IMPLEMENTATION OF RNA INTERFERENCE (RNAi) TECHNOLOGY TO UNRAVEL THE MECHANISTIC ACTION OF PLANT-DERIVED COMPOUNDS

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Gent, August 2018

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TABLE OF CONTENT

LIST OF ABBREVIATIONS ........................................................................................................................ vi

LIST OF TABLES ........................................................................................................................................ vii

LIST OF FIGURES ........................................................................................................................................ viii

ABSTRACT ................................................................................................................................................... ix

CHAPTER 01: INTRODUCTION .................................................................................................................. 1
1.1. Background information ......................................................................................................................... 1
1.2. Research problem identification and justification ................................................................................... 1
1.3. Objectives of the study ........................................................................................................................... 3

CHAPTER 02: REVIEW OF LITERATURE .................................................................................................... 4
2.1. Global shrimp production – an overview ................................................................................................. 4
2.2. Constraints in shrimp aquaculture ......................................................................................................... 5
  2.2.1. Diseases as a major constraint in aquaculture ...................................................................................... 6
2.3. Major diseases in shrimp aquaculture ..................................................................................................... 6
  2.3.1. Vibriosis in shrimp aquaculture ........................................................................................................... 6
  2.3.2. Vibrio harveyi and diseases ................................................................................................................... 7
2.4. Disease prevention, control, and treatment ............................................................................................ 7
  2.4.1. Green water technique ....................................................................................................................... 8
  2.4.2. Probiotics .............................................................................................................................................. 8
  2.4.3. Phage therapy .................................................................................................................................... 8
  2.4.4. Immunostimulants ............................................................................................................................ 9
2.5. Heat Shock Proteins ............................................................................................................................... 10
  2.5.1. The function of heat shock proteins and mechanism of action .......................................................... 10
  2.5.2. Hsp families ....................................................................................................................................... 13
  2.5.3. Hsp 70 family .................................................................................................................................... 14
2.6. Polyphenolic compounds and Phloroglucinol ......................................................................................... 15
  2.6.1. What are polyphenolic compounds? ................................................................................................ 15
  2.6.2. Functions of polyphenolic compounds ............................................................................................ 16
  2.6.3. Natural polyphenolic compounds as Hsp inducers ........................................................................... 16
  2.6.4. Phloroglucinol .................................................................................................................................... 17
2.7. RNA interference (RNAi) ...................................................................................................................... 18
  2.7.1. Molecular mechanism ..................................................................................................................... 18
  2.7.2. Disease control .................................................................................................................................. 19
2.8. Artemia as a model organism for studying diseases in aquaculture ...................................................... 20
  2.8.1. Life cycle ........................................................................................................................................... 20
  2.8.2. Artemia as a model organism ........................................................................................................... 21
CHAPTER 03: MATERIALS AND METHODS ......................................................... 22
3.1. Materials .................................................................................................. 22
   3.1.1. Experimental site .................................................................................. 22
   3.1.2. Experimental animals .......................................................................... 22
   3.1.3. Bacterial strains .................................................................................... 22
   3.1.4. Culture media ...................................................................................... 22
   3.1.5. Reagents ................................................................................................ 22
   3.1.6. Sea water, other equipment and glassware ........................................... 22
3.2. Methods ..................................................................................................... 23
   3.2.1. Culturing of bacteria for challenge assay .............................................. 23
   3.2.2. Hatching of axenic brine shrimp larvae ............................................... 23
   3.2.3. Brine shrimp challenge assays .............................................................. 23
      3.2.3.1. Dose response relationship ............................................................. 23
      3.2.3.2. Unravelling the mode of action ..................................................... 24
   3.2.4. Assay of heat shock protein 70 family (hsp70) genes, prophenoloxidase (proPO) and transglutaminase (tgase) gene expression by quantitative real-time PCR (RT-qPCR) analysis ................................................................. 26
      3.2.4.1. Pretreatment, challenge and sample collection ......................... 26
      3.2.4.2. RNA extraction and cDNA synthesis ......................................... 26
      3.2.4.3. Quantitative real-time PCR (RT-qPCR) ....................................... 26
   3.2.5. Knockdown of hsp70 and hsc70 genes by RNA interference (RNAi) ................................................................. 28
      3.2.5.1. Culture of Artemia for microinjection .................................... 28
      3.2.5.2. Preparation of dsRNA ................................................................. 29
      3.2.5.3. Microinjection of A. franciscana female with ds RNA ................. 29
      3.2.5.4. Challenge assay ............................................................................. 29
      3.2.5.5. Protein extraction and analysis of Hsp70 and Hsc70 proteins .... 31
      3.2.5.6. Analysis of hsp70 and hsc70 genes .............................................. 31
   3.2.6. Curative effect of phloroglucinol against Vibrio harveyi ................. 31
3.2.7. Statistical analysis .................................................................................. 32

CHAPTER 04: RESULTS ......................................................................................... 33
4.1. Phloroglucinol pretreatment of axenic brine shrimp larvae protects them against subsequent V. harveyi challenge ................................................................. 33
4.2. Phloroglucinol generated prooxidant activity mediates the protective response in brine shrimp larvae ......................................................................................... 34
4.3. Brine shrimp larvae pretreated with phloroglucinol show a tendency to hsp70 family genes induction ................................................................. 35
4.4. Phloroglucinol elicits protective immune responses in the brine shrimp larvae ................................................................. 39
4.5. Phloroglucinol treatment protects axenic brine shrimp larvae challenged with V. harveyi ................................................................. 41

CHAPTER 05: DISCUSSION ......................................................................................... 43
5.1. Dose response effect of phloroglucinol pretreatment on axenic brine shrimp larvae ................................................................. 44
5.2. Phloroglucinol generated prooxidant activity and the protective response in brine shrimp larvae ......................................................................................... 44
5.3. Phloroglucinol generated prooxidant activity and its association with \textit{hsp}70 family and immune genes ..............................................45
5.4. Knockdown of \textit{hsp}70 and \textit{hsc}70 genes by RNA interference (RNAi) ..............................................47
5.5. Treatment effect phloroglucinol on axenic brine shrimp larvae challenged with \textit{V. harveyi}48

\textbf{CHAPTER 06: CONCLUSIONS AND RECOMMENDATIONS} .............................................49
6.1. Conclusions ..................................................................................................................49
6.2. Recommendations ....................................................................................................49

\textbf{CHAPTER 07: REFERENCES} ..................................................................................51
### LIST OF ABBREVIATIONS

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>BIP</td>
<td>Binding Immunoglobulin Protein</td>
</tr>
<tr>
<td>BLIS</td>
<td>Bacteriosin-like substances</td>
</tr>
<tr>
<td>bp</td>
<td>Base pair</td>
</tr>
<tr>
<td>°C</td>
<td>Celcius</td>
</tr>
<tr>
<td>cDNA</td>
<td>Complementary Deoxyribonucleic acid</td>
</tr>
<tr>
<td>C&lt;sub&gt;T&lt;/sub&gt;</td>
<td>Cycle threshold</td>
</tr>
<tr>
<td>dsRNA</td>
<td>Double stranded Ribonucleic acid</td>
</tr>
<tr>
<td>µE</td>
<td>Micro Einstein</td>
</tr>
<tr>
<td>ECP</td>
<td>Extracellular products</td>
</tr>
<tr>
<td>EF1&lt;sub&gt;α&lt;/sub&gt;</td>
<td>Elongation factor 1α</td>
</tr>
<tr>
<td>FAO</td>
<td>Food and Agriculture Organization</td>
</tr>
<tr>
<td>FASW</td>
<td>Filtered autoclaved sea water</td>
</tr>
<tr>
<td>g</td>
<td>Gram</td>
</tr>
<tr>
<td>gfp</td>
<td>Green fluorescence protein</td>
</tr>
<tr>
<td>GOAL</td>
<td>Global Aquaculture Alliance</td>
</tr>
<tr>
<td>h</td>
<td>Hour</td>
</tr>
<tr>
<td>Hsc</td>
<td>Heat shock cognate</td>
</tr>
<tr>
<td>Hsp</td>
<td>Heat shock protein</td>
</tr>
<tr>
<td>HSPA4</td>
<td>Heat shock protein family A (Hsp70) member</td>
</tr>
<tr>
<td>Hspi</td>
<td>Heat shock protein inducer</td>
</tr>
<tr>
<td>HPV</td>
<td>Hepatopancreatic parovirus</td>
</tr>
<tr>
<td>IHNV</td>
<td>Infectious hypodermal and hematopoietic necrosis virus</td>
</tr>
<tr>
<td>IL</td>
<td>Interleukin</td>
</tr>
<tr>
<td>kDa</td>
<td>Kilodalton</td>
</tr>
<tr>
<td>l</td>
<td>Litre</td>
</tr>
<tr>
<td>LD&lt;sub&gt;50&lt;/sub&gt;</td>
<td>Lethal dose 50%</td>
</tr>
<tr>
<td>LPS</td>
<td>Lipopolysaccharides</td>
</tr>
<tr>
<td>m</td>
<td>Meter</td>
</tr>
<tr>
<td>MBV</td>
<td>Monodon baculo virus</td>
</tr>
<tr>
<td>mg</td>
<td>Milli gram</td>
</tr>
<tr>
<td>MHC</td>
<td>Major histocompatibility complex</td>
</tr>
<tr>
<td>min</td>
<td>Minute</td>
</tr>
<tr>
<td>ml</td>
<td>Millilitter</td>
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<tr>
<td>mM</td>
<td>Millimole</td>
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<tr>
<td>ng</td>
<td>Nanogram</td>
</tr>
<tr>
<td>µM</td>
<td>Micromole</td>
</tr>
<tr>
<td>PDI</td>
<td>Protein disulfide isomerase</td>
</tr>
<tr>
<td>proPO</td>
<td>Prophenoloxidase</td>
</tr>
<tr>
<td>RNAi</td>
<td>RNA inteference</td>
</tr>
<tr>
<td>s</td>
<td>Seconds</td>
</tr>
<tr>
<td>ssRNA</td>
<td>Single stranded RNA</td>
</tr>
<tr>
<td>SOD</td>
<td>Superoxide dismutase</td>
</tr>
<tr>
<td>tgage</td>
<td>Transglutaminase</td>
</tr>
<tr>
<td>TLR</td>
<td>Toll-like receptors</td>
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<tr>
<td>TNF</td>
<td>Tumor necrosis factor</td>
</tr>
<tr>
<td>TSV</td>
<td>Taura syndrome virus</td>
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<tr>
<td>UV</td>
<td>Ultra violet</td>
</tr>
<tr>
<td>WSSV</td>
<td>White spot syndrome virus</td>
</tr>
<tr>
<td>YHV</td>
<td>Yellow Head Virus</td>
</tr>
</tbody>
</table>
LIST OF TABLES

Table 2.1:  Major Hsp families ................................................................. 14
Table 3.1:  Nucleotide sequence of the primers used in RT-qPCR .................. 28
# List of Figures

<table>
<thead>
<tr>
<th>Figure</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.1</td>
<td>World production of shrimps – Capture fisheries and Aquaculture</td>
<td>4</td>
</tr>
<tr>
<td>2.2</td>
<td>Issues and challenges in shrimp aquaculture</td>
<td>5</td>
</tr>
<tr>
<td>2.3</td>
<td>Bacteriophage lytic cycle</td>
<td>9</td>
</tr>
<tr>
<td>2.4</td>
<td>Two functions of heat shock proteins</td>
<td>12</td>
</tr>
<tr>
<td>2.5</td>
<td>Upregulation of heat shock protein</td>
<td>13</td>
</tr>
<tr>
<td>2.6</td>
<td>Chemical structure of Phloroglucinol</td>
<td>17</td>
</tr>
<tr>
<td>2.7</td>
<td>Schematic representation of the siRNA pathway in Crustacea</td>
<td>19</td>
</tr>
<tr>
<td>2.8</td>
<td>Artemia life cycle</td>
<td>20</td>
</tr>
<tr>
<td>3.1</td>
<td>Experimental design for dose response relationship</td>
<td>24</td>
</tr>
<tr>
<td>3.2</td>
<td>Experimental design for mode of action</td>
<td>25</td>
</tr>
<tr>
<td>3.3</td>
<td>Experimental design for gene expression of hsp70 family and immune genes</td>
<td>27</td>
</tr>
<tr>
<td>3.4</td>
<td>Experimental design RNAi experiment</td>
<td>30</td>
</tr>
<tr>
<td>4.1</td>
<td>Survival (%) of phloroglucinol-pretreated brine shrimp larvae after 48 h of challenge with V. harveyi BB120</td>
<td>33</td>
</tr>
<tr>
<td>4.2</td>
<td>Survival (%) of brine shrimp larvae pretreated with antioxidant enzyme (catalase)</td>
<td>34</td>
</tr>
<tr>
<td>4.3</td>
<td>Survival (%) of brine shrimp larvae pretreated with antioxidant enzymes (catalase + super oxide dismutase)</td>
<td>35</td>
</tr>
<tr>
<td>4.4</td>
<td>Expression of the (A) hsp70, (B) hsc70, (C) hsc70.5, (D) PDI and (E) HSPA4 genes in brine shrimp larvae</td>
<td>36</td>
</tr>
<tr>
<td>4.5</td>
<td>Expression of (A) the prophenoloxidase (proPO) and (B) transglutaminase I (tgase I) genes in brine shrimp larvae</td>
<td>39</td>
</tr>
<tr>
<td>4.6</td>
<td>Survival (%) of brine shrimp larvae challenged with V. harveyi BB120 and exposed (continuously) to different doses of phloroglucinol</td>
<td>42</td>
</tr>
</tbody>
</table>
ABSTRACT

The phenolic compound phloroglucinol has been extensively studied as a health-care agent for application in (aquatic) animal production system. It is because the compound has to possess a wide variety of biological properties, such as antioxidant, anti-cancer, antimicrobial, anti-tumor etc. In our study, it was aim to verify whether phloroglucinol could induce prophylactic effect on the shrimp model Artemia against Vibrio harveyi, an important bacterial pathogen which cause detrimental effects to shrimp aquaculture worldwide. Subsequently, the mechanistic action of phloroglucinol against Vibrio harveyi BB120 using the axenic brine shrimp and V. harveyi as host-pathogen model system was investigated. Furthermore, the therapeutic effect of phloroglucinol against V. harveyi infection was also studied. The gnotobiotic test system represents an exceptional system for carrying out such studies because it eliminates any possible interference of microbial communities (naturally present in the experimental system) in mechanistic studies and furthermore facilitates the interpretation of the results in terms of a cause effect relationship. The findings from this study provided clear evidences suggesting that phloroglucinol pretreament, at an optimum concentration (30 μM), induced protective effects in the brine shrimp against V. harveyi challenge. By pretreating brine shrimp with the compound in the presence or absence of an antioxidant enzyme mixture (catalase and superoxide dismutase), it was shown that the Vibrio-protective effect of the compound was caused by its prooxidant action (e.g. generation of hydrogen peroxide). Furthermore, phloroglucinol-mediated Hsp70 might be responsible for generating protective innate immunity through regulating the expression of t gase I immune gene in brine shrimp larvae. Besides its prophylactic effect, our results also suggested that phloroglucinol has a therapeutic effect against V. harveyi BB120 as treatment of axenic brine shrimp with dose of 2 μM, and simultaneously challenged with V. harveyi caused a significant enhancement in the survival. Taken together, our results suggest that the ability of phloroglucinol to induce protective immunity and treatment effect makes it a potential natural anti-infective agent for application in the shrimp aquaculture system.

Keywords: Axenic brine shrimp, Vibrio harveyi, phloroglucinol, heat shock protein, prooxidant
CHAPTER 01
INTRODUCTION

1.1. Background information

Shrimp production by aquaculture is a high value activity worldwide (Baruah et al., 2012). According to the FAO statistics shrimps and prawns are the second most important commodity in terms of value, and it accounted for 16% of the total value of internationally traded fish products (FAO, 2016). While global catches of shrimp have been stabilizing since 2012, shrimp aquaculture production is rapidly increasing, with the contribution of 54% to the world shrimp supply by aquaculture in 2014 which was 28% in 2000 (FAO 2016; GOAL 2014, 2016). However, despite all these success the biggest obstacle of shrimp aquaculture practices is the occurrence of diseases caused by pathogenic or opportunistic bacteria such as *Vibrio* spp. and not only it hampers the sustainable development of the sector but also cause indirect impact on the environment. Historically, crucial collapses have been occurred in shrimp aquaculture industry due to disease outbreaks especially in the top producing countries, such as China, Thailand, Indonesia, Taiwan and Ecuador (Lucas and Southgate, 2012).

1.2. Research problem identification and justification

In shrimp aquaculture system, vibrios are among the normal bacterial flora of cultural populations and the habitats (Otta and Karunasagar, 1999; Shakibazadeh et al., 2012) from which *V. harveyi*, *V. alginolyticus* and *V. parahaemolyticus* are most frequently isolated (Sung et al., 2001; Chitov et al., 2009). *V. harveyi* is one of the most significant pathogens which cause devastating effects to wide range of invertebrates including Penaeid shrimp (Abraham and Palaniappan 2004; Vezzulli et al., 2010). Though most *Vibrio* spp are regarded as opportunistic pathogens, *V. harveyi* could be considered as a primary pathogen. They can cause luminescent vibriosis and mass mortalities to *Penaeus monodon* (Karunasagar et al., 1994), *Litopenaeus vannamei* (Aguirre-Guzm’an et al., 2001), *P. japonicas* (Liu et al., 1996), *P. merguensis* (Sae-Oui et al., 1987). During the last few decades, mass mortalities of Penaeid shrimp occurred due to *V. harveyi* infections were reported frequently in hatcheries and grow-out pond (Uma et al., 2008; Vandenberghe et al., 1999; Vezzulli et al., 2010; Chrisolite et al., 2008) particularly in South America, Ecuador, and Asia (Chatterjee and Haldar, 2012). *V. harveyi* is a marine Gram-negative luminous organism (Farmer et al., 2005). Virulence of this pathogen may be due to the production of a number of virulence determinants including biofilms (Karunasagar et al., 1994), extracellular products (ECPs) notably proteases and haemolysins (Liu et al., 1996; Soto-Rodriguez et al., 2003), lipopolysaccharides (LPS) (Montero and Austin 1999), and interaction with bacteriophage (Oakey and Owens 2000) and bacteriocin-like substance (BLIS) (Prasad et al., 2005), which are regulated by quorum sensing (Henke and Bassler, 2004). However, it is unclear which one is the most important virulent determinant and possibly, there are strain differences, and virulence may be attributable to any of a number of factors (Austin and Zhang 2006).
Chemotherapy is the conventional approach of preventing, controlling and treating bacterial diseases in aquaculture (Vaseeharan et al., 2004; Reverter et al., 2014) and it is effective, rapid and help in maintaining healthy population of animals (Sekkin and Kum, 2011). However, chemotherapy including antibiotics has limited application due to severe adverse effects on the environment and the living organisms (Hameed and Balasubramaniam, 2000). Particularly, development of resistant pathogens due to inappropriate use of antibiotics (Defoirdt, 2007), contamination of the environment and accumulation in tissues of sea food (Vadstein, 1997; Reverter et al., 2014). Moreover, V. harveyi has the ability to form biofilms with resistance to disinfectants and antibiotics (Karunasagar et al., 1994). Thus, eco-friendly prophylactic bio-control methods, alternative to chemotherapeutics, is gaining momentum for further development of more sustainable aquaculture.

Recently many studies are focusing on the application of heat shock proteins (Hsps) as disease prevention strategy owing to their multi-functional features. Hsps are soluble intracellular chaperones present in all organisms from prokaryotes to eukaryotes (Srivastava, 2002; Robert, 2003) and are expressed constitutively and/or induced by stress (Cimino et al., 2002; Feder, 1999; Feder and Hofmann, 1999). Hsps perform essential biological function under both normal and stressful conditions by assisting in the folding of nascent proteins, assembly and disassembly of multi-subunit protein complexes, translocation of proteins across membranes and degradation of proteins (Cimino et al., 2002; Feder, 1999; Buchanan, 2000). The families of Hsps are classified according to their molecular mass in kilodaltons (kDa) such as Hsp110, Hsp90, Hsp70, Hsp60, Hsp21, and Hsp10. Hsp70 has gained attention in many studies and this protein can be found in both constitutive and inducible forms (Baruah et al., 2014). Various environmental stressors such as temperature shock, oxidative stress, nutritional deficiencies, ultraviolet radiation, chemicals, various biotic stressors and anoxia triggers the upregulation of hsp70 mRNA and increased Hsp70 production (Kumar et al., 2018; Norouzitallab et al., 2015; Baruah et al., 2010; Rahman et al., 2004; Pockley, 2003).

Some synthetic and plant-derived small molecules are identified as Hsp inducers to prevent disease conditions (Putics et al., 2008; Ohtsuka et al., 2005, Soti et al., 2005; Westerheide and Morimoto, 2005). These compounds are referred to as heat shock protein inducing compounds (HSPI). It is interesting to investigate natural phenols and polyphenols as Hsp inducing agents since they are safe, easily available at low cost and can be easily administered. Most of phenolic compounds derived from traditional medicinal plants have antioxidant and anti-inflammatory properties, but only few of them are potent Hsp activators/modulators (Mait and Manna, 2014). Recently, several bioactive compounds including curcumin, celastrol, gambogic acid, (Mait and Manna, 2014), Tex-OE® (Niu et al., 2014; Baruah et al., 2012), carvacrol (Baruah et al., 2017), pyrogallol (Baruah et al., 2015), phloroglucinol (Kumar et al., 2018) have been identified as Hsp modulators.
Thus, development of natural phenol and polyphenol products that protect the shrimp against vibrios by enhancing the immune reactivity against the pathogen is necessary. However, to study the anti-pathogenic effects of a polyphenol plant-based compound in vivo, elimination or extrication of the effect of naturally occurring microbial communities associated with the host is important (Baruah et al., 2017) since in germ-associated conditions, the compound of interest is either metabolized by microbial communities or influences the physiology of host-associated microbes, thereby making it difficult to understand the host response toward tested compound (Baruah et al., 2015). Consequently, the selection of appropriate animal model system is vital. Considering all these requirements the brine shrimp (A. franciscana) that can be reared under gnotobiotic conditions (allowing full control over the host-associated microbial communities) with relatively low space and cost, is an appropriate model system for conducting such study. Moreover, this characteristically small, highly osmotolerant (Triantaphyllidis, 1998) brine shrimp has a rapid generation cycle (cyst to adult in 20–30 days), well-characterized developmental stages, the ability to form cyst that can be stored and used and established molecular techniques like RNA interference (RNAi). All these traits make brine shrimp an exceptional experimental system to study the biological activity of plant-based compounds (King and MacRae, 2012; King et al., 2013; Baruah et al., 2015; Iryani et al., 2017).

1.3. Objectives of the study

Using brine shrimp as a model system, this study aimed to investigate the following objectives:

- To verify whether phloroglucinol could induce prophylactic effect against *V. harveyi* BB120 strain.
- To unravel the underlying mechanism of action of phloroglucinol behind the possible prophylactic effect.
- To investigate the possible association of Hsp70 family genes and immune genes for the induction of protective responses in brine shrimp against pathogenic *V. harveyi* BB120 strain.
- To investigate the effect of phloroglucinol as a treatment against *V. harveyi* BB120 strain.
CHAPTER 02
REVIEW OF LITERATURE

2.1. Global shrimp production – an overview

Shrimp has become a popular cuisine and a good protein source with high international market value. After being the most important commodity traded in value term for decades, shrimps and prawns now rank second in value term since 2013 and accounted for about 16% of the total value of internationally traded fish products in 2016 (FAO, 2016). Global catches of shrimp have been stable at 3.5 million tonnes since 2012 (3.3 million tonnes in 2012 and 3.5 million tonnes in 2016), with the exception of Argentine red shrimp (*Pleoticus muelleri*) (FAO 2016). While shrimp capture fisheries are stagnating shrimp aquaculture is increasing with the contribution of 54% to the world shrimp supply by aquaculture in 2014 which accounted 28% in 2000 (FAO 2016; GOAL 2014, 2016). In 2015, brackish and marine water shrimp production was recorded at 4.13 million tonnes while freshwater prawn production was 0.74 million tonnes (FAO 2015).

Figure 2.1: World production of shrimps – Capture fisheries and Aquaculture (Source: GOAL, 2016)

Shrimps and prawns are mainly produced in developing countries, and much of this production enters international trade. In 2016 shrimp and prawns gained a share of 6.47% of the total fish exports in the world which is almost 50% increment compared to 1996 (3.73%). However, it shows a decline compared to 2006 (6.50%) as economic conditions improve, growing domestic demand in these countries is leading to lower exports and due to the shrimp disease occurred in recent years, in particular in Asia (FAO 2016). China is the number one shrimp producer in the world followed
Black tiger shrimp, *P. monodon* was the major species of shrimp aquaculture with a contribution of more than 50% of total cultured shrimp production increasing yearly from 1985 to 1995. Due to the occurrence of disease problems, mainly the outbreaks of white spot syndrome virus (WSSV) and yellow head virus (YHV), production of *P. monodon* was decreased dramatically after 1995 (Le Hong, 2008). In order to overcome the persistent production losses of black tiger shrimp, a continuous expansion of the production of Pacific white shrimp, *L. vannamei* is driven in many countries (Briggs et al., 2004, Sellars et al., 2015). Favorable features of *L. vannamei* such as fast growth, low production cost, the potentiality for intensive culture and resistance to diseases make it one of the mostly used species in aquaculture at present.

Since 1969, it has been cultured in America and it was introduced to Asia after 2000. *L. vannamei* production accounted for more than 75% of Asian shrimp production from 2007 (Wyban, 2009) and was the dominant species farmed in Thailand, China, and Indonesia. In 2016, *L. vannamei* production was 4.1 million tonnes while the *P. monodon* contributed with only 0.7 million tonnes (FAO, 2016).

### 2.2. Constraints in shrimp aquaculture

According to the reports by Global Aquaculture Alliance 2016 survey, shrimp aquaculture sector is constrained by several challenges and issues including disease, feed cost, seed stock quality and availability, access to disease-free brood stock, international market prices etc. However, a major obstacle in shrimp aquaculture is the diseases which are considered a significant problem to the development of a sector with severe economic losses worldwide (Chatterjee and Haldar, 2012).

![Figure 2.2: Issues and challenges in shrimp aquaculture](Source: GOAL, 2016)
2.2.1. Diseases as a major constraint in aquaculture

Diseases continue to challenge the aquaculture sector globally and disease outbreaks have cost significant production losses (Brown and Poulain, 2013; Cattermoul et al., 2014). Since the disease is one of the primary hindrances to aquaculture development of many species, focus on aquatic biosecurity and health management is continuously increasing worldwide (FAO, 2015). In recent years, although global farmed shrimp production has increased, major producing countries, in particular in Asia, has experienced a decline in output because of shrimp disease. For instance, in 2014, Viet Nam became the third major exporter, overtaking Thailand due to the substantial decline in exports since 2013, mainly linked to reduced shrimp production as a result of disease problems (FAO 2016).

2.3. Major diseases in shrimp aquaculture

Diseases in shrimp aquaculture include syndromes with infectious and non-infectious etiologies. Infectious diseases caused by pathogen, such as virus, bacteria, fungal, protistan and metazoan and noninfectious diseases including diseases due to environmental extremes, nutritional imbalances, toxicants, and genetic factors are with greater economic importance for shrimp aquaculture (Lightner, 1988, 1993a, 1996; Brock and Lightner, 1990a; Fulks and Main, 1992; Johnson, 1995). Viral diseases which include at least 20 different viruses are crucial among all the infectious diseases (Dastidar et al., 2013). Examples of such diseases include white spot syndrome (WSSV), infectious hypodermal and hematopoietic necrosis (IHHNV), yellow head virus (YHV), hepatopancreatic parvovirus (HPV), Taura syndrome virus and many others (FAO, 2007; Israngkura and Sae-Hae, 2002). The monodon baculovirus (MBV) in Taiwan in the mid-1980s was the first widely reported epidemic shrimp disease (Liao et al., 1992) and it was followed by infectious hypodermal and hematopoietic necrosis virus (IHHNV) in the Americas (Lightner, 1996), yellow head virus (YHV) in Thailand (Flegel, 1997) and Taura syndrome virus (TSV) in the Americas (Brock, 1997), all creating big economical losses for both P. monodon and L. vannamei. (FAO 2015). The economic losses due to viral diseases in shrimp culture have significantly contributed to the profit margin and this value range from a level of US$ 17.5 million to US$ 3.2 billion (FAO, 2007).

2.3.1. Vibriosis in shrimp aquaculture

Bacterial diseases cause a range of problems to shrimp aquaculture ranging from mass mortalities to growth retardation and sporadic mortalities. Several bacterial species are responsible for the major bacterial diseases of shrimps. Among them, Vibrio species are the vital bacterial pathogens of shrimp which cause deleterious effects to the shrimp production. They are widely distributed in freshwater, brackish water, and marine water environments. Among the identified Vibrio species V. harveyi, V. splendidus, V. penaeacea, V. anguillarum, V. parahaemolyticus, V. vulnificus, V. campbelli etc are the major pathogens of aquatic animals including shrimps (Brock and Lightner, 1990; Lightner 1996; Otta et al., 1999a, 2001). Vibrio disease in shrimp is described as vibriosis, penaeid bacterial septicemia, penaeid vibriosis, luminescent vibriosis or red-led disease (Aguirre-
Signs of the disease include lethargy, tissue and appendage necrosis, slow growth, slow metamorphosis, body malformation, bioluminescence, muscle opacity and melanization of the exoskeleton (Aguirre-Guzman et al., 2004). Some Vibrio species have been identified as opportunistic pathogens which cause disease when the host is stressed or immune suppressed while some species are primary pathogens (Lavilla-Pitogo et al., 1990; Peddie and Wardle, 2005).

Due to disease outbreaks, especially the AHPND outbreaks caused by V. parahaemolyticus in China, Thailand and Vietnam, shrimp production in East and Southeast Asia declined between 2011 and 2015. The loss of revenue due to AHPND in Southeast Asia has so far been estimated at over US$ four billion (FAO, 2016).

2.3.2. Vibrio harveyi and diseases

V. harveyi are luminous bacteria widely distributed in the marine environment in association with surface and gut of marine and estuarine organisms and also in shrimp pond water and sediment (Karunasagar et al., 1994; Otta et al., 1999a, 2001; Sawabe et al., 2007). It causes disease in wild and cultured aquatic organisms including penaeid shrimps, finfish and molluscs causing severe losses to aquaculture (Karunasagar et al., 1994; Sawabe et al., 2007). Though most Vibrio spp are regarded as opportunistic pathogens, V. harveyi could be considered as a primary pathogen. They can cause luminescent vibriosis resulting mass mortalities to P. monodon (Karunasagar et al., 1994), L. vannamei (Aguirre-Guzm’an et al., 2001), P. japonicas (Liu et al., 1996), P. merguensis (Sae-Oui et al., 1987). Luminescent vibriosis has become a major constraint to shrimp production in South America and Asia (Austin and Zhang, 2006). Also, it has contributed to the big collapse in shrimp grow out in the Philippines in 1993 (Lavilla-Pitogo, 2003). One of the major health threats for shrimp aquaculture in India is vibriosis caused by V. harveyi, V. parahaemolyticus, V. splendidus (Mohan, 1996). Losses due to luminescent vibriosis in Indonesian hatcheries in 1991 have been reported as high as US$100 million (Defoirdt et al., 2007a). A study conducted by Karunasagar et al. (1994) noted that certain strains of V. harveyi isolated from sea water had high LD$_{50}$ for P. monodon larvae, while isolates from moribund larvae had a low LD$_{50}$ suggesting that V. harveyi strains may vary in virulence.

2.4. Disease prevention, control, and treatment

Chemotherapy is the conventional way of preventing, controlling and treating bacterial diseases including Vibriosis in aquaculture (Vaseeharan et al., 2004; Reverter et al., 2014). Use of these chemical drugs such as antibiotics is effective, rapid and help in maintaining a healthy population of animals (Sekkin and Kum, 2011). However, chemotherapy imparts severe adverse effects on the environment and the living organisms (Hameed and Balasubramaniam, 2000). For example, development of resistant pathogens due to inappropriate use of antibiotics (Defoirdt, 2007), contamination of the environment and accumulation in tissues of sea food (Vadstein, 1997; Reverter et al., 2014).
et al., 2014). Thus, environmental friendly prophylactic alternatives and bio control methods gain momentum.

2.4.1. Green water technique

The green water technique is mainly based on the addition of selected micro algal species in closed aquaculture systems and it’s a most commonly used bio technique to control disease, demonstrating promising results with aquaculture organisms. *Isochrysis galbana* enhances survival of sea bass larvae (Cahu et al., 1998) and *Tetraselmis* species protects fish and shrimp against the detrimental effects of pathogenic bacteria by producing antibacterial substances (Austin and Day, 1990; Austin et al., 1992; Salvesen et al., 2000). According to Cohen, (1999) addition of cyanobacteria attenuates disease caused by bacteria and virus by stimulating secretion of an antioxidative substance and immunologically active molecules and virostatic compound. Although these findings suggest using green water technique attenuate disease in diverse ways, effectiveness in controlling disease still have to be proven (Defoirdt et al., 2007).

2.4.2. Probiotics

As per the FAO/WHO (2001) definition probiotics are ‘live micro-organisms which when administered in adequate amounts, confer a health benefit on the host’. However, probiotics in aquaculture can be live or dead preparations, including cellular/extracellular components of the micro-organism(s), administered either as a feed supplement or to the rearing water (Sharifuzzaman and Austin, 2017). Upon addition, probiotics improve the health of the organism, enhance larval survival and prevent the proliferation and colonization of opportunistic and pathogenic bacteria in intensive aquaculture rearing systems (Fanzafar, 2006). Further, it contributes to intestinal microbial balance (Talwalkar and Kailasapathy, 2003; Fanzafar, 2006), produces inhibitory compounds and chemical substances such as acid, hydrogen peroxide and bacteriocins that antagonize pathogen growth (Reid, 1999; Vazquez et al., 2005). Probiotics also compete for adhesion sites in the gut reducing colonization of opportunistic bacteria (Strom and Ringo, 1993) and enhance the immunity (Vaseeharan and Ramasamy, 2003). In the case of shellfish, probiotics demonstrated effectiveness against *V. alginolyticus*, *V. coralliilyticus*, *V. harveyi*, *V. parahaemolyticus* and *V. splendidus* infections in penaeid shrimp, scallop (*Pecten maximus*) and Pacific oyster (*Crassostrea gigas*) (Kesarodi-Watson et al., 2012; Li et al., 2008; Preetha et al., 2007; Tseng et al., 2009). By all these means probiotics help in disease prevention of the organisms and improve the health status.

2.4.3. Phage therapy

In phage therapy bacteriophages (viruses that infect bacteria) specifically, kill a certain pathogen. Bacteriophages were discovered as viral infections of bacteria in the early 1920s and their value for antibacterial therapy was almost immediately recognized (Nakai and Park, 2002). The mechanism of bacteriophage through the process called lysis (lytic cycle) is as explained in Figure 2.3. A major advantage of phage therapy is that non-target microbiota is not affected because the phages usually
have a narrow host range rather than a broad spectrum (Defoirdt et al., 2011). However, many phages are strain specific rather than species-specific (Defoirdt et al., 2011), thus phages for use as biocontrol agents to treat luminescent vibrios should be selected on their capability to infect a wide range of luminescent vibrios (Defoirdt et al., 2007).

![Figure 2.3: Bacteriophage lytic cycle](http://top-img.com/b/bacteriophage-lytic-cycle)

**Figure 2.3: Bacteriophage lytic cycle** - (1) Phage attaches to a specific host bacterium and (2) injects its DNA, (3) disrupting the bacterial genome and killing the bacterium, and (4) taking over the bacterial DNA and protein synthesis machinery to make phage parts. (5) The process culminates with the assembly of new phage, and (6) the lysis of the bacterial cell wall to release a hundred new copies of the input phage into the environment (Thiel, 2004) (Image: http://top-img.com/b/bacteriophage-lytic-cycle).

Phage therapy has been already applied successfully in aquaculture practices for treating some bacterial infections (Karunasagar et al., 2007; Imbeault et al., 2006; Vinod et al., 2006; Nakai and Park, 2002; Nakai et al., 1999). Interestingly, the phage treatment performed much better than the daily addition of antibiotics (5 mg/l oxytetracycline and 10 mg/l kanamycin), in which the shrimp survival was only 40%.

Some constrains in phage therapy such as carrying virulence factors (Austin et al., 2003; Khemayan et al., 2006) and rapid development of resistance to phage attachment, which renders bacteria resistant to phage attack (Fischetti et al., 2006) can be overcome by testing whether they carry any virulence genes and safety, and by applying cocktails of phages or by using phage components instead of intact phage (Defoirdt et al., 2007).

### 2.4.4. Immunostimulants

Immunostimulants are biological and synthetic chemicals that stimulate the immune response (Bagni et al., 2005) and which are administered as dietary supplements by injection or immersion (Smith et al., 2003). Immunostimulants receiving most attention comprise live bacteria, killed bacteria, glucans, peptidoglycans and lipopolysaccharide (Smith et al., 2003). Immunostimulants
are vital especially for crustaceans such as shrimps which rely only on the innate immune system (Rowley and Powell, 2007). The innate immune mechanisms in crustaceans include phagocytosis, encapsulation, nodule formation, blood coagulation and clot formation, the release of antimicrobial peptides, and melanization through the prophenoloxidase (proPO) cascade (Rowley and Powell, 2007).

Several reports have mentioned the use of immunostimulants in aquaculture, for example, to control luminescent vibriosis in shrimp, resulting in increased prophenoloxidase and phenoloxidase activities and hemocyte counts, and significantly increased survival after experimental infection with luminescent vibrios (Alabi et al., 1999; Thanardkit et al., 2002; Marques et al., 2006). *Aeromonas hydrophilia* enhances survival of the Artemia upon *V. campbellii* challenge (Marques et al., 2005). Although immunostimulation shows promise it has some drawbacks such as immunostimulation might be too intense and can harm or even kill the host (Smith et al., 2003; Vadstein, 1997), the response is likely to be short in duration since there is no memory component involved and long-term administration of such agents seems to decrease the immunostimulant effect and does not always promote disease resistance (Sakai, 1999; Smith et al., 2003).

Previous studies on immunostimulants such as PHB (Baruah et al., 2015), marine algae (Felix and Rajeev, 2004) and natural herbs (Citarasu et al., 2006), showed that there were some protective innate immune responses, especially the proPO immune system possibly mediated by immunostimulants induced Heat shock proteins (HSPs). Clear evidence suggested that heat shock proteins (HSPs) can mediate responses similar to that of immunostimulants (Baruah et al., 2013; 2012; 2010; Zügel and Kaufmann, 1999).

### 2.5. Heat Shock Proteins

#### 2.5.1. The function of heat shock proteins and mechanism of action

Heat shock proteins are highly conserved proteins which also referred to as molecular chaperones, found in all cellular organisms when they are exposed to cellular stress (Whitley et al., 1999; Welch 1993). They are of varying molecular weight from 16 – 100kDa (Welch 1993). Observation of focal swelling (puffs) on polytene chromosomes of fruit flies (*Drosophila*) salivary glands after heat shock exposure led to the first discovery of Heat shock proteins (Whitley et al., 1999; Roberts et al., 2010). Eventually it was found that these swellings were the sites of transcriptional induction of genes encoding for a particular group of proteins, which were designated HSPs (Tissieres et al., 1974), it’s not a unique phenomenon to fruit flies but occur in mammals and occur in all evolutionary levels (Lindquist and Craig, 1988) and HSPs response is universal to all cells and that other stressors such as anoxia ischaemia, toxins, protein degradation, hypoxia, acidosis and microbial damage which lead to their up-regulation (Chiang et al., 1989; Whitley et al., 1999; Welch 1993) hence, termed also as stress proteins which are more elucidate Locke, 1997; Whitley et al., 1999).
Intracellular counterparts of HSPs referred to as chaperones, constitutive chaperones or heat shock cognates (HSCs) generally residing in the cytosol, nucleus, and mitochondria. They play a major role in numerous homoeostatic functions, including maintenance of protein structure and folding, supporting and repairing damaged cytoskeleton elements, assisting in the production and folding of intra-cellular proteins to eventually achieve functional shape, enzymes and hormone receptors and maintenance of mitochondria, and nuclear and cell wall lipoprotein membranes (Beckman et al., 1990). In response to any kind of cellular stress including cold, heat, UV radiation, toxins, pathogens, nutritional deficiency, protein degradation, hypoxia, acidosis, microbial damage constitutive HSCs are upregulated to produce newly formed HSPs which can be detected in the cells at concentrations two or three times those of the constitutive chaperones as well as in tissue fluids (Chiang et al., 1989; Locke, 1997) and they assist in the repair of denatured proteins or promote their degradation after stress or injury. They have been referred to as “molecular chaperones” because of this function.

Moreover, Hsps are released into the extracellular compartments under certain physiological conditions (Pockley, 2003) and play significant regulatory roles in both innate and adaptive immunity (Phohaszka et al., 2002; Robert, 2003). Extracellular Hsps are postulated to mediate production of cell surface peptides and aid in the immune system to recognize diseased cells (Deane et al., 2004). By stimulating Toll-like receptors TLR2 and TLR4 (Asea et al., 2002; Vabulas et al., 2002), Hsp70 transduces inflammatory danger signals to immune cells (Srivastava, 2002a; Fleshner and Johnson, 2005). It also stimulates the secretion of inflammatory cytokines, nitric oxide (NO) synthase, tumor necrosis factor α (TNF-α), Interleukin (IL)-1β and IL-6 from macrophages and neutrophils (Asea et al., 2002; Campisi and Fleshner, 2003; Johnson and Fleshner, 2006). During adaptive immunity Hsps may play a role in major histocompatibility complex (MHC)- class II-peptide complex assembly (DeNagel and Pierce, 1993) which involved in antigen presentation and subsequently activating T cells to destroy pathogens as well as malfunctioning cells (Srivastava, 2002a, 2002b).
Figure 2.4: Two functions of heat shock proteins. *Top:* As new polypeptide chains (proteins) are being produced by ribosome within the cell, heat shock proteins assist in the correct folding of polypeptide chain into a functional protein. Presence of heat shock protein (*purple*) assures that the new protein will assume its functional three-dimensional configuration. *Bottom:* After stress event, heat shock proteins also assist in refolding or degradation of damaged or denatured proteins (Source: Whitley *et al.*, 1999).

Regulation of HSP gene transcription is mediated by the interaction of heat shock factors (HSFs) with heat shock elements in gene promoter regions (Voellmy 1994; Pockley 2003). Though the structural similarity of HSFs of plants and animals is considerable, there are significant differences in the complement and activity of HSF family members in different groups of organisms (Feder and Hofmann 1999). For example, most of the insects have only HSF1, finfish and other vertebrates have three or four HSFs while HSF1, HSF2, HSF4 commonly expressed and HSF3 is avian specific (Roberts *et al.*, 2010). Different heat shock transcription factor family are activated by specific stresses. Inactive heat shock factors which exist as monomers become trimers once they are activated and these trimerized active form of HSFs are capable of binding to the promoter site of the stress protein gene. This binding initiate transcription and translation (Fig 5) (Whitley *et al.*, 1999).
Figure 2.5: Upregulation of heat shock protein. Many types of stress are capable of increasing expression of some heat shock proteins (inducible). Stress results in activation of heat shock factor (HSF) monomers (red). HSF monomer moves from cytosol to nucleus where it combines with other monomers to form a trimer. A trimer of HSFs attaches to the operator (promotor site) on heat shock gene (DNA). Attachment of trimer to operator results in the production of heat shock protein messenger RNA (green), and transcription occurs in minutes. Messenger RNA moves to the cytosol where heat shock protein is formed (translation) (Source: Whitley et al., 1999).

2.5.2. HSP families

Several families of Hsps are identified in eukaryotes and they are categorized according to their function, sequence homology and molecular mass in kilo-Daltons (kDa) (Whitley et al., 1999; Roberts et al., 2010). The families primarily include Hsp100, Hsp90, Hsp70, Hsp60, Hsp40 and several smaller Hsp groups (Table 1) (Whitley et al., 1999; Roberts et al., 2010). Many family members of the Hsp families have counter parts referred to as heat shock cognates (Hscs) which are expressed in the cell under normal non-stress conditions. They play a major role in the regulation of normal protein synthesis (Roberts et al., 2010).
### Table 2.1: Major Hsp families (modified from Lindquist 1992)

<table>
<thead>
<tr>
<th>Protein</th>
<th>Members</th>
<th>Monomer mass (kDa)</th>
<th>Cell localization</th>
<th>Function</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hsp 100</td>
<td>ClpA, ClpB, ClpC, Hsp104</td>
<td>80 - 110</td>
<td>Cytoplasm, nucleolus, nucleus, chloroplast</td>
<td>Thermotolerance, ethanol tolerance, long-term spore viability</td>
</tr>
<tr>
<td>Hsp 90</td>
<td>Hsp82, Grp94, HtpG</td>
<td>82 – 96</td>
<td>Cytoplasm, nucleus</td>
<td>Essential for viability; increased concentration required for growth at high temperatures</td>
</tr>
<tr>
<td>Hsp 70</td>
<td>DnaK, grp78, hsc70, BIP, Kar2, ssa, ssb, ssc, ssd</td>
<td>67 - 76</td>
<td>Cytoplasm, nucleus, mitochondria, chloroplasts, endoplasmic reticulum</td>
<td>Chaperone required for protein assembly, secretion, protein import into the endoplasmic reticulum and organelles; growth at high temperatures</td>
</tr>
<tr>
<td>Hsp 60</td>
<td>GroEL, Hsp65, cpn60, Rubisco-binding protein</td>
<td>58 – 65</td>
<td>Mitochondria, chloroplasts</td>
<td>Chaperonin, assembly of oligomeric proteins and folding of monomeric proteins; high concentration required for growth at elevated temperatures</td>
</tr>
<tr>
<td>sHsps</td>
<td>Many</td>
<td>18-40</td>
<td>Cytoplasm, nucleus</td>
<td>Protection from stress, apoptosis inhibition</td>
</tr>
</tbody>
</table>

### 2.5.3. HSP 70 family

Hsp70s are a group of highly conserved proteins of which expression is constitutive or inducible under different conditions (Sanders, 1993). The constitutively expressed protein shares about 95% sequence homology (identity of the DNA sequence) with the inducible form of Hsp70. They are molecular chaperones that play key roles in many vital cellular functions under both normal and stressful conditions, and those include assisting in the folding of nascent proteins, translocation of these proteins between cell organelles, assembly and disassembly of multi-subunit complexes, refolding or degradation of denatured proteins due to stresses, dissolution of pathological protein aggregates, and other processes enhancing the survival of normal and diseased cells (Roberts et al., 2010). Further, Hsp 70 family is the largest stress protein family with at least 121 proteins have been characterized (Sanders, 1993). Higher degree of sequence homology can be seen in prokaryotic and eukaryotic Hsp70 (about 60% and 46% amino acid sequence identity) and might have the same
biological activity in all Hsp70 proteins regardless of the source (Baruah et al., 2010; Pockley et al., 2008) especially in relation to their protective properties against bacterial infections.

Hsp70 has gained considerable attention as a potential agent for the prevention and treatment of diseases in human as well as in farmed terrestrial and aquatic animals (Nagai et al., 2010; Roberts et al., 2010; Westerheide and Morimoto 2005) and members of the Hsp70 family are the most extensively studied group of stress proteins to date. For example study of Baruah et al., 2017 showed the induction of Hsp72 using phenolic compound carvacrol is associated with the generation of resistance in Artemia larvae against lethal heat stress or pathogenic Vibrio harveyi.

Another study found that Poly-β-Hydroxybutyrate (PHB) confer protection to Artemia host against Vibrio campbellii by a mechanism of inducing heat shock protein (Hsp) 70 and this effect of PHB is associated with the generation of protective innate immune responses, especially the prophenoloxidase and transglutaminase immune systems (Baruah et al., 2015). According to Kumar et al., 2018 phloroglucinol induced Hsp70 mediates the survival of brine shrimp larvae against V. paraahaemolyticus infection. Yung et al., 2008 showed that Hsp 70 induction by combined hypothermic/hyperthermic shock followed by recovery at ambient temperature increases the thermostolerance and protect against infection by V. campbellii in Artemia larvae. Non-lethal heat shock (NLHS) enhance the shrimp tolerance to V. paraahaemolyticus AHPND infection and this is likely mediated by the induction of HSP70, HSP90 and subsequent activation of the proPO system (Junprung et al., 2017). The survival of Artemia nauplii knocked down Hsp70 by RNAi, as compared to those containing the Hsp, was reduced 41% by heat stress and 34% upon V. campbellii infection (Iryani et al., 2017).

### 2.6. Polyphenolic compounds and Phloroglucinol

#### 2.6.1. What are polyphenolic compounds?

Polyphenols are secondary metabolites present in all vascular plants (in fruits, vegetables, cereals) and constitute a large family of ubiquitous and varied substances, from simple molecules to complex structures (Munin and Edwards-Levy, 2011). They are mostly considered as secondary metabolites and they play an important role in plant growth, reproduction, fruits color, sensory characteristics and as defense mechanisms against ultraviolet radiation and pathogens (Balasundram et al., 2006). To date, several thousands of polyphenolic compounds have been characterized in plants and grouped together in various classes. Variations in the basic chemical structure determine the degrees of oxidation, hydroxylation, methylation, glycosylation and the possible connections to other molecules (primary metabolites such as carbohydrates, lipids, proteins, or phenolic secondary metabolites) (Munin and Edwards-Levy, 2011).

Natural polyphenols have been widely studied and found to possess many important bioactivities (Arts and Hollman, 2005) such as controlling gene expression, induce apoptosis, decrease platelet
aggregation, increase blood vessel dilation, modulate intercellular signaling and modulate enzyme activities within the body (Rodrigo et al., 2011).

2.6.2. Functions of polyphenolic compounds

Since last decade, there has been much more interest in the potential health benefits of dietary plant polyphenols as antioxidants. Several epidemiological studies and associated meta-analyses strongly suggest that long-term consumption of diets rich in plant polyphenols offer protection against development of cancers, cardiovascular diseases (CVDs), diabetes, osteoporosis and neurodegenerative diseases (Watson et al., 2013; Habauzit and Morand, 2011; Vauzour et al., 2010; Pandey and Rizvi, 2009).

Among the notable bioactivities of phenolic compounds, the antioxidant activities have been widely studied, including scavenging of free radicals, inhibition of lipid oxidation, reduction of hydroperoxide formation, and etc (Sato et al., 1996). Postprandial hyperlipemia and oxidative stress, a well-defined risk factor for atherosclerosis, could be reduced by polyphenols due to its antioxidant activity (Li et al., 2014). Numerous studies have shown that dietary polyphenols could reduce the risk of thrombosis, which is one of the leading causes for myocardial infarction, ischemic heart disease, etc. (Santhakumar et al., 2013; 2015; Singh et al., 2008). Polyphenols could play an important role in anticancer. The anticancer effects of polyphenols have been observed at mouth, stomach, duodenum, colon, liver, lung, mammary gland or skin. Many polyphenols, such as proanthocyanidins, flavonoid, resveratrol, tannins, epigallocatechin-3-gallate, gallic acid, and anthocyanin, have been tested; all of them showed protective effects in some models although their mechanisms of action were found to be different (Johnson et al., 1994). Polyphenols have been demonstrated potential antibacterial, antifungal and antiviral activities (Baydar et al., 2006; Chavez et al., 2005; Jung et al., 2005). Some studies have shown the sensitivity of different bacterial species such as Staphylococcus aureus, Pseudomonas aeruginosa, Bacillus subtilis, Escherichia coli, Salmonella and Listeria monocytogenes to various natural polyphenols. The destabilization of the outer membrane of Gram-negative microorganisms, as well as interactions with the cell membrane, might be one of the specific mechanisms behind the antibacterial action.

Although several biological effects based on epidemiological studies can be scientifically explained, the mechanism of action for some effects of polyphenols is not well understood. The complete role of polyphenols in human health is yet to be investigated.

2.6.3. Natural polyphenolic compounds as Hsp inducers

Some synthetic and plant-derived small molecules are identified as HSP inducers to prevent disease conditions (Putics et al., 2008; Ohtsuka et al., 2005, Soti et al., 2005; Westerheide and Morimoto, 2005). These compounds are referred to as heat shock protein inducing compounds (HSPi). It is interesting to investigate natural polyphenols as HSP inducing agents. Polyphenolic compounds are safe, easily available at low cost and can be easily administered. Most of the phenolic compounds derived from
traditional medicinal plants have antioxidant and anti-inflammatory properties, but only a few of them are potent HSPs activators/modulators (Mait and Manna, 2014). Recently, several bioactive compounds including curcumin, celastrol, gambogic acid, (Mait and Manna, 2014), Tex-OE® (Niu et al., 2014; Baruah et al., 2012), carvacrol (Baruah et al., 2017), pyrogallol (Baruah et al., 2015) have been identified as HSPs modulators.

For example, a study carried out using phenolic compound carvacrol, an approved food component, and using the well-established gnotobiotic Artemia model system, authors have determined whether carvacrol could induce Hsp72 in vivo, whether this putative effect could generate resistance in Artemia against biotic/abiotic stress and also unraveled the mechanism behind the possible HSP72-inducing effect of carvacrol. Here it had been shown in vitro study carvacrol act as a co-inducer of HSP72, enhancing HSP72 production only in combination with a bona fide stress compared to the compound alone. Induction of HSP72 was associated with the generation of resistance in Artemia larvae against subsequent lethal heat stress or pathogenic Vibrio harveyi (Baruah et al., 2017).

Moreover, another study carried out by the same authors have shown that pretreating gnotobiotically cultured brine shrimp with an optimized dose of the phenolic compound pyrogallol (the functional unit of many polyphenols) has induced protective effects in the brine shrimp against V. harveyi infection. Also, they have shown that generation of prooxidant is linked to the induction of heat shock protein Hsp70, which is involved in eliciting the prophenoloxidase and transglutaminase immune responses (Baruah et al., 2015).

### 2.6.4. Phloroglucinol

Phloroglucinol C₆H₃(OH)₃ is also called phloroglucine, 1,3,5-trihydroxy benzene, 1,3,5-benzenetriol (Ullmann et al., 1985) is a member of the polyphenol of the organic chemical group. It is one of the phlorotannin components that is abundantly found in Ecklonia cava (edible brown algae), that belongs to the Laminariaceae family (Ryu et al., 2013).

![Chemical structure of Phloroglucinol](https://pubchem.ncbi.nlm.nih.gov/compound/phloroglucinol#section=Top)
A study has been carried out to determine whether phloroglucinol is effective against the AHPND strain *V. parahaemolyticus* MO904 using gnotobiotic *Artemia* model. It has found that pretreatment with phloroglucinol, at an optimum concentration (30 μM), protects axenic brine shrimp larvae against *V. parahaemolyticus* infection and induced heat shock protein 70 (Hsp70) production (two folds or more) as compared with the control. The protective effect of phloroglucinol was caused by its prooxidant effect and is linked to the induction of Hsp70. Further, RNA interference has used to confirm that phloroglucinol-induced Hsp70 mediates the survival of brine shrimp larvae against *V. parahaemolyticus* infection. The study has validated in xenic *Artemia* model and in a *Macrobrachium rosenbergii* system. Thus, the phloroglucinol treatment might become part of a holistic strategy to control AHPND in shrimp (Kumar et al., 2018).

### 2.7. RNA interference (RNAi)

RNA interference (RNAi) is a biological process where the expression of a specific gene is silenced post-transcriptionally by messenger RNA (mRNA) degradation or translational inhibition after the introduction of gene-specific double-stranded RNA (dsRNA) into a cell or organism (Fire et al., 1998; Meister and Tuschl, 2004; Marc et al., 2010). Since its discovery in 1998, RNAi has been used extensively in research, where it is applied as a functional genomics tool. Additionally, other possible applications, such as the use of RNAi in crop protection as well as its therapeutic use in animals and humans, are under development already (Huvenne and Smagghe, 2010). In Crustacea, RNAi has also already been used successfully as an antiviral strategy in aquaculture (Lima et al., 2013).

#### 2.7.1. Molecular mechanism

While the RNAi mechanism shows a high degree of evolutionary conservation throughout eukaryotes, differences in RNAi pathway components are found between different taxonomical groups. For RNAi, double-stranded RNA (dsRNA), which is introduced into the cell, is processed into small regulatory RNAs consisting of 20–30 nucleotides by an RNase-III-like enzyme called Dicer (Zamore et al., 2000). These small regulatory RNAs are then taken up by the RNA-inducing silencing complex (RISC), which is comprised of various proteins, and which unwinds the small regulatory RNAs into single-stranded molecules. The antisense strand, called the guide strand, is loaded into the RISC complex. The other strand, called the passenger strand, is degraded as a RISC complex substrate. Finally, this complex is coupled to the target mRNA, based on specific base-pairing, to induce endonucleolytic cleavage causing the destruction of mRNA molecules. The RNAi mechanism in shrimp is known to play a role in antiviral immunity.
Figure 2.7: Schematic representation of the siRNA pathway in Crustacea. The long dsRNAs are processed in the cytoplasm by the Dicer-2 RNase into siRNAs. The resulting siRNAs are taken up by the RNA-induced silencing complex (RISC). The duplexed siRNA which is bound to the Ago2 protein, the central component of RISC, is unwound, and the passenger strand rapidly dissociates. Finally, this complex is coupled to the target mRNA, based on specific complementary base-pairing, to induce endonucleolytic cleavage causing the degradation of mRNA molecules (Source – Nguyen et al., 2016)

2.7.2. Disease control

Aquaculture, and more specifically the shrimp industry, is often faced with outbreaks of disease caused by bacteria and viruses. The discovery of RNAi has enabled studies on immune mechanisms and the function of genes involved in the process of fighting bacteria in shrimp species (Nguyen et al., 2016). Mechanisms that prevent bacteria from proliferation are believed to be related with prophenoloxidase. Knockdown of the inactive precursor of prophenoloxidase by RNAi resulted in a significantly increased bacterial load in *M. japonicas* (Faguto et al., 2009). Viral disease outbreaks are a major concern in the development of the shrimp aquaculture industry. RNAi also has been widely used as a powerful tool in identifying the genes that participate viral infection (Li et al., 2007) as well as defense from the host (Pndaro et al., 2013; Wang et al., 2013). One of the potential antiviral therapeutics to be considered is using RNAi to inhibit the replication of the RNA viruses as well as DNA viruses by knocking down virus-specific genes or downregulating host genes that are related to viral replication mechanisms. The direct targeting of viral genes also allows to inhibit viral replication in the host.
2.8. Artemia as a model organism for studying diseases in aquaculture

Artemia also known as brine shrimp belong to the phylum Arthropoda and class Crustacea. Artemia populations are typically found in natural salt lakes and man-made salterns scattered throughout the tropical, subtropical and temperate climatic zones, but they also inhabit brackish and hypersaline waters since they are highly osmotolerance (Van Stappen, 1996). In their natural environment, they have the ability to tolerate harsh environmental conditions such as extreme temperatures, high ultra-violet radiation and changing aeration (Clegg, 2007). Artemia includes several species worldwide namely; A. franciscana, A. sinica, A. urmiana, A. salina, A. tibetiana (Van Stappen, 1996).

2.8.1. Life cycle

Both oviparity (in extreme conditions) and ovoviviparity (in favorable environmental conditions) can be found in Artemia strains and female can switch in between these two reproduction methods (Van Stappen, 1996). Upon immersion in sea water under appropriate temperature and aeration, Artemia cyst hydrate and hatch within 24 hours releasing free-swimming larvae called as instar I nauplius (Van Stappen, 1996). Nauplii thrive completely on yolk sac reserves during this stage and the digestive system is partially functional. Subsequently after one molt (in 8 hours) they become instar II nauplius which can ingest small food particles and then proceed through about 15 molts. 10th Instar stage onwards different morphological and functional changes including sex differentiation occur. Adult Artemia 1cm in length has an elongated body with two stalked complex eyes, a linear digestive tract, sensorial antennulae and 11 pairs of functional thoracopods.

![Figure 2.8 - Artemia life cycle](image-url)
2.8.2. *Artemia* as a model organism

Other than its global popularity as a fish and shrimp larval feed, *Artemia* are widely used for scientific research as a result of several special attributes such as ability to culture under axenic condition, short hatching and generation time, quick stage transition, possibility for being fed using different feed types (algae, bacteria, yeast, detritus), high availability of cyst that can be stored for years, ability to culture with simple techniques and etc (Norouzitallab *et al*., 2014; Baruah *et al*., 2010; Soltanian, 2007).

Critical problem in studying the anti-pathogenic effects of a polyphenol plant-based compound *in vivo* is the difficulty to either eliminate or extricate the effect of the microbial communities that occur naturally in the system (Baruah *et al*., 2017). In addition, in germ-associated conditions, the compound of interest is either metabolized by microbial communities or influences the physiology of host-associated microbes, thereby making it difficult to understand the host response toward tested compound (Baruah *et al*., 2015). Other than the above mentioned traits, especially the ability to culture *Artemia* under axenic conditions make it an exceptional experimental system to study the biological activity of polyphenol plant-based compounds as it allows to distinguish the direct effect on the host (by pre-exposing axenic brine shrimp larvae to the compound for a certain duration) from indirect effects (King and MacRae, 2012; King *et al*., 2013; Baruah *et al*., 2015; Iryani *et al*., 2017).

Moreover, *Artemia* cysts were used in space radiation experiments due to its ability to remain dormant for long period and resist stress (Demets, 1995). They are greatly useful for studying diseases in crustaceans (Overton and Band, 1981; Marques *et al*., 2005; Baruah *et al*., 2010; Sung *et al*., 2009; Soltanian *et al*., 2007;), stress response studies (Clegg *et al*.; 2000a; MacRae 2003), feed quality analysis (Marques *et al*., 2004a, b), probiont testing (Marques *et al*., 2005, 2006c). Moreover, it can be used as a model organism for studying the regulation of gene expression during embryonic development in crustaceans (Soltanian, 2007) and nutraceuticals/therapeutics test mainly against well-known pathogens including *V. alginolyticus*, *V. parahaemolyticus*, *V. harveyi*, *V. campbellii*, *V. proteolyticus*, *V. vulnificus*. (Baruah *et al*., 2015; 2013; 2011; 2010; 2009; Defoirdt *et al*., 2013; 2007; 2006).
CHAPTER 03
MATERIALS AND METHODS

3.1. Materials

3.1.1. Experimental site
All experiments were conducted at the Laboratory of Aquaculture & Artemia Reference Centre (ARC), Faculty of Bioscience Engineering, Department of Animal Sciences and Aquatic Ecology, Ghent University, Belgium. The study was started in February 2018 and carried out until July 2018.

3.1.2. Experimental animals
The experiments were carried out using the model organism Artemia franciscana. The cysts of Artemia originating from the Great Salt Lake, Utah, USA (EG® Type, batch 21452, INVE Aquaculture, Dendermonde, Belgium) were used for the experiments.

3.1.3. Bacterial strains
Vibrio harveyi BB120 strain, available in the collection of the Laboratory of Aquaculture & Artemia Reference Center was used for the challenge tests on Artemia.

3.1.4. Culture media
Marine Broth (Carl Roth GmbH+Co. KG) was used as medium for culturing of V. harveyi species. 20 g of marine broth was dissolved in 500 ml of distilled water and autoclaved at 121 °C, 15 psi for 20 minutes.

3.1.5. Reagents
Phloroglucinol (C₆H₆O₃, melting point =215-220 °C, molecular weight =126.11 g/mol) was used as the testing compound for the experiments. The compound Phloroglucinol (≥99%) purchased from Sigma-Aldrich (Diegem, Belgium) was dissolved in sterile distilled water at 0.4 g/l (3.17 mM). Superoxide dismutase (SOD) (2,000–6,000 units/mg protein) and catalase (2,000–5,000 units/mg protein) were also obtained from Sigma-Aldrich. Catalase was dissolved in sterile distilled water at 0.5 g/l as stock solution. All the stock solutions were prepared fresh for each experiment.

3.1.6. Sea water, other equipment and glassware
Filtered autoclaved sea water (FASW) of 35 g/l salinity was prepared for all the experiments using instant ocean® synthetic sea salt (Aquarium Systems, Sarrebourg, France). All the necessary equipment and glassware including beakers, spatulas, sieves, erlenmeyer flasks, Eppendorf tubes, pipette tips and glass bottles were autoclaved at 121 °C and 15 psi for 20 minutes prior to use.
3.2. Methods

3.2.1. Culturing of bacteria for challenge assay

V. harveyi stored at -80 °C in Marine Broth containing 30% glycerol was inoculated (10 μl) in 100 ml erlenmeyer flasks containing 20 ml of autoclaved Marine Broth. The culture was incubated with continuous shaking for 20 h (till log phase) at 28 °C. The number of bacteria was determined spectrophotometrically at 550 nm following McFarland equation, where an OD of 1.0000 corresponded to 1.2 x 10⁹ cells/ml.

3.2.2. Hatching of axenic brine shrimp larvae

Axenic instar II brine shrimp larvae were obtained following decapsulation and hatching process as described by Baruah et al. (2009). In brief, 2 g of Artemia cysts were hydrated in 89 ml of distilled water for 1 h. Decapsulation was done using 3.3 ml NaOH (32%) and 50 ml NaOCl (50%) and then the decapsulation was stopped after 2 min by adding 50 ml autoclaved Na₂S₂O₃ (10 g/l). During the reaction, 0.2 μm filtered aeration was provided. All manipulations were carried out under a laminar flow hood and all tools were sterilized. The decapsulated cysts were washed with FASW containing 35 g/l of instant ocean®. The cysts, suspended in 2 l glass bottles containing FASW, were incubated at 28 °C for 24 h for hatching with constant illumination of approximately 27 μE/m² and 0.2 μm filtered aeration. Hatched larvae at developmental stage instar II (mouth is opened to ingest particles) were collected for the experiments.

3.2.3. Brine shrimp challenge assays

In total, three separate challenge tests were performed.

3.2.3.1. Dose response relationship

In the first test, dose response relationship of phloroglucinol (protective effect) was determined. Axenically hatched brine shrimp larvae at developmental stage II (in which their mouth is open to ingest food particles) were collected, counted volumetrically and thereafter transferred to 500 ml sterile glass bottles containing sterile seawater. The axenic larvae were pretreated with increasing concentrations of phloroglucinol (10, 20, 30, 40 and 50 μM) for a fixed time (2 h) at 28 °C. They were rinsed repeatedly with FASW to wash away the compound and then allowed to recover for 2 h at 28 °C. Brine shrimp larvae that did not receive phloroglucinol pretreatment served as control. Following recovery period, groups of 20 larvae were transferred to sterile 40 ml glass tubes that contained 10 ml of FASW (35 g/l salinity). Subsequently, it was verified whether such pretreatment can protect the larvae against subsequent challenge with pathogenic V. harveyi at 10⁷ cells/ml. The survival of the larvae was scored after 48 h of challenge as described by Smith (2008). As control groups, non-pretreated larvae that were not challenged with V. harveyi (negative control) or challenged (positive control) were used. Each treatment and control was performed in quintuplicate.
3.2.3.2. **Unravelling the mode of action**

The second and the third tests were performed to determine the mode of action of phloroglucinol. In the second test, axenic larvae were pretreated with an optimized dose of phloroglucinol (dose which gave maximum protection to challenged larvae in the dose response assay), the antioxidant enzyme catalase (10 mg/l, Sigma-Aldrichs, Belgium) or a mixture of antioxidant enzyme catalase and phloroglucinol. The larvae in the control group did not undergo any pretreatment. Following pretreatment, the larvae were counted, distributed into sterile 40-ml glass tubes and then challenged with *V. harveyi* as described in the dose response study. Survival of the larvae was scored after 48 h of challenge. Brine shrimp larvae that did not receive phloroglucinol pretreatment either challenged with *V. harveyi* or not served as negative and positive controls. Each treatment and control were performed in five replicates.
In the third test, the axenic brine shrimp larvae were pretreated with an optimized dose of phloroglucinol, a mixture of the antioxidant enzymes catalase (10 mg/l) and SOD (75 units), or a combination of phloroglucinol and antioxidant enzymes mixture in a similar manner as described above. The larvae in the control group did not undergo any pretreatment. Groups of 20 larvae were counted and distributed in sterile 40-ml glass tubes and then challenged with *V. harveyi* as described in the dose response study. The survival of larvae was scored 48 h after the addition of pathogen. Brine shrimp larvae that did not receive phloroglucinol pretreatment either challenged with *V. harveyi* or not served as negative and positive controls. Each treatment and control were performed in five replicates. These challenge test data were obtained from a previous study.

![Figure 3.2: Experimental design for mode of action](image-url)

Figure 3.2: Experimental design for mode of action
3.2.4. Assay of heat shock protein 70 family \((hsp70)\) genes, prophenoloxidase \((proPO)\) and transglutaminase \((tgase)\) gene expression by quantitative real-time PCR (RT-qPCR) analysis

3.2.4.1. Pretreatment, challenge and sample collection

Axenically hatched brine shrimp larvae were subsequently divided into two groups: treatment and control. The treatment group was pre-treated with a pre-optimized dose of phloroglucinol \((30 \mu M)\) for \(2\) h at \(28°C\).

Following \(2\) h pre-treatment with phloroglucinol, brine shrimp larvae were repeatedly rinsed with FASW to wash away the compound. They were further subdivided in to two groups, with one group challenged with \(V.\) harveyi at \(10^7\) cells/ml, and the other group was not challenged. Control group was also subdivided in to two groups, with one group challenged with \(V.\) harveyi at \(10^7\) cells/ml and the other group remain unchallenged. The pre-treated brine shrimp larvae that were either challenged with \(V.\) harveyi at \(10^7\) cells/ml or not were maintained as main treatment and control, respectively. The non pre-treated (control) brine shrimp larvae that were either challenged with \(V.\) harveyi at \(10^7\) cells/ml or not were maintained as positive and negative controls, respectively. Each treatment and control was done in triplicate. Samples were collected from each control and treatment for the determination of gene expression of \(hsp70\) family genes and immune genes. All the samples were collected at \(0\), \(6\) and \(12\) h post challenge, rinsed in distilled water, immediately frozen in liquid nitrogen and stored at \(-80°C\).

3.2.4.2. RNA extraction and cDNA synthesis

Total RNA was extracted from brine shrimp larvae samples using RNaseasy® Plus Mini Kit (Qiagen, Germany) according to the manufacturer’s protocol. RNA concentrations were determined by NanoDrop spectrophotometer (ThermoFisher Scientific, Belgium). First strand cDNA was synthesized from \(500\)ng total RNA using the RevertAid First Strand cDNA synthesis kit (Thermo Fisher Scientific) according to the manufacturer’s instructions. The purity and quantity of the cDNA were determined by \(2\%\) agarose gel electrophoresis and NanoDrop spectrophotometer (Thermo Fisher Scientific, Belgium), respectively.

3.2.4.3. Quantitative real-time PCR (RT-qPCR)

The expression of \(hsp70\), \(hsc70\), \(hsc70-5\), \(PDI\) and \(HSPA4\); \(hsp70\) family genes and \(proPO\), \(tgase\) I immune genes in brine shrimp were analyzed by RT-qPCR using a pair of specific primers (Table 3.1). The qPCR amplifications were carried out in a total volume of \(10\) \(\mu l\), containing \(3\) \(\mu l\) of nuclease free water, \(1\) \(\mu l\) of forward and reverse primers, \(5\) \(\mu l\) of Maxima SYBR Green/Rox qPCR Master mix (Thermo Fisher Scientific) and \(1\) \(\mu l\) of cDNA template. The qPCR was performed in a StepOne Real Time PCR System (Applied Biosystems) using a two-step amplification protocol: initial denaturation (\(10\) min at \(95°C\)); \(40\) cycles of denaturation (\(15\) s at \(95°C\)) and
annealing/extension (60 s at 60 °C). Melting curve analysis was performed from 55 to 95 °C with continuous fluorescent reading every 0.5 °C increments to confirm that only one product was amplified. Elongation factor 1 α (EF1α) gene (Table 3.1) was used as reference gene. The fluorescent signal intensities were recorded at the end of each cycle. The cycle threshold (C_T) values for each hsp70 family and other immune genes were recorded. Relatively to the expression of EF1α transcripts, the relative expression was calculated by the 2^ΔΔC_T method (Livak and Schmittgen, 2001). The amplification was done in duplicate for each sample.

![Experimental design for gene expression of hsp70 family and immune genes](image)

**Figure 3.3:** Experimental design for gene expression of hsp70 family and immune genes
Table 3.1: Nucleotide sequence of the primers used in RT-qPCR

<table>
<thead>
<tr>
<th>Primer name</th>
<th>Sequence (5’-3’)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HSC70-F</td>
<td>ACTTACTCCGACAATCAGCC</td>
</tr>
<tr>
<td>HSC70-R</td>
<td>CAAATGTCACCTCGATTGGAGGA</td>
</tr>
<tr>
<td>HSC70-5-F</td>
<td>GAAGGCTAAAGAAACACC</td>
</tr>
<tr>
<td>HSC70-5-R</td>
<td>TTCAAAATCGACCAATCAAA</td>
</tr>
<tr>
<td>HSPA4-F</td>
<td>TGATTTTGGAATACTCGCTTTAC</td>
</tr>
<tr>
<td>HSPA4-R</td>
<td>CGATATTGCTTAAATTTCTCCGA</td>
</tr>
<tr>
<td>HSP70-F</td>
<td>CGATAAAGGGCGTCTCTCCA</td>
</tr>
<tr>
<td>HSP70-R</td>
<td>CAGCTTCAGGAATTTGCTCTTG</td>
</tr>
<tr>
<td>PDI-F</td>
<td>AGCGTTCATTGAAAGATAATGAAGT</td>
</tr>
<tr>
<td>PDI-R</td>
<td>CATCACAACGTCATGATCTGC</td>
</tr>
<tr>
<td>tgase 1-F</td>
<td>GCAAGGAGCGTTGGAATGGGT</td>
</tr>
<tr>
<td>tgase 1-R</td>
<td>TGTTTGGGAGTATCGACTGT</td>
</tr>
<tr>
<td>proPO-F</td>
<td>TCTGCAAGGAGGATTAAGGAGA</td>
</tr>
<tr>
<td>proPO-R</td>
<td>TGACTGACAAAGGAGATGGGAC</td>
</tr>
<tr>
<td>EF1α-F</td>
<td>TCGACAAGAGAAACCATTGAAA</td>
</tr>
<tr>
<td>EF1α-R</td>
<td>ACGCTACGCTTTAAGTTGTCC</td>
</tr>
</tbody>
</table>

3.2.5. Knockdown of hsp70 and hsc70 genes by RNA interference (RNAi)

3.2.5.1. Culture of Artemia for microinjection

Brine shrimp cysts were hatched in xenic condition, following decapsulation and hatching procedures. 1.5 g of *A. franciscana* cysts were hydrated in 89 ml of distilled water for 1 h. Brine shrimp larvae were obtained via decapsulation using 3.3 ml NaOH (32%) and 50 ml NaOCl (50%). During the reaction, aeration was provided and all the manipulations were carried out in non-sterile conditions. Decapsulation was stopped after 2 min by adding 50 ml Na2S2O3 at 10 g/l. The decapsulated cysts were washed with non-sterile seawater of 35 g/l. The cysts, suspended in 50 ml falcon tubes containing sea water, were incubated in a rotor for 24 h for hatching at 28 °C with constant illumination of approximately 27 μE/m²s. Hatched larvae were collected and transferred to aquariums containing sea water. The larvae were fed daily with non-axenic green algae (*Tetraselmis suecica*) and grown to adults in a controlled temperature room (28 °C) with constant illumination of approximately 27 μE/m²s.


### 3.2.5.2. Preparation of dsRNA

The dsRNA specific to the *A. franciscana hsp70* (GenBank: AF427596.1) and *hsc70* gene transcript were amplified (436bp) using gene specific primers (*hsp70* - forward primer: 5’ GATGCAGGTGCCATTGC 3’ and reverse primer: 5’ AGCTCCTCAAACGGGC 3’ and *hsc70* - forward primer: 5’ TAATACGACTCATAAGGGAGGTTGGAGTGTAGACTCCTT 3’ and reverse primer: 5’ TAATACGACTCATAAGGGAGAAGCAGTATGATTCGAGAGCA 3’), each primer included the T7 promoter (5’ TAATACGACTCACTATAGGG 3’) at their 5’ ends. The PCR was performed at 94 °C for 3 min, followed by 35 cycles of 94 °C for 25 sec, 53 °C for 25 sec and 72 °C for 2 min, and then final extension at 72 °C for 5 min. The purified PCR product was used as a template to generate dsRNA by using the MEGAscript RNAi kit (Ambion, USA) and the in vitro transcription reaction was performed according to the manufacturer’s instructions. Briefly, PCR purified product was incubated overnight at 37° C with four ribonucleotides (ATP, CTP, GTP and UTP), T7 enzyme mix and reaction buffer. Then the dsRNA was incubated at 37 °C for 1 h with DNase I and RNase for nuclease digestion to remove any template DNA and ssRNA that did not anneal. The *hsp70* and *hsc70* dsRNA, quantified in Nanodrop spectrophotometer (ThermoFisher Scientific, Belgium) were subjected to 1.5% agarose gel electrophoresis in order to check the integrity and efficiency of duplex formation. In addition, a green fluorescence protein (gfp) fragment (455bp) was amplified to prepare dsRNA of *gfp*, which served as a negative control using primers (forward 5’ AGAGCGCTTCTCGTTGGGG 3’ and reverse 5’ AGACCTGAAGTTCATCTGC 3’); each primer included the T7 promoter (5’ TAATACGACTCACTATAGGG 3’) at their 5’ ends.

### 3.2.5.3. Microinjection of *A. franciscana* female with ds RNA

The dsRNA specific to *hsp70, hsc70, hsp70 + hsc70* (double interference) and *gfp* were mixed separately in 1:10 ratio (v/v) with 0.5% phenol red in Dulbecco’s phosphate buffered saline (DPBS) (Sigma-Aldrich). The diluted dsRNA (250 nl of solution containing approximately 80 ng dsRNA) was injected with a FemtoJet® microinjector (Eppendorf, USA) using Femtotips II microinjection capillary tips (Eppendorf, USA) to egg sacs of adult brine shrimp female while viewing under stereomicroscope. Total twenty numbers of females were injected with dsRNA for each *hsp70, hsc70, hsp70 + hsc70* and *gfp*. Injected females were observed for 2 h, and the animals which retained dye, remained healthy and could swim properly were employed for further experiments. Mating pairs i.e., injected adult female and male were transferred to individual well in a 6-well plate containing 35 g/l FASW. The brine shrimp pairs were fed daily with green algae, *Tetraselmis suecica* and maintained in a controlled temperature room (28 °C) with constant illumination of approximately 27 μE/m2 s. After 5 days, nauplii were collected from each mating pairs for further analysis.

### 3.2.5.4. Challenge assay

The brine shrimp larvae obtained from single female injected with dsRNA specific to *hsp70, hsc70, hsp70 + hsc70* (double knockdown) or *gfp* were collected, counted and thereafter transferred to
sterile 50 ml falcon tubes containing 30 ml FASW. The larvae were pretreated with optimized dose of phloroglucinol for 2 h at 28 °C. They were rinsed repeatedly with FASW to wash away the compound and then allowed to recover for 2 h at 28 °C. Following recovery, the larvae were transferred to sterile 40 ml glass tubes containing 10 ml of FASW. Subsequently, the tubes were inoculated with *V. harveyi* BB120 strain at 1x10^7 cells/ml. The survival of brine shrimp larvae was scored 48 h after the addition of pathogen. The brine shrimp larvae, obtained from dsRNA injected females that did not receive phloroglucinol pretreatment, and either challenged with *V. harveyi* or not served as positive and negative controls. Subsequently, the larvae were washed with sterile distilled water, counted (20 nos.) and collected in 1.5 ml sterile eppendorf tubes.

Figure 3.4: Experimental design RNAi experiment
3.2.5.5. Protein extraction and analysis of Hsp 70 and Hsc 70 proteins

Brine shrimp larvae samples were homogenized in cold buffer K (150 mM sorbitol, 70 mM potassium gluconate, 5 mM MgCl₂, 5 mM NaH₂PO₄, 40 mM HEPES, pH 7.4) (Clegg et al., 2000), supplemented with protease inhibitor cocktail (Sigma-Aldrich®, USA). Subsequently the samples were centrifuged at 2200 x g for 1 min, and supernatant protein concentration was determined by the Bradford method using bovine serum albumin as standard (Bradford, 1976).

Supernatant samples were then diluted to obtain the concentration of 10 µg/µl, combined with 5 µl of loading buffer, vortexed, heated for 5 min at 95 °C and electrophoresed in 10% SDS-PAGE gel (BioRad, Belgium), with each lane receiving equivalent amount of protein. HeLa (heat shocked) cells (Enzo Life Sciences, USA) (6 µg) were loaded on to one well to serve as a positive control. Gels were then either stained for 1 h with Coomassie Biosafe (BioRad Laboratories, USA) for SDS PAGE or transferred to polyvinylidene fluoride membrane (PVDF) (BioRad Immuno-BlotTM PVDF) for Western blot. Stained gels were destained with distilled water for overnight and the images were taken by a ChemiDoc MP imaging system (BioRad, Belgium).

For western blot analysis, sandwich was prepared consisting of fiber and filter paper soaked in western blot running buffer for 1 h, gel and the PVDF membrane soaked in absolute methanol for 15 mins. Then the gel for the western blot was transferred into PVDF membrane by running in western blot buffer with pH 8.3 on 100 v for 1 h. The membranes were washed with washing buffer trissaline (1.2 g Tris, 9 g NaCl and 1 ml Triton X-100 with pH 7.6) for 3 times. Then the membranes were incubated with blocking buffer (50 ml of 1 x phosphate-buffered saline containing 0.2 % (v/v) tween 20 and 5 % (w/v) bovine serum albumin) for 1 h at room temperature and then with mouse monoclonal anti-Hsp70 antibody (3A3) (Affinity BioReagents Incl., Golden, CO), Hsp70 mouse monoclonal IgG and Hsc70 mouse monoclonal IgG₂₈ for three gels at the dilution of 1:500 for overnight at 4 °C. Horseradish peroxidase conjugated donkey anti-mouse IgG (Affinity BioReagents Incl., Golden, CO) was used as secondary antibody at the dilution of 1:5000 for the membrane incubated with anti-Hsp70 antibody (3A3) and 1:2000 for the membranes incubated with Hsp70 mouse monoclonal IgG and Hsc70 mouse monoclonal IgG₂₈. The membranes were then incubated with clarity western ECL substrate (chemiluminescence reagent) (BioRad Laboratories) for 5 min and the signals were detected by a ChemiDoc MP imaging system (BioRad, Belgium).

3.2.5.6. Analysis of hsp70 and hsc70 genes

Detection of hsp70 and hsc70 mRNA was carried out as described in the 3.2.4.2 and 3.2.4.3 sections.

3.2.6. Curative effect of phloroglucinol against Vibrio harveyi

In this experiment, curative effect of phloroglucinol (as a treatment) was determined. Axenically hatched brine shrimp larvae at developmental stage II (in which their mouth is open to ingest food particles) were collected, counted and groups of 20 larvae were transferred to sterile 40 ml glass
tubes that contained 10 ml of FASW (35 g/l salinity). Then they were challenged with *V. harveyi* at 10⁷ cells/ml. Challenged larvae were exposed (continuously) to different doses of phloroglucinol (2, 4, 6, 8 and 10 µM) for 48 h, rinsed to wash away the compound, and then allowed to recover for 2 h. Larvae that were either challenged with *V. harveyi* (+) or unchallenged (-) without subsequent exposure to phloroglucinol served as controls. Each treatment and control was performed in quintuplicate. These data were obtained from a previous study.

**3.2.7. Statistical analysis**

Survival data were arcsin transformed to satisfy normality and homoscedasticity requirements as necessary. The data were then subjected to one-way analysis of variances (ANOVA) followed by Duncan’s multiple range test using statistical software statistical package for the social sciences version 20.0. *P* values ≤ 0.05 were considered significant.

RT-qPCR data (2⁻ΔΔCₜ values) in the control group was regarded as 1.0 and thereby the expression ratio of the treatment was expressed in relation to the control and then the expression ratios were log transformed to satisfy normality and homoscedasticity requirements. The expression ratios were then subjected to one-way analysis of variances (ANOVA) followed by Duncan’s multiple range test to determine the significance differences between control and treatments, using statistical software statistical package for the social sciences version 20.0. *P* values ≤ 0.05 were considered significant.
4.1. Phloroglucinol pretreatment of axenic brine shrimp larvae protects them against subsequent *V. harveyi* challenge

In the first experiment, we investigated whether phloroglucinol could confer protection to the host *Artemia* against *V. harveyi* BB120 strain. To this end, we pretreated axenic larvae with varying concentrations of phloroglucinol for 2 h, after which the phloroglucinol was washed away, and the larvae were subsequently challenged with *V. harveyi*. As shown in Figure 4.1, brine shrimp larvae that received phloroglucinol pretreatment in the range of 30 and 40 μM exhibited a significant increase in the survival compared to (positive) control. However, the maximum survival was observed at a concentration of 30 μM. By contrast, the survival of brine shrimp larvae pretreated with phloroglucinol higher than 30 μM appeared to decrease. This indicates that phloroglucinol at an optimal concentration shows a protective effect against *V. harveyi* infection.

![Figure 4.1: Survival (%) of phloroglucinol-pretreated brine shrimp larvae after 48 h of challenge with *V. harveyi* BB120.](image)

The larvae were pretreated with phloroglucinol at the indicated doses for 2 h, rinsed to wash away the compound, and then allowed to recover for 2 h. The larvae were subsequently challenged with *V. harveyi* at $10^7$ cells/ml of rearing water. Non-pretreated larvae that were either challenged with *V. harveyi* (+) or unchallenged (-) served as controls. Error bars represent the standard error of five replicates; different letters indicate significant differences ($P<0.05$).
4.2. Phloroglucinol generated prooxidant activity mediates the protective response in brine shrimp larvae

We next sought to investigate the mechanism of action of phloroglucinol induced protection against *V. harveyi*. Previous studies reported that phenolic compounds possess prooxidant characteristics resulting in the generation of ROS (e.g. H$_2$O$_2$), and this induces protective responses. Here, we verified whether phloroglucinol induces *Vibrio*-protective effect through its prooxidant effect. We elucidated the involvement of ROS in phloroglucinol-induced protection in brine shrimp against *V. harveyi*. To verify this assumption, the experiment shown in Fig. 4.2 legend was performed. Consistent with the results of our previous challenge test (Figure 4.1) pretreatment of brine shrimp larvae with the optimized dose of phloroglucinol (30 μM) led to significantly increased survival upon challenge compared to positive control, whereas co-pretreatment with antioxidant enzyme catalase partially neutralized the phloroglucinol induced protection (Figure 4.2). Importantly, the survival of challenged larvae that were only pretreated with antioxidant enzyme did not show a significant difference in survival compared with the non-pretreated challenged larvae (positive control).

![Figure 4.2: Survival (%) of brine shrimp larvae pretreated with antioxidant enzyme (catalase), phloroglucinol, or in combination with both (antioxidant enzyme and phloroglucinol) after 48 h of challenge with *V. harveyi* BB120. The larvae were pretreated with antioxidant enzyme (catalase, 10 mg/l), phloroglucinol (30 μM), and mixture of antioxidant enzyme and phloroglucinol for 2 h, rinsed to wash away the compound, and then allowed to recover for 2 h. The larvae were subsequently challenged with *V. harveyi* at 10$^7$ cells/ml of rearing water. Non-pretreated larvae that were either challenged with *V. harveyi* (+) and those not challenged with *V. harveyi* (-) served as controls. Error bars represent the standard error of five replicates; different letters indicate significant differences ($P < 0.05$).](image)

To further substantiate the prooxidant activity in phloroglucinol induced protection against *V. harveyi*, we carried out additional *in vivo* survival assay with two antioxidant enzymes (catalase and SOD). Pretreatment of brine shrimp larvae with the optimized dose of phloroglucinol (30 μM) led to significantly increased survival upon challenge, whereas co-pretreatment with antioxidant...
enzymes (catalase + SOD) fully neutralized the phloroglucinol induced protection (Figure 4.3). Interestingly, the survival of challenged larvae that were pretreated with phloroglucinol was significantly higher than the survival of challenged larvae of both treatments; pretreated with antioxidant enzymes and a mixture of antioxidant enzymes and phloroglucinol.

Taken together, these results indicate that phloroglucinol generated H$_2$O$_2$ is at least in part responsible for the protection of brine shrimp larvae against _V. harveyi_.

![Figure 4.3: Survival (%) of brine shrimp larvae pretreated with antioxidant enzymes (catalase + super oxide dismutase), phloroglucinol, or in combination with both (antioxidant enzymes and phloroglucinol) after 48 h of challenge with _V. harveyi_ BB120. The larvae were pretreated with antioxidant enzymes (catalase, 10 mg/l and superoxide dismutase (SOD), 75 units), phloroglucinol (30 μM), and mixture of antioxidant enzymes and phloroglucinol for 2 h, rinsed to wash away the compound, and then allowed to recover for 2 h. The larvae were subsequently challenged with _V. harveyi_ at $10^7$ cells/ml of rearing water. Non-pretreated larvae that were either challenged with _V. harveyi_ (+) and those not challenged with _V. harveyi_ (-) served as controls. Error bars represent the standard error of five replicates; different letters indicate significant differences ($P < 0.05$).

4.3. Brine shrimp larvae pretreated with phloroglucinol did not show a significant increase in _hsp70_ family gene expression

Consequently, we further determined whether induction of _Hsp70_ family genes could be a mechanism for increased resistance of phloroglucinol-pretreated brine shrimp larvae against _V. harveyi_. To address this, we analyzed the expression profile of inducible _hsp70_ family mRNA _hsp70, hsc70, hsc70-5, PDI and HSPA4_ by RT-qPCR.

In the expression profile of _hsp70_, the brine shrimp larvae pretreated with phloroglucinol and challenged with _Vibrio_ showed 1.8 fold ($P > 0.05$) increase in _hsp70_ mRNA compared to the control, immediately after the challenge (0 h) (Figure 4.4-A). At 6 h post challenge it tend to decrease and remain constant at 12 h post challenge. 1.55 fold ($P > 0.05$) considerably higher _hsp70_ expression could be observed at 6 h post treatment in brine shrimp larvae pretreated with phloroglucinol and non-challenged with _Vibrio_. Brine shrimp larvae without pretreatment and challenged with _Vibrio_...
exhibited a similar trend with more downregulation in gene expression at 12 h post challenge. However, the expression levels were not significantly different from those in the corresponding controls.

Figure 4.4 | Continued
Figure 4.4 | Continued
Figure 4.4: Expression of (A) hsp70, (B) hsc70, (C) hsc70.5, (D) PDI and (E) HSPA4 gene in brine shrimp larvae. Two groups of larvae were pretreated with optimized dose (30 µM) of phloroglucinol for 2 h at 28 °C and two groups were maintained without pretreatment. After 2 h recovery period challenged with V. harveyi at 10^7 cells/ml of rearing water. The pre-treated brine shrimp larvae that were either challenged with V. harveyi or not were maintained as treatment 1 (P+V+) and treatment 2 (P+V-) respectively. The non pre-treated (control) brine shrimp larvae that were either challenged with V. harveyi (P-V+) or not (P-V-) were maintained as positive and negative control respectively. Samples were collected for gene expression assay at 0, 6 and 12 h after Vibrio challenge. At each time point, the expression of hsp70, hsc70, hsc70.5, PDI, HSPA4 mRNA using in the control group was set at 1.0 and all other data points were normalized accordingly. The EF1α gene was used as an internal control. Error bars represent the standard errors of three biological replicates.

For hsc70 expression profile, non-pretreated brine shrimp larvae with phloroglucinol (P-V+), at 12 h post challenged exhibited 2.7 fold increase (P > 0.05) compared to the control whilst a it was considerably higher in the larvae pretreated with phloroglucinol and non-challenged (P+V-) with 2.5 (P > 0.05) and 3.0 (P > 0.05) fold at 6 h and 12 h, respectively (Figure 4.4-B). Compared to these two treatments gene expression of brine shrimp larvae which were both pretreated and challenged with Vibrio (P+V+) showed a lower hsc70 expression. Immediately after the challenge (0 h) it was 1.74 fold (P > 0.05) and subsequently it tend to decrease in 6 h and 12 h compared to the corresponding controls. For any treatment the expression levels were not significantly different from those in the corresponding controls.

A considerable increase in hsc70-5 gene expression level (1.9 fold, P > 0.05) compared to the control was presented only in brine shrimp larvae pretreated with phloroglucinol without subsequent Vibrio challenge (P+V-) at 12 h (Figure 4.4-C).
As shown in Figure 4.4-D expression of mRNA for \textit{PDI} indicated considerable increase in brine shrimp larvae pretreated with phloroglucinol without \textit{Vibrio} challenge (P+V-) (2.9 fold, \( P > 0.05 \)) and non-pretreated brine shrimp larvae with \textit{Vibrio} challenge (P-V+) (2.6 fold, \( P > 0.05 \)), at 12 h time point compared to the corresponding controls. However, none of the treatment was significantly different from the corresponding controls.

In the expression profile of \textit{HSPA4}, the brine shrimp larvae pretreated with phloroglucinol and challenged with \textit{Vibrio} (P+V+) showed 2.1 fold increase (\( P > 0.05 \)) in \textit{HSPA4} mRNA compared to the control, immediately after the challenge (0 h) (Figure 4.4-E). At 6 h and 12 h it has gradually decreased to 1.0 fold and 0.6 fold respectively (\( P > 0.05 \)). Considerably higher \textit{HSPA4} expression (2.9 fold, \( P > 0.05 \)) could be observed at 12 h post challenge in non-pretreated brine shrimp larvae challenged with \textit{Vibrio}. Brine shrimp larvae pretreated with phloroglucinol and non-challenged appeared to increase (2.9 fold, \( P > 0.05 \)) mRNA expression at 12 h post challenge. However, the expression levels were not significantly different from those in the corresponding controls.

As an overview, Hsp70 family mRNA expression appear to increase in brine shrimp larvae pretreated with phloroglucinol and either challenged with \textit{Vibrio} or not. Nevertheless, the increase of the expression is not significant at any treatment or time point. Interactive effect of phloroglucinol pretreatment and \textit{V. harveyi} challenge was determined using a two-way ANOVA. However, a significant interaction effect was not evident from the statistical analysis (\( P > 0.05 \)). These results indicate that phloroglucinol-mediated Hsp70 might be responsible for generating protective innate immunity in brine shrimp larvae.

\textbf{4.4. Phloroglucinol elicits protective immune responses in the brine shrimp larvae}

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure45.png}
\caption{Relative expression of \textit{proPO} gene in brine shrimp larvae.}
\end{figure}

\textbf{Figure 4.5 | Continued}
Figure 4.5: Expression of (A) the prophenoloxidase (*proPO*) and (B) transglutaminase I (*tgase I*) genes in brine shrimp larvae. Two groups of larvae were pretreated with optimized dose (30 µM) of phloroglucinol for 2 h at 28 °C and two groups were maintained without pretreatment. After 2 h recovery period challenged with *V. harveyi* at 10^7 cells/ml of rearing water. The pre-treated brine shrimp larvae that were either challenged with *V. harveyi* or not were maintained as main treatment (P+V+) and security control (P+V-) respectively. The non pre-treated (control) brine shrimp larvae that were either challenged with *V. harveyi* (P-V+) or not (P-V-) were maintained as positive and negative control respectively. Samples were collected for gene expression assay at 0, 6 and 12 h after *Vibrio* challenge. At each time point, the expression of *proPO* and *tgase I* mRNA in the control group was set at 1.0 and all other data points were normalized accordingly. The EF1α gene was used as an internal control. Error bars represent the standard errors of three biological replicates. Significant differences when compared to the control group are indicated with asterisk marks (*: P < 0.05)

Accumulating evidences suggested that use of powerful innate immune effectors such as *proPO*, *tgase*, ferritin might also be one of the underlying mechanisms to defend against the bacterial infections in invertebrates including brine shrimp ([Baruah et al., 2015a, 2015b; Junprung et al., 2017]. Thus, we also aimed to determine temporal expression of the innate immunity-related genes. We addressed this by analyzing the temporal expression of prophenoloxidase (*proPO*) and transglutaminase I (*tgase I*) by RT-qPCR.

As shown in Fig. 4.5-A, *proPO* gene expression in brine shrimp larvae non-pretreated and challenged with *Vibrio* (P-V+) tend to increase at 0 and 6 h with 1.6 and 1.8 fold respectively (p > 0.05). In the group pretreated with phloroglucinol and non-challenged with *Vibrio*, 3.4 fold (p > 0.05) higher gene expression was observed at 12 h post treatment. The expression of the *proPO* gene in the brine shrimp larvae pretreated with phloroglucinol and challenged with *Vibrio* displayed a different pattern. At 0 h, the expression of *proPO* in the larvae was considerably higher (2.5 fold) (p > 0.05) compared to the control. However, at 6 h post challenge, brine shrimp larvae exhibited a decrease in the *proPO* mRNA levels (P > 0.05) and again 3.0 fold increase (p > 0.05) at 12 h post
A pattern similar to what was observed in pretreated and non-challenged (P+V-) and pretreated and challenged (P+V+) brine shrimp larvae mRNA expression in proPO (Figure 4.5-A) could be discerned for all the treatments in tcase I mRNA (Figure 4.5-B) expression. More specifically, non-pretreated and challenged brine shrimp larvae mRNA expression has considerably increased soon after challenge (0 h) (1.5 fold, P > 0.05), slightly decreased at 6 h (1.0 fold, P > 0.05) and at 12 h significantly increased to 2.5 fold (P < 0.05) compared to the control. Brine shrimp larvae which were pretreated and non-challenged (P+V-) also showed a marked increase of 1.9 fold (P > 0.05) expression at 0 h and then decreased noticeably at 6 h (0.6 fold, P > 0.05). However, at 12 h it has slightly deviated from the observed pattern showing a similar expression level (0.7 fold, P > 0.05) as observed at 6 h. Corresponding to the trend observed in P-V+ treatment, brine shrimp larvae pretreated and challenged with Vibrio, tcase I mRNA expression increased markedly (2.5 fold, P > 0.05) at 0 h, decreased to 0.7 fold (P > 0.05) at 6 h and then significantly increased at 12 h by 3.2 fold (P < 0.05) compared to the corresponding controls.

In order to determine whether there is an interactive effect of phloroglucinol pretreatment and V. harveyi challenge, a two-way ANOVA was performed. However, a significant interaction effect was not evident from the statistical analysis (P > 0.05). These results suggest that phloroglucinol pretreatment induced resistance in the brine shrimp larvae against V. harveyi infection is by mediating an induced expression of tcase I.

4.5. Phloroglucinol treatment protects axenic brine shrimp larvae challenged with V. harveyi

We have previously shown the prophylactic effect of phloroglucinol pretreatment for axenic brine shrimp larvae against V. harveyi. Next we sought to investigate the treatment effect of phloroglucinol using axenic brine shrimp larvae and V. harveyi as host-pathogen model. To this end, we challenged axenic larvae with V. harveyi and then exposed continuously to varying concentrations of phloroglucinol for 48 h, after which the phloroglucinol was removed. As shown in Figure 4.6, brine shrimp larvae that received phloroglucinol treatment in the range of 2 to 10 μM exhibited a significant increase in the survival compared to (positive) control. However, the maximum survival was observed at a concentration of 2 μM. The survival of brine shrimp larvae treated with phloroglucinol higher than 2 μM appeared to decrease gradually. This indicates that phloroglucinol treatment shows an optimal protective effect against V. harveyi infection at 2 μM.
Figure 4.6: Survival (%) of brine shrimp larvae challenged with *V. harveyi* BB120 and exposed (continuously) to different doses of phloroglucinol. The larvae were challenged with *V. harveyi* at 10^7 cells/ml of rearing water and exposed (continuously) to different doses of phloroglucinol for 48 h, rinsed to wash away the compound, and then allowed to recover for 2 h. Larvae that were either challenged with *V. harveyi* (+) or unchallenged (-) without subsequent exposure to phloroglucinol served as controls. Error bars represent the standard error of five replicates; different letters indicate significant differences (P<0.05).
CHAPTER 05
DISCUSSION

The shrimp aquaculture industry gained tremendous growth during the last few decades (FAO 2016; GOAL 2014, 2016), and this was associated with the rise in the novel forms of intensive aquaculture practices. However, intensification has led to the increase of various forms of anthropogenic stress in the aquaculture ecosystems, which have led to the emergence of many (new) diseases in aquatic farming species (Walker and Winton, 2010). In the shrimp aquaculture system, the biggest setback of such practices has been the occurrence of diseases caused by pathogenic or opportunistic bacteria, such as Vibrio spp., which adversely affect the sustainable development of shrimp aquaculture. Among major Vibrio species, V. harveyi is one of the most significant pathogens which cause devastating effects to wide range of invertebrates including Penaeid shrimp (Abraham and Palaniappan, 2004; Vezzulli et al., 2010). Although most Vibrio spp are regarded as opportunistic pathogens, V. harveyi could be considered as a primary pathogen. They can cause luminescent vibriosis and mass mortalities to P. monodon (Karunasagar et al., 1994), L. vannamei (Aguirre-Guz’man et al., 2001), P. japonicas (Liu et al., 1996), P. merguensis (Sae-Oui et al., 1987). Despite its role as a serious pathogen, pathogenicity of V. harveyi is yet to be fully elucidated (Austin and Zhang, 2006). Findings from a few studies, however, have suggested that the virulence of this pathogen may be attributed to the production of a number of virulence determinants including biofilms (Karunasagar et al., 1994), extracellular products (ECPs) notably proteases and haemolysins (Liu et al., 1996; Soto-Rodriguez et al., 2003), lipopolysaccharides (LPS) (Montero and Austin 1999), and interaction with bacteriophage (Oakey and Owens 2000) and bacteriocin-like substance (BLIS) (Prasad et al., 2005), which are regulated by quorum sensing (Henke and Bassler, 2004).

At present, eco-friendly prophylactic bio-control methods are gaining attention due to several drawbacks in the use of conventional approaches, such as disinfectants and antibiotics. Inappropriate use of antibiotics, for instance, had led to the development of resistant pathogens (Defoirdt, 2007), environmental contamination and accumulation in seafood tissues (Vadstein, 1997; Reverter et al., 2014). Moreover, V. harveyi form biofilms to resist against disinfectants and antibiotics (Karunasagar et al., 1994). The production of Hsps in fish and shellfish has been shown to have profound protective effects under detrimental conditions, and thus several studies have been carried out on the use of Hsps as alternative anti-infective agent to chemotherapeutics (Baruah et al., 2010, 2011, 2012, 2014, 2015; Iwama et al., 1998; Roberts et al., 2010; Sung et al., 2007, 2008; Kumar et al., 2018). For instance, in an Artemia model it was shown that induction of Hsp70, upon exposure of the animals to a non-lethal heat shock, led to a significant increase in the resistance of the animal towards subsequent challenge with vibrios (Sung et al., 2007, 2008). In a shrimp aquaculture system, however, non-lethal heat shock is possibly not the best way to enhance Hsp70 production because acute temperature shifts could be detrimental, adversely affecting physiological balance and causing significant mortality. It would therefore be useful and beneficial to find less traumatic approaches.
to induce Hsp level in aquatic organisms. There is currently more and more interest to look for natural compounds (such as phenolic compounds) as Hsp inducers since they are considered to be safe, easily available at low cost and can be easily administered.

In the present study, using axenic brine shrimp and *V. harveyi* as host-pathogen model system, it was aim to study whether phloroglucinol could induce prophylactics effects in brine shrimp larvae against *V. harveyi*. Subsequently, the mode of action of the compound behind the possible *Vibrio*-protective effect was studied. Furthermore, we also focused on the expression of Hsp 70 family genes and immune-related genes to study their possible involvement. Other than the prophylactic effect of phloroglucinol, therapeutic effect of the compound was also evaluated.

5.1. Dose response effect of phloroglucinol pretreatment on axenic brine shrimp larvae

In the first experiment we investigated whether phloroglucinol could confer protection to brine shrimp against *V. harveyi* BB120 strain and the optimum dose that confer the protection by carrying out a dose response study (Figure 4.1). Results showed that phloroglucinol at a dose of 30 μM conferred maximum protection to the larvae against *V. harveyi*. Even though, the survival of brine shrimp larvae at 40 μM exhibited a significant increase compared to (positive) control, it appeared to decrease beyond 30 μM suggesting that phloroglucinol could be detrimental at higher doses. Supporting our results, a study carried out by Kumar *et al.*, 2018, the maximum survival was reported at 30 μM in brine shrimp larvae pretreated with pholoroglucinol and subsequently challenged with *V. parahaemolytics*. Moreover, survival in pretreatment concentrations higher than 30 μM appeared to decrease gradually. However, these authors have tested the cytotoxic effect of phloroglucinol in the axenic brine shrimp larvae and it has shown that this negative effect only becomes apparent in the presence of *Vibrio* and is not evident in the germ-free larvae, suggesting that in the absence of the pathogen the larvae can cope with high phloroglucinol exposure and that the decrease in survival is not due to the cytotoxic effect of the compound.

5.2. Phloroglucinol generated prooxidant activity and the protective response in brine shrimp larvae

Next we sought to investigate the mechanism of action of phloroglucinol behind the protective effect against *V. harveyi*. Several studies have reported that phenolic compounds possess prooxidant characteristics resulting in the generation of ROS (eg. H$_2$O$_2$), and this induces protective responses by Hsp upregulation. Thus, by adding a ROS-scavenging enzyme catalase, it was assumed that the released ROS like superoxide anion and/or H$_2$O$_2$ will be inactivated by the enzyme. As we expected, pretreatment of brine shrimp larvae with the optimized dose of phloroglucinol (30 μM) led to a significantly increased survival upon challenge, whereas co-pretreatment with antioxidant enzyme catalase partially neutralized the phloroglucinol-induced protection (Figure 4.2). Then to further substantiate the prooxidant activity of phloroglucinol, an additional in vivo survival assay was carried out with two ROS-scavenging enzymes (catalase and SOD) exhibited an interesting result.
The survival of challenged larvae pretreated with phloroglucinol was significantly higher than the survival of challenged larvae with the mixture of antioxidant enzymes and phloroglucinol, indicating that pretreatment with two antioxidant enzymes fully neutralized the phloroglucinol induced protection (Figure 4.3). To further rule out the possibility that the decreased survival in the group pretreated with a combination of antioxidant enzyme/s and phloroglucinol was due to a negative effect of the antioxidant enzyme/s, we tested the effect of antioxidant enzyme/s on the survival of axenic brine shrimp larvae and apparent adverse effect could not be observed. From this compendium of evidences, it can be suggested that ROS generated during pretreatment is at least in part responsible for inducing protective effect in brine shrimp larvae. Consistent with the results, the study of Kumar et al. (2018) using axenic brine shrimp-V. parahaemolyticus host-pathogen model, showed that the Vibrio-protective effect of phloroglucinol was caused by its prooxidant effect. In another studies (Baruah et al., 2014, 2015), the polyphenol compounds pyrogallol and Tex-OE were shown to cause Vibrio-protective effect by mechanism of prooxidant action. Moreover, the authors has revealed, an initial burst of H2O2 and/or superoxide anion by the compound is contributing to the induction of Hsp70 and hence the protective effect. Production of ROS by phloroglucinol also elucidate the adverse effect of phloroglucinol beyond an optimum dose, as observed in the dose response assay. This detrimental effect could be explained by an over exposure to ROS, result in oxidative stress inflecting e.g. cellular damage (but other toxic effects may occur as well) (Baruah et al., 2014).

5.3. Phloroglucinol generated prooxidant activity and its association with hsp70 family and immune genes

The inducible Hsp70 has been shown to elicit protective immunity against bacterial infections has been previously described (Baruah et al., 2017, 2015a, 2015b, 2013, 2011; Sung et al., 2011; Kumar et al., 2018). Next we aimed to determine whether the induction of Hsp70 may be the underlying mechanism behind the induction of resistance against V. harveyi challenge in phloroglucinol-treated brine shrimp larvae. To this end, the expression profile of inducible hsp70 family mRNA hsp70, hsc70, hsc70-5, PDI and HSPA4 were analyzed by RT-qPCR. Further, accumulating evidences suggested that use of powerful innate immune effectors such as proPO, tgase, ferritin might also be one of the underlying mechanisms to defend against the bacterial infections in invertebrates including brine shrimp ((Baruah et al., 2015a, 2015b; Junprung et al., 2017). Thus, we also aimed to determine temporal expression of the innate immunity-related genes. We addressed this by analyzing the temporal expression of prophenoloxidase (proPO) and transglutaminase I (tgase I) by RT-qPCR.

Results showed that any of the hsp70 family genes were not significantly upregulated in response to phloroglucinol pretreatment. The expression of the immune-related gene proPO in response to phloroglucinol also did not significantly upregulated. On the contrary, expression of tgaseI mRNA significantly upregulated at 12 h time point in brine shrimp larvae pretreated with phloroglucinol and challenged with Vibrio. This suggests that tgase I might be involved in inducing protective
effect against *V. harveyi* in brine shrimp larvae pretreated with phloroglucinol. Moreover, absence of significant upregulation in other immune (*proPO*) and *hsp70* family mRNA levels, does not necessarily indicate those gene expression levels are not elevated. The generation of protective immunity is an energy consuming process (Babu *et al*., 2013; Ganz, 2009). In invertebrates, each of the effector systems involved in the immune response may carry a different cost when activated and their relative expression may shape the cost of the whole immune response to a standard challenge and/or immunostimulant (Ong *et al*., 2006). As a result, it is possible that the immune-related genes in phloroglucinol-pretreated larvae are expressed in a sequential manner and some genes might have expressed at the early hours and some at the latter hours post challenge. The possible variations in the degrees of expression between the immune effectors might contribute to resistance of the host against subsequent pathogenic challenge, while minimizing the potential costs of the immune response (Ong *et al*., 2006). Apart from that, we must also acknowledge the possible upregulation of other molecular chaperones, such as Hsp40, Hsp60 and Hsp90 by phloroglucinol and their implication in conveying resistance to brine shrimp against *Vibrio* challenge likely exist as well (Baruah *et al*., 2014; Norouzitallab *et al*., 2014) explaining the absence in significantly increased level of *hsp70* family and *proPO* mRNA levels.

A recent study reported that phloroglucinol is an effective inducer of Hsp70 and the protective effect of phloroglucinol was associated with Hsp70 induction (Kumar *et al*., 2018). However, it is worth mentioning that this induction was observed at the protein level but not at the mRNA level. Thus, the studied *hsp70* family and *proPO* mRNA might have translated to the corresponding proteins thereby conferring protection against *Vibrio*. The observed lack of correlation between *hsp70* mRNA and protein concentration can be explained by the different lifetimes of the molecules (Vogel and Marcotte, 2012): *hsp70* mRNA half-life is short (about 50 min) in cells after stress, even shorter in cells already containing Hsp70 protein (De Maio, 1999; Theodorakis *et al*., 1999) and few copies per cell are produced, causing their concentrations in cells to fluctuate much more than those of the longer-lived (about 2 h) corresponding protein (Danxi and Duncan, 1995).

Even though, the expression of *hsc70*, *hsc70*-5, *PDI* and *HSPA4* mRNA expression indicate a marked elevation in positive control (P-V+) or/and pretreated and non-challenged (P+V-) brine shrimp larvae, it does not appeared to be statistically significant. This could be possibly due to high standard errors. Further, the interactive effect of phloroglucinol pretreatment and *V. harveyi* challenge was statistically analyzed, however, a significant interaction effect was not apparent. Another point for discussion is that *hsp70* expression appeared to increase at all the tested time points in brine shrimp larvae pretreated and challenged with *Vibrio*. This could be a likely explanation for the higher survival of brine shrimp larvae which had received this treatment (P+V+), despite the fact that there is no significant elevation in expression.

Brine shrimps being an invertebrate, depend on their innate immune factors to build up resistance against pathogens since they lack adaptive immunity. The *proPO* system is a major innate defense
system that composed, among other proteins, of the prophenoloxidase enzyme, which is the zymogen of phenoloxidase (PO) (Cerenius and Soderhall, 1994; Jiravanichpaisal et al., 2006). The PO induces its protective effect by its role in cuticular melanization, sclerotisation, wound healing, encapsulation and eventual killing of the pathogens (Cerenius et al., 2008; Gao et al., 2009). Our results exhibited that the induction effect was more prominent when brine shrimps were treated with phloroglucinol in conjunction with Vibrio (P+V+) (2.5-fold, soon after challenge and 3-fold at 12 h post challenge) and phloroglucinol alone (P+V-) (3.5-fold, soon after pretreatment), compared to positive control. This result suggests that there might be a correlation with the elevated levels of hsp70 mRNA transcripts in same treatments (P+V+ and P+V-). However, there is no sufficient evidence to prove this hypothesis since it is not statistically significant.

When it comes to the invertebrates defense mechanisms, coagulation system play a vital role (Baruah et al., 2015). As some invertebrates including brine shrimp are having an open circulatory system, it is essential to quickly prevent the loss of blood or equivalent fluids through inflicted injuries (caused by pathogen) since these losses create a great impact on their survival (Cerenius and Soderhall, 2011). Further, there is a need to prevent microbes that gained access to the body through the wound from disseminating throughout the open circulatory system (Baruah et al., 2015). The coagulation system prevent such injuries from having serious consequences. The defense molecule TGase is a major component of this system responsible for catalyzing the clotting reaction (Chen et al., 2005; Lin et al., 2008). Our result revealed that tgase I mRNA expression in brine shrimp larvae with subsequent Vibrio challenge with (P+V+) or without (P-V+) phloroglucinol pretreatment enhanced significantly at 12 h post challenge although it was only considerably increased at 0 h in brine shrimp pretreated and non-challenged with Vibrio (P+V-). Interestingly, survival data explained the higher survival of phloroglucinol pretreated group that were subsequently challenged with V. harveyi (P+V+) treatment. It is possible that the functional protein Tgase encoded by the phloroglucinol-mediated tgase I assists in host defense by preventing tissue damage and simultaneously by blocking (further) progression of Vibrio infection in brine shrimp. Similar observations have been reported by Babu et al. (2013), where shrimp fed a diet containing immunostimulants showed significantly higher expression of tgase I and had higher protection against the shrimp pathogen white spot syndrome virus.

### 5.4. Knockdown of hsp70 and hsc70 genes by RNA interference (RNAi)

According to previous study, there exists a strong correlation between the amount of induced Hsp70 and the degree of improved protective response in brine shrimp larvae pretreated with phloroglucinol against V. parahaemolyticus (Kumar et al., 2018). Along with this finding, to gain insight into the molecular bases for the observed protective effect in brine shrimp larvae pretreated with phloroglucinol and challenged with V. harveyi, we used RNAi to assess the role of two hsp70 family genes in protecting brine shrimp larvae against V. harveyi BB120 strain. Moreover, in order to elaborate the above mentioned finding and determine whether this correlation is solely depend on hsp70 or any other hsp70 family gene, we focused on both hsp70 and hsc70. RNAi-mediated
gene-silencing technique has been very frequently used to investigate the function of critical genes involved in the immune response (Iryani et al., 2017; Baruah et al., 2014; Hannon and Rossi, 2004; Mao et al., 2007; Junprung et al., 2017). We prepared dsRNA specific to Hsp70 and Hsc70 and injected A. franciscana females in order to eliminate Hsp70 and Hsc70 mRNA and protein from larvae released from these females.

Subsequently, as we mentioned in the materials and methods we aspired to see the effect of hsp70, hsc70 and both hsp70 and hsc70 (double interference) gene knockdown on the survival of brine shrimp larvae produced by the adult brine shrimp females, after pretreating with phloroglucinol and challenging with V. harveyi. In addition to that, Hsps at mRNA and protein level were intended to be analyzed through RT-qPCR and western blot analysis. However, due to several technical and logistic constrains this experiment was not be able to carried out during the intended period. Efforts are currently underway in our laboratory to carry out the RNAi experiment to address the above-mentioned investigations.

5.5. Treatment effect of phloroglucinol on axenic brine shrimp larvae challenged with V. harveyi

The prophylatic effect of phloroglucinol pretreatment for axenic brine shrimp larvae against V. harveyi was evident from our previous results. In vitro and in vivo experiments by various investigators have suggested a wide range of potential therapeutic effects associated with phloroglucinol treatment, and that includes antioxidant (Kim et al., 2012), anticancer (Lopes-Costa et al., 2017; Kim et al., 2015; Pia Schiavone et al., 2014), antimicrobial (Pia Schiavone et al., 2014; Lee et al., 2009), anti-tumor (Kwon et al., 2012) and anti-spasmodic (Annahazi et al., 2014). Thus, we investigated the treatment effect of phloroglucinol using axenic brine shrimp larvae and V. harveyi as host-pathogen model. Brine shrimp larvae that received phloroglucinol treatment in the range of 2 to 10 μM exhibited a significant increase in the survival compared to (positive) control. However, the maximum survival was observed at a concentration of 2 μM suggesting that it is the optimum protective concentration of phloroglucinol as a treatment for V. harveyi infected brine shrimp. A previous study has reported that the extracts and compounds including phloroglucinol from rhizome of a thick-stemmed wood fern (Dryopteris crassirhizoma) were highly active against Gram-positive bacteria such as methicillin-resistant Staphylococcus aureus KCTC 1928, Streptococcus mutants and Bacillus subtilis (Lee et al., 2009). The survival of brine shrimp larvae treated with phloroglucinol higher than 2 μM appeared to decrease gradually. Thus, our study strongly suggest the treatment effect of phloroglucinol against V. harveyi which can be a safe, low cost, easily available and eco-friendly alternative for chemotherapeutics.
CHAPTER 06
CONCLUSIONS AND RECOMMENDATIONS

6.1. Conclusions
In the present study, using the axenic brine shrimp-\textit{V. harveyi} host-pathogen model, mechanistic action of a plant-derived phenolic compound, phloroglucinol was unraveled. Based on the results of these experiments, the following conclusions can be made:

- The compound phloroglucinol at an optimum dose could significantly enhance the survival of axenic brine shrimp challenged with \textit{V. harveyi} BB120. The optimum dose for phloroglucinol was 30 μM.
- The \textit{Vibrio} protective effect of phloroglucinol was, at least in part, caused by the prooxidant activity of the compound.
- Phloroglucinol-mediated Hsp70 might be responsible for generating protective innate immunity through regulating the expression of \textit{tgase I} immune gene in brine shrimp larvae.
- Phloroglucinol also has a therapeutic effect against \textit{V. harveyi} BB120 and by treating axenic brine shrimp, challenged with \textit{V. harveyi} at an optimum dose of 2 μM, a significant enhancement in the survival can be achieved. Thus, phloroglucinol is a compound with preventive and curative effect against \textit{V. harveyi} BB120.

6.2. Recommendations

- In our study, the obtained \textit{hsp} mRNA levels were not significantly higher. This might be due to the fact that these mRNA might have expressed at early hours or latter hours than tested. Thus, temporal induction of the mRNA expression should be carried out over a period of time.
- Hsp70 induction might have occurred at the protein level. Thus, to confirm this hypothesis a western blot analysis can be done.
- To gain more insights into the functional properties of phloroglucinol, more detailed study should be carried out by employing RNAi technique to knockdown hsp70 family genes, as we planned in our study.
- In our study, we evaluated the treatment effect of phloroglucinol against \textit{V. harveyi}, further, mode of action of this treatment effect should also be carried out.
This experiment was carried out using brine shrimp as the model organism at the laboratory scale under axenic conditions. To validate the findings, it would be interesting to carry out similar studies under conventional (non-axenic) conditions using commercially important aquaculture organisms.
CHAPTER 07
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