regard some novel mechanisms have been discovered, and this is in part due to the current popularity of the application of –omics technologies that address fundamental and applied research questions related to genetic material, gene expression, protein expression, and low molecular weight metabolites (Gómez-Chiarri et al., 2015).

Recent advances in molecular techniques and studies, such as the improvement of genome databases for a number of species, development of mass spectrometry-based proteomics and the improvement and diversification of bioinformatics tools, have yielded new mechanisms for the investigation of –omics studies. For instance, the availability of molecular sequences, from genes and proteins, has transformed both the theory and practice of experimental biology (Ashburner et al., 2000).

However, protein identification using a mass spectrometry-based approach generally requires a sequenced and annotated genome of the species under investigation (Romero-Rodriguez et al., 2014). Although some species already have their genome fully sequenced, especially model species, several others are still not fully sequenced, particularly invertebrate organisms. Even without a full genome sequence, molecular research can still be performed in those species based on homologue and orthologue sequences from closely related species (Gómez-Chiarri et al., 2015). Unlike gene sequences, which rarely match between species, protein sequences are more conserved, and are more likely to be similar among closely related species. This makes high-throughput identification of proteins from non-model organisms more efficient. In this regard, a proteomics approach is a worthy consideration for the study of non-model species (Carpentier et al., 2008).

The molecular techniques mentioned above are useful for the annotation of protein descriptions and biological functions, as well as predicting the molecular pathways and networks involving target proteins. Therefore, we took this opportunity to investigate proteome changes when *H. midae* was immune stimulated with a probiotic-supplemented diet. In this regard, the study aimed to: (i) identify and quantify all proteins expressed in *H. midae* haemocytes using a large-scale proteomics approach (iTRAQ coupled with LC-MS/MS); thereafter, annotate them, distinguish differentially expressed proteins, and validate the differentially expressed proteins using an orthogonal approach; (ii) describe the biological function of the proteins identified using bioinformatics tools; thereafter, identify groups of proteins that presented similar immune-related classes over the course of the experiment, and predict the interaction networks and molecular pathways involving the differentially expressed proteins; and (iii) characterise a protein that is most suitable for inclusion in future work intending to improve the understanding of the probiotic effect on the *H. midae* immune system.

Initially, the two probiotic strains used in this research, *Vibrio midae* SY9 and *Debaryomyces hansenii* AY1, were cultured and incorporated into a kelp-based feed. Incorporation was at a level previously shown to stimulate the immune system of this species of abalone as discussed by Macey and Coyne (2005). Several studies have investigated the effect of probiotics in host immune systems over time, ranging from a few hours to approximately 14 days. This study focused on the first hours of probiotic feeding; hence, we analysed protein samples during the initial 36 hours of host exposure to a probiotic-supplemented diet (Chapter 2).

One of the ways of assessing the status of the abalone immune system is to conduct an analysis of the level of phagocytic activity of the circulating haemocytes. The assessment of phagocytic activity during host immune studies has been used widely in aquaculture research. In this study, the level of phagocytic activity of the circulating haemocytes showed an increase at six hours post abalone feeding of a probiotic-supplemented diet. While phagocytosis may be considered as merely a mechanism of waste disposal, an alternate description is that its function is to generate the phagosomes, organelles that are required for the effective initiation of host defense and other crucial homeostatic processes (Stuart and Ezekowitz, 2008). Phagocytic cells are fundamental to the innate defense mechanism in both invertebrate and vertebrate organisms (Roth and Kurtz, 2009). Interestingly, some of the differentially expressed proteins identified in this study (Ras-related protein and V^{+}ATPase) have been reported to play an important role

in the membrane activity and phagosome maturation pathway, which are processes related to phagocytosis; and this is discussed further in this chapter.

As the timing of probiotic stimulation of the abalone immune system was determined with the help of the phagocytosis assay, iTRAQ analysis, a mass spectrometry-based proteomics protocol, was employed to investigate the protein profiles of haemocyte samples taken over a 36 hour period and to evaluate whether there was any variation in the haemocyte proteome of abalone fed a probiotic-supplemented diet. Among the several proteins identified in this study, hemocyanins and Ras-related proteins were the most abundant and over-represented. Some key findings were the identification of proteins such as calmodulin 2 and heat shock protein 70 that were up-regulated at all sampling time-points. Other proteins such as regulatory of differentiation 1 and septin-2 showed early up-regulation only in the first sampling time-point, and their expression levels then returned to those recorded at the control time-point (0 hour sample).

The above findings emphasise that the proteome is complex and dynamic, and confirm that each protein may behave differently. Therefore, the above-mentioned proteins may be included in future studies to increase our understanding of the abalone haemocyte proteome with regard to the immune system of this mollusc. Additionally, special attention was given to the following proteins as they were differentially expressed when abalone were fed a probiotic-supplemented diet: annexin, COP9 signalosome complex subunit 4, phosphorylase, T-complex protein 1 subunit gamma, V-type proton ATPase subunit B, Rab 1 and Ras-related protein Rab 1A. Among these proteins, COP9 signalosome subunit 4 and Ras-related protein 1A were successfully validated using western blot analysis, an orthogonal proteomic method.

Although the validation process of the iTRAQ analysis confirmed the quantitative and qualitative expression of the target proteins, it is important to mention that some issues arose during this validation process. The first issue pertained to the unavailability of specific antibodies with which to analyse abalone protein samples. Therefore, antibodies raised against human proteins, which showed high homology to our target protein, were purchased. The target protein detected with the anti-COP9 antibody had an observed molecular mass that differed to the predicted size of COP9 signalosome subunit 4. However, further two-dimensional SDS-PAGE and protein spot identification by LC-MS/MS confirmed that the identified protein

identity was indeed COP9 signalosome subunit 4. The validation of proteins from non-model organisms using immunoblot techniques is sometimes hampered by the lack of species-specific antibodies (Glückmann et al., 2007), resulting in an inability to detect target proteins, binding to proteins with an unusual molecular mass or to different protein, and binding to several proteins or isoforms at the same time (as noted during the validation of V^{+}ATPase subunit B). On the other hand, the advantage of western blot analysis is that it elucidates the physicochemical characteristics of a protein, specifically the molecular mass and immuno-affinity (Ghosh et al., 2014; Hughes and Herr, 2012). In this regard, the use of western blots for validation of mass spectrometry data reduces false positive results between on-target and off-target signals (Hughes et al., 2014). It is for these reasons that several studies have used western blot techniques in an attempt to complement and corroborate their results (Brackley and Grantham, 2010; Kodippili et al., 2014; Mamali et al., 2009; Zoppino et al., 2010).

Nevertheless, the identification and validation of the putative Ras-related protein Rab 1A is the most significant achievement arising from this study. The expression of this protein in haemocytes was found to be affected when abalone were fed a probiotic-supplemented diet, in that it was down-regulated during the entire experiment. This pattern was confirmed by both iTRAQ analysis and the western blot assay. Ras-related proteins are known mainly for their central role in membrane trafficking; additionally, several studies have recognised their importance in immune activity and defense response against pathogenic microorganisms (Barr, 2013; Jordens et al., 2005; Pereira-Leal and Seabra, 2000; Stein et al., 2003).

Due to the role that Ras-related proteins plays in immune activity, the Ras-related protein 1A was further investigated in Chapter 4. The protein was found to be present both in the membrane and in the cytosol fractions of the abalone haemocyte, and the probiotic-supplemented diet fed to the abalone seemed to mediate a reduction of Ras-related protein 1A in both haemocyte fractions. This finding is quite novel and unexpected. According to the literature, host immune stimulation likely induces the activation of members of Ras-related proteins, causing their attachment to the membrane and, consequently, increasing its expression in the membrane fraction (Barr, 2013; Fukuda, 2008; Szatmári and Sass, 2014).

Contrary to our expectation, the expression of Ras-related protein was down-regulated in the membrane fraction of haemocytes sampled from abalone fed the probiotic-supplemented diet.

We speculate that during probiotic-induced immune stimulation, Ras-related protein Rab 1A was translocated from the cytosol to the membranes in order to interact with its effector proteins. This interaction possibly resulted in a post-translational modification that prevented recognition of the protein by the anti-Rab antibody during the western blot assay. A possible explanation of the iTRAQ analysis, which also showed that this protein is down-regulated, could be that only soluble proteins were evaluated by iTRAQ LC-MS/MS. Thus, iTRAQ analysis of haemocyte membrane proteins must be carried out in order to confirm our hypothesis that the Ras-related proteins Rab 1A is translocated from the cytosol to the membrane when abalone are stimulated with probiotics supplied through the diet.

The other aim of this study was to use bioinformatics tools to analyse the abalone haemocyte proteome data and identify groups of proteins showing similar expression patterns, especially with regard to those that belonged to immune-related GO terms. Of the 12 sub-groups (sub-clusters) identified, only four were catalogued to specific immune-related GO terms. Some of the immune-related GO terms were response to stimulus, endocytosis, stress response, cell adhesion, defense response to bacteria, and apoptosis. The aggregation of these immune-related GO terms in specific sub-groups of proteins confirmed our hypothesis that proteins with similar functions may be regulated mutually and, therefore, may be identified within the same sub-groups during an immune stimulation.

Functional analysis of the protein-protein interactions of the differentially expressed abalone haemocyte proteins mapped the target proteins to a single protein interaction network, supporting the supposition that these proteins are functionally related. Interestingly, neurotrophic tyrosine kinase receptor (NTRK) seemed to interact directly with most of the target proteins. According to Terry et al. (2011), NTRK signalling in human lungs promoted cell survival and proliferation, whereas inhibition of NTRK signalling induced apoptosis and reduced clonogenicity. Although NTRK is a protein that is reported to be present in vertebrates (Benito-Gutiérrez et al., 2006; Braunger et al., 2014), our results suggest that this protein may have a homologue in invertebrates as well. This information may provide new insight to the existing knowledge of abalone immune system proteins. However, our hypothesis needs to be confirmed with further work. On the other hand, when the differentially expressed proteins were analysed in order to determine which biochemical pathway they are mostly related to, phagosome maturation was the most significantly canonical pathway identified, with members

of Ras-related protein Rab and V^+ATPases playing a crucial role in this pathway. Again, these results confirm the importance of members of the small GTPases family (Ras-related proteins) in the abalone immune system. Nevertheless, these results should be interpreted as the first overview of the putative proteins, interaction networks and molecular pathways affected by the probiotic-supplemented diet provided to farmed *H. midae*.

5.1.1. Limitations of this study and suggestions for further research

One of the main restrictive factors of this study was the lack of full genome sequences from *Haliotis sp* – thus resulting in several unidentified peptide spectra and, consequently, uncharacterised proteins. Therefore, future research should take into account the unidentified proteins from this study that were differentially expressed, as new updates may possibly be made to the molluscan database, which could identify more proteins affected by probiotic immune stimulation in abalone and probably address the technical gaps encountered in this study.

There are no commercially available antibodies that have been raised specifically against abalone proteins. As a result, we had to use polyclonal antibodies that were raised against human homologues of the abalone proteins; hence, only three out of seven proteins could be validated. Although the antibodies used in this study presented high homology with our target proteins, we encountered some issues during the validation process, such as the lack of specificity to the target proteins. Accordingly, in future work, a greater effort must be made to confirm cross-reactivity between commercially available antibodies and a number of the other proteins identified in this study, especially those that showed high fold changes, as the validation component of this study only focussed on the statistically significant, differentially expressed proteins.

Even though we were able to use bioinformatics to elucidate the interaction network and molecular pathways involving differentially expressed haemocyte proteins from abalone fed a probiotic-supplemented diet, there were limitations imposed on our ability to analyse the entire data set of identified abalone proteins, as human orthologues of some of the abalone proteins

were absent from protein databases. Hence, it was not possible to determine their putative cellular function. In this regard, future work will need to include a greater effort in functionally characterising the proteins identified in abalone haemocytes in order to enhance the information regarding invertebrate proteins in global protein databases, particularly with regard to molecular interaction networks and cellular biochemical pathways.

While approximately five hundred proteins were identified in this study, roughly more than one hundred proteins were common to the two independent experiments performed and the common proteins were, consequently, the focus of this study. Thus, future work should focus on those proteins that were identified in only one experiment (especially those that showed high fold changes), as they may provide new insights into the effect of a probiotic-supplemented diet on the abalone immune system. However, accurate validation of the data must be possible in order to have full confidence in the identification of these target proteins.

Several proteins such as calmodulin 2, peroxiredoxin-1, serine/threonine-protein phosphatase and Ras-related protein Rab 14 showed high fold changes in comparison to the control sampling time-point. However, further statistical analysis indicated that the differences were not statistically significant. Nevertheless, we hypothesise that this biological variation may be important to our understanding of the changes that occur in the abalone haemocyte proteome during a probiotic-feeding experiment. Therefore, in further studies, alternate proteomics approaches should be used to investigate the expression of these proteins in similar experiments.

Ras-related protein Rab was identified as a protein that should be included in further studies aimed at increasing our understanding of the effect of a probiotic-supplemented diet on the abalone haemocyte proteome. Therefore, future studies should investigate the expression of this protein in stressed abalone and in abalone challenged with pathogenic bacterium. This is supported by several reports that have mentioned the involvement of Ras-related protein Rab in the immune activity of some invertebrates during pathogenic infection (Wang et al., 2013b; Wu and Zhang, 2007; Zong et al., 2008).

5.1.2. Conclusion

The main objectives of this study were met since (i) quantification of the proteins identified in haemocytes from *H. midae* fed a probiotic-supplemented diet using iTRAQ analysis was successful. Although several proteins seemed to be regulated over the course of the experiment, seven proteins were statistically differentially expressed and only three proteins (COP 9 signalosome subunit 4, Ras-related protein Rab A1 and V^{+}ATPase subunit B) out of the seven were qualitatively and quantitatively validated by western blot, due to the unavailability of suitable antibodies; (ii) further bioinformatics analysis revealed that those proteins that presented similar expression patterns showed related biological functions, and the differentially expressed proteins are likely to interact and participate in related immune activities; (iii) and the characterisation of Ras-related protein A1 showed that this protein is an appropriate candidate for further studies aimed at increasing our understanding of the abalone immune system.

Several novel hypotheses were suggested in this research that still need to be addressed in order to gain a better understanding of the proteome variation in haemocytes of *H. midae* fed a probiotic-supplemented diet. This may improve abalone farming practices and help abalone farmers' better deal with stress and diseases affecting farmed *H. midae*, ultimately contributing to the sustainability of the abalone industry. To the best of our knowledge, this is the first molecular investigation of the abalone immune response during host stimulation with a probiotic-supplemented diet. Therefore, this study unlocks new avenues for future studies on the invertebrate immune system using a mass spectrometry-based approach which will increase our understanding of the proteome and the effect of probiotics in cultured invertebrate species.

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APPENDIX A – MEDIA AND SOLUTIONS

The glassware used to prepare all media, solutions and buffers was washed in distilled water and autoclaved at 121 ^{0}C for 40 min prior to use. Distilled water was purified using a Milli-RO Plus (Millipore) water purification system. To sterilise purified water, it was autoclaved at 121 ^{0}C for 40 min before use. Ultrapure water for all mass spectrometry-based experiments was obtained by further purifying distilled water using a Milli-Q Plus (Millipore) water purification system. After preparation, all media, solutions and buffers were autoclaved at 121 ^{0}C for 40 min, before being stored at room temperature, unless otherwise stated. The list of the following media, solutions and buffers is presented according to their sequence of mention in this thesis.

A.1. Media

A.1.1. Tryptone soya broth (TSB), pH 7.3

Tryptone casein (pancreatic)	17 g
Dipotassium hydrogen phosphate (K_2HPO_4)	2.5 g
Glucose Monohydrated	2.5 g
Sodium Chloride	30 g
Soya peptone (Peptone powder)	3 g
Make in distilled water to	1000 ml (and autoclave)

For TSB agar add 20g Bacteriological agar

A.1.2. Yeast peptone D-glucose (YPD) broth, pH 7.3

Glucose monohydrate	20 g
Yeast extract	10 g
Peptone	20 g
Make in distilled water to	1000 ml (and autoclave)

YPD agar, add of 20 g Bacteriological agar before autoclave.

A.2. Solutions

A.2.1. Sea salt solution (SSS)

NaCl	30.0 g
$MgCl_2.6H_2O$	2.3 g
KCl	0.3 g
Make in distilled water to	1000 ml (and autoclave)

A.2.2. Modified Hank´s Balance Salt Solution (MHBSS), pH 7.2

Glucose Monohydrated	10.4 g
NaCl	11.2 g
KCl	0.41 g
KH_2PO_4	0.1 g
$CaCl_2$	0.355 g
$MgCl_2$	1.31 g
$MgSO_4$	1.57 g
EGTA (Sigma)	0.015 g
Make in distilled water to	500 ml (and autoclave)

A.2.3. 50% Acetic acid

100% Glacial acetic acid	500 ml
Make in distilled water to	1000 ml

Do not autoclave

A.2.4. 0.1 M $NaHCO_3$, pH 9.0

$NaHCO_3$	0.84 g
Water to	100 ml (and autoclave)

A.2.5. 0.1 M Fluorescein 5-isothiocyanate, isomer 1 (FITC), Sigma

FITC	0.5 g
0.1 M $NaHCO_3$	5 ml

A.2.6. 10x Phosphate buffered saline (PBS), pH 7.4

NaCl	87.0 g
$Na_2 HPO_4$ (Merck)	22.5 g
KH_2PO_4	2.0 g
Make in distilled water to	1000 ml (and autoclave)

Dilute 10x stock solution to 1x working concentration in autoclaved distilled water

A.2.7. 100 µg.ml^{-1} Ethidium bromide

Ethidium bromide (Sigma)	1 mg
Make in PBS to	10 ml

Shake well to dissolve. Caution, Ethidium bromide is a powerful mutagen and gloves should be worn at all times when handling the solution.

A.2.8. 0.5 M Triethylammonium bicarbonate (TEAB) Sigma

1 M TEAB	10 ml
Make in Ultrapure water to	20 ml

Make up fresh before every use.

A.2.9. Lysis buffer

Sodium dodecyl sulfate (SDS) (Merck)	100 mg
Make in 0.5 M TEAB to	10 ml

Make up fresh before every use.

A.2.10. 0.5 M Tris(2-carboxyethyl)phosphine (TCEP)

1 M TCEP	5 ml
Make in Ultrapure water to	5 ml

Aliquot into 1 ml fractions into Eppendorf tubes and store at -20 ºC.

A.2.11. 0.2 M Methyl-methanethiosulfonate (MMTS) Fluka

1 M MMTS	20 µl
Make in 100 % isopropanol (MS-grade, Fluka) to	100 ml

Aliquot out into 20 µl fractions into sterile Eppendorf tubes and store at -20^{0}C.

A.2.12. UT Buffer

Urea	4.8 g
Make in 0.5 M TEAB to	10 ml

Make up fresh before use.

A.2.13. UT Buffer – 0.15 M MMTS

UT Buffer	92.5 µl
0.2 M MMTS	7.5 µl

Make up fresh before use.

A.2.14. Trypsin Gold

Trypsin Gold (Promega)

50 mM Acetic acid (v/v)

Reconstitute trypsin lyophilised powder in 50 mM (v/v) acetic acid to a concentration of 2 µg.µl^{-1} and aliquot 20 µl fractions into sterile Eppendorf tubes. Store at -80 ^{o}C. Thaw on ice prior to use.

A.2.15. 5x Sample application buffer (SAB)

Glycerol	250 µl
Tris-HCl, pH 6.8	250 µl
DTT (1,4-Dithiothreitol)	0.1 g
SDS	50 mg
Bromophenol blue (grain)	tips

Store at -20 ^{o}C.

A.2.16. 4 % Stacking gel

Tris-Cl/SDS, pH 6.8	0.5 ml
40 % Acrylamide	0.4 ml
10 % AMPS	25 µl
TEMED (last to be added)	2 µl
Make in sterile water	3.073 ml

Store at 4 ^{o}C.

A.2.16.1. Tris-Cl/SDS, pH 6.8

Tris	6.05 g
SDS	0.4 g
Make in sterile water to	100 ml

Filter sterilize (0.45 µm). Store at 4 ^{o}C.

A.2.16.2. 10 % AMPS (Ammonium PerSulphate)

AMPS	0.1 g
Make in sterile water to	1 ml

Store at 4 ^{o}C.

A.2.17. 12 % Polyacrylamide separating gel

Tris-Cl/SDS, pH 8.8	2.5 ml
Acrylamide (40 %)	2.49 ml
10 % AMPS	50 µl
TEMED (last to be added)	10 µl
Make in sterile water	4.95 ml

Store at 4 ^{o}C.

A.2.17.1. Tris-Cl/SDS, pH 8.8

Tris	91 g
SDS	2 g
Make in sterile water to	500 ml

Filter sterilize (0.45 µm). Store at 4 ^{o}C.

A.2.18. 5x SDS electrophoresis buffer

Tris	15.1 g
Glycine	72 g
SDS	5 g
Make in water to	1000 ml

Dilute in water 1 in 5 to make 1x SDS electrophoresis buffer.

A.2.19. Towbin buffer

Tris	6.06 g
Glycine	28.84 g
Methanol (20% in total volume)	400 ml
Make in water	2000 ml

Store at 4 ^{o}C.

A.2.20. Ponceau S stain

Ponceaus powder	0.1 g
Glacial acetic acid	5 ml
Make in sterile water	100 ml

Light sensitive. Re-usable.

A.2.21. 10x Tris buffered saline (TSB), pH 7.5

Tris	2.5 ml
NaCl	2.49 ml
Make in water to	500 ml

Autoclave. Dilute in water 1 in 10 to make 1x TBS

A.2.22. Blocking buffer

Skim milk powder	5 g
Make in TSB to	100 ml

A.2.23. Primary antibodies solution (rose in rabbit)

A.2.23.1. Anti-COP 9 signalosome subunit 4 – 1:500

Anti-COP 9 signalosome subunit 4	20 µl
Make in blocking buffer to	10 ml

A.2.23.2. Anti-Ras-related protein Rab 1A – 1:250

Anti-Ras-related protein Rab 1A	40 µl
Make in blocking buffer to	10 ml

To make 1:100, use 0.4 µl of the antibody and make in blocking buffer to 200 µl.

A.2.23.3. Anti-V+ATPase subunit B – 1:500

Anti-V+ATPase subunit B	20 µl
Make in blocking buffer to	10 ml

Store at -20 °C. Defrost on ice before use. Re-usable, but discard when hybridisation signal begins to fade.

A.2.24. Tris buffered saline – Tween (TSBT)

1x TSB	190 ml
Tween 20	100 µl

A.2.25. Secondary antibody solution

A.2.25.1. Anti-rabbit secondary antibody solution – 1:10 000

Goat anti-rabbit (peroxidase conjugate)	1 µl
Make in blocking buffer to	10 ml

A.2.25.2. Anti-rabbit secondary antibody solution – 2:10 000

Goat anti-rabbit (peroxidase conjugate)	2 µl
Make in blocking buffer to	10 ml

Store at -20 °C. Defrost on ice before use. Re-usable, but discard when hybridisation signal begins to fade

A.2.26. 80 % Acetone

Acetone	100 ml
Make in water to	500 µl

A.2.27.Urea lysis buffer (ULB)

Urea	24 g
Thiourea	7.612 g
CHAPS	1 g
Make in water to	50 ml

Heat to aid dissolving. Aliquot out into Eppendorf tubes store at -20°C.

A.2.28. Rehydration solution

Protein	100 ml
DTT	0.5 g
Ampholytes	1.35 µl
ULB	6.65 µl
Bromophenol blue	Tip

A.2.29. Equilibration buffer 1

Equilibration buffer stock solution	5 ml
DTT	50 g
Bromophenol blue	Tip

Prepare immediately before use. Vortex well prior to use.

A.2.29.1. Equilibration buffer stock solution

Urea	18.02 g
SDS	1 g
Glycerol	15 ml
Make in Tris-Cl, pH 8.8 to	50 ml

A.2.29.1.1. Tris-Cl, pH 8.8

Tris	90.83 g
Make in water to	500 ml

A.2.30. Equilibration buffer 2

Equilibration buffer stock solution	5 ml
IAA	0.24 g
Bromophenol blue	Tip

Make up fresh before use. Vortex well prior to use.

A.2.31. 0.5 % Agarose in 1x SDS electrophoresis buffer

Agarose (Lonza)	0.5 g
1x SDS electrophoresis buffer	100 ml
Bromophenol blue	Tip

Heat to aid dissolving. Prior to use, re-melt to about 40 ^{o}C before use.

A.2.32. Coomassie blue R250

Coomassie blue R250	0.5 g
Methanol	225 ml
Glacial acetic acid	50 ml
Make in water	225 ml

A.2.33. Destaining solution

Methanol	300 ml
Glacial acetic acid	100 ml
Make in water to	1000 ml

A.2.34. 2 % Paraformaldehyde

Paraformaldehyde (Sigma)	2 g
Make in PBS	100 ml

Dissolve paraformaldehyde powder in 80 ml of PBS on a heated stirring plate set at 70 ^{o}C for approximately 20 – 30 minutes. Some drops of 1M NaOH may help the solution to become completely clear). Allow to cool to room temperature, just adjust the pH to 7.4 (pH strips), and make up to final a volume of 100 ml with PBS. Filter sterilize and store at 4 ^{o}C (light sensitive) for up to 2 weeks.

A.2.35. Permeabilization solution – Triton X-100

Triton X-100	0.5 g
PBS	1000 ml

A.2.36. Blocking solution – Triton X-100

BSA	1 g
Triton X-100	100 µl
PBS	100 ml

A.2.37. Secondary antibody solution for immunochemistry assay – 1:100

Anti-rabbit conjugated to fluorophores ALEXA 488	1 µl
Make in blocking solution to	500 µl

Keep in 4 ^{o}C. Light sensitive.

A.2.38. Hoechst Nuclear stain (1:5000)

Hoechst Nuclear stain

PBS

Hoechst Nuclear stain stock at a concentration of 0.5 $\mu g.ml^{-1}$ was diluted to a working concentration of 1:5000 in PBS. Light sensitive.

A.2.39. 100 nM Rhodamine Phalloidin – cytoskeleton stain (F-actin)

Rhodamine phalloidin

Methanol

PBS

Reconstitute Rhodamine phalloidin (Cytoskeleton, Cat. # PHDR1) in 500 µl of 100% methanol to a final 14 µM stock concentration. Aliquot into 50 µl amounts and store at -20 ^{0}C (up to 6 months). Light sensitive. Prior to use, dilute 3.5 µl of the 14 µM labelled stock into 500 µl of PBS to make a working concentration of 100 µM.

A.2.40. Mowiol working stock

Mowiol

anti-fading agent (n-propylgallate, Sigma),

Thaw 1 – 5 ml of mowiol (in an Epperndorf) at room temperature and, add some (tip of small spatula) anti-fading agent, warm to 50 ^{o}C for 1 hour and the invert tube frequently to dissolve anti-fading agent. Centrifuge to sediment any remaining particles and to remove air bubbles. The final mixture is stable for several weeks at 4 ^{o}C. Light sensitive. Do not use mowiol once the mixture has turned a yellowish colour.

A.2.41. 25 mM ABC/50 % ACN

50 mM ABC	2.5 ml
100 % ACN (Acetonitrile)	2.5 ml

A.2.41.1. 50 mM ABC (Ammonium bicarbonate)

ABC	80 mg
Make in Ultrapure water to	20 ml

A.2.42. 10 mM DTT/ABC

DTT	10 mg
25 mM ABC	6.67 ml

Make up fresh before use.

A.2.42.1. 25 mM ABC

50 mM ABC	10 ml
Make in Ultrapure water to	20 ml

A.2.43. 55 mM IAA/ABC

IAA (Iodoacetamide)	50 mg
25 mM ABC	5 ml

Make up fresh before use.

A.2.44. Trypsin buffer for protein in gel digestion

Trypsin vial (20 µg)

Re-suspension solution

Dissolve a single vial of trypsin in 200 µl of the re-suspension solution provided in the trypsin kit. Divide into aliquots of 20 µl each in Eppendorf tubes and store at -80 ^{o}C. The re-suspension buffer has a pH less than 3. To activate trypsin prior to use the pH must be raised to 8 and temperature to above 30 ^{o}C. Once activated, trypsin must be discarded after use.

A.2.45. 70 % ACN

100 % ACN	1400 µl
Make in Ultrapure water to	2 ml

APPENDIX B – RECIPES AND STANDARD METHODS

B.1. Recipes

B.1.1. Kelp cakes

Allow 100 g of dried kelp (*Ecklomia maxima*) to swell overnight in 1000 ml of SSS (Appendix A.2.1). Mix the following components:

Dried Kelps (Kelpak)	300 g (wet)
Agar	15 g
Make in SSS to	1000 ml

Mix the three components and autoclave for 20 min at 121 ºC. Then cool (50 ºC) and pour into petri dishes. For **kelp cakes supplemented with probiotics**, harvest 4 l of each of the strains, concentrate using a centrifuge and add in an autoclaved and cooled kelp cake mixture before pouring this into petri dishes.

B.2. Standard methods

B.2.1. FITC-labelled *Vibrio anguillarum*

V. anguillarum was cultured for 24 hours at 22 ºC in Tryptone Soya Broth (TSB). The bacteria was heat killed at 65 ºC water bath for 10 minutes, and pelleted by centrifugation at 10 000 rpm, for 15 min, at 4°C. Cells were washed twice in sterile PBS before re-suspension in 0.1 M $NaHCO_3$ pH 9.0 containing 0.1 $mg.ml^{-1}$ fluorescein 5-isothiocyanate, isomer 1 (FITC, Sigma). Cells were allowed to label in whilst shaking for approximately 16 hour at 25 ºC in the absence of light, then centrifuged at 10 000 rpm for 15 min, at 4°C and re-suspended in 10 ml PBS. The bacteria were counted using a haemocytometer and a light microscope. The concentration was adjusted to $10^8.ml^{-1}$ bacteria in 1x PBS. Aliquots of the FITC-labelled bacteria were stored at -20 ºC until needed.

B.2.2. Protein in gel tryptic digestion

Gel pieces containing protein spots were place inside and Eppendorf tube.

(i) A volume of 200 µl of water was added into the tube and incubated in a vortex-low setting for 5 minutes. The water was removed using a pipette. This step was repeated for a total of 3 times to wash the gel pieces.

(ii) A volume of 200 µl of 25 mM ABC/50 % ACN (Appendix A.2.41) was added into the tube and incubated in a vortex-low setting for 5 minutes. The supernatant was removed using a pipette. This step was repeated for a total of 4 times to discolour the gel pieces.

(iii) A volume of 100 µl of 100 % ACN was added into the tube and incubated in a vortex-low setting for 10 minutes (the spots turned white). The supernatant was removed using a pipette.

(iv) The gel pieces were dried in a Speed-vac for approximately 30 minutes.

(v) A volume of 100 µl of 10 mM DTT/ABC (Appendix A.2.42) was added into the tube and incubated for 30 minutes at 37 ^{o}C. The supernatant was removed using a pipette.

(vi) A volume of 200 µl of 25 mM ABC/50% ACN was added into the tube and incubated for 2 minutes. The supernatant was removed using a pipette.

(vii) A volume of 100 µl of 55 mM IAA/ABC (Appendix A.2.43) was added into the tube and incubated for 60 minutes in dark. The supernatant was removed using a pipette.

(viii) A volume of 200 µl of 25 mM ABC/50% ACN was added into the tube and incubated in a vortex-low setting for 10 minutes. The supernatant was removed using a pipette.

(ix) The gel pieces were dried in a Speed-vac for approximately 30 minutes.

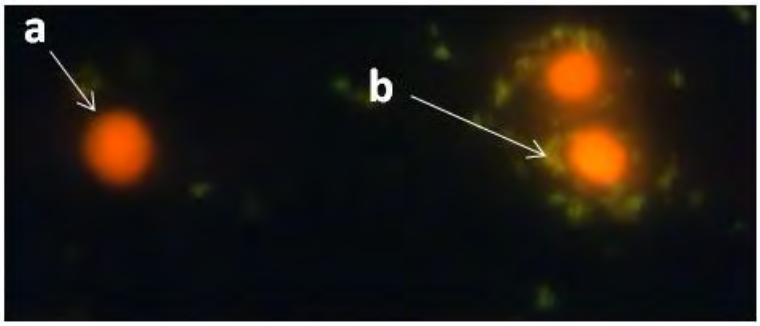
(x) A volume of 20 µl of trypsin buffer for protein in gel digestion (Appendix A.2.44) was added into the tube (it is important the trypsin buffer just cover the gel pieces, excess trypsin will result in autolysis). It was incubated for 16 hours inside a thermostat at 37 ^{o}C.

(xi) To extract the peptides from the gel pieces, a volume of 20 ul of 70 % ACN (Appendix A.2.45) was added to the tube and incubated in vortex-low setting for 30 minutes. The supernatant was removed and placed in a low-bind Eppendorf tube. This step was repeated for a total of 2 times (it is important to pool the supernatant into the same tube).

(xii) Supernatant was totally dried in a Speed-Vac and stored at -80 ^{o}C until being sent to mass spectrometry analysis (dried peptides are stable for approximately 2 months).

APPENDIX C – SUPPLEMENTARY INFORMATION

C.1. Image taken during the phagocytosis assay



Haemocytes cells challenged with pathogenic bacteria. Orange represents the nuclei of the haemocytes (labelled with Ethidium Bromide) and the green is the *Vibrio anguillarium* (labelled with FITC). (a) Haemocyte with no vibrio (non-phagocytic cell) and (b) haemocyte with vibrio attached to the nuclei (phagocytic cell).

C.2. Summary of LC-MS/MS analysis of the haemocytes proteome from *H. midae.*

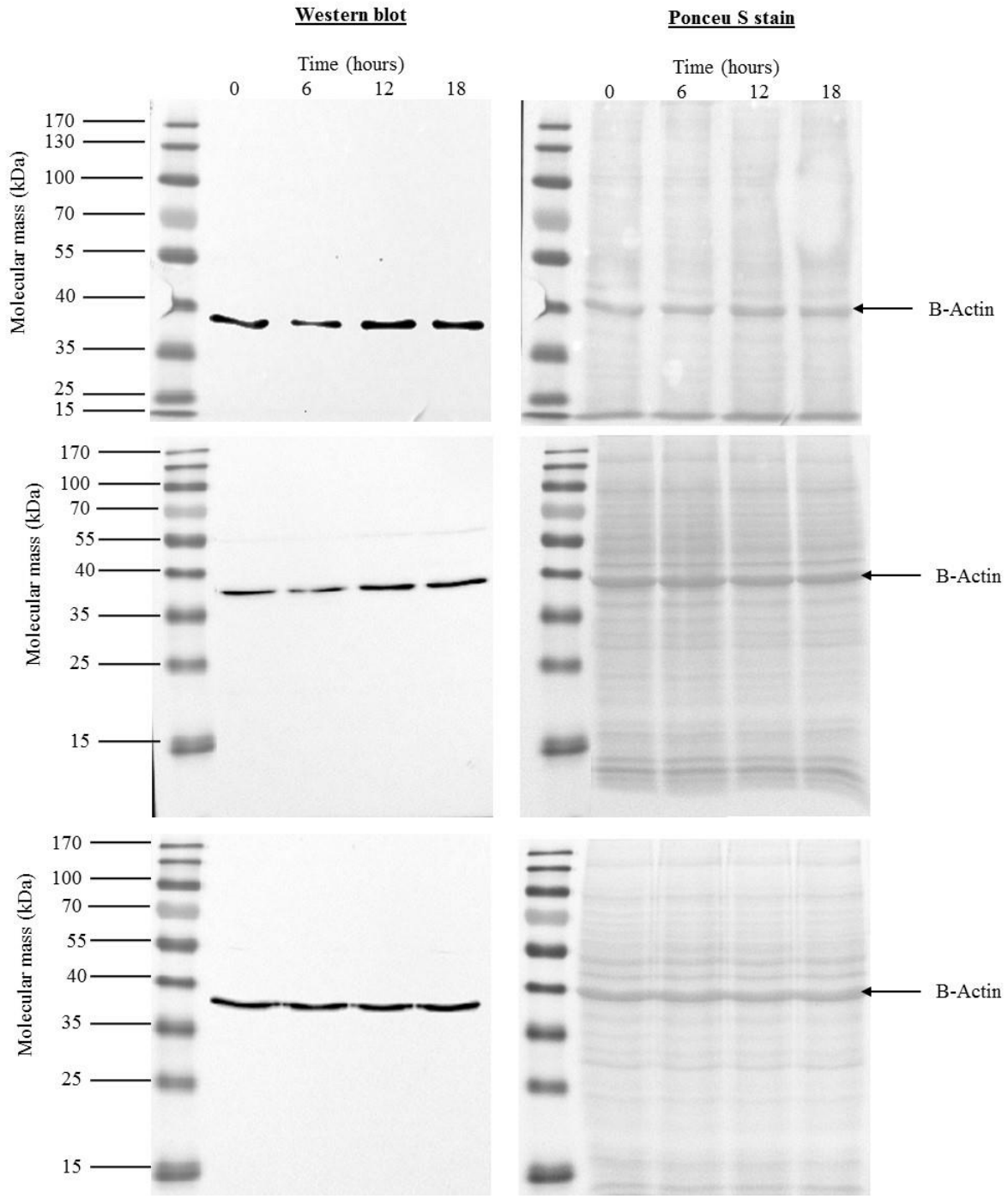
Description	iTRAQ 1	iTRAQ 2
MS scans	218140	126497
MS/MS scans	37717	65070
Peptide-Spectrum Matches	6199	3498
Peptide sequences	1138	844

C.3. Mass spectrometry identification of COP9 signalosome subunit 4 from gel protein spot

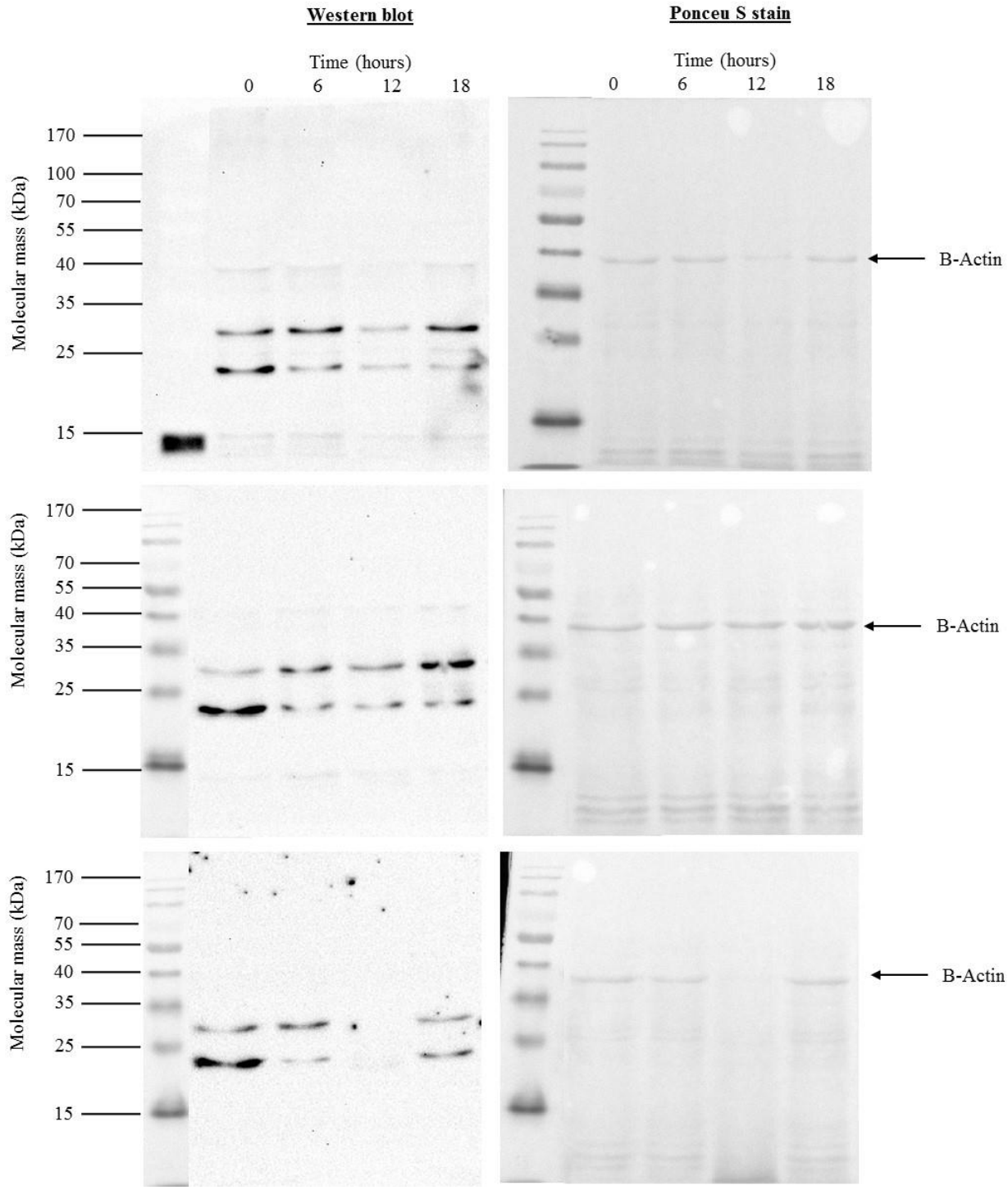
Description	Identification
Protein	COP9 signalosome complex subunit 4 (*Crassostrea gigas*)
Accession number	tr|K1R3V8|K1R3V8_CRAGI
Percentage of coverage	53
Number of unique peptides	2
Molecular mass (kDa)	46,498

C4. Validation of protein expression using western blot analysis

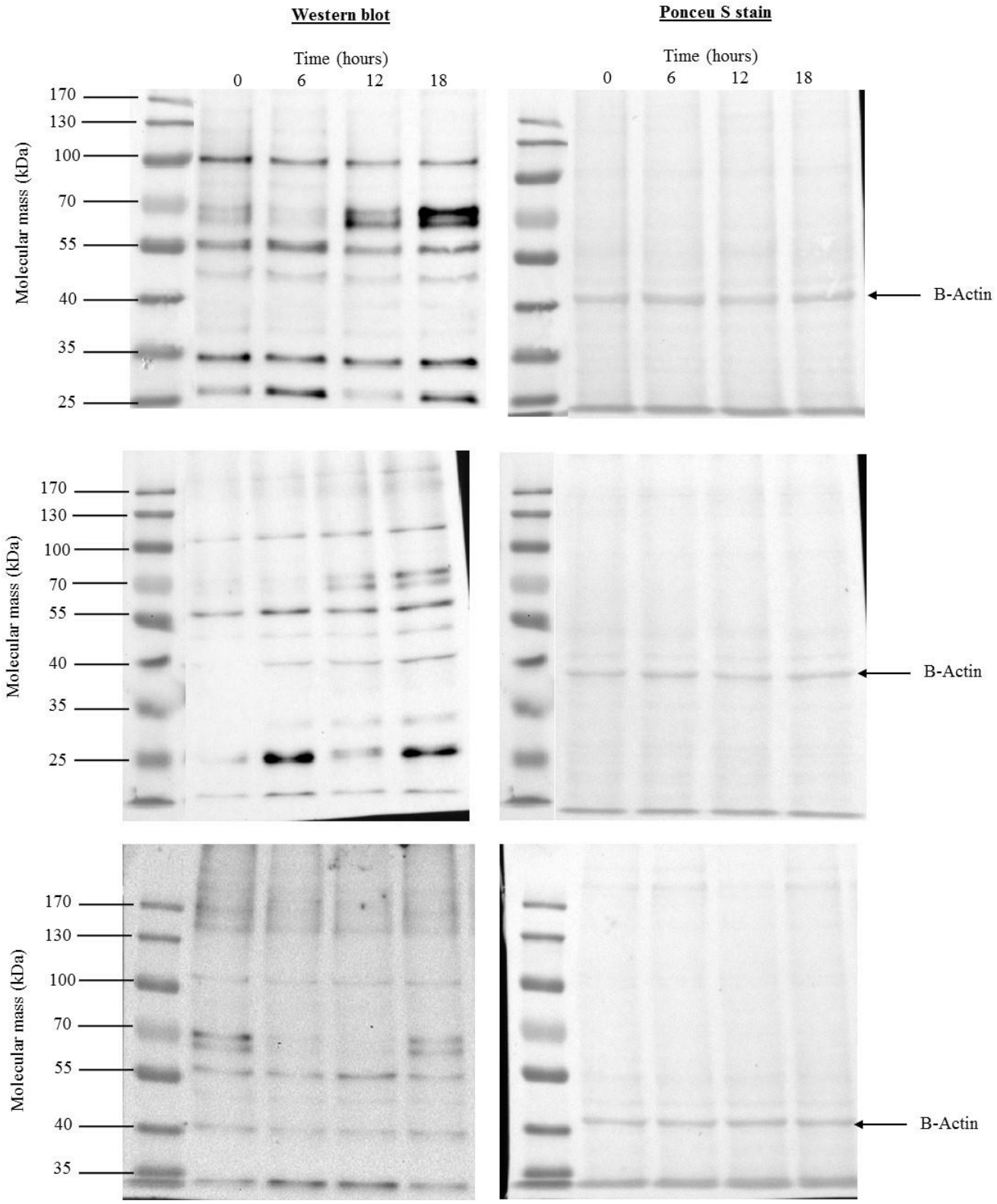
C4.1. COP9 signalosone subunit 4 (46 kDa), three biological replicates.



C4.2. Ras-related protein Rab 1A (23 kDa), three biological replicates.



C.4.3. V^+ATPase subunit B (58 kDa), three biological replicates.



C.5. Putative human orthologues proteins blasted from Uniprot database

C.5.1. Cluster "a" – down-regulated

Sub cluster	Identities			Protein description = species in which was found
	Molluscs	Human	Align (%)	
1	K1QB83_CRAGI	B4DJ75	95.7	Serine/threonine-protein phosphatase OS=Crassostrea gigas
1	K1S4Q2_CRAGI	P50991	76.8	T-complex protein 1 subunit delta (Fragment) OS=Crassostrea gigas
1	K1QX02_CRAGI	P46531	26.7	Tenascin-X OS=Crassostrea gigas
2	A7L3I9_HALCR	P62820	99.3	RAB1 (Fragment) OS=Haliotis cracherodii
2	K1QX44_CRAGI	Q15907	97.1	Ras-related protein Rab-11B OS=Crassostrea gigas
2	B6RB23_HALDI	P62820	92.2	Ras-related protein Rab-1A OS=Haliotis discus discus
2	K1QD28_CRAGI	P62491	86.3	Ras-related protein Rab-11A OS=Crassostrea gigas
2	K1PY30_CRAGI	Q15019	71.0	Septin-2 OS=Crassostrea gigas
2	K1PJ46_CRAGI	P14618	66.2	Pyruvate kinase OS=Crassostrea gigas
2	D7EZG9_CRAGI	Q01518-2	56.4	Adenylyl cyclase-associated protein OS=Crassostrea gigas
2	B3TK64_HALDV	Q9H0C2	49.7	ADP_ATP carrier protein (Fragment) OS=Haliotis diversicolor
2	K1QQ39_CRAGI	Q9NRW1	38.7	Ras-related protein RHA1 OS=Crassostrea gigas
3	K1QG58_CRAGI	P60709	96.8	Actin OS=Crassostrea gigas
3	B6RB76_HALDI	Q15286-2	90.6	RAB protein OS=Haliotis discus discus
3	B6RB29_HALDI	P62826	88.5	Ran-1-prov protein OS=Haliotis discus discus
3	K1QLP5_CRAGI	E9PK34	85.2	Coatomer subunit delta OS=Crassostrea gigas
3	K1Q122_CRAGI	O14950	81.8	Myosin regulatory light chain sqh OS=Crassostrea gigas
3	K1RLC5_CRAGI	P48643	75.3	T-complex protein 1 subunit epsilon OS=Crassostrea gigas
3	K1QCC1_CRAGI	P00558	72.8	Phosphoglycerate kinase OS=Crassostrea gigas
3	Q6SQL9_9GAST	P63261	55.2	Actin (Fragment) OS=Patella pellucida
3	B6RB97_HALDI	P06396	44.7	Gelsolin OS=Haliotis discus discus
3	B3TK70_HALDV	Q92804-2	42.4	Putative RNA-binding protein OS=Haliotis diversicolor
3	K1P339_CRAGI	P18206	36.9	Vinculin OS=Crassostrea gigas
3	Q53IP9_MEGCR	_____	0.0	Hemocyanin 1 OS=Megathura crenulata
3	Q6KC56_MEGCR	_____	0.0	Keyhole limpet hemocyanin1 OS=Megathura crenulata
4	Q86DH9_APLCA	P60953	89.5	Cdc42 OS=Aplysia californica
4	Q8ITH0_BIOGL	P12814	70.0	Alpha-actinin (Fragment) OS=Biomphalaria glabrata
4	K1QQR1_CRAGI	Q14764	65.4	Major vault protein OS=Crassostrea gigas
4	Q5XLV2_ENTDO	_____	0.0	Hemocyanin A-type (Fragment) OS=Enteroctopus dofleini
4	B5RHQ6_9MOLL	_____	0.0	Hemocyanin fgh (Fragment) OS=Falcidens crossotus
4	Q9BJ58_ENTDO	_____	0.0	Hemocyanin G-type (Fragment) OS=Enteroctopus dofleini
4	HCYG_ENTDO	_____	0.0	Hemocyanin G-type_ units Oda to Odg OS=Enteroctopus dofleini
4	C7FEG7_HALDV	_____	0.0	Hemocyanin isoform 1 (Fragment) OS=Haliotis diversicolor
4	B0RZD4_PECMA	_____	0.0	Vitellogenin OS=Pecten maximus
5	I1SKJ3_APLCA	P62805	99.0	Histone H4 OS=Aplysia californica
5	D2XEB0_CRAHO	Q5PY61	98.7	Ubiquitin OS=Crassostrea hongkongensis
5	Q8T6A1_APLCA	P62987	93.5	Ubiquitin/ribosomal L40 fusion protein (Fragment) OS=Aplysia californica
5	Q70MM6_CRAGI	P23396	92.5	Ribosomal protein S3 (Fragment) OS=Crassostrea gigas
5	A9LMJ6_HALDI	P46781	91.8	Ribosomal protein S9 OS=Haliotis discus discus
5	K1QWC3_CRAGI	P23396	88.1	40S ribosomal protein S3 OS=Crassostrea gigas

5	B6RB72_HALDI	P84077	87.3	ADP-ribosylation factor 2 OS=Haliotis discus discus
5	K1Q9V3_CRAGI	P38606	85.5	V-type proton ATPase catalytic subunit A OS=Crassostrea gigas
5	Q45Y88_HALRU	Q05639	84.2	Elongation factor 1-alpha (Fragment) OS=Haliotis rufescens
5	K1PNR3_CRAGI	Q00610	83.7	Clathrin heavy chain 1 OS=Crassostrea gigas
5	E1B300_9BIVA	H7C3T4	77.0	Thioredoxin peroxidase OS=Cristaria plicata
5	K1RH70_CRAGI	P52209	76.3	6-phosphogluconate dehydrogenase_ decarboxylating OS=Crassostrea gigas
5	G9K380_HALDV	P39023	75.4	Putative 60S ribosomal protein L3 OS=Haliotis diversicolor
5	B6RB96_HALDI	P46777	74.1	Ribosomal protein l5 (Fragment) OS=Haliotis discus discus
5	B6RB30_HALDI	P04406	73.6	Glyceraldehyde-3-phosphate dehydrogenase OS=Haliotis discus discus
5	B6RB90_HALDI	Q0QF37	72.8	Malate dehydrogenase (Fragment) OS=Haliotis discus discus
5	D3K380_PINFU	Q13162	72.7	Peroxiredoxin OS=Pinctada fucata
5	K1QVK0_CRAGI	P37837	63.5	Transaldolase OS=Crassostrea gigas
5	Q45R40_APLCA	P35580-2	62.9	Nonmuscle myosin II (Fragment) OS=Aplysia californica
5	K1Q273_CRAGI	P50914	57.0	60S ribosomal protein L14 OS=Crassostrea gigas
5	K7ZQ52_PTEPN	_____	0.0	Pif OS=Pteria penguin
6	K1PZ08_CRAGI	C9J592	92.7	Ras-related protein Rab-7a OS=Crassostrea gigas
6	K1R6S5_CRAGI	P46781	86.6	40S ribosomal protein S9 OS=Crassostrea gigas
6	K1R4S7_CRAGI	P61026	81.5	Ras-related protein Rab-10 OS=Crassostrea gigas
6	K1RW85_CRAGI	P23526	78.8	Adenosylhomocysteinase OS=Crassostrea gigas
6	K1PK85_CRAGI	Q86VP6	72.6	Cullin-associated NEDD8-dissociated protein 1 OS=Crassostrea gigas
6	A6MD73_HALDI	P20591-2	50.0	Mx OS=Haliotis discus discus

C.5.1. Cluster "b" – up-regulated

Sub cluster	Identities			Protein description = species in which was found
	Molluscs	Human	Align (%)	
7	CALM_PATSP	P62158	96.6	Calmodulin OS=Patinopecten sp.
7	K1PWR0_CRAGI	Q01105-2	75.9	Protein SET OS=Crassostrea gigas
7	K7R2X9_9BIVA	Q01105-2	63.6	SET nuclear oncoprotein (Fragment) OS=Scrobicularia plana
7	Q9U9B6_MYTED	Q14764	58.4	Major vault protein (Fragment) OS=Mytilus edulis
8	K1QCB0_CRAGI	P46782	96.9	40S ribosomal protein S5 OS=Crassostrea gigas
8	B0B039_MYTED	P0CG47	95.6	Ubiquitin (Fragment) OS=Mytilus edulis
8	Q4H451_CRAGI	P46782	89.8	Ribosomal protein S5 OS=Crassostrea gigas
8	K4INQ5_9BIVA	P62195	88.8	Proteasome 26S subunit (Fragment) OS=Solen grandis
8	K1PTH4_CRAGI	C9J1Z8	84.1	ADP-ribosylation factor OS=Crassostrea gigas
8	K1R3N2_CRAGI	D6RIS5	83.8	Methylmalonic aciduria type A protein_ mitochondrial OS=Crassostrea gigas
8	K1R1E2_CRAGI	O15144	82.0	Actin-related protein 2/3 complex subunit 2 OS=Crassostrea gigas
8	B6RB18_HALDI	Q53HU0	72.5	Chaperonin containing tcp1 OS=Haliotis discus discus
8	K1QHK9_CRAGI	Q14204	71.9	Dynein heavy chain_ cytoplasmic OS=Crassostrea gigas
8	B6RB17_HALDI	P08865	71.4	40S ribosomal protein SA OS=Haliotis discus discus
8	E6Y2Z7_HALDV	Q08170	65.2	Splicing factor arginine/serine-rich 4 OS=Haliotis diversicolor supertexta
8	K1RBC9_CRAGI	Q9H0I9	63.1	Transketolase-like protein 2 OS=Crassostrea gigas
8	B6RB63_HALDI	P07237	61.3	Protein disulfide isomerase OS=Haliotis discus discus
8	Q7YZR4_HALAI	P06754	55.5	Tropomyosin 1 OS=Haliotis asinina
8	B7XC62_HALDI	P06753	55.2	Tropomyosin OS=Haliotis discus discus
8	Q7YZR3_HALAI	P06753	54.1	Tropomyosin 2 OS=Haliotis asinina
8	K1R0Y9_CRAGI	P05141	52.5	ADP_ATP carrier protein OS=Crassostrea gigas
8	B6RAZ8_HALDI	P78417	43.9	Omega class glutathione-s-transferase 1 OS=Haliotis discus discus
8	K1PV35_CRAGI	Q8NBH2-2	33.0	Kyphoscoliosis peptidase OS=Crassostrea gigas
8	B3SND4_HALDV	Q9Y281	30.2	Actin depolymerisation factor/cofilin OS=Haliotis diversicolor
9	A2CI32_9BIVA	Q9BTM1	92.6	Histone H2A OS=Azumapecten farreri
9	B3TK56_HALDV	D6RI02	92.3	40S ribosomal protein S3a (Fragment) OS=Haliotis diversicolor
9	K1QI28_CRAGI	P21281	89.1	V-type proton ATPase subunit B OS=Crassostrea gigas
9	K1PVA1_CRAGI	P55072	88.7	Transitional endoplasmic reticulum ATPase OS=Crassostrea gigas
9	K1R6Z7_CRAGI	P25705-2	85.7	ATP synthase subunit alpha OS=Crassostrea gigas
9	K1S6V7_CRAGI	P30153	82.3	Serine/threonine-protein phosphatase 2A 65 kDa regulatory subunit A alpha isoform OS=Crassostrea gigas
9	B8XW76_HALDV	P27635	81.9	QM-like protein OS=Haliotis diversicolor supertexta
9	K1R6F1_CRAGI	O14818	80.8	Proteasome subunit alpha type OS=Crassostrea gigas
9	I1VYX2_MYTTR	P48735-2	78.6	Isocitrate dehydrogenase [NADP] OS=Mytilus trossulus
9	K1QNG9_CRAGI	P62750	75.8	60S ribosomal protein L23a OS=Crassostrea gigas
9	B3TK66_HALDV	P62241	75.0	40S ribosomal protein S8 OS=Haliotis diversicolor
9	B3TK20_HALDV	P04075	70.0	Fructose-bisphosphate aldolase (Fragment) OS=Haliotis diversicolor
9	K1R466_CRAGI	P49368	68.4	T-complex protein 1 subunit gamma OS=Crassostrea gigas
9	K1Q615_CRAGI	Q06830	64.3	Peroxiredoxin-1 OS=Crassostrea gigas

9	H9LJ34_CRAAR	Q9H0I9	62.5	Transketolase-like protein 2 (Fragment) OS=Crassostrea ariakensis
9	C7EAA2_HALAI	O15523	61.4	PL10-like protein OS=Haliotis asinina
9	K1RQC1_CRAGI	P47989	57.7	Xanthine dehydrogenase/oxidase OS=Crassostrea gigas
9	K1PR25_CRAGI	A0A024R163	57.0	Regulator of differentiation 1 OS=Crassostrea gigas
9	B6RB35_HALDI	P37802	39.7	Transgelin OS=Haliotis discus discus
9	E4W3F6_HALDV	P78417	38.0	Glutathione-S-transferase OS=Haliotis diversicolor supertexta
9	Q8I0U4_HALTU	P14679-2	29.2	H2 protein (Fragment) OS=Haliotis tuberculata
10	K1QLZ1_CRAGI	P61158	84.5	Actin-related protein 3 OS=Crassostrea gigas
10	A2TF45_9BIVA	P54652	83.2	Heat shock protein 70 OS=Laternula elliptica
10	B4E3Z5_9BIVA	E9PQQ4	79.8	Heat shock protein 70kDa A (Fragment) OS=Laternula elliptica
10	B1N693_HALDI	Q13162	79.3	Thioredoxin peroxidase 1 OS=Haliotis discus discus
10	H9AWU2_HALDH	A0A024RDL1	77.6	Chaperonin containing T-complex polypeptide subunit zeta OS=Haliotis discus hannai
10	J9U877_9BIVA	P27797	71.2	Calreticulin OS=Hyriopsis cumingii
10	Q45Y86_HALRU	P60174	69.4	Triosephosphate isomerase (Fragment) OS=Haliotis rufescens
10	K1PUJ1_CRAGI	P35241	66.3	Radixin OS=Crassostrea gigas
10	K1PYW8_CRAGI	P08133	44.9	Annexin OS=Crassostrea gigas
10	Q9GP18_HALTU	_____	0.0	Hemocyanin (Fragment) OS=Haliotis tuberculata
10	Q1MVA1_MEGCR	_____	0.0	Hemocyanin 2 OS=Megathura crenulata
10	Q27Q57_SEPOF	_____	0.0	Hemocyanin subunit 1 OS=Sepia officinalis
10	Q6KC55_MEGCR	_____	0.0	Keyhole limpet hemocyanin2 OS=Megathura crenulata
11	K1RAJ1_CRAGI	P17987	73.0	T-complex protein 1 subunit alpha OS=Crassostrea gigas
11	K1PM29_CRAGI	P11413	71.3	Glucose-6-phosphate 1-dehydrogenase OS=Crassostrea gigas
11	K1S151_CRAGI	P50395	65.1	Rab GDP dissociation inhibitor beta OS=Crassostrea gigas
11	KARG_HALMK	P12277	40.5	Arginine kinase OS=Haliotis madaka
12	K1R3V8_CRAGI	Q9BT78	77.5	COP9 signalosome complex subunit 4 OS=Crassostrea gigas
12	K1Q948_CRAGI	P11217	68.7	Phosphorylase OS=Crassostrea gigas
12	B3TK26_HALDV	P62158	27.7	Calmodulin 2 OS=Haliotis diversicolor
	K1QC78_CRAGI	P61106	87.9	Ras-related protein Rab-14 OS=Crassostrea gigas
	B3TK60_HALDV	Q9NZT1	28.0	Calmodulin-dependent protein kinase (Fragment) OS=Haliotis diversicolor

C.6. List of all biological processes related to immune classes from proteins identified in *H. midae* haemocytes

ATP metabolism
DNA metabolism
RNA biosynthesis
RNA metabolism
amide biosynthesis
apoptotic process
aromatic compound biosynthesis
biosynthesis
carbohydrate derivative metabolism
carbohydrate metabolism
carboxylic acid metabolism
catabolism
cell adhesion
cellular amide metabolism
cellular aromatic compound metabolism
cellular biosynthesis
cellular carbohydrate metabolism
cellular catabolism
cellular glucan metabolism
cellular macromolecule biosynthesis
cellular macromolecule catabolism
cellular macromolecule metabolism
cellular metabolism
cellular nitrogen compound biosynthesis
cellular nitrogen compound metabolism
cellular polysaccharide metabolism
cellular protein metabolism
cellular protein modification process
cellular response to DNA damage stimulus
cellular response to endogenous stimulus
cellular response to stress
coenzyme metabolism
cofactor metabolism
death
defense response
endocytosis
energy derivation by oxidation of organic compounds
energy reserve metabolism
gene expression
generation of precursor metabolites and energy
glucan metabolism
glycosyl compound metabolism
heterocycle biosynthesis
heterocycle metabolism
immune response
immune response-regulating signaling pathway
innate immune response
macromolecule biosynthesis
macromolecule catabolism
macromolecule metabolism
macromolecule modification
metabolism
modification-dependent macromolecule catabolism
monosaccharide metabolism
negative regulation of cellular metabolism
negative regulation of metabolism
nicotinamide nucleotide metabolism
nitrogen compound metabolism
nucleic acid metabolism
nucleobase-containing compound biosynthesis
nucleobase-containing compound metabolism
nucleobase-containing small molecule metabolism
nucleoside monophosphate metabolism
nucleoside phosphate metabolism
nucleoside triphosphate metabolism
nucleotide metabolism
organic acid metabolism
organic cyclic compound biosynthesis
organic cyclic compound metabolism
organic substance biosynthesis
organic substance catabolism
organic substance metabolism

organonitrogen compound biosynthesis
organonitrogen compound metabolism
organophosphate metabolism
oxidation-reduction process
oxidoreduction coenzyme metabolism
oxoacid metabolism
peptide biosynthesis
peptide metabolism
phosphate-containing compound metabolism
phosphorus metabolism
phosphorylation
polysaccharide metabolism
positive regulation of catalytic activity
positive regulation of cellular protein metabolism
positive regulation of defense response
positive regulation of metabolism
positive regulation of nitrogen compound metabolism
positive regulation of protein metabolism
primary metabolism
proteasomal protein catabolism
protein catabolism
protein metabolism
protein modification by small protein conjugation or removal
protein modification process
proteolysis
proteolysis involved in cellular protein catabolism
purine nucleoside metabolism
purine nucleoside monophosphate metabolism
purine nucleotide metabolism
purine ribonucleoside metabolism
purine ribonucleotide metabolism
purine-containing compound metabolism
pyridine-containing compound metabolism
regulation of RNA metabolism
regulation of biosynthesis
regulation of catabolism
regulation of catalytic activity
regulation of cellular biosynthesis
regulation of cellular catabolism
regulation of cellular metabolism
regulation of cellular protein catabolism
regulation of cellular protein metabolism
regulation of defense response
regulation of gene expression
regulation of innate immune response
regulation of macromolecule metabolism
regulation of metabolism
regulation of nitrogen compound metabolism
regulation of phosphate metabolism
regulation of phosphorus metabolism
regulation of primary metabolism
regulation of protein catabolism
regulation of protein metabolism
regulation of protein modification process
regulation of proteolysis
regulation of response to stress
regulation of transcription, DNA-templated
response to endogenous stimulus
response to external stimulus
response to stress
ribonucleoside metabolism
ribose phosphate metabolism
single organism cell adhesion
single-organism biosynthesis
single-organism carbohydrate metabolism
single-organism catabolism
single-organism metabolism
small molecule metabolism
toll-like receptor signaling pathway
transcription from RNA polymerase II promoter
transcription, DNA-templated
translation
translational elongation

C.7. General functional immune classification of the proteins identified in proteins, the number of counts and the percentage that they represent.

GO Terms	Biological function	Number of counts	Fraction (%)
GO:0008152	metabolism	175	55.91
GO:0006950	stress response	42	13.42
GO:0019538	protein metabolism	28	8.95
GO:0006955	immunology, immune response	21	6.71
GO:0009056	catabolism	20	6.39
GO:0005975	carbohydrate metabolism	9	2.88
GO:0016265	death	7	2.24
GO:0006915	apoptosis	4	1.28
GO:0009719	response to endogenous stimulus	2	0.64
GO:0007155	cell adhesion	2	0.64
GO:0009605	response to external stimulus	1	0.32
GO:0006897	endocytosis	1	0.32
GO:0042981	regulation of apoptosis	1	0.32

C.7.1. List of the immune classes identified in each of the 12 sub-clusters and the respective percentage (down-regulated proteins).

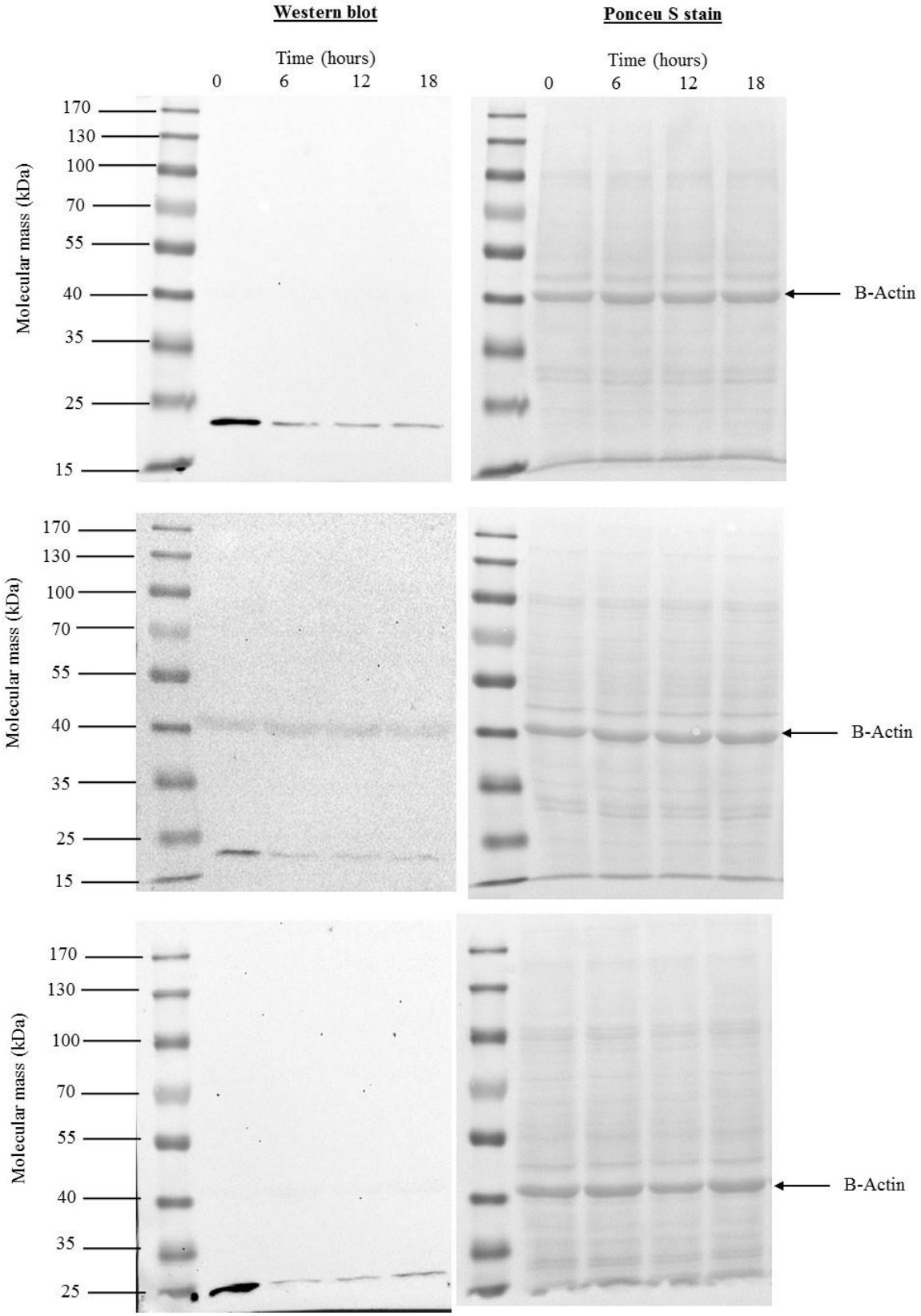
Sub-cluster	Number of proteins	Correlation	GO terms	Immune classe (biological function)	Fraction (%)
1	3	0.95	GO:0008152	metabolism	80.0
			GO:0019538	protein metabolism	20.0
2	8	0.98	GO:0008152	metabolism	43.2
			GO:0006950	stress response	12.6
			GO:0009605	response to external stimulus	11.7
			GO:0019538	protein metabolism	7.2
			GO:0009607	response to biotic stimulus	5.4
			GO:0016032	viral life cycle	3.6
			GO:0001816	cytokine production	3.6
			GO:0007155	cell adhesion	3.6
			GO:0046879	hormone secretion	2.7
			GO:0009056	catabolism	2.7
			GO:0050663	cytokine secretion	1.8
			GO:0042742	defense response to bacteria	0.9
			GO:0006897	endocytosis	0.9
3	12	0.95	GO:0008152	metabolism	85.7
			GO:0007155	cell adhesion	14.3
4	9	0.99	GO:0008152	metabolism	100.0
5	21	0.98	GO:0008152	metabolism	66.1
			GO:0006950	stress response	8.8
			GO:0019538	protein metabolism	6.2
			GO:0016265	death	5.7
			GO:0006915	apoptosis	5.3
			GO:0009056	catabolism	4.9
			GO:0042981	regulation of apoptosis	1.8
			GO:0005975	carbohydrate metabolism	1.3
6	6	0.99	GO:0008152	metabolism	85.7
			GO:0019538	protein metabolism	14.3

C.7.2. List of the immune classes identified in each of the 12 sub-clusters and the respective percentage (up-regulated proteins).

Sub-cluster	Number of proteins	Correlation	GO terms	Immune classe (biological function)	Fraction (%)
7	4	0.97	_____	_____	__
8	20	0.97	GO:0008152	metabolism	64.6
			GO:0009056	catabolism	16.0
			GO:0019538	protein metabolism	14.6
			GO:0006950	stress response	1.9
			GO:0016032	viral life cycle	1.9
			GO:0007155	cell adhesion	1.0
9	21	0.95	GO:0008152	metabolism	60.8
			GO:0019538	protein metabolism	15.9
			GO:0009056	catabolism	6.5
			GO:0016265	death	6.1
			GO:0006915	apoptosis	5.6
			GO:0042981	regulation of apoptosis	2.3
			GO:0006950	stress response	1.9
			GO:0005975	carbohydrate metabolism	0.9
10	3	0.96	GO:0008152	metabolism	82.5
			GO:0007155	cell adhesion	7.5
			GO:0019538	protein metabolism	5.0
			GO:0009056	catabolism	5.0
11	6	0.96	GO:0008152	metabolism	77.8
			GO:0019538	protein metabolism	22.2
12	3	0.96	GO:0008152	metabolism	100.0

C.8. Analysis of Rab 1A (23 kDa) expression in cytosolic and membrane fraction of abalone haemocytes using western blot approach.

C.8.1. Rab 1A from cytosolic fraction, three biological replicates.



C.8.2. Rab 1A from membrane fraction, three biological replicates.