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EASTERN OYSTER LARVAL TRANSCRIPTOMES IN RESPONSE TO PROBIOTIC AND PATHOGENIC BACTERIA

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EASTERN OYSTER LARVAL TRANSCRIPTOMES IN RESPONSE TO PROBIOTIC AND PATHOGENIC BACTERIA

BY

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ABSTRACT

Oysters are described as keystone species serving an important ecological role. As filter-feeders they help in maintaining water quality. Oyster reefs provide refuge and support to different organisms. The eastern oyster, *Crassostrea virginica,* native to the East Coast of United States and Gulf of Mexico is a part of the rapidly growing aquaculture industry. Aquaculture production depends on a healthy and constant supply of oyster larvae that are provided by hatcheries. Several hatcheries on the east coast that provide *C. virginica* seed to oyster farms face significant losses owing to *Vibrio* infections causing massive larval mortalities. Use of antibiotics is avoided due to possibility of development of antibiotic resistance. The probiotic bacteria, *Phaeobacter inhibens* S4 and *Bacillus pumilus* RI06-95 have been shown to successfully protect *C. virginica* larvae from *V. coralliilyticus* RE22 infection. Use of these probiotics in hatcheries can reduce mortalities due to disease thereby avoiding significant economic losses. In order to design best practices for probiotic use it is crucial to understand their mechanisms of action. There has been great progress in understanding the components of oyster immune system, its functioning in response to various stimuli and its uniqueness as compared to other organisms. This is in part due to availability of sophisticated tools like high throughput sequencing and various –omics analyses such as proteomics, genomics and transcriptomics and partly due to interest in controlling diseases affecting aquaculture. As such most of our knowledge is based on studies that focus on oyster-pathogen or oyster-environmental stimuli interaction. Little is known about the effect of bacteria other than pathogens on the oysters. Moreover, very little about larval immunity of eastern oyster, *C. virginica*. This is the first study to investigate

the effect of both pathogen and probiotic bacteria on *C. virginica* larval immunity using transcriptomes. The aim of this study is to test the safety and efficacy of formulated probiotic *Bacillus pumilus* RI06-95 in a hatchery, understand the mechanisms of action of both probiotics and to characterize the effect of *V. coralliilyticus* RE22 infection on the larval immune system of eastern oysters.

Chapter 1 reviews the current knowledge of oyster immune system and the mechanisms of action of probiotics especially mechanisms related to immunomodulation of innate immunity. Previous studies have demonstrated successful protection of *C. virginica* larvae from *V. coralliilyticus* RE22 infection in a laboratory based setting as well as in a hatchery using laboratory grown cultures of probiotics. The ultimate use of the probiotics is in a hatchery setting, which would require easy to use and stable formulation of the probiotics instead of time consuming laboratory-grown probiotic cultures that are viable for only a short duration of time.

Chapter 2 discusses methods of formulation of probiotic *Bacillus pumilus* RI06-95, testing the formulation in a hatchery and its effect on larval survival at the hatchery and post *V. coralliilyticus* RE22 experimental challenge in the laboratory. A spray dried formulation of *Bacillus pumilus* RI06-95 was both shelf-stable and effective in protecting *C. virginica* larvae from *V. coralliilyticus* RE22 challenge. The formulation did not show any adverse effects on the larvae during the course of the trial.

Chapter 3 investigates the host–pathogen interaction between *C. virginica* larvae and *V. coralliilyticus* RE22 using transcriptomes produced after experimental challenge. Exposure of larvae to the pathogen for 6 hours provided information of the changes in the larval oysterimmune system brought about by the pathogen in the early stages of

disease. Overall, despite upregulation of several pattern recognition receptors, immune signaling pathways leading to the production of antimicrobial effectors, such as protease inhibitors and the pore forming protein perforin-2, were suppressed by *V. coralliilyticus* RE22. The transcriptomic evidence suggests that lack of an adequate immune response to thwart the infection of RE22, combined with a high metabolic load and decreased feeding, leads to large-scale mortalities of *C. virginica* larvae. This research allows for a better understanding of the disease process caused by *V. coralliilyticus* RE22 in larval eastern oysters.

Chapter 4 investigates the effect of exposure to non-pathogenic probiotic bacteria *P. inhibens* S4 and *B. pumilus* RI06-95 on the immune system of the host, *C. virginica* larvae. It presents evidence of immunomodulation of *C. virginica* larval immunity by both probiotic organisms. High upregulation of immune effectors such as serine protease inhibitors is seen in larval oysters after short exposures to the probiotic (6 and 24h) in the laboratory as well as after exposure for several days during a hatchery trial. Other important modulations that help larvae protect themselves from *V. coralliilyticus* RE22 infection include activation of pathogen receptors and signaling pathways, modulation of mucin genes, and upregulation of pore-forming protein perforin-2.

Chapter 5 summarizes and advocates the of use of probiotics in the larviculture of *C. virginica* and suggests their potential role in limiting vibriosis.

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Dedication

I dedicate this dissertation to my parents Dr. Harshvardhan Modak and Shriya Modak, my husband Aniket Kulkarni and my sons Ishir and Akaash Kulkarni. Thank you mum and dad for exposing me to science in an early age and kindling that passion ever since. Thank you Aniket, without your constant support, patience, enthusiasm and encouragement, I could not have seen this day. Thank you, little bubs I owe this to you both. I hope I have sparked the same love for curiosity and science that my parents sparked in me.

PREFACE

This dissertation was written in accordance with the manuscript format guidelines established by the Graduate School of the University of Rhode Island. The dissertation includes an introduction and the following three manuscripts:

1. "Use of formulated probiotic *Bacillus pumilus* RI06-95 for preventing vibriosis in larviculture of the eastern oyster *Crassostrea virginica*." prepared for submission to Journal of Shellfish Research.
2. "Characterizaton of *Crassostrea virginica* larval response to *Vibrio coralliilyticus* RE22" prepared for submission to Developmental and Comparative Immunology.
3. "Immunological response of *Crassostrea virginica* larvae to probiotics *Bacillus pumilus* RI06-95 and *Phaeobacter inhibens* S4" prepared for submission to Developmental and Comparative Immunology.

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CHAPTER 1

LITERATURE REVIEW: IMMUNITY IN OYSTERS AND GENERAL MECHANISMS OF ACTION OF PROBIOTICS

Abstract

Oysters are a unique model of immunology since they lack a classic adaptive immune system and only possess an innate immune system. Some research suggests presence of memory although a lot more remains to be elucidated. Oysters possess a wide variety of pattern recognition receptors. Most of the components of signaling pathways like TLR, NF-kB and MAPK are known while others like the complement system are not fully known yet. Immune effectors like antimicrobial peptides and enzymes like lysozyme are important in oysters especially in mucosal immunity. Exposure to probiotics leads to modulation of host immune genes that eventually provide protection from pathogens. Probiotics can modulate expression of receptors, signaling pathways and production of effectors in specific hosts. Immunomodulation as a mechanism of action of probiotics is seen in a variety of organisms including invertebrates and vertebrates alike and therefore may play an important role in the mechanism of probiotics in oysters.

Immunity in oysters

Oysters are sessile filter feeding animals that provide important ecological and economical services. As such immunological studies to understand disease resistance and improve aquaculture practices has given a boost in our understanding of the oyster immunology. Although some research suggests presence of immunological memory (Green et al., 2015) it is generally recognized that oysters lack adaptive immunity and only possess innate immunity. The circulating phagocytic hemocytes form the cellular branch of the innate immunity in oysters. The production of antimicrobial effectors via activated signaling pathways due to recognition of PAMPs (pathogen-associated molecular patterns) by PRRs (pattern recognition receptors) forms the humoral branch of innate immunity (Wang et al., 2018). Current research of the oyster immunity is reviewed below with emphasis on (i) Recognition (ii) Signaling pathways (iii) Effectors (iv) Apoptosis and autophagy and (v) Mucosal immunity. Immune-related genes in the eastern oyster *Crassostrea virginica* are illustrated in Fig 1.

Recognition

Recognition of non-self is achieved via PRRs that comprise of peptidoglycan recognition proteins (PGRPs), lectins, toll-like receptors (TLRs), Gram-negative binding proteins (GNBPs), scavenger receptors (SRs) and fibrinogen-related proteins (FREPs) (Gerdol et al., 2018, Wang et al., 2018). Lectins are further classified into major families including C-type, F-type, R-type, H-type, P-type, X-type, I-type lectins, pentraxins, galectins (formerly S-type lectins), ficolins, and others (Vasta et al. 2007).

Several of these PRRs are highly diversified in oysters (Zhang et al., 2014, Zhang et al., 2015). C-type lectins require a special mention since they are not only involved in pathogen recognition but also in activation of complement cascade.

Signaling pathways

Signals transmitted by receptors allow activation of several signaling pathways like TLR signaling pathway, NF-kB signaling pathway, mitogen-activated protein kinase (MAPK) signaling cascade, prophenol/phenol oxidase cascade and complement pathway in oysters. Sophisticated tools like whole genome sequencing and –omics analysis have led to tremendous progress in understanding molecules involved in these pathways that are common with other organisms as well unique to oysters. The TLR/NF-kB signaling pathway is a crucial pathway that upon recognition by TLR receptors activate transcription factors facilitating production of effectors like cytokines, interleukins, antimicrobial peptides (AMPs) and others (Gerdol et al., 2018). MyD88 serves as a critical cytosolic adaptor modulating TLR signaling pathway and Pacific oyster genome encodes an expanded set of 10 MyD88 genes (Zhang et al., 2015). MAPK pathway comprises of many protein kinases and its active involvement in oyster immunity is evidenced by their activation upon bacterial exposures (Qu et al., 2016). Although studies support existence of a complement pathway in bivalves (Gerdol et al., 2015, Li et al., 2015) the exact components and mechanisms of activation remain to be identified (Gerdol et al., 2018).

Effectors

Broad ranged effectors are produced upon induction of signaling pathways by PRR recognition and function in elimination of pathogens. These include antimicrobial peptides (AMPs), defensins, lysozymes, cytokines, protease inhibitors, antioxidant enzymes and acute phase proteins. Serine protease inhibitors have been identified for their role as important effectors in granting resistance to pathogens (La Peyre et al. 2010, Xue et al. 2006, McDowell et al., 2014). Enzymes, such as superoxide dismutase, catalase and glutathione peroxidase defend oysters by eliminating reactive oxygen species (ROS). They are important especially during increased oxidative stress caused by pathogen infection. Another important member of effectors are the heat shock proteins (HSPs) that help oysters modulate stress response and protect them from environmentally induced cellular damage caused by a variety of stressors (Wang et al., 2018).

Apoptosis and autophagy

Apoptosis, programmed cell death is an extremely important process in oysters involved in immune system homeostasis and function, defense against parasite and pathogens and self/non-self recognition. The baseline apoptosis rates observed in circulating and resident hemocytes in oysters is high (Sokolova, 2009). Apoptosis in oysters has two major pathways intrinsic and extrinsic. The main players consist of caspases and inhibitors of apoptosis (IAPs) that regulate the process. Apoptosis limits the spread of pathogen while preventing inflammatory damage of surrounding tissues (Sokolova, 2009). Although apoptosis has been studied for a long time the exact functional

relevance of its modulation by biotic and abiotic factors is still unknown in bivalves (Gerdol et al., 2018, Wang et al., 2018).

Autophagy plays a housekeeping role in organisms and is important in innate immunity. It is activated in oysters in response to bacterial, viral and environmental stimuli (Gerdol et al., 2018, Wang et al., 2018). Its role in protecting Pacific oysters from viral and bacterial challenge was demonstrated recently (Moreau et al., 2015) but a lot more remains to be investigated.

Mucosal immunity

Mucus forms an external barrier of defense and plays a key role in host-microbe interactions. Mucus consisting of crosslinked glycoproteins forms a physical barrier to microbes and contains a myriad of effectors that defend the host from infection (Allam and Espinosa, 2016). These include enzymes like lysozymes, hydrolases and proteases, AMPs, antioxidants and lectins to name a few (Espinosa et al., 2016). Mucus composition can affect pathogen adhesion and production of components is often regulated by them (Linden et al., 2008, Allam and Espinosa, 2016). This understudied topic is a crucial part of the innate immunity in oysters and needs further exploration.

Most of the knowledge of oyster immunity is based on a large body of research that is centered on bacterial and viral pathogens and environmental stressors but we know very little about the impact of friendly or beneficial bacteria on the immune system of oysters. Addressing this dearth of knowledge might reveal important novel insights in the oyster

immune system. The next section of this review discusses the effect of probiotics on different organisms focusing especially on their impact on immune system.

General mechanisms of action of probiotics

Probiotics, as defined by Food and Agricultural Organization and World Health Organization, are live microorganisms that when administered in adequate amounts confer a health benefit on the host (FAO and WHO 2006). Although probiotic uses for better growth, digestion, immunity and disease resistance of the host have been known their mechanisms have not been fully elucidated yet. Some of the known mechanisms of action are summarized in Fig 2 and discussed in detail below.

Nutrient availability

Probiotics improve the utilization of feed by the host by producing or stimulating production of exoenzymes that digest ingredients in feed such as carbohydrates, proteins and fat. This aid in increased digestibility of feed, boosts host growth. Probiotics *Bacillus subtilis, Lactococcus lactis* and *Saccharomyces cerevisiae* showed increased secretion of amylase, trypsin, protease and lipase in sea bass (*Labeo rohita)* (Tovar-Ramirez et al., 2002, Mohapatra et al., 2012). Application of probiotic strains of Bacillus in white shrimp (*Litopenaeus vannamei*) and *Fenneropenaeus indicus* feed, improved feed digestibility resulting in increased size of the shrimp (Heizhao et al., 2004). In fact, production of chitinases, proteases, cellulases, lipases and trypsin by the bacteria isolated from the digestive tract of various aquatic organisms have been shown contribute to fish nutrition (Vine et al., 2006, Ray et al. 2012). Increasing nutrient

availability and stimulation of growth through increased volatile fatty acids production by probiotics has been studied in poultry industry as well (Ajuwon., 2016).

Production of inhibitory compounds

Probiotics produce or stimulate production of several non-specific compounds that are effective in inhibiting pathogen growth including, antimicrobial compounds (hydrogen peroxide, nitric acid and bacteriocins), siderophores, proteases and lysozymes. A non-pathogenic strain *Vibrio mediterranei* 1 produces bacteriocin-like inhibitory substance against *Vibrio parahaemolyticus* spp (Carraturo et al., 2006). In fact, bacteriocin production allows probiotics to compete within complex microbial communities and influence the health of the host (Dobson et al., 2012). Probiotics administered to tilapia (*Oreochromis niloticus*) increased lysozyme activity in host (Taoka et al., 2006). *Phaeobacter inhibens* S4 produces tropodiethic acid (TDA) that kill pathogenic *V. coralliilyticus* RE22, *Vibrio harveyi* BB120 and *Alioseovarius crassostreae* CV919-312^{T} in oysters (Karim et al., 2013, Zhao et al., 2016). *Enterococcus durans* strain LAB18s showed antimicrobial and antioxidant activity against several pathogenic bacteria (Pieniz et al., 2014).

Competitive exclusion of pathogenic bacteria

Probiotics often compete with pathogenic bacteria for space and nutrients that hinder their proliferation. Direct inhibition of pathogens by production of inhibitory compounds as discussed above is one way they competitively exclude pathogens. Other mechanisms include formation of biofilms, blocking adhesion sites and profuse

probiotic growth. An oyster probiotic, *P. inhibens* S4 produces biofilms that inhibit the growth of pathogens *V. coralliilyticus* and *V. anguillarum* (Zhao et al., 2016). *Lactobacilli* reduced the adhesion of rainbow trout pathogens (Balcazar et al., 2007). Exclusion of pathogenic bacteria by competition from probiotic bacteria was also shown in poultry. Native bacteria from adult chickens were used to protect chicks from infestation of *Salmonella infantis* (Rantala and Nurmi., 1973) as well as other enteropathogens (Schneitz, 2005). Porcine probiotics *Lactobacilli* and *Bifidobacteria* compete for attachment sites on epithelial cells and exclude pathogens in the intestine (Gross et al., 2008).

Enhancement of the Epithelial Barrier

Gut is in constant contact with a large number of bacteria and its integrity is often one of the most important barriers against invading pathogens. Increased expression of genes involved in tight junction signaling due to probiotic treatment reinforces this barrier (Anderson et al., 2010). *Escherichia coli* Nissle 1917 (EcN1917) has been shown to not only prevent disruption of the mucosal barrier by enteropathogenic *E. coli*, but also restore mucosal integrity (Anderson et al., 2010). Probiotics differentially modulate epithelial cell responses via activation or suppression of distinct signaling pathways in a strain-dependent manner (Llewellyn et al., 2017).

Immunity

Effects on mucosal immunity

Mucus is made up of polymerized mucins that protect hosts from pathogens, enzymes, toxins, dehydration and abrasion (Hardy et al., 2013). *Lactobacillus plantarum* 299v and *Lactobacillus rhamnosus* GG have been shown to up-regulate production of MUC2 and MUC3 intestinal mucins that weakens the adherence of pathogenic *Escherichia coli* O157:H7 (Mack et al., 1999). Probiotics mediate modulation of mucin expression as a strategy for intestinal colonization of beneficial microbes to the host (Caballero-Franco et al., 2007). Mucus contains lysozymes, antimicrobial substances, antibodies and enzymes that have added benefits in controlling pathogenic invasion. Production of these substances can be modulated by presence of probiotics. Probiotic treatment led to increase in lysozyme production in Japanese flounder (Ye et al., 2011). Probiotic strains such as *Lactobacillus* GG, *Bifidobacterium actis* Bb-12 (Rautava et al., 2006) and *Saccharomyces boulardii* (Rodrigues et al., 2000) have been demonstrated to enhance IgA production and secretion.

Immunomodulation

Probiotic research shows mounting evidence of probiotic-host communication through pattern recognition receptors resulting in modulation on key signaling pathways such as NF-kB and MAPK to enhance or suppress activation and influence downstream pathways (Bermudez-Brito et al., 2012, Hardy et al., 2013, De et al., 2014). Probiotics and pathogens share PAMPs/MAMPs that can induce innate inflammatory pathways. Secondary and chronic exposure to probiotics induce suppressive /tolerogenic response that modulate NF-kB and MAPK pathways (Llewellyn et al., 2017). Effect in humans for some example probiotics is illustrated in Fig 3. *L. casei* CRL 431 interacts with

epithelial cells through TLR2 and induces an increase in the number of CD-206 and TLR2 receptors in the cells involved in the innate immune response in humans (Vinderola et al., 2005). *Lactobacillus* stimulates TLR9 that induces cytoplasmic accumulation of ubiquitinated IkB and inhibition of NF-kB activation (Lee et al., 2006). *L. reuteri* and *L. casei* engage with C-type lectin, prime dendritic cells and that lead to increased production of IL-10 (Smits et al., 2005). In contrast, *L. reuteri* strains DSM 17938 and ATCC PTA 4659 downregulates expression of TNF-α, TLR4 and NF-kB and upregulates IL-10 expression in rats (Bermudez-Brito et al., 2012). Along with the influence on innate immunity probiotics also have impacts on adaptive immunity (Hardy et al., 2013).

In addition, increase in phagocytic activity in probiotic fed Nile tilapia (*Oreochromis niloticus*) (Vieira et al., 2010) and increase in total hemocyte count and serum agglutination activity in probiotic fed and challenged marine shrimp (Sayed et al., 2011) are also documented. Probiotics have also been shown to confer protection against many cellular stresses, which include oxidative stress-mediated apoptosis (Llewellyn et al., 2017).

Thus, probiotic bacterial strains can be generalized to exert immune-activation, -deviation or -regulation/suppression responses (Hardy et al., 2013). Selection of probiotic strains especially in combination along with prebiotics can have beneficial effects on hosts. However, it is crucial to gain full knowledge of their modulatory capabilities and formulate their use with careful consideration.

Goals of this study

There has been much progress in understanding immunity in mollusks especially in bivalves but we still lack knowledge of larval immunity in eastern oyster *C. virginica.* There is also a dearth of understanding in the effect of bacteria on larval immunity. Probiotics protect several organisms from *Vibrio spp* infection including crayfish (*Cherax tenuimanus)* (Ambas et al., 2013), brine shrimp (*Artemia franciscana)* (Giarma et al., 2017), oyster (*C. virginica)* (Karim et al., 2013), turbot (*Scophthalmus maximus*) (Villamil et al., 2002) as well as humans (Carraturo et al., 2006) using mechanisms like antibiotic production and indications of immunomodulation. However, we need a thorough investigation of the nature of their immunomodulatory ability.

The overall goal of this study was to understand the mechanism of action of probiotics *B. pumilis* RI06-95 and *P. inhibens* S4 against pathogen *V. coralliilyticus* RE22 and formulate them for use in the field.

Laboratory grown bacterial culture of *B. pumilis* RI06-95 was previously shown to protect *C. virginica* larvae from infection of *V. coralliilyticus* RE22 (Karim et al., 2013). The first aim was to formulate the probiotic such that it can be effectively used in hatcheries and to test their efficacy. A series of formulations were prepared and tested in lab as well as in hatcheries to establish their efficacy.

The second specific aim was to understand the immunological response of *C. virginica* larvae to both probiotics *B. pumilis* RI06-95 and *P. inhibens* S4 in order to understand if immunomodulation is one of the mechanisms of action of these probiotics. Next generation RNA sequencing technology was used to obtain the transcriptomic response of *C. virginica* larvae to probiotics in a lab controlled and a hatchery environment to thoroughly investigate their effect on several larval genes at a time.

The third specific aim was to understand the immunological response of *C. virginica* larvae to pathogen *V. coralliilyticus* RE22 in order to understand its pathogenesis. To investigate this, larval transcriptomes generated post challenge with *V. coralliilyticus* RE22 were compared to control transcriptomes.

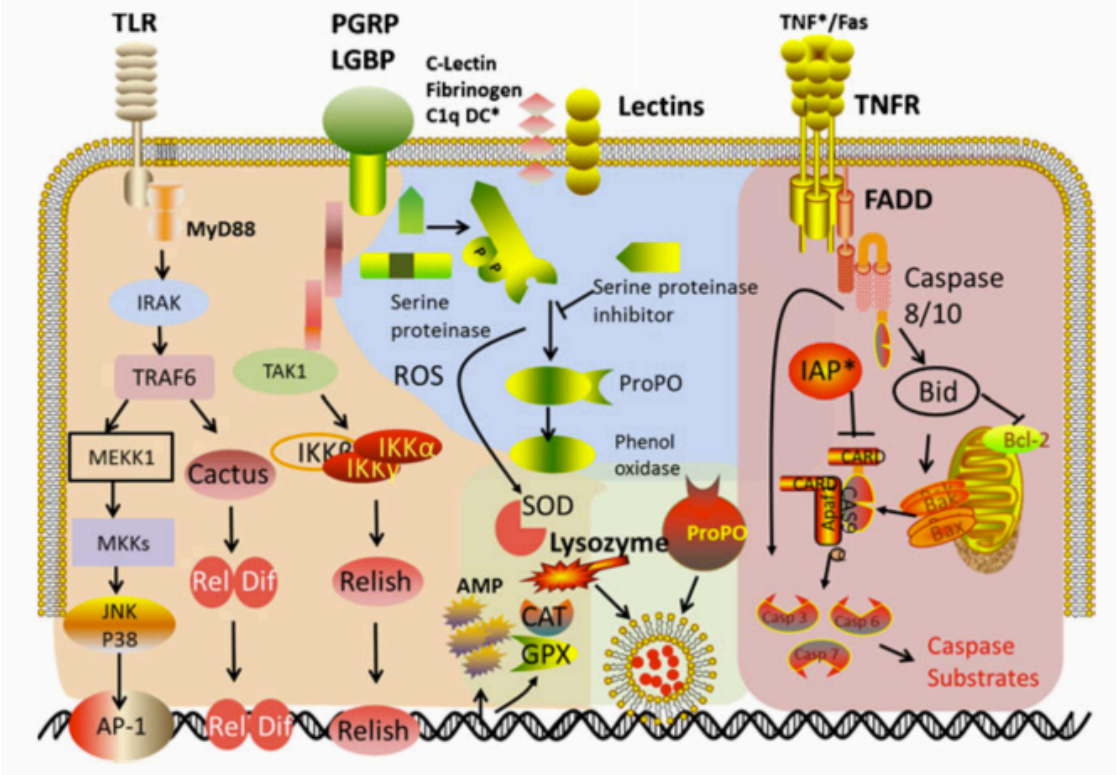

Figure 1: Immune-related genes present in the eastern oyster, *Crassostrea virginica*. Adapted from Zhang et al., 2014.

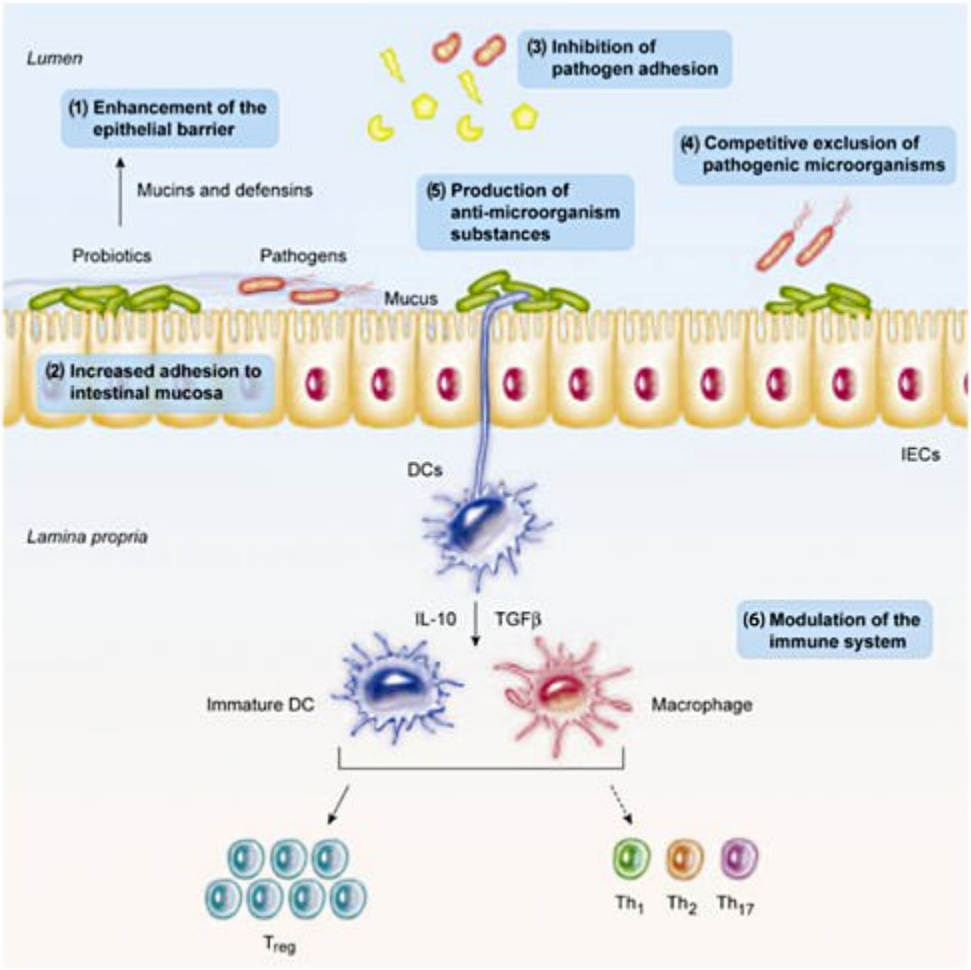

Figure 2: Major mechanisms of action of probiotics. Illustration adapted from Bermudez-Brito et al., 2012.

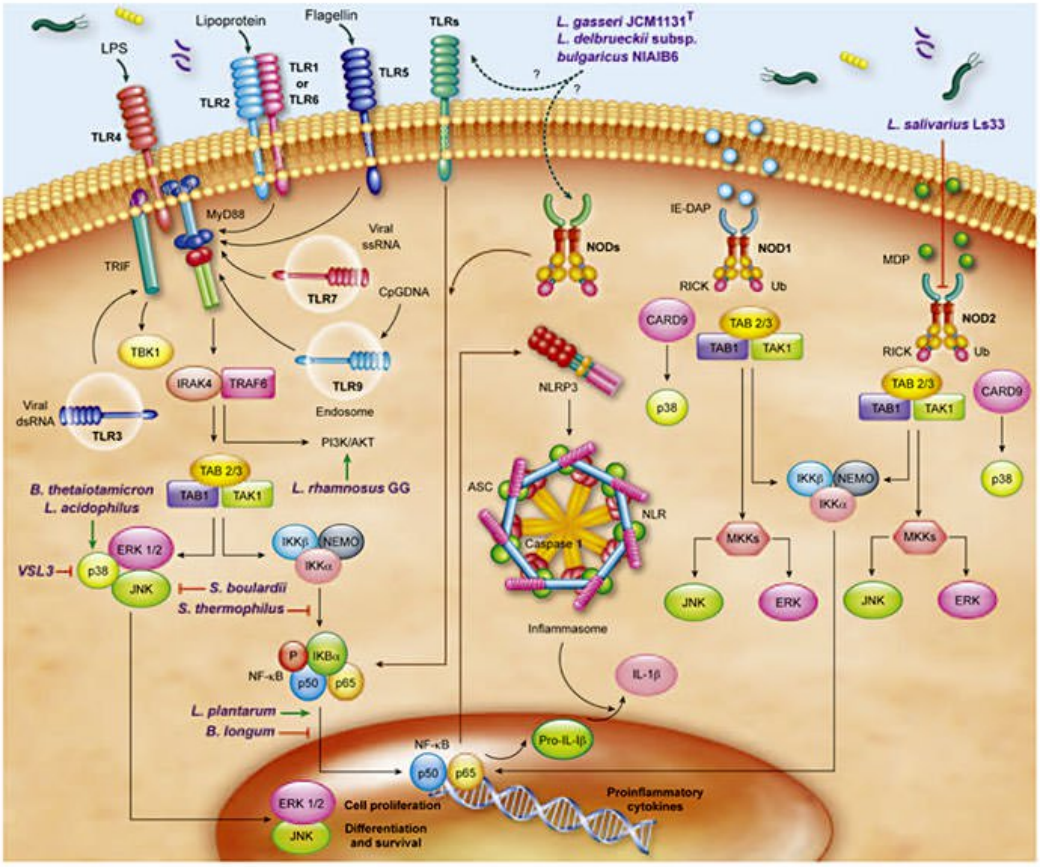

Figure 3: Examples of modulation of innate immune response by probiotics. Adapted from Bermudez-Brito et al., 2012.

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CHAPTER 2

USE OF FORMULATED PROBIOTIC *BACILLUS PUMILUS* RI06-95 FOR PREVENTING VIBRIOSIS IN LARVICULTURE OF THE EASTERN OYSTER *CRASSOSTREA VIRGINICA*

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T. Modak contributions: Design and performance of hatchery trial with spray-dried formulation (Envera); further compilation with previous results for final manuscript.

Abstract

Vibriosis is a major disease affecting larval eastern oysters, *Crassostrea virginica,* causing sudden and massive larval mortalities. A candidate probiotic strain, *Bacillus pumilus* RI06-95, was previously investigated as a disease prevention method and found to reduce mortality due to vibriosis in oyster larvae and juveniles. The goal of this research was to develop a stable formulation of probiotic RI06-95 to facilitate storage and delivery in a hatchery setting. Three types of formulations (granulated, lyophilized and spray dried) of RI06-95 were developed. Cell viability of all formulations remained above 10^5 colony forming units (CFU) per mL for up to 8 weeks of storage. The granulated and spray-dried formulation had no adverse impacts on larval oyster survival and provided protection against challenge with the bacterial pathogen *Vibrio coralliilyticus* RE22 (Relative Percent Survival, RPS, as compared to probiotic untreated control: 69 ± 1 % and 52 ± 35 % respectively). However, treatment of larval oysters with the lyophilized formulation led to a significant decrease in survival compared to non-treated controls and afforded no protection. Daily treatment of oyster larvae with the spray dried formulation in pilot-scale hatchery trials provided significant protection against laboratory challenge with RE22 (RPS 43 ± 4 %). These results demonstrate that a sprayed-dried formulation for probiotic RI06-95 can be safely and effectively used for disease prevention in shellfish hatcheries.

Introduction

The bivalve shellfish (oysters, clams, scallops, and mussels) industry is an important and rapidly expanding area of aquaculture production. The total landings for oysters, clams and scallops in United States alone valued at $859 million (NMFS 2016). A primary requisite for the aquaculture of most bivalve shellfish species is an abundant, reliable, and inexpensive supply of seed/small juveniles (Helm *et al.* 2004). Shellfish larvae, however, are prone to infectious diseases, which can result in a rapid and high rate of larval mortality in commercial hatcheries (Elston 1998), leading to substantial economic losses. For instance, pathogenic strains from several *Vibrio* spp. including *V. alginolyticus, V. anguillarum, V. coralliilyticus, V. ordalii, V. splendidus, V. tubiashii,* and others, cause bacillary necrosis of larval bivalve shellfish. Clinical signs of vibriosis in bivalves include necrosis of mantle epithelium, clumping of the cilia, and rapid mortality (Tubiash *et al.* 1965; Berthe 2004; Gomez-Leon *et al.* 2005; Kesarcodi-Watson *et al.* 2009).

Given the absence of an adaptive immune system in bivalves allowing for the use of vaccines as disease prevention tools, the use of probiotics is one of the most promising management strategies for shellfish disease prevention and control (Elston 1998; Verschuere *et al.* 2000; Prado *et al.* 2010). Probiotics are defined as live, non-pathogenic microorganisms which, when administered in adequate amounts, confer a health benefit to the host (Food and Agricultural Organization of the United States 2006). The most widely used probiotics in human and animal health belong to *Bacillus*

spp., *Bifidobacterium* spp., and lactic-acid bacteria such as *Lactobacillus* spp. (Hong *et al.* 2005, Cutting S 2011). In particular, *Bacillus* spp. are attractive for commercial products because they are aerobic, spore-forming bacteria. Spores are capable of surviving extreme conditions such as the high temperatures and pressure conditions sometimes used for formulating a commercial probiotic product. Formulations of *Bacillus* spp. are stable for long periods without significant loss in viability because spores enable survival in harsh conditions until germination and proliferation occur in more favorable environments (Lalloo *et al.* 2010; Cutting 2011; Azevedo de & Tavares Brag 2012; Sorokulova 2013; Edna *et al.* 2014).

We previously reported that marine *Bacillus pumilus* RI06-95, a producer of the antibiotic amicoumacin (Socha 2008), antagonized growth of the shellfish pathogen *V. coralliilyticus* RE22 *in vitro* and protected eastern oyster *Crassostrea virginica* and bay scallop *Argopecten irradians* larvae against experimental challenge with *V. coralliilyticus* RE22 (Karim *et al.* 2013b; Sohn *et al.* 2016a). It was also shown that daily treatment of larval rearing tanks in a hatchery with RI06-95 led to a decline in the levels of *Vibrio* spp. on tank surfaces and an increase in the survival of larval oysters when challenged with a pathogen (Sohn *et al.* 2016a). *Bacillus* spp., have shown promise as probiotic bacteria to improve host survival, growth, and development in aquaculture (Queiroz & Boyd 1998; Luis-Villaseñor *et al.* 2011; Martínez Cruz *et al.* 2012; Li *et al.* 2014). Additionally, some bacilli exhibit antagonistic effects against pathogenic *Vibrio* spp. (Decamp & Moriarty 2006; Vaseeharan & Ramasamy 2003). Whole genome analysis of RI06-95 reveals that it is most closely related to *B. pumilus* SAFR-32 (Hamblin *et al.* 2015), a strain isolated as a contaminant in spacecraft

assembly facilities (Gioia *et al.* 2007). *B. pumilus* strains have been isolated from a wide range of habitats, from aquatic and terrestrial hosts (Hill *et al.* 2009) to desert basalt (Benardini *et al.* 2003), and have been suggested as probiotics for plants, humans, crustaceans, and finfish (Duc *et al.* 2004; Hill *et al.* 2009; Sun *et al.* 2010; Murugappan *et al.* 2013).

Although many studies have shown promising results for the use of probiotics in shellfish aquaculture, no commercial products are available with demonstrated safety and efficacy in bivalve larviculture. Probiotics typically are available in several types of commercial formulations, including dry materials (such as wettable powders, dusts, and granules) and liquid products (such as cell suspensions in water, oils, and emulsions) (Austin *et al.* 1995; Schisler *et al.* 2004; Salinas *et al.* 2006; Savini *et al.* 2010; Dagá *et al.* 2013). An appropriate formulation should offer several advantages in addition to host protection, including: the stabilization of microorganisms during distribution and storage; ease in handling and delivery of the product; protection of the microbes from adverse environmental factors; and safety to the aquaculture species. Therefore, the successful development of an appropriate probiotic formulation requires testing for efficacy, safety, and stability, especially in bivalve hatchery facilities.

Here, we evaluate three novel formulations of the candidate shellfish probiotic *B. pumilus* RI06-95. We determine storage and usage potential, and test each formulation along with fresh cultures of the same probiotic bacterium for safety and host protection in both laboratory and in semi-commercial scale hatchery experiments. While all three formulations resulted in stable products with suitable shelf lives, only a spray-dried formula provided a high level of safety and efficacy desired for a

commercially viable product. Our results demonstrate a safe, stable, and easy-to-use formulation for *C. virginica* larval aquaculture production.

Methods

Oyster larvae

Laboratory challenge experiments: For the bacterial challenge experiments, eastern oysters, *C. virginica*, (4 - 6 day old) were obtained from the Blount Shellfish Hatchery at Roger Williams University (Bristol, RI, USA) or Virginia Institute of Marine Science (VIMS) (Wachapreague, VA, USA). Oyster larvae were transported to the laboratory at the University of Rhode Island (Kingston, RI, USA) and acclimated at room temperature (~20°C) for at least 24 h before treatment. The larvae were fed instant algae Shellfish Diet 1800^{TM} (Reed Mariculture Inc., San Jose, CA. USA) during the experiments.

Hatchery trials: Adult eastern oysters were spawned at the Blount Shellfish Hatchery for Trials I, II, III and V and at the VIMS Shellfish Hatchery at the Aquaculture Genetics & Breeding Technology Center (ABC), VIMS for Trial IV. Larval oysters were distributed into 100-120 L conical tanks at the Blount Shellfish Hatchery 2 days after fertilization and fed live microalgae, a mix of *Tisochrysis lutea* (CCMP1324; formerly *Isochrysis* sp., Tahitian strain) and *Pavlova lutheri* (CCMP1325), daily. Larvae were distributed into 60 L tanks at the VIMS Shellfish hatchery, and fed *Pavlova* sp. days 1 - 4 and a mix of *Pavlova* sp. and *Chaetocerus gracilis* from day 5 on.

Pathogen and probiotic strains

V. coralliilyticus RE22 was supplied by H. Hasegawa, Department of Biomedical Sciences, Oregon State University (USA). The freshly cultured *B. pumilus* RI06-95 for comparison with formulated versions was cultured in the laboratory. Both bacteria were maintained as stocks in 50% glycerol at -80 °C until use. They were cultured on yeast peptone with 3% NaCl (YP30) media (5 g L^{-1} of peptone, 1 g/L of yeast extract, 30 g/L of ocean salt (Red Sea Salt, Ohio, USA)) at 28 °C with shaking at 175 rpm as described in Karim *et al.* 2013a.

Formulation process

Granulated Product Formulation (RI-G)

Probiotic *B. pumilus* RI06-95 was incubated in 2.25% NaCl (YP22.5) broth (yeast extract 1 g/L, peptone 5 g/L, 22.5 g/L ocean salt, Instant Ocean) at 25 °C and 175 rpm. An initial culture was incubated for 2 d, then transferred to fresh YP22.5 and incubated for 4 d. The culture was partitioned into 50 mL sterile centrifuge tubes and centrifuged for 10 min at 2,350 × g. After centrifugation, cell pellets were transferred into a sterile petri dish (100 × 15 mm), and dishes were swirled with 2-3 mL culture media to ensure that the surfaces were completely covered in cells. The dishes were then covered with single ply, light duty paper (Kimwipes®) and placed in a convection oven to dry at 30 °C with constant airflow for 24-48 h, depending on initial volume. The dry cell mass was extruded through three particle size (40s, 80s, and 325s) USA Standard Sieve stainless steel screens (Cole Palmer, Illinois, USA), yielding products with average particle sizes of 43, 177, and 420 μm, respectively. The resulting granulated products were transferred into sterile glass vials and stored at 4 °C. For hatchery trials the granulated formulations were scaled up following the same formulation procedure

as above except bacterial cultures were centrifuged at 9,300 × g for 10 min and the final cell pellet was dried at room temperature (22 ± 3 °C) for approximately 2 days.

Lyophilized Product Formulation (RI-L)

Probiotic *B. pumilus* RI06-95 was cultured from frozen stocks and then centrifuged as above. After discarding the liquid supernatant, 25 mL of Sugar Salt Solution (SSS) (2.5 g/L Instant Ocean, 200 mM sucrose, filtered deionized (DI) water (pre-filtered through a 0.2 μm filter)) was added to each tube, and the cell pellet was re-suspended using a vortex. The re-suspended cells were frozen at -20 °C for 12 h, and then lyophilized for 48 h (Labconco FreeZone 4.5 lyophilizer, Kansas City, MO, USA). The tubes were stored at 4 °C until use. 100 mM sucrose was used as a cryoprotectant during the lyophilization process. For hatchery trials, individual tubes with a single dose of formulation for a target dose of 5×10^4 CFU/mL for 100 L were prepared.

Spray-dried formulations (RI-SD)

Spray-dried formulations were prepared by Envera LLC (West Chester, PA) using a proprietary formula. Computer controlled fermentation vessels were used to grow the probiotic and pasteurized to make 100% spore-based product. After pasteurization, the probiotic was centrifuged and spray dried into a fine powder that can be easily hydrated with seawater. The final concentration of the probiotic in the formulation was 8.6×10^{11} CFU/mg of powder. For the hatchery trial, tubes of the appropriate amount of formulation for a target dose of 5×10^4 CFU/mL in each 100 L tank were prepared. At the hatchery seawater was added to the tubes and mixed thoroughly. The mixed formulation was then added to the tanks daily during feeding.

Fresh culture controls (RI)

In order to determine the influence of the formulation process itself on the effectiveness of the probiotic *in vivo*, we tested simultaneous treatments of freshly cultured *B. pumilus* RI06-95 (cultures prepared as described in Sohn *et al.* 2016b) alongside formulated treatments in all lab and hatchery trials.

Viability and stability of formulated products

Viability and stability of each formulation was measured by counting colony forming units (CFU) on 2.5% yeast peptone agar plates using serial dilutions. Pre-formulation cell concentrations in CFU/mL were measured from culture aliquots directly before centrifugation. The RI-L product was re-suspended in 50 mL filtered sterile seawater (FSSW). The RI-G was suspended at 5 mg/mL in FSSW for 10 min and then vortexed for 1 min. The RI-SD was suspended using 0.1 g into 50 mL FSSW, followed by 10-fold serial dilutions. The percent cell viability in the formulations was calculated as follows: % Viability = [(sample formulation CFU/mL) / (pre-formulation CFU/mL)] × 100%

RI-L was stored at 4 °C, while samples of RI-G were stored at either room temperature or 4 °C and RI-SD stored at room temperature. The stability of the formulated probiotics was measured immediately after formulation (t = 0) and 1, 2, 5, and 8 weeks after formulation, except RI-SD. Each assay was performed in triplicate.

Laboratory pathogen challenge experiments

Laboratory challenge assays were conducted following protocols outlined in (Karim *et al.* 2013a). Briefly, larval oysters were placed into six-well plates with 5 mL of filtered sterile sea water (FSSW, 28 psu). Probiotic treatments were added to the larvae at a concentration of 10^4 CFU/mL and incubated at room temperature with gentle

shaking. After 24 h, the larvae were placed onto a 42 µm nylon mesh and washed gently using FSSW, then placed back into the original wells. Finally, *V. coralliilyticus* RE22 was added to each well, with the exception of the non-challenged controls, at a final concentration of 10^5 CFU/mL. Larval survival was quantified ~ 24 h after the pathogen was added using the neutral red technique (Gómez-León et al. 2008). Survival was calculated by using the formula: Survival (%) = 100 × (number of live larvae/total number of larvae).

The relative percent survival (RPS) of probiotic pretreatment compared to the challenged control was calculated using the formula: RPS (%) = [1 - (% survival challenged control treatment / % survival challenged treatment)] × 100 as described in Karim et al., 2013.

Hatchery trials

Hatchery experiments were conducted at Roger William University (RWU), Bristol, RI or the Aquaculture Breeding Center at the Virginia Institute of Marine Sciences (ABC), following standard operating procedures at each hatchery. For each trial, twelve 60 L (ABC) or 100 L (RWU) conical larval rearing tanks were used. We performed four independent trials at RWU between January 2014 and July 2016, and one trial at VIMS in June 2015 (Trial IV), testing each of the formulations at least once. Each trial was initiated by adding 8 -10 larvae/mL (800,000 to 1,000,000 initial larvae) per tank 1-2 d post-fertilization to the conical tanks. Tanks were randomly assigned to treatments and probiotic formulations were added daily at the time of feeding mixed with the algal food. Larvae were kept in static conditions and tanks were drained-down

every other day, cleaned, and re-stocked with fresh water. Treatments, number of tanks per treatment, and trial duration for each trial is shown in Table 1.

Larval survival and growth during hatchery trial

Data was collected at the time of selected drain-down events. Oyster larvae were passed through different sized mesh screens (35, 55, 75, and/or 105 μm for Trials I, II, and III; 35, 48, or 63 μm for Trial IV; 35 or 75 μm for Trial V) depending on the age and size of the larvae. Oyster larvae retained in each of the screens were collected in a container, seawater was adjusted to a fixed volume (1 – 5 L depending on the amount of larvae), and aliquot samples (1 mL each) were placed in Sedgewick Rafter counting chambers (Graticules ® S50). Larvae were fixed with Lugol's iodine (Trials I-III) or temporarily immobilized with a 2:1 mixture of freshwater: 70% isopropyl alcohol (Trials IV, V). Larvae were counted under a microscope and the presence of live and dead larvae were recorded. After counting, 50 larvae from each tank (25 from top screen, 25 from bottom screen) from Trial I and 25 larvae from each tank from Trials II, III, and V were randomly selected from the slides and photographed with an Olympus BX51 microscope (Olympus) and measured using an Olympus DP25 camera and CellSens Standard 1.6 image software (Olympus). During Trial IV, 5 larvae from each tank were randomly selected and measured on a Nikon E200 microscope. A random sample from each culture was photographed using a Nikon DS-Fi2 camera and DS-L3 camera control unit. Interval survival rate was determined by dividing the number of live larvae at each time point by the number of live larvae returned to the tank on the previous time point.

Laboratory pathogen challenge of probiotic-treated larvae from hatchery

An aliquot of larvae from each tank collected at selected drain-down events was transported to the laboratory at University of Rhode Island. Oysters (about 40 – 50 larvae) were placed in six-well plates and then challenged with *V. coralliilyticus* RE22 at a final concentration of 10^5 CFU/mL following the methods described in the laboratory challenge section. Oyster larvae from Trial IV could not be challenged since very low number of oyster larvae were left in the probiotic treated groups at the hatchery.

Determination of levels of *Vibrio* spp in the hatchery

Total number of *Vibrio* spp. was evaluated using a plate count method on thiosulfate-citrate-bile salts-sucrose medium (TCBS, Difco) (Sohn et al. 2016a). Samples were collected from water in the rearing tank (3 x 10 mL), tank surfaces (by swabbing), and oysters (about 1,000) when the tanks were drained. Swab samples (3 per tank) of tank surfaces (about 48 cm in length in total) were collected from each tank for all except Trial V. Each cotton swab was placed into a sterile Falcon tube containing 1 ml of FSSW and then mixed vigorously. Oyster larvae were rinsed with FSSW, homogenized using a sterile pestle, and suspended in FSSW. Ten-fold serial dilutions of each sample were prepared in triplicate, and then triplicate 10 µL of each dilution were plated on TCBS agar plates. After a ten-fold serial dilution, 10 µL samples of each of the dilutions were spotted evenly onto TCBS agar plates in triplicate for all except trial 5. The inoculated plates were incubated for 16 - 20 h at 28 ˚C and the colony forming units (CFU) were calculated. Results are expressed as CFU/mL, where 1 mL corresponds to 1 mL of water in the tank, 1 mL of swab suspension, or 1 mL of water

contacting about 1,000 larvae. Determination of *Vibrio* spp. levels could not be performed on larvae from trial IV due to very low numbers of surviving larvae.

Statistical Analysis

Larval oyster survival data were subjected to arcsine square root transformation prior to statistical analysis. The one-way analysis of variance (ANOVA) was used to determine significance between treatments within each time point. The two-way ANOVA was also used to determine significance between groups with time and treatment as factors. The Tukey's or Sidak's multiple comparison tests were used for post-hoc pairwise comparisons. A p-value < 0.05 was considered to be statistically significant.

Formulation cell viability data were analyzed by two-way ANOVA followed by Tukey's Test for each temperature and each time point. All statistical analyses were performed using Graphpad Prism, version 6.0 (Graphpad Software, Inc.). Differences were considered to be significant at values of $p < 0.05$.

Results

Viability and stability of formulated products

The stability of the three formulated products was assessed after storage for 8 weeks (RI-G and RI-L) (Figure 1) or 16 weeks (RI-SD) at ambient temperature. The three formulated products varied in their final CFU/ml following storage. RI-L and RI-G had similar pre-formulation concentrations of 1×10^8 CFU/mL and 1.27×10^8 CFU/mL, respectively. We observed a loss in viability immediately after the RI-G formulation process (data for RI-SD not available), and then again one week after storage at both 4

and 27 °C. However, we note strong stability after this initial loss. The spray dried formulation had a concentration ~200-250-fold higher at 2.65×10^{10} CFU/mL 16 weeks post formulation.

Effectiveness of *B. pumilus* RI06-95 formulations at promoting survival after a pathogen challenge

Laboratory challenge experiments: Pretreatment with fresh or formulated probiotic had no adverse impact on larval survival (i.e. in the absence of pathogens) over a 48h period aside from a single instance (L, Fig. 2B), where the formulation reduced larval survival by 46% compared to the unchallenged control. In the same trial, treatment of sucrose alone also showed significant reduction in larval survival (L, Fig. 2B). The ability of formulations to protect oyster larvae from exposure to the pathogen *V. coralliilyticus* RE22 was either higher or equal to that of fresh cultures in all experiments conducted, except in the one instance where sucrose alone was shown to reduce larval survival (Table 2, L, Fig. 2B). Larval survival was significantly greater in both fresh and formulated treatments versus controls for one of the three L treatments (Fig.2D), the G treatment (Fig.2A), and both SD treatments (Fig. 2E, F) (Table 2). In only two instances was there a significantly higher protection by formulation against the pathogen challenge than the fresh culture (Table 2, L III and SD II, Fig. 2D and 2F respectively).

Hatchery Trials:

Effect of daily treatment with probiotics in the hatchery on larval growth and survival

Based on successful protection from pathogen challenges in laboratory trials, all three formulations were tested in a hatchery. Treatments included in each hatchery trial and the length of treatment is described in Table 1. The formulations did not have an effect on the larval growth as shown by larval size measurements compared to control except for Trial IV with RI-L (Figure 3). None of the formulations had a significant detrimental impact on survival in the hatchery (Figure 4) except Trial I (RI-G) that showed a significant negative effect of the probiotic additions. None of the trials showed a significantly positive effect on larval survival due to probiotic addition (Figure 4). Of the three formulations, the spray-dried product had the smallest impact on larval survival. Thus, the SD-formulation is safe for use with oyster larvae in the hatchery.

Effect of daily treatment with probiotics in the hatchery on larval survival to bacterial challenge

Larvae from the hatchery experiments were tested for improved survival following challenge with *V. coralliilyticus* RE22. Since pathogens could not be introduced into the hatchery, larvae were collected and subjected to laboratory challenges as described in methods section. Larvae exposed to the granulated or lyophilized probiotics in the hatchery did not show significantly higher survival to a 24 h bacterial (*V. coralliilyticus* RE22) challenge as compared to non-treated challenged larvae (One-way ANOVA; $p > 0.05$) (Figure 5). A fresh culture of RI06-95 offered some protection on day 12 in Trial II (One-way ANOVA; $p < 0.05$; Figure 5 D). Relative percent survival (RPS) provided by the fresh culture of RI06-95 in this trial was 36 ± 6 % on day 12 (Table 3). On the other hand, trial V showed significantly improved survival both with RI-SD and fresh culture of RI06-95 as compared to controls (One-way ANOVA; $p < 0.05$; Figure 5 G).

The relative percent survival (RPS) with fresh culture was 28 ± 6% and RI-SD was 43±4% (Table3).

Effect of daily treatment with probiotics in the hatchery on levels of total Vibrio spp.

In general, daily treatment of tanks with either formulation of *B. pumilus* RI06-95 did not lead to a significant decrease in the levels of total *Vibrio* spp. in water, tank surfaces, or oyster larvae as compared to control groups at each of the time points (Figure 6 and Figure 7). High levels of variability were observed between tanks and trials within treatments. Interestingly, levels of *Vibrios* in the water were lower than 10^3 CFU/mL in Trial I (Figure 6A) and none were detected on the tank surfaces during this trial (Figure 6D). Trial I was performed in January, a month in which lower levels of *Vibrios* are present in coastal waters in the region (and therefore in water being pumped into the hatchery) (Duan & Su 2005, Parveen et al. 2008). Similarly, very low levels of *Vibrios* were found in Trial V in the water (Fig 6G). Levels of *Vibrios* on tank surfaces and larvae were not measured during Trial V. Overall the results show that certain days probiotic treated tanks (formulated or fresh) show reduced level of *Vibrios spp.* as compared to control but there is no significant trend to specifically ascertain that effect.

Discussion

We outline three formulation protocols, a granulation process (G), a lyophilization process (L), and a commercial spray-dried process (SD). Variation in terms of success

was achieved for each of the formulations, with the spray-dried formulation showing overall the best performance.

Granulation process: A traditional approach for formulating microorganisms is air-convective drying, which is a cost-effective process for the dehydration of microorganisms (Fu & Chen 2011, Guergoletto et al. 2012). Granulation after an air-convective drying is necessary to prevent segregation of the constituents of the powder and to provide consistent particulate sizes. The loss of viability of probiotic bacteria during granulation is associated with granulation operating conditions such as temperature, mechanical and moisture stress, and the characteristics of the selected microorganisms (Hiolle et al. 2010). This process did cause an immediate loss in viability from fresh cultures over the short term, as dehydration of bacterial cells poses serious physiological challenges to the survival of cells, such as conformational and chemical changes in structural proteins and membrane lipids (Ananta 2005, Santivarangkna et al. 2008, Ohtake & Wang 2011). However, after these initial short-term losses the cell count stabilized and remained consistent over 8 weeks. Storage conditions such as temperature and humidity have also been shown to affect the stability of granulated probiotic product (Ananta 2005). Mortality of probiotic bacteria during storage is associated with various stress factors such as temperature, oxygen/air, light, moisture/humidity, and package material, a combination of which tends to damage or destroy cells (Wang et al. 2004, Ananta 2005, Chávez & Ledeboer 2007). Our results, however, suggest that beyond an initial decrease in viability, the granulated product of RI06-95 could be stored at either 4 °C or room temperature and maintain viability for up to for 8 weeks. The stability of the granulated product during storage may be due to

the adaptation of *Bacillus* spp. to extreme environmental stress by spore-formation characteristics (Desmond et al. 2002, Driks 2002, Hong et al. 2005, Cutting 2011).

RI-G showed protection in laboratory experiments and did not show any detrimental effect on the larvae in any of the laboratory assays. However, in the hatchery trial formulation treated larvae showed reduced survival as compared to control and freshly grown probiotic. It demonstrated protection from pathogen challenge in laboratory trials but was unsuccessful in doing so in the hatchery trial. Despite the favorable results from viability and storage of the granulation protocol, research on the granulated product was discontinued in this study due to a negative influence on survival of larval oysters in hatchery settings.

Lyophilization: The lyophilized formulation (L) did not significantly impact cell viability after the formulation process. Lyophilization has previously been investigated as a way of preserving and formulating *Bacillus* spp. as probiotic products (Henn et al. 2015). To ensure sufficient viability after freeze-drying, a disaccharide cryoprotectant such as sucrose or trehalose is typically added to provide structural support to cell membranes and proteins (Leslie et al. 1995). We successfully used sucrose at a concentration of 100 mM that provided stability and viability over time.

RI-L led to variable results in larval survival in hatchery trials. It failed to provide protection from pathogen challenge in the 2 out of 3 laboratory experiments and the hatchery trial. It produced no observable negative effect on water quality. Our results suggest that the addition of sucrose may be responsible for the negative impact on larval survival, as sucrose alone (without *B. pumilus* RI06-96) lowered larval survival in 2 of

4 trials where it was investigated. Because a wide range of bacterial taxa can readily use sucrose, we suggest that its addition to the formulated product may encourage antagonistic bacterial growth, and presents greater risks than advantages.

Spray drying: Of the three formulations tested, the commercially prepared spray dried formulation was found to maintain the highest concentration at room temperature over time while also showing no negative impact on larval oysters in the laboratory or in the hatchery trials. After 16 weeks at room temperature, the SD-product still contained $>2.65 \times 10^{10}$ CFU/g. Previous research has shown that probiotic concentrations of *Bacillus* products at around 1×10^4 CFU/ml provide optimal performance (Karim *et al.* 2013a; Sohn *et al.* 2016a), meaning to reach a final target concentration of 1×10^4 CFU/ml in a 1,000 L commercial tank, only ~0.4 g of RI-695 would need to be added. This would be extremely cost effective for use at a larger scale. Another added benefit of the formulation is its ease of use. The powder quickly suspends in seawater and is added to the tank very easily.

The spray-dried formulation was also shown to perform as well or better than freshly prepared *B. pumilus* RI06-95 in both laboratory experiments and hatchery trials. In hatchery experiments, RI-SD showed no significant reduction in larval survival, water quality or larval growth. In fact, it increased survival compared to freshly prepared culture in the hatchery trial by day 12. RI-SD also performed well in pathogen challenge experiments, increasing survival of larvae after the challenge at the same rate or greater as compared to freshly prepared culture.

As seen in previous hatchery experiments (Sohn et al. 2016), high levels of variability were seen between tanks and trials within a treatment. The variation in results within and/or between experiments in this study could be due to several factors: (1) a different quality and health status of larvae from each trial; (2) the impact of various environmental and biological factors such as salinity, pH, temperature/season at the hatchery; (3) variability in the characteristics of different rearing systems, such as tank, source or treatment of water, and location of hatchery (Balcazar et al., 2006; Gatesoupe, 1999; Martínez Cruz et al., 2012; Utting and Millican, 1997); and 4) the effect of variability in the composition of microbial communities and how these communities may interact with the probiotic. Due to above factors the variability is more pronounced in hatchery trials than controlled laboratory experiments. Although variability is seen within and/or between experiments in this study for RI-G and RI-L, it is highly minimized in the trials using RI-SD. More importantly there is consistency in the goal of achieving protection from pathogen challenge with use of RI-SD.

The use of probiotics as a disease control mechanism has particular and critical relevance to shellfish hatcheries, where disease losses are high, vaccination is not possible and use of antibiotics is not recommended. Our results demonstrate successful formulation of the candidate probiotic *B. pumilus* RI06-95 for its use in shellfish hatcheries using the spray drying method. It also demonstrates the challenge in formulating the probiotic and the need of thorough testing in both laboratory and hatchery setting to confirm the effect of formulation. The laboratory and hatchery trials confirm that the RI-SD formulation is stable over a long term, remains viable and shows comparable performance to freshly grown cultures of the probiotic. It is suitable for

storage, transportation and can be easily applied in a hatchery by mixing with sea water. Although the addition of RI-SD did not show reduction in *Vibrio* spp. in general, this might not be a strategy used by the probiotic as its mechanism of action. Probiotics are known to modulate the immune system of the host (Hardy et al., 2013, Mortha et al., 2014, Sanchez et al., 2015). This could be one the strategies used by *B. pumilus* RI06-95 to provide protection in the event of vibriosis.

Future research in mechanism of action of the probiotic would help in optimization of the use formulation in terms of dosage timing and frequency.

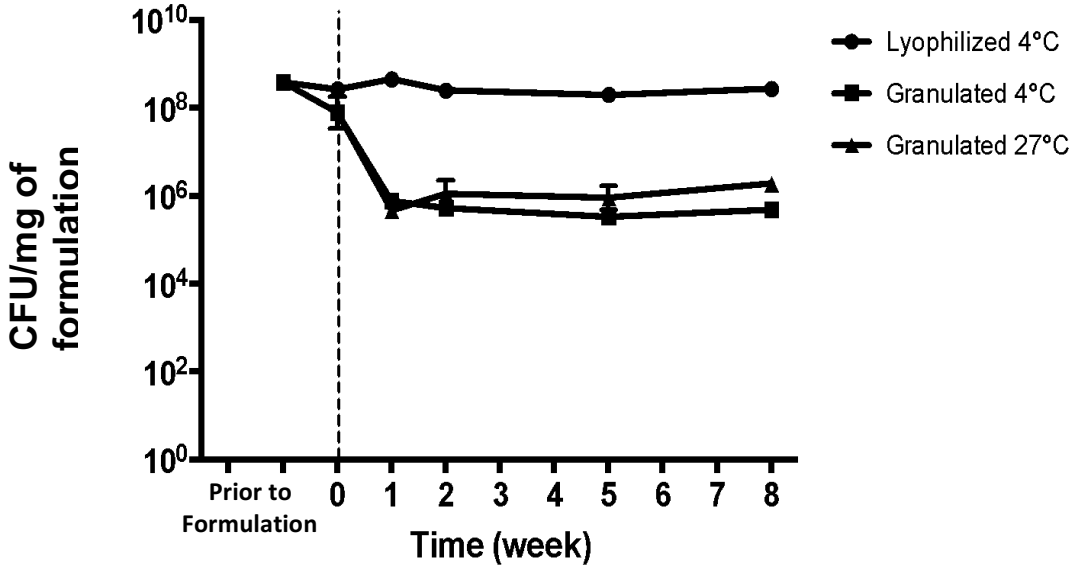


Figure 1. Impact of formulation processing (granulation or lyophilization) and temperature storage on the stability of *Bacillus pumilus* RI06-95. Cell count in the reconstituted formulation after storage for up to 8 weeks was determined using a plating method. Data expressed as mean ± SEM of CFU/mg of formulation.

Hatchery Trial	Treatments	Tanks per treatment	Treatment period (days)	Dates performed
I	Control, RI-G	6	14	01/03/14 – 01/24/14
II	Control, ConwS, RI, RI-L	3	12	01/29/15 – 02/10/15
III	Control, ConwS, RI, RI-L	3	12	02/22/15 – 03/06/15
IV	Control, RI-L	4 (control), 3(RI-L)	10	06/24/15 – 07/08/15
V	Control, RI, RI-SD	3	12	06/06/16-06/17/16

Table 1: Treatments included in each hatchery trial and the total length of treatment in days. Abbreviations: controls (no probiotic provided); ConwS = 100 mM sucrose (no

probiotic, control for lyophilized formulation); RI-G = granulated formulation; RI-L = lyophilized formulations (in 100 mM sucrose); RI-SD = spray-dried formulation; RI = RI06-95 freshly cultured in lab.

	Treatment	Relative Percent Survival (RPS, % ± SEM)	Plots
Granulated	RI-G	69 ± 1	(**Figure 2** A)
Lyophilized I	RI	26 ± 5	(**Figure 2** B)
	RI-L	-93 ± 86	
Lyophilized II	RI	22 ± 11	(**Figure 2** C)
	RI-L	25 ± 6	
Lyophilized III	RI	56 ± 4	(**Figure 2** D)
	RI-L	74 ± 1	
Spray-dried I	RI	23 ± 6	(**Figure 2** E)
	RI–SD	21 ± 13	
Spray- dried II	RI	75 ± 5	(**Figure 2** F)
	RI–SD	83 ± 2	

Table 2. Laboratory challenged experiments results: Effect of pre-incubation of oyster larvae for 24 h with RI06-95 formulated products on survival (RPS, % ± SEM) after challenge with *V. coralliilyticus* RE22. Survival was measured 24 h after challenge and 48 h after addition of the probiotic. Data is expressed as Relative Percent Survival (RPS, % ± SEM) of challenged oysters exposed to probiotics compared to *V. coralliilyticus* RE22 challenged control. Abbreviations: RI-G = granulated formulation; RI-L = lyophilized formulation (in 100 mM sucrose); RI-SD = spray dried formulation, RI = fresh; RE22 = *V. coralliilyticus* RE22.

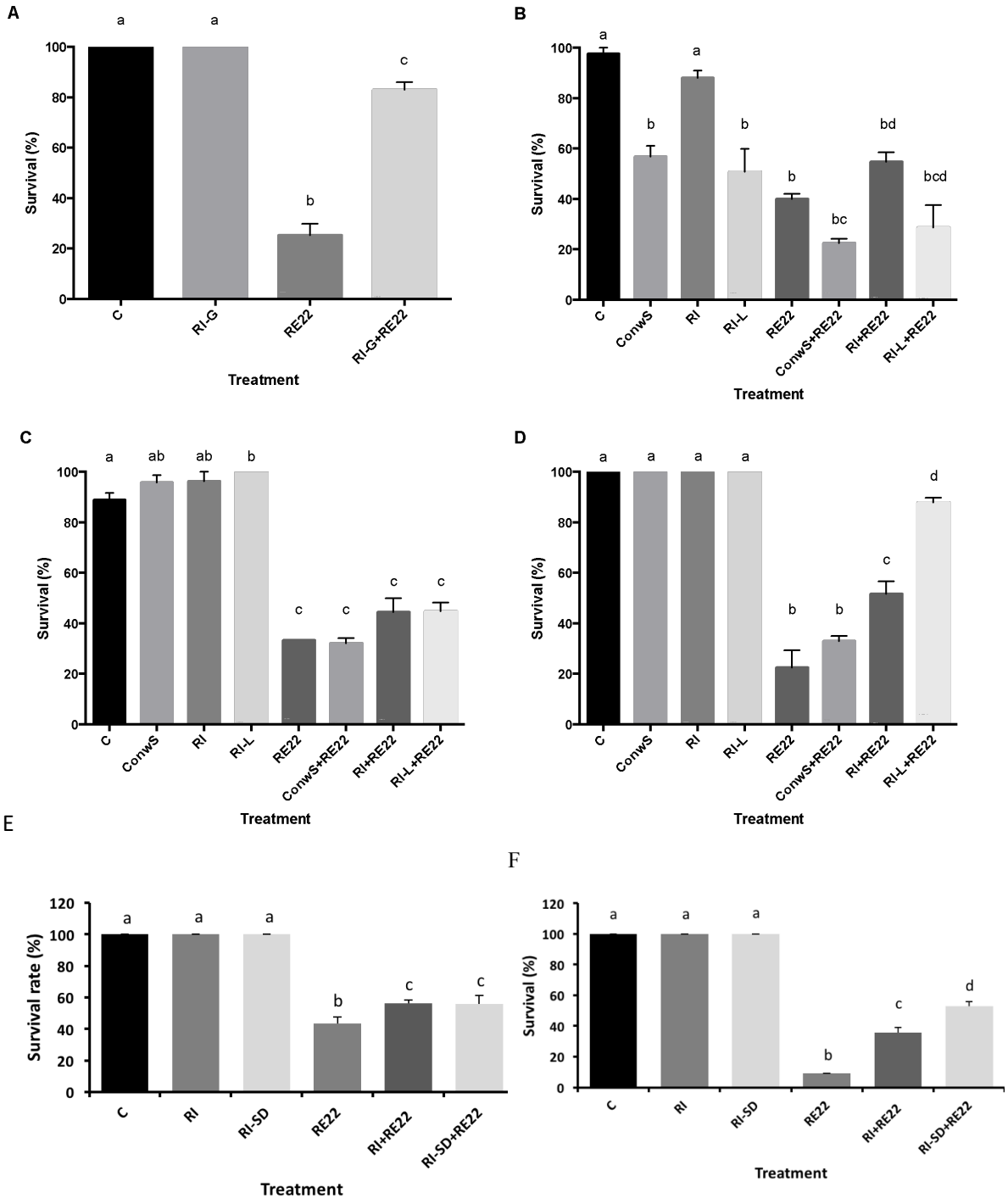


Figure 2. Laboratory challenged experiments results: Effect of pre-incubation of oyster larvae with *Bacillus pumilus* RI06-95 formulated products for 24 h on survival (% ± SEM) after challenge with *V. coralliilyticus* RE22. Survival was measured 24 h after challenge and 48 h after addition of the probiotic. (A) Exposure to a granulated product of *Bacillus pumilus* RI06-95; (B), (C), and (D) Exposure to lyophilized formulations

(representative experiments) (E) and (F) Exposure to spray dried formulations. Abbreviations: C = no probiotic; ConwS = 100 mM sucrose; RI-G = granulated formulation; RI-L = lyophilized (in 100 mM sucrose) formulation 5; RI = fresh RI06-95; RE22 = *V. coralliilyticus* RE22. Different letters indicate statistically significant differences between the treatments.

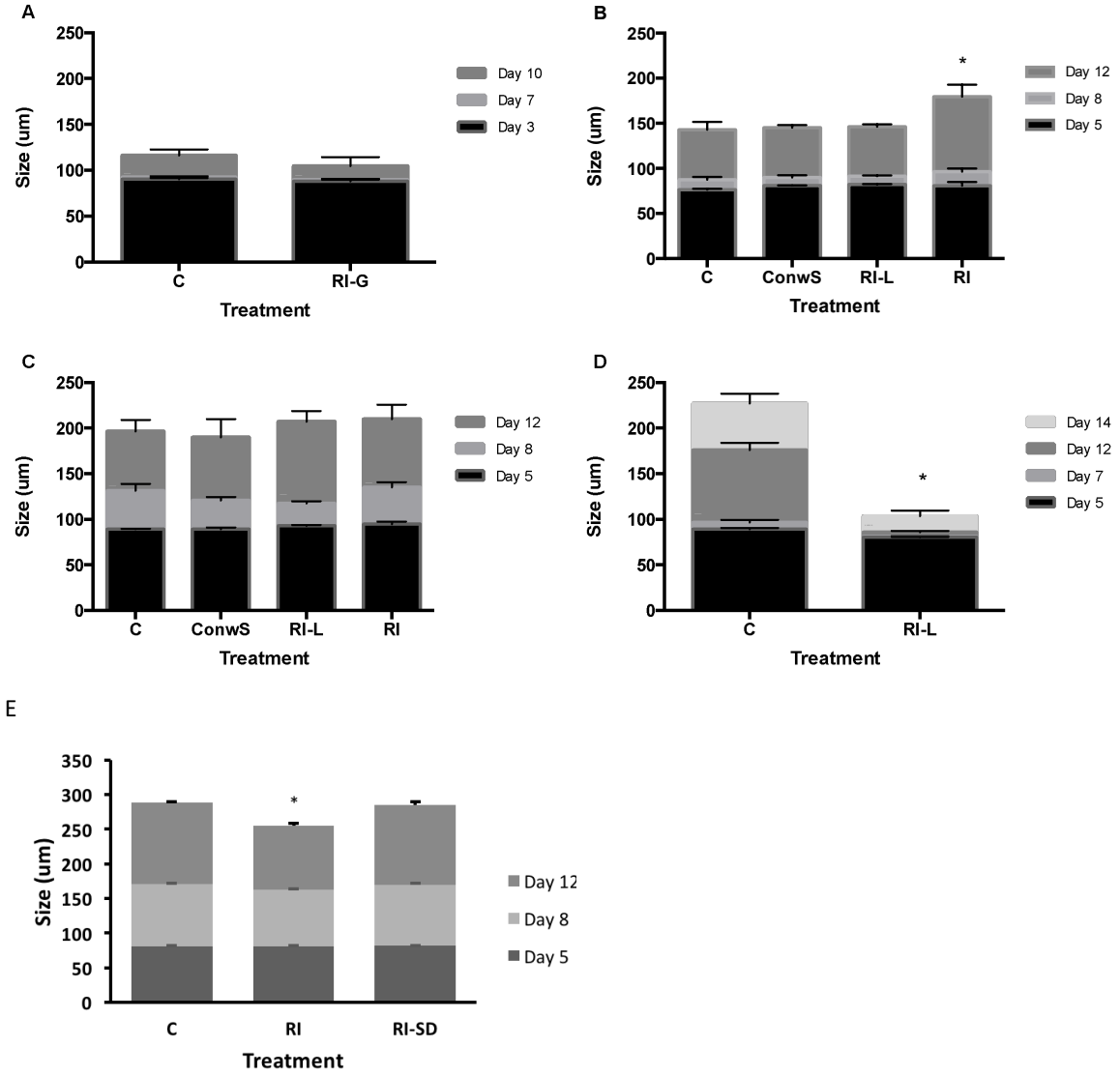


Figure 3. Effect of daily treatment with different formulations of *Bacillus pumilus* RI06-95 of larval eastern oysters (*Crassostrea virginica*) in the hatchery on mean larval size (µm ± SEM) at selected time points. (A) Trial I; (B) Trial II; (C) Trial III; (D) Trial IV and (E) Trial V. Abbreviations: C = no probiotic; ConwS = 100 mM sucrose; RI-G = granulated formulation; RI-L = lyophilized (in 100 mM sucrose) formulation; RI-SD

= formulation RI = fresh RI06-95; RE22 = *V. coralliilyticus* RE22. An asterisk (*) indicates statistical significances compared to controls.

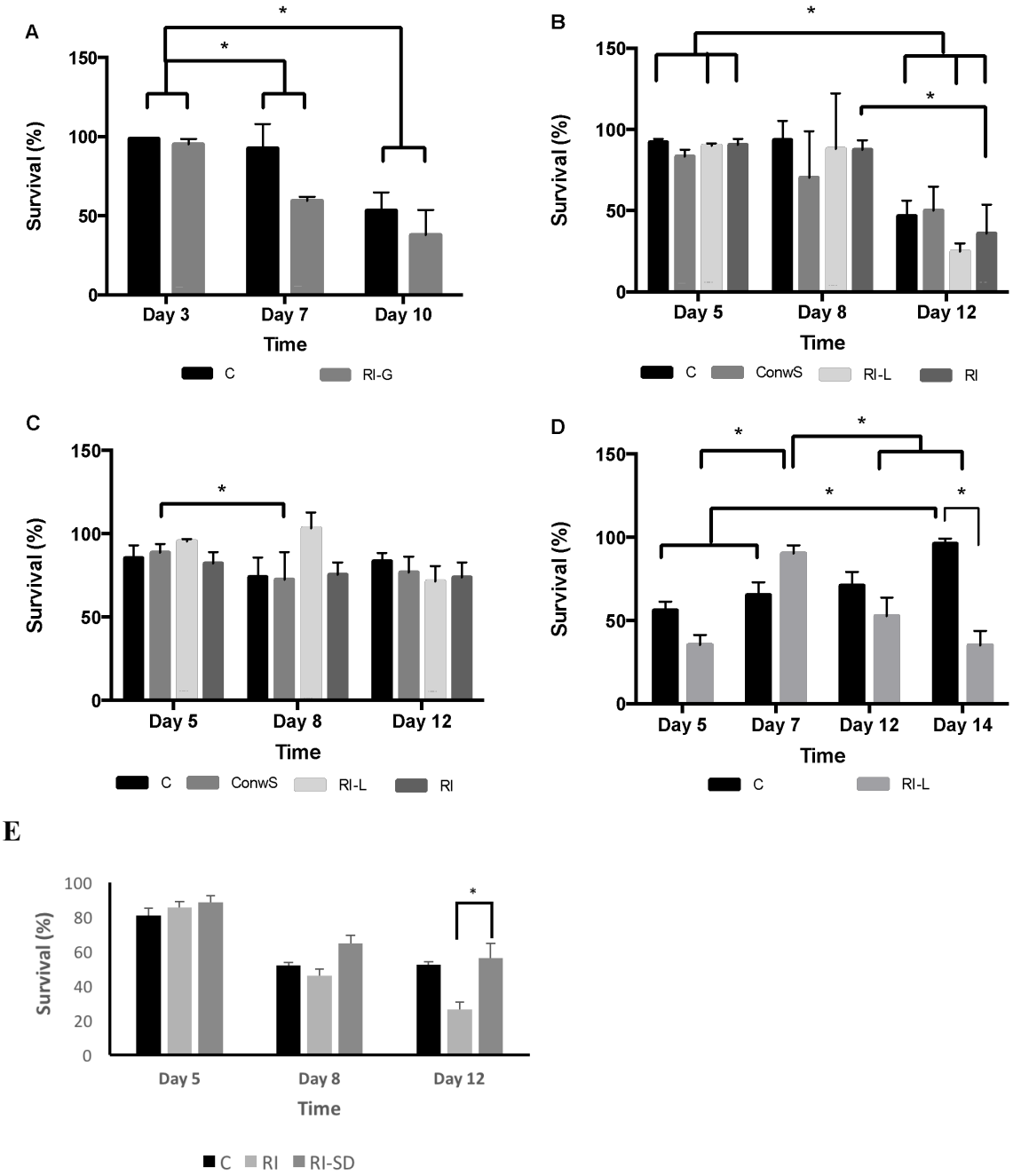
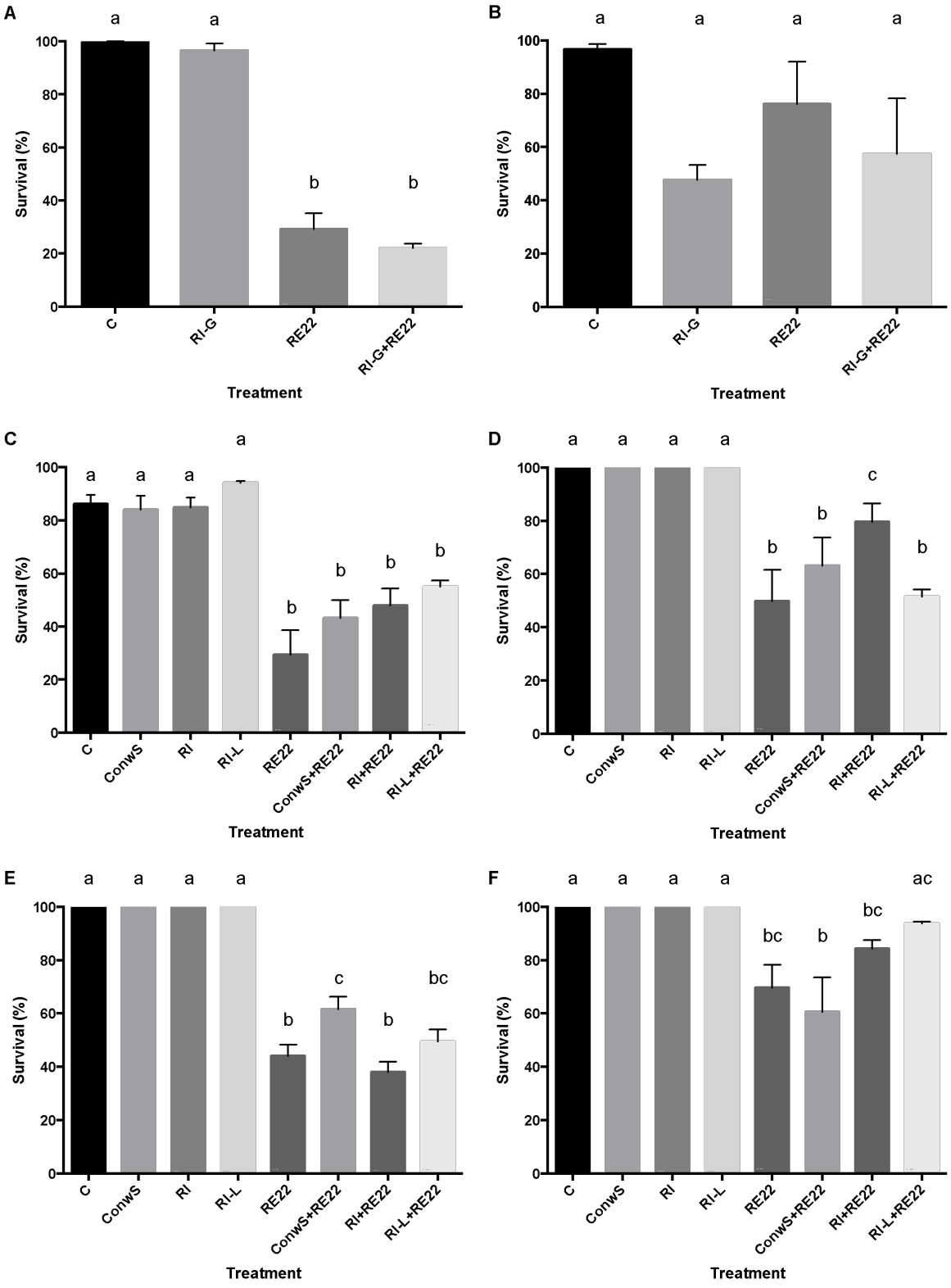


Figure 4. Effect of daily treatment with probiotics in the hatchery on interval survival (% ± SEM) of oyster larvae between selected time points. (A) Trial I; (B) Trial II; (C) Trial III; (D) Trial IV and (E) Trial V. Abbreviations: C = no probiotic; ConwS = 100 mM sucrose; RI-G = granulated formulation; RI-L = lyophilized (in 100 mM sucrose)

formulation; RI-SD = spray dried formulation; RI = fresh RI06-95. An asterisk (*) indicates statistical significances between treatments.



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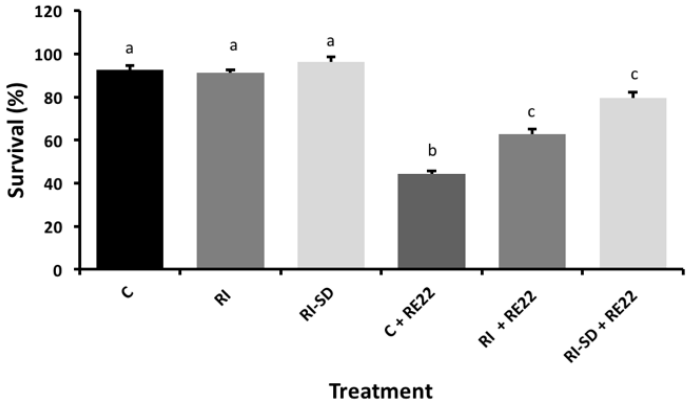
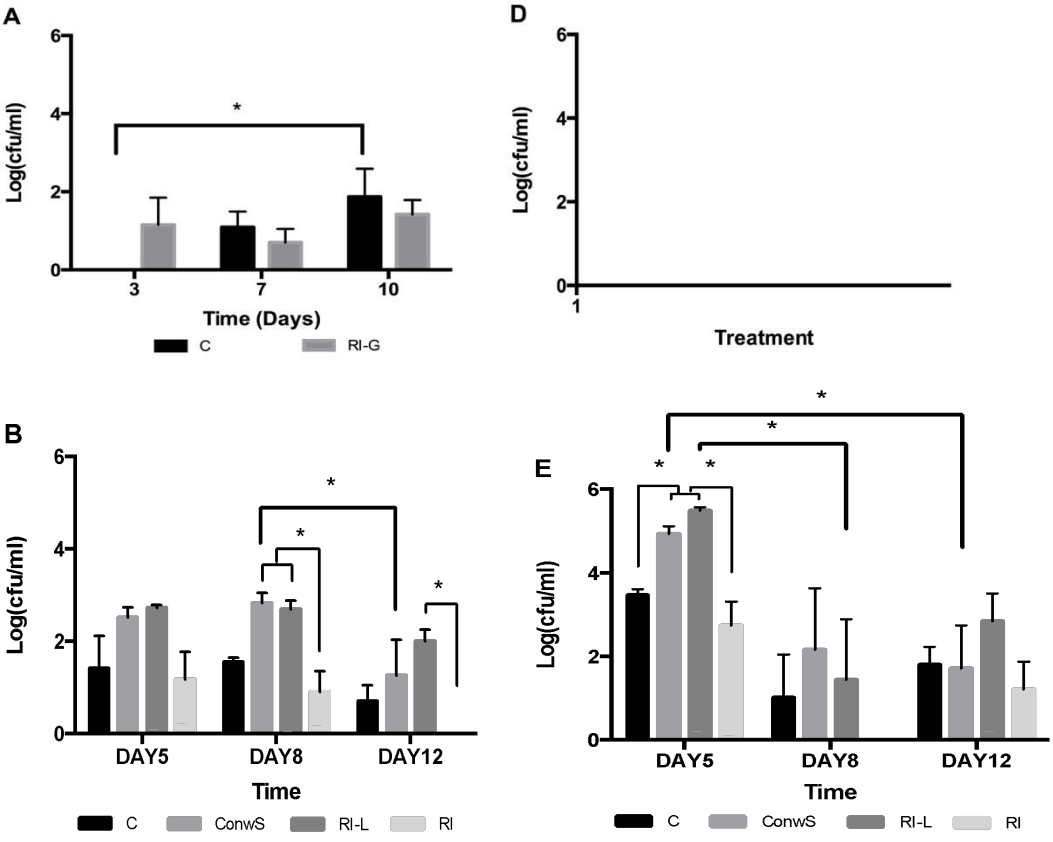


Figure 5. Effect of daily probiotic treatment in the hatchery on larval survival to a laboratory challenge with the pathogen *Vibrio coralliilyticus* RE22. Larvae were brought to the laboratory and survival was measured 24 h after challenge with RE22. (A) Larvae collected on Day 3 after fertilization in Trial I; (B) Day 7 in Trial I; (C) Day 5 in Trial II; D) Day 12 in Trial II; E) Day 5 in Trial III; F) Day 12 in Trial III. (G) Day 8 in Trial V. Abbreviations: C = no probiotic; ConwS = 100 mM sucrose; RI-G = granulated formulation; RI-L = lyophilized (in 100 mM sucrose) formulation; RI-SD = spray-dried formulation; RI = fresh RI06-95; RE22 = *V. coralliilyticus* RE22. A different letter indicates a significant difference between treatments (One-way ANOVA; $p < 0.05$).

Trial	Treatments	Relative Percent Survival (RPS, % ± SEM)	
		Day 3	**Day 7**
I	RI-G+RE22	-10 ± 2	-78 ± 88
		Day 5	**Day 12**
II	RI+RE22	36 ± 9	36 ± 6
	RI-L+RE22	46 ± 3	2 ± 5

III	RI+RE22 RI-L+RE22	2 ± 13 - 36 ± 28	16 ± 3 26 ± 1
IV	**No challenge data due to low survival of larvae**		
		Day 8	
V	RI + RE22 RI-SD + RE22	28 ± 6 43 ± 4	

Table 3. Effect of daily exposure to formulations of *B. pumilus* RI06-95 in the hatchery on larval oyster survival (%) 24 h after challenge with *Vibrio coralliilyticus* RE22. Data is expressed as Relative Percent Survival (RPS, % ± SEM) of challenged oysters exposed to probiotics compared to *V. coralliilyticus* RE22 challenged control. Abbreviations: C = no probiotic; ConwS = 100 mM sucrose; RI-G = granulated formulation; RI-L = lyophilized (in 100 mM sucrose) formulation; RI-SD = spray dried formulation, RI = fresh RI06-95; RE22 = *V. coralliilyticus* RE22



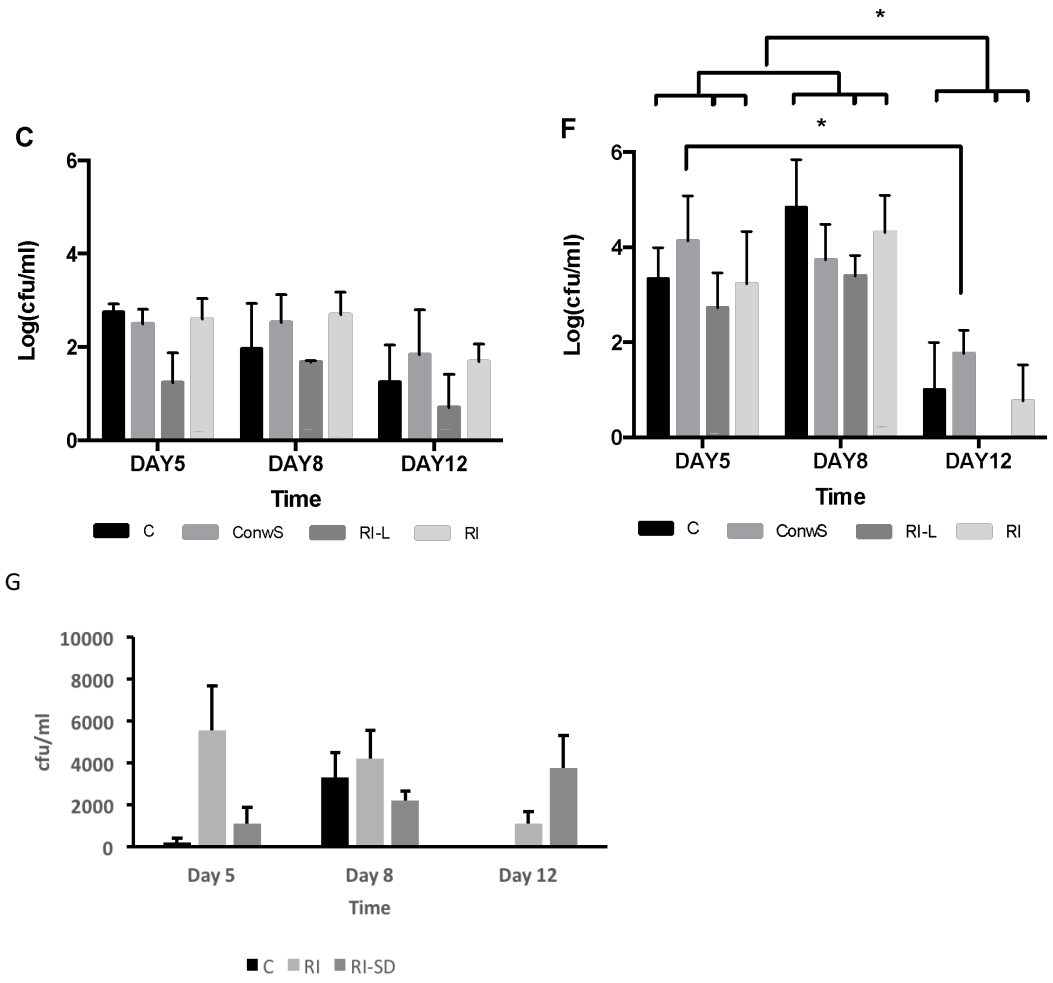


Figure 6. Effect of daily treatment with probiotics on total vibrio levels (Log_{10}(CFU/mL) ± SEM) in water (A, B, C, G) and tank surfaces (D, E, F) in a hatchery. (A and D) Trial I (no bacteria were detected in tank surfaces in Trail I); (B and E) Trial II; and (C and F) Trial III. (G) Trial V. Abbreviations: C = no probiotic; ConwS = 100 mM sucrose; RI-G = granulated formulation; RI-L = lyophilized (in 100 mM sucrose) formulation;RI-SD = spray-dried formulation; RI = fresh RI06-95; RE22 = *V. coralliilyticus* RE22. An asterisk (*) indicates significant differences between treatments (mean ± SEM, $p < 0.05$; Two-way ANOVA).

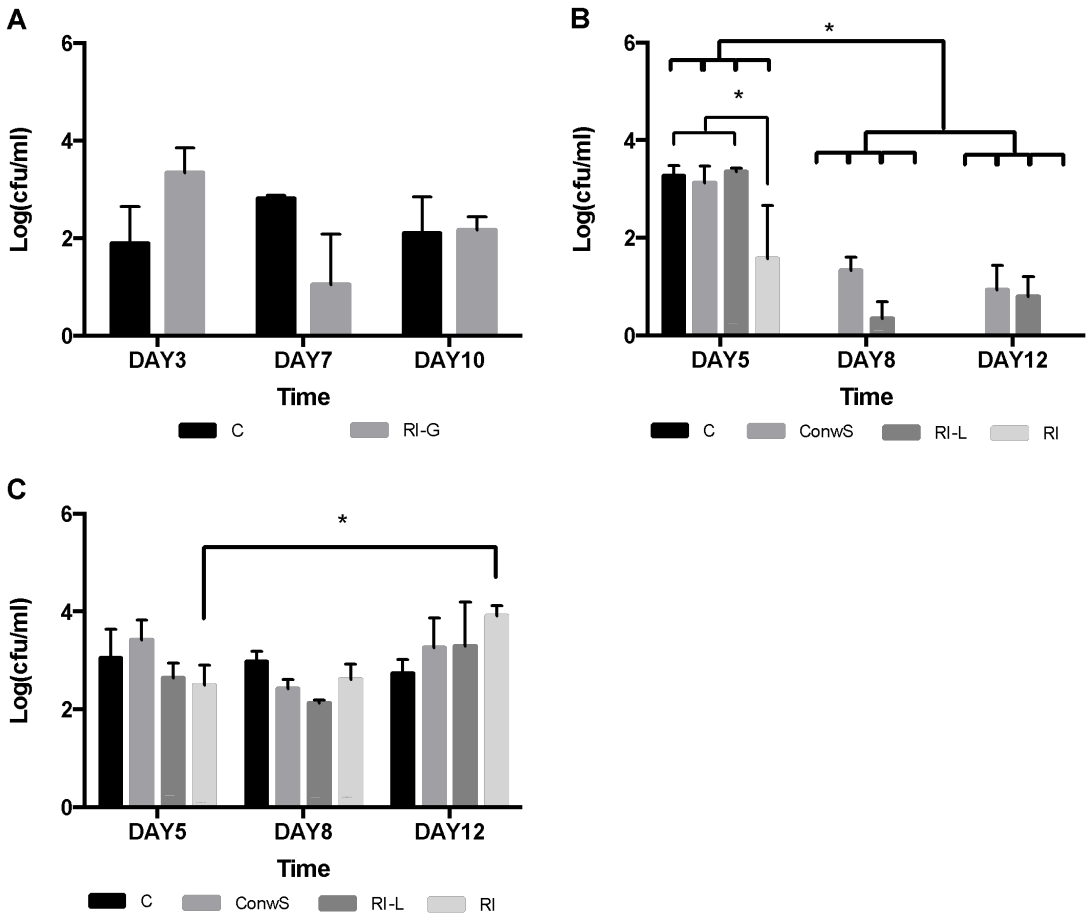


Figure 7. Effect of daily treatment with probiotics on total vibrio levels (Log_{10}(CFU/mL) ± SEM) on oyster larvae in the hatchery. (A) Trial I; (B) Trial II; and (C) Trial III. Abbreviations: C = no probiotic; ConwS = 100 mM sucrose; RI-G = granulated formulation; RI-L = lyophilized (in 100 mM sucrose) formulation; RI = fresh RI06-95; RE22 = *V. coralliilyticus* RE22. An asterisk (*) indicates significant differences between treatments (mean ± SEM, $p < 0.05$; Two-way ANOVA).

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CHAPTER 3

CHARACTERIZATON OF *CRASSOSTREA VIRGINICA* LARVAL RESPONSE TO *VIBRIO CORALLIILYTICUS* RE22

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Abstract

Vibrio spp. are ubiquitous in marine environments and, in the case of pathogenic species, responsible for causing disease in several marine organisms. *Vibrio coralliilyticus* has emerged as a pathogen affecting a variety of invertebrate species. In corals, certain strains cause bleaching, while *V. coralliilyticus* RE22 causes massive and rapid mortality of eastern oysters (*Crassostrea virginica*) larvae. Such mortality events in hatcheries where oyster larvae are reared, lead to heavy losses and subsequent shortage of oyster seed for the grow-out industry. A better knowledge of oyster-pathogen interactions and the mechanisms involved in RE22 pathogenicity may aid the development of effective management strategies. This study aims to characterize the larval immune response to experimental challenge by *V. coralliilyticus* RE22. Six to ten-day old *C. virginica* larvae were exposed to *V. coralliilyticus* RE22 for 6 hours to understand the host response in the early stages of the disease. Transcriptomes were obtained by high throughput sequencing of cDNA from three replicate experiments. Comparison of RE22 treated larval transcriptomes to untreated control larvae yielded 1,534 differentially expressed transcripts ($p \leq 0.05$). Overall, transcriptomic data showed evidence of suppression of key immune signaling pathways but possibly activated antiviral pathways. The larval response to RE22 lacked production of protease inhibitors, hypothesized to be involved in providing protection against the proteases that are a key virulence factor of RE22. In addition, transcriptomic data suggests modulation of mucus and cytoskeletal components. The transcriptomic response was also

characterized by differential expression of metabolic genes, suggesting high metabolic demand and oxidative stress contributing to larval mortality. This study fills a major gap in our knowledge on the immune responses in larval stages of this economically and ecologically important species. This information could aid in developing solutions to control disease and design better management practices for hatcheries.

1. Introduction

Vibrio spp. are common pathogens causing disease in a wide variety of aquatic species, including several species of mollusks. Strains of *V. coralliilyticus* also cause disease in corals, leading to bleaching (Ben-Haim et al., 2003, Wilson et al., 2013). *V. coralliilyticus* RE22, previously known as *V. tubiashii* RE22, causes vibriosis in bivalve larvae (Richards et al., 2015). The disease resulted in heavy mortalities that severely affected oyster seed production of shellfish hatcheries (Elston et al., 2008).

Infection by vibrios in bivalve larvae is dramatically rapid in progression and characterized with signs of bacillary necrosis, reduced feeding, and swarming of bacteria around the moribund larvae (Tubiash et al., 1965). An investigation of the colonization and infection process in Manila clam (*Ruditapes philippinarum*) larvae using a GFP-tagged *Vibrio* sp. showed pathogen entry through ingestion, with infection quickly spreading to other organs and followed by colonization and proliferation in the entire body (Dubert et al., 2016). The genome of *V. coralliilyticus* RE22 shows that it encodes several extracellular metalloproteases, serine proteases, hemolysins and type secretion systems as virulence factors (Hasegawa et al., 2008, Hasegawa et al., 2009, Spinard et al., 2015). Experimental infection of *C. virginica* larvae and juveniles with *V. coralliilyticus* RE22 showed differences in susceptibility based on the age and genetic background of the oysters (Gómez-León et al., 2008).

V. coralliilyticus YB1 specifically infects coral *Pocillopora damicornis* causing coral tissue lysis at higher seawater temperatures (26-29°C) and its virulence factors include

a potent extracellular protease (Ben-Haim and Rosenberg, 2002, Ben-Haim et al., 2003a) whose production is also temperature regulated (Ben-Haim et al., 2003b). *V. coralliilyticus* P1 genome and mutant studies demonstrated presence of 17 metalloproteases, serine protease, hemolysin-related protein RbmC, chitinase and effector genes including *vgrG, hlyA* and *hcp* (Santos et al., 2011). Transcriptomic studies investigating the responses of coral *Pocillopora damicornis* to *V. coralliilyticus* YB1 reported immunosuppression of the host as a pathogenesis strategy of YB1 (Vidal-Dupiol., et al., 2014) including repression of the antimicrobial damicornin (Vidal-Dupiol et al., 2011a). Innate immunity related genes involved in *P. damicornis* responses to *V. coralliilyticus* YB1 include lectins, cystatin B, ferretin, and selenium-binding protein (Vidal-Dupiol et al., 2011b).

Several studies have characterized changes in gene expression patterns in larval stages of bivalves during development including *Pinctada fucata* (Li et al., 2016), *C. angulata* (Qin et al., 2012) and in response to vibrio infection in *Crassostrea gigas* (Hasegawa et al., 2008) and *C. virginica* (Genard et al., 2012). This study aims to enhance knowledge on bivalve-vibrio interactions by analyzing the transcriptomic response of larval eastern oysters, an economically and ecologically important species, to infection with *V. coralliilyticus* RE22, a bacterial pathogen capable of causing rapid and high levels of mortality in bivalve hatcheries. The goals of this study are to (1) characterize the response of *C. virginica* larvae to experimental challenge with *V. coralliilyticus* RE22; and (2) provide hypotheses on possible strategies used by *V. coralliilyticus* RE22 to overcome larval immune defenses. This information will aid in developing solutions to control disease and design better management practices for hatcheries.

2. Materials and methods

2.1 Vibrio coralliilyticus RE22 culture:

The pathogen (supplied by H. Hasegawa, Department of Biomedical Sciences, Oregon State University) was maintained and stored in 50 % glycerol stocks at -80°C until use. Inocula from freezer stocks were plated on yeast peptone with 3%NaCl (YP30; 5 g L^{-1} of peptone, 1 g L^{-1} of yeast extract, 30 g L^{-1} of ocean salt, Instant Ocean) agar plates for 2 d, then transferred to 5 mL of YP3 broth incubated at 25°C on a shaker (134 rpm) for 1 d. Cultures were washed using Artificial Filtered Sterile Seawater (AFSW, 28-30 psu salinity) twice by centrifugation at 23,000 rpm for 10 min. The OD at 550 nm was measured and the stock was diluted such as to obtain a sub lethal concentration of 5×10^4 CFU mL^{-1} for transcriptome analysis and a lethal concentration (Karim et al., 2013) of 5×10^5 CFU mL^{-1} for disease progression analysis.

2.2 Oyster larvae:

C. virginica larvae were obtained from shellfish hatcheries on the east coast of United States. Larvae 6-10 days old were collected at the hatchery and shipped overnight to the lab at the University of Rhode Island on a wet filter. Upon arrival to the laboratory, larvae were washed with AFSW on top of a 40 μm nylon mesh and placed in stock containers containing AFSW. Larvae were acclimatized to the laboratory environment (room temperature) for 24 h prior to the experiments.

2.3 Effect of V. coralliilyticus RE22 on mortality of C. virginica larvae

In order to understand the rate of progression of disease, *C. virginica* larvae were experimentally challenged with 5 x 10^5 CFU mL^{-1} *V. coralliilyticus* RE22 (Karim et al., 2013). Larval density (larvae/mL) of the stock was determined using a Nikon E200 microscope. Larvae (~100) were distributed in wells of a 6-well plate with 5 mL AFSW and maintained at 22 - 23 °C with gentle rocking. Two treatments (control and challenge) were each conducted in triplicate. Larval mortality was recorded at 6, 9, 14, 18 and 20h post addition of *V. coralliilyticus* RE22 by evaluation of active swimming and/or gut and cilia movement using a Nikon E200 microscope.

2.4 Effect of pathogen exposure on larval gene expression

2.4.1 Experimental set up

For biological replicates, the complete set up as explained below was performed using larvae from three different hatcheries (n = 3 experiments, operationally designated as K, M, and V). Larvae from the stocks were distributed into tissue culture flasks (~10,000 per flask) in 500 mL AFSW and kept on a shaker with gentle shaking at ~50 rpm at room temperature. Larvae were acclimatized to the experimental set up for an additional 24h prior to challenge. Each treatment (control and challenge) was conducted in duplicate to serve as technical replicates. Larvae were fed with 1 mL of instant algae Shellfish Diet 1800™ (20,000 cells/mL; Reed Mariculture Inc., San Jose, CA. USA) immediately prior to treatment in order to promote pathogen ingestion. Challenge with *V. coralliilyticus* RE22 was performed with a sub lethal concentration of 5 × 10^4 CFU mL^{-1} for 6h.

2.4.2 Larval Collection post treatments

Control larvae were collected at 0h and RE22 treatments were collected 6h post challenge. Larvae were aspirated gently from the flasks using a 100mL serological pipette, and filtered through a 40 μm sterile filter for collection. Since dead larvae settle to the bottom, the last 25 mL of each flask was not collected to avoid bias in transcriptomic response. Larvae were washed with 2mL of AFSW on a 40μm filter, followed by a wash using 2mL of RNAlater™ , aspirated from the filter using a pipette, placed in labeled 2 ml RNase free microfuge tubes, and held at 4°C for 24h in RNAlater™ followed by storage at -20°C.

2.4.3 RNA extraction, cDNA prep and sequencing

Tri-reagent™ (Sigma-Aldrich) was used for extracting total RNA from all the samples following manufacturer's instructions (TRI Reagent™ Protocol, Sigma-Aldrich). RNA extracts were DNase treated using the DNA-free™ DNA removal kit from Ambion and purity and concentration of RNA was assessed using a Nanodrop 8000 spectrophotometer (Thermo Scientific). RNA from technical replicates was pooled at equimolar concentration. The quality and quantity of the pools were assessed using Agilent 2100 Bioanalyzer and High Sensitivity D1000 ScreenTape®. RNA samples were selectively enriched for poly-A containing mRNA and cDNA libraries were prepared using the PrepX RNAseq library Prep Kit (Takara Bio USA, inc). Samples were sequenced on Illumina HiSeq platform with 2x125 reads at a targeted sequencing

coverage of 20-30M per sample at the Harvard University, FAS Center for Systems Biology, MA.

2.4.4 Assembly, annotation and analysis

Raw reads obtained from sequencing were filtered, trimmed and adapters were removed using bbduk program in BBTools suite from Joint Genome Institute and viewed in FASTQC (Andrews, 2010). Processed reads were aligned to *C. virginica* reference genome (version 3.0) via HISAT2 2.1.0 (Kim et al., 2015) and assembly was performed using Stringtie (Pertea et al., 2016) using default parameters. To compare the depth of sequencing across all samples preseq package was used (Daley and Smith., 2013). Differential gene expression analysis was performed by comparing transcript counts between RE22 6 h treatment (replicates K, M, V) vs control 0 h (replicates K, M, V) using DESeq2 (Love et al., 2014). Transcripts with Benjamini-Hochberg adjusted p value ≤ 0.05 and log fold change of ≥ 2 or ≤ -2 were considered significantly differentially expressed. This analysis design only allowed for the most conservative estimates and only showed differentially expressed genes representing all the biological replicates. Annotation for differentially expressed genes (DEGs) was performed by mapping to NCBI protein non-redundant (NR) database using BLASTx (Altschul et al., 1997) with an e-value cutoff of $1e^{-3}$ and hit number threshold of 20. Mapping DEGs to GO terms was conducted using BLAST2GO v4.1.9 (Conesa et al., 2005) and functional enrichment was done using topGO (Alexa et al., 2006) with default parameters. ReviGO (Supek et al., 2011) was used to plot and visualize results obtained from topGO with default parameters (allowed similarity was set to medium). Significantly enriched GO

terms were obtained by using Fishers exact test ($p \leq 0.01$). Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway annotations were also obtained using the KEGG Automatic Annotation Server (KAAS).

3. Results

3.1 Effect of V. coralliilyticus RE22 on mortality of C. virginica larvae

Mortality in larval oysters exposed to 5×10^5 CFUmL of *V. coralliilyticus* RE22 was initially seen at 14h after challenge, increasing exponentially after that (Figure 1a). The larvae appeared normal at 6h, but 9h after challenge many showed reduced motility and feeding (Figure1b).

3.2 Transcriptome alignment

Depth of sequencing for all the transcriptomes ranged from 16,617,375 – 39,681,499 paired end reads. Sequencing saturation curves for all transcriptomes were close to full saturation, indicating that all but the rarest transcripts would be represented in the transcriptome (Figure 2). The alignment rate to the *Crassostrea virginica* reference genome using HISAT2 ranged from 85 – 89 % (Table 1).

3.3 Differential expression Analysis

Comparison of transcriptomes obtained from RE22 treated (6h) larvae to control (0h) larvae using DESeq yielded 1,534 differentially expressed transcripts ($p \leq 0.05$, upregulation: log fold change ≥ 2, downregulation: log fold change ≤ -2). Refer to supplementary data tables in appendix for descriptions and log fold change values.

3.4 GO and KEGG annotation

A Gene Ontology (GO) term enrichment analysis was performed on all the differentially expressed transcripts in response to RE22 challenge. There were 22 biological processes significantly enriched ($p<0.05$) that mainly belonged to metabolism and signaling, but none related to immunity (Table 2, Figure 3); 17 metabolic functions significantly enriched ($p<0.05$) including "receptor activity" (Figure 4) and membrane related terms significantly enriched in the cellular component (CC). The highest number of DEGs mapped to KEGG annotations belonged to signal transduction (Table 3).

3.6 Differentially expressed immune genes in response to RE22

3.6.1 Immune related genes

Described below are some of the important immune-related genes showing differential expression in RE22 exposed larvae (6h) as compared to control (0h) (Table 4).

Transcripts corresponding to immune receptors upregulated in response to RE22 included TLR receptors (TLR4, TLR13 and TLR Tollo isoform X2), lectin and fucolectin, and leucine-rich repeats (LRRs). Transcripts identified as scavenger receptor, complement C1q-like protein 2 and 4, LRR9, and fibronectin type III domain-containing protein 2 were downregulated.

Transcripts related to the TLR signaling pathway, including myeloid differentiation primary response protein MyD88-like (MyD88), TNF receptor-associated factor 4-like (TRAF4), mitogen-activated protein kinase kinase kinase 7-like (TAK1) and toll-interacting protein-like (TOLLIP), showed downregulation in response to RE22 exposure. Important members of the NF-kB pathway that were downregulated included

NF-kappa-B-activating protein-like (NKAP) and IkB-alpha. An essential component of the MAP kinase signal transduction pathway, dual specificity mitogen-activated protein kinase kinase 7-like (MKK7), was upregulated. Surprisingly, transcripts related to antiviral pathways including stimulator of interferon genes protein-like (STING) and ubiquitin carboxyl-terminal hydrolase 25-like isoform X3 (USP25) and some members of the JAK-STAT pathway were upregulated in response to RE22.

In terms of immune effectors, some mucin transcripts were differentially expressed in response to RE22, showing a mixed response (both up and downregulation).

In addition, cytoskeleton related transcripts including cytoplasmic actin and septin-11-like were downregulated, but dynamin-1-like transcripts showed high levels of upregulation.

3.6.2 Cell death

Transcripts corresponding to autophagy related gene ATG9A were highly upregulated in response to RE22. Several transcripts that belong to the apoptosis pathway were differentially expressed in response to RE22 including transcripts identified as death domain-containing protein CRADD-like, caspases (1, 2, 6, 7-like) and IAP3 were upregulated while caspase 3 and IAP2 were downregulated (Table 4).

3.6.3 Metabolism and oxidative stress

Transcripts involved in metabolism that were differentially expressed included Cyt p450 and Cyt c subunits I and III. Heat shock proteins HSP12A and HSP12B were

highly upregulated, while a few limited antioxidant enzymes were upregulated in response to RE22 (Table 4).

4. Discussion

Both differential expression and functional enrichment analyses of oyster larvae 6h after challenge with the bacterial pathogen *V. coralliilyticus* RE22 suggest increased metabolic demand and activated pattern recognition receptors but repression of immune signaling pathways, preventing production of immune effectors against RE22. This pattern of gene expression is in line with the rapid disease progression observed, with clinical signs evident 14h after challenge, and heavy mortality by 24h. This acute pattern of infection allows for a very short window to activate immune responses. Therefore, the host likely relies on a strong constitutive response and a rapid induction of immune effectors to combat infection. Such rapid progression of disease in larvae is characteristic of *Vibrio* spp. (Tubiash et al., 1965, Dubert et al., 2016). These results are in accordance with immunosuppressive response to *V. coralliilyticus* YB1 as seen in coral *Pocillopora damicornis* (Vidal-Dupiol et al., 2011a, Vidal-Dupiol et al., 2014) as well as those seen in *C. gigas* in response to virulent *Vibrio* spp (Decker and Saulnier, 2011).

4.1 Differentially expressed immune genes in response to RE22

Highlights of the immunological response of *C. virginica* larvae to *V. coralliilyticus* RE22 at 6h of exposure include pathogen detection via activated pathogen recognition receptors. However, along with an increased expression of immune receptors, an overall

suppression of key immune signaling pathways and lack of specific immune effectors against RE22 was seen, suggesting that the pathogen is able to neutralize the immune response of the larval host.

Pattern recognition receptors (PRRs) are extremely important to innate immune system that recognize conserved pathogen-associated molecular patterns (PAMPs) and trigger signaling pathways that produce a variety of antimicrobials (Akira et al., 2006). Activation of TLR receptors indicate larvae can detect presence of bacteria especially via TLR4, which detects LPS (Chow et al., 1999) and hence Gram-negative pathogens like RE22. Activation of TLRs (Lorgeril et al., 2011, Zhang et al., 2011, Wang et al., 2016b), lectin (Chen et al., 2011, Genard et al., 2013) and C1q domain containing proteins (Lv et al., 2018) by several *Vibrio* spp. and parasitic exposures (Tanguy et al. 2004) have been demonstrated in bivalves. Lectins can activate the complement system and promote phagocytosis and killing of potential pathogens (Fujita et al., 2004). However, downregulation of complement C1q-like protein 2 and 4 in response to RE22 suggest suppression of recognition via C1q proteins by RE22.

Consistent with the observed response to *V. coralliilyticus* YB1 in coral *Pocillopora damicornis* (Vidal-Dupiol et al., 2014), key immune signaling pathways in larval oysters such as TLR, NF-kB, and IL-17 were also downregulated by RE22. Myeloid differentiation primary response protein 88 (MyD88) is currently the only known adaptor protein in bivalves (Gerdol et al., 2017) that modulates functioning of TLR pathway to promote activation of NF-kB pathway (Janssens and Beyaert, 2002). Downregulation of this fundamental signaling mediator suggests suppression of TLR pathway. However, MyD88 was upregulated at 24h and TRAF at 48h post challenge

with *V. coralliilyticus* LPI 06/210 (10^4 bacteria/mL in final concentration) in *C. gigas* larvae with 13 and 17% mortality rate in challenged larvae as compared to 5 and 7% in control at 24 and 48h respectively (Genard et al., 2013). It is possible that a later upregulation of these transcripts upon *V. coralliilyticus* RE22 exposure may also occur in eastern oysters, but our analysis was limited to the early time points. Disturbance of host immune responses leading to downregulation of immune genes was reported in 2yr old *C. gigas* post challenge with virulent *Vibrio* sp, *V. splendidus* LGP32-GFP and *V. aesturianus* 02/041 during first 6h of challenge (Decker and Saulnier, 2011).

4.2 Unexpected differentially expressed immune genes in response to RE22

4.2.1 Conflicting immune gene responses:

Along with the general agreement of suppressed immune recognition and signaling pathways based on the differentially expressed transcripts, there are some results that deviate from this observation. Interestingly, toll-interacting protein (TOLLIP), an important regulator of TLR pathway that represses the TLR pathway (Zhang and Ghosh., 2002) was downregulated. Zhang et al., (2015) also found downregulation of TOLLIP at 6h of *V. anguillarum* infection in Yesso scallop (*Patinopecten yessoensis*) but upregulated in the acute phase at 3h. It is possible that our experiment missed the acute stage of the disease and the very early responses to infection.

TRAFIP2 plays a role similar to MyD88 leading to NF-kB activation through IRAK in the TLR signaling pathway and it can mediate MAPK pathway via MAPK9 or cJun N-terminal kinase (Rosani et al., 2015). Its upregulation in response to RE22 may suggest activation of NF-kB pathway and production of pro-inflammatory cytokines (Gu et al.,

2013). This contradicts the earlier notion of suppressed NF-kB pathway. Upregulation of MKK7 as seen here can lead to activation of MAPK pathway via stimulation of JNK followed by c-Jun transcriptional activity (Lu et al.,1997). This is in contrast to abalone challenge with virulent *V. harveyi* ORM4 and response of coral *P. damicornis* to *V. coralliilyticus* YB1 where induction of the MAPK pathway was delayed (Travers et al., 2009, Vidal-Dupiol et al., 2014). Both MAPK and NF-kB activation was seen in surviving *C. gigas* post challenge with virulent *Vibrio* spp., suggesting their importance in host defense (Lorgeril et al., 2011). Since this transcriptome is obtained from a pool of larvae perhaps these conflicting signals are derived from the presence of both susceptible and resistant larvae to RE22 exposure in the pools of oysters used in our experiments.

4.2.2 Antiviral immune gene responses:

Although, differentially expressed transcripts in response to RE22 indicate majority of the key immune signaling pathways to be suppressed, antiviral pathways seem to remain active. STING is a key regulator for sensing intracellular single- or double-stranded nucleic acids. STING via the cGAS-STING pathway complex with TAK1 and trigger expression of interferon genes. cGAS is activated whenever foreign DNA (both bacterial and viral nucleic acids) is detected in the cytoplasm (He et al., 2015, Gerdol, 2017). These results suggest the possibility of an intracellular invasion by RE22 that could lead to activation of STING or effectors of type secretion systems of RE22 (T6SS or T1SS) inadvertently leading to activation of these pathways. A special STING homolog LvSTING was activated in shrimp in response to *V. parahaemolyticus* infections that participates in antimicrobial peptide production (Li et al., 2017).

Similarly, activation of JAK-STAT pathway has been reviewed in bivalves as an antiviral response (Green et al., 2015) but microbial activation of this pathway has been shown in Chinese mitten crab *Eriocheir sinensis* (Li et al., 2013).

4.2.3 Effectors

Extracellular metalloproteases in *V. coralliilyticus* RE22 are shown to be important in its pathogenicity to *C. gigas* larvae (Hasegawa et al., 2008). Therefore, the observation of lack of serine protease inhibitors in challenged larvae, as well as the lack of upregulation of other types of protease inhibitors, was unexpected. It is possible that protease inhibitors are not differentially expressed at the time point tested (6 h) but might be at a later time point. Expression of metalloprotease of *V. tubiashii* 07/118 T2 was shown to be downregulated during early infection stage in *C. gigas* larvae (3 - 6 h) but significantly upregulated (20 fold) at 16 -18 h post infection with ~60% mortality at 24h post-infection (Mersni-Achour et al., 2015).

Mucus is the first line of defense in oysters besides the closed oyster shell. Mucus was one of the few immune effectors shown to be upregulated in larval oysters exposed to *V. coralliilyticus* RE22. Some pathogenic *Vibrio* spp. require binding to mucin in the gut epithelium as a part of their pathogenesis (Bhowmick et al., 2008, Jang et al., 2016), so it is possible that modulation of host mucus production or composition may allow RE22 to bind better and breach host defenses in larval oysters.

4.3 Cytoskeletal reorganization

Downregulation of septin-11 associated with the cytoskeleton in response to RE22 suggests possible disruption of cytoskeleton by RE22, but the functional implications

of this downregulation is not clear. Both actin and septin 8B were shown to be upregulated by challenge with *V. splendidus* LGP32 in *C. gigas* (Duperthuy et al., 2011) and soft-shell clams, *Mya arenaria* (Araya et al., 2010) for hemocyte invasion. Cytoskeletal disruption using upregulation of ß-actin due to *V. tapetis* challenge in *Ruditapes philippinarum* has also been demonstrated (Brulle et al., 2012). We need to know more about nature of RE22 pathogenesis in cytoskeletal modulation to fully understand this.

4.4 Cell death

It is difficult to interpret whether apoptosis is inhibited or enhanced in response to RE22 treatment due to modulation of both pro (caspases) and anti-apoptotic (apoptosis inhibitor) genes. This was also the case in surviving *C. gigas* on exposure of different strains of virulent *Vibrio* spp. (Lorgeril et al., 2011). IAPs were modulated in both susceptible and resistant *C. virginica* families in response to *A. crassostreae* (McDowell et al., 2014). The mechanisms underlying pathogen-induced modulation of apoptosis in mollusks are not well understood.

4.5 Metabolism and oxidative stress

Differential expression of heat shock proteins and cytochrome oxidases during RE22 challenge suggests that larvae are experiencing stress and high metabolic demand due to the inability to rapidly clear RE22 infection. Higher stress levels and lower metabolic rates have been seen in late responses (24-48h) of *V. coralliilyticus* LPI 06/210 in *C. gigas* (Gernard et al., 2013). Moreover, no increase in expression of antioxidant

enzymes, necessary to deal with oxidative stress from activated metabolism, was seen in our study. These results suggest that oyster larvae, which already possess a high metabolic demand to sustain the processes of rapid growth and development, may not be able to cope with the additional metabolic demand associated with infection. Moreover, reduced feeding in infected moribund larvae may not allow replenishment of energy to mount an expensive immune response (Gernard et al., 2011). It has been shown for several *V. tubiashii* strains affecting bivalves that the pathogen enters the host through ingestion, proliferates in the gut, then spreads to other organs, including the cilia that are involved in swimming and capturing particles (Tubiash et al., 1965).

5. Conclusion:

The observed absence of induced expression of protease inhibitors, antimicrobial peptides or other immune effectors able to block RE22 virulence factors, along with other indications of a suppressed immune system, suggest that larvae are left highly susceptible to disease and then succumb to infection. Additionally, differential gene expression analysis indicative of a high metabolic demand and oxidative stress are consistent with the rapid mortality observed during RE22 infection in oyster larvae. Further in-depth studies are required to tease out details of the mechanisms used by RE22 to manipulate the immune system of oyster larvae.

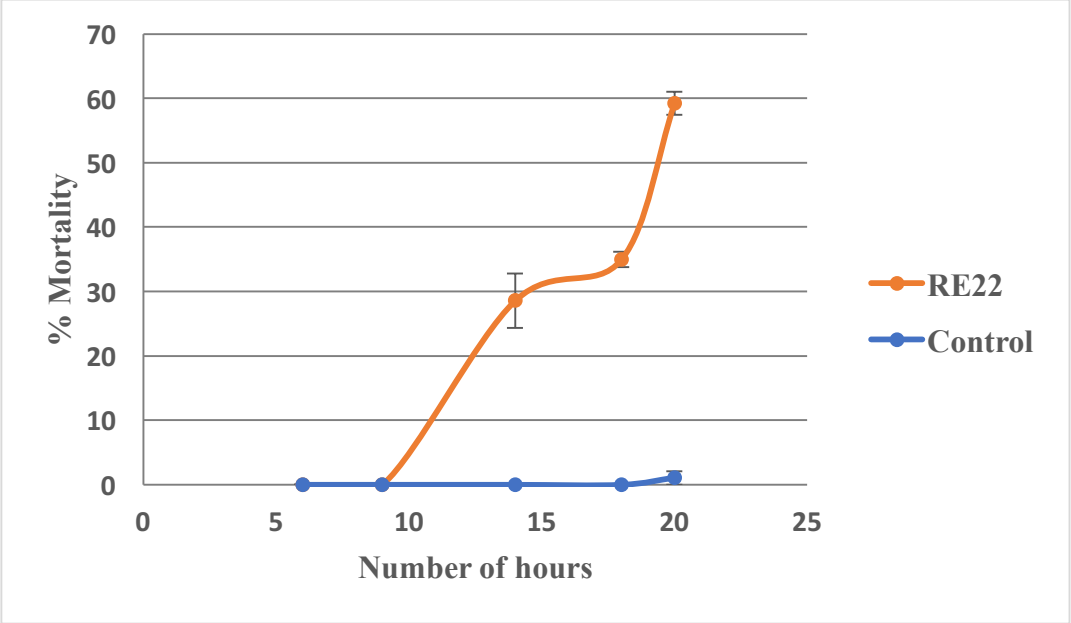

Figure 1a: Effect of challenge with *V. coralliilyticus* RE22 on mortality of *C. virginica* larvae. Cumulative percent mortality +/- standard error in oyster larvae after 6 – 20 h of challenge with $5x10^5$ CFU/mL of RE22. Data was averaged over six replicates. Mortality was first observed at 14 h after challenge, and rapidly increasing thereafter.

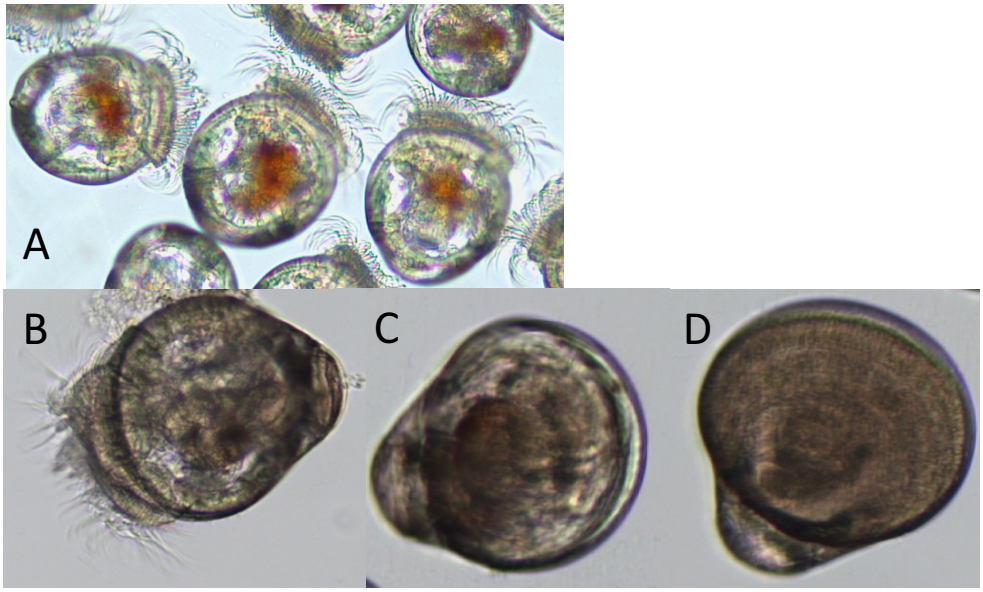

Figure 1b: Effect of *Vibrio coralliilyticus* RE22 on mortality of *C. virginica* larvae. (A) Actively swimming healthy control larvae (B) Active larva with cilia showing signs of some clumping at 6h (C) Moribund larva with retracted cilia showing reduced movement at 9h (D) Dead larva with empty shell at 14h

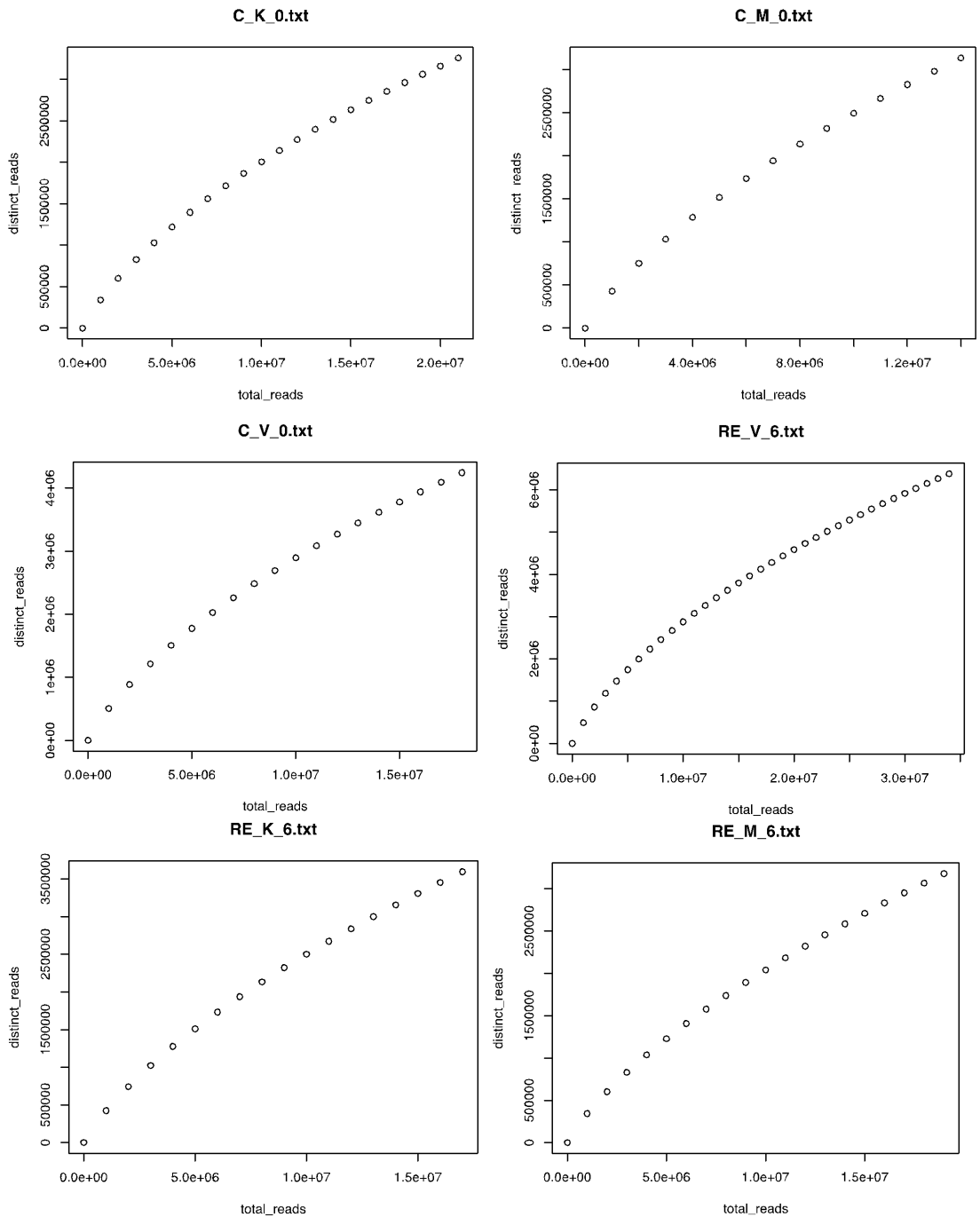

Figure 2: Sequencing saturation curves for control and challenged larval transcriptomes showing comparable depth of sequencing for all transcriptomes. Three independent experiments (K, M, V) with two treatments (Control, C; RE22 treatment, RE22) were performed.

Table 1. Oyster larval transcriptomes in response to a 6h challenge with *Vibrio coralliilyticus* RE22 challenge. Number of paired end reads per sample and % alignment rate to *Crassostrea virginica* reference genome using HISAT2. Three independent experiments (K, M, V) with two treatments (Control, C; RE22 treatment, RE22) were performed in duplicate.

Sample	# paired reads	% Alignment to *Crassostrea virginica* genome
C_K_0	22,963,376	89
C_M_0	16,617,375	87
C_V_0	20,674,506	86
RE_K_6	19,379,823	86
RE_M_6	21,118,821	89
RE_V_6	39,681,499	85

Table 2: Gene Ontology (GO) terms of biological functions significantly ($p<0.05$) enriched in oyster larvae in response to pathogen challenge (RE22).

GO Term	Significant number of transcripts mapped	classicFisher p value
macromolecule modification	83	0.0074
cellular protein modification process	81	0.0098
protein modification process	81	0.0098
cellular protein metabolic process	104	0.0125
biological regulation	201	0.0144
cellular macromolecule catabolic process	17	0.0202
regulation of biological process	189	0.0204
protein metabolic process	127	0.0221
regulation of cellular process	169	0.0271
regulation of cell communication	29	0.0319
regulation of signaling	29	0.0319
phosphorus metabolic process	86	0.0339
phosphate-containing compound metabolic process	86	0.0339

protein catabolic process	14	0.0406
cellular protein catabolic process	14	0.0406
proteolysis involved in cellular protein...	14	0.0406
cell communication	112	0.0409
protein phosphorylation	39	0.0416
signaling	111	0.0451
single organism signaling	111	0.0451
regulation of signal transduction	27	0.0453
positive regulation of cellular process	27	0.0453

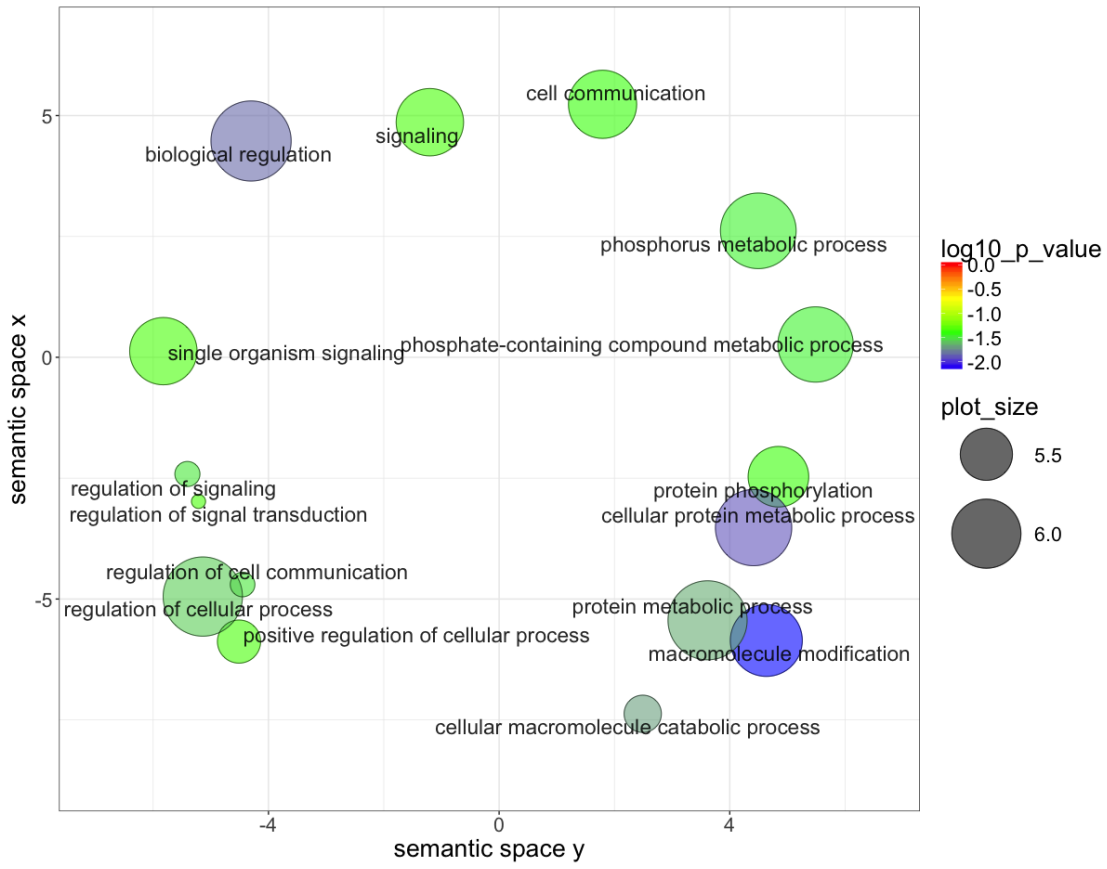

Figure 3: Functional enrichment of differentially expressed transcripts using Gene Ontology terms in Biological Process. The color scale in the legend shows level of significance, with colder colors indicating higher significance and the size of the bubble corresponds to the number of significant transcripts mapped to the term.

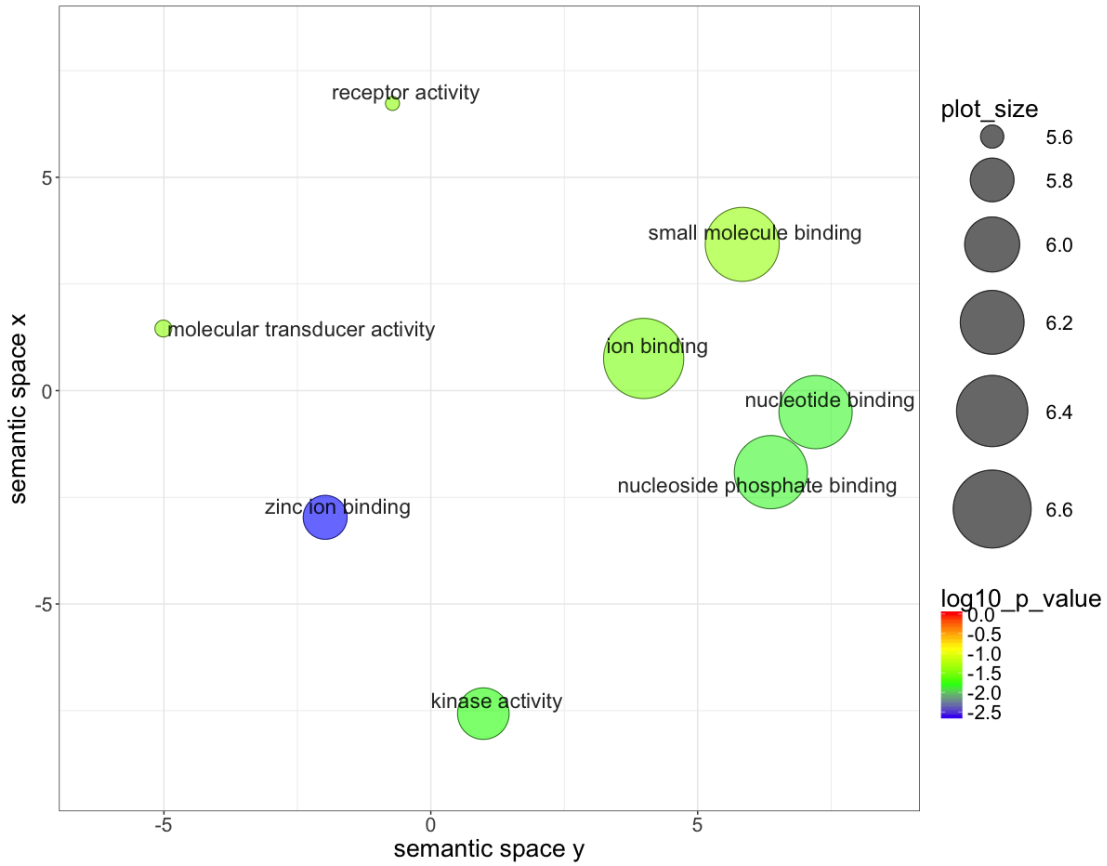

Figure 4: Functional enrichment of differentially expressed transcripts using Gene Ontology terms in Metabolic Function. The color scale in the legend shows level of significance, with colder colors indicating higher significance and the size of the bubble corresponds to the number of significant transcripts mapped to the term.

Table 3: Mapping of differentially expressed transcripts to biological pathways in the Kyoto Encyclopedia of Genes and Genomes (KEGG) database. Numbers indicate number of transcripts mapped to each category.

	RE22(6h)
Metabolism	
Carbohydrate metabolism	29
Energy metabolism	5
Lipid metabolism	36
Nucleotide metabolism	21
Amino acid metabolism	25
Metabolism of other amino acids	8
Glycan biosynthesis and metabolism	12
Metabolism of cofactors and vitamins	7
Metabolism of terpenoids and polyketids	4
Biosynthesis of other secondary metabolites	4
Xenobiotics and biodegradation metabolism	7
Genetic information processing	
Transcription	10
Translation	16
Folding sorting and degradation	13
Replication and repair	3
Environmental processing	
Membrane transport	2
Signal transduction	199
Signaling molecules and interaction	10
Cellular processes	
Transport and catabolism	41
Cell growth and death	39
Cellular community-eukaryotes	42
Cellular community-prokaryotes	2
Cell motility	6
Organismal systems	
Immune system	93
Endocrine system	112
Circulatory system	20

Digestive system	37
Excretory system	11
Nervous system	44
Sensory system	23
Development	19
Aging	8
Environmental adaptation	17

Table 4: Comparison of expression of selective differentially expressed genes (DEGs) as compared to control categorized by immune processes ($p \leq 0.05$, upregulation: log fold change ≥ 2, downregulation: log fold change ≤ -2). Colors denote level of expression as compared to control. Red: all transcripts upregulated, orange: some transcripts upregulated while some downregulated and yellow: all transcripts downregulated. DEGs with * denote highly differentially expressed gene.

DEGs	**Expression as compared to control**
Recognition	
TLRs	
TLR4	
TLR4 isoform X1	
TLR13	
TLR Tollo isoform X2	
TOLLIP (toll-interacting protein-like isoform X3)	
protein toll-like	
myeloid differentiation primary response protein MyD88-like	
Lectins	
lectin-like *	
fucolectin-like	
Scavenger receptors	
scavenger receptor class B member 1 isoform B	
LRR	
leucine-rich repeat transmembrane neuronal protein 3-like isoform X1	
leucine-rich repeat-containing protein 74B-like isoform X2	
leucine-rich repeat-containing protein 9-like isoform X2	
Fibronectin type III domain	

fibronectin type III domain-containing protein 2-like isoform X3	
Complement	
complement C1q-like protein 2	
complement C1q-like protein 4	
Metabolic Enzymes with New Role of Carbohydrate Binding	
hexokinase-2-like isoform X2	
B cell receptor	
dapp1 dual adaptor for phosphotirosine*	
Signaling pathways in Immune response	
TLR pathway	
myeloid differentiation primary response protein MyD88-like	
TNF receptor-associated factor 4-like isoform X5 (TRAF4)	
mitogen-activated protein kinase kinase kinase 7-like isoform X3 (TAK1)	
JAK-STAT	
tyrosine-protein kinase JAK2-like (JAK)	
son of sevenless homolog 2-like * (SOS2)	
tyrosine-protein phosphatase non-receptor type 4-like isoform X4 (PTPN4)	
tyrosine-protein phosphatase non-receptor type 23-like (PTPN23)	
NF-kB signaling pathway	
NF-kappa-B-activating protein-like (NKAP)	
NF-kappa-B inhibitor alpha-like isoform X1 (IkB)	
TNFAIP3-interacting protein 1-like * (TNIP1)	
adapter protein CIKS-like isoform X4 (TRAF3IP2/Act1/CIKS)	
Mitogen-Activated Protein Kinases (MAPK) pathway	
dual specificity mitogen-activated protein kinase kinase 7-like isoform X1 (MKK7)	
mitogen-activated protein kinase kinase kinase 7-like isoform X3 (TAK1)	
cGAS-STING pathway	
stimulator of interferon genes protein-like (STING)	
Signal transduction	
death domain-containing protein 1-like	
death domain-containing protein CRADD-like *	
ubiquitin carboxyl-terminal hydrolase 14-like	
ubiquitin carboxyl-terminal hydrolase 25-like isoform X3 * (USP25)	
1-phosphatidylinositol 4,5-bisphosphate phosphodiesterase gamma-1-like isoform X4 (PLCG1)	

Effectors	
Signaling mucin HKR1	
mucin-12-like *	
mucin-2-like	
mucin-5B-like	
mucin-19-like, partial	
septin-11-like isoform X2	
Apoptosis	
Caspase 1	
Caspase 2	
Caspase 3	
Caspase 6	
Caspase 7	
Caspase 7 Isoform X1	
Caspase 7 Isoform X3	
baculoviral IAP repeat-containing protein 2-like	
baculoviral IAP repeat-containing protein 3-like isoform X1 *	
putative inhibitor of apoptosis*	
death domain-containing protein CRADD-like *	
XK-related protein 8-like isoform X2 *	
XK-related protein 6, partial *	
cAMP-dependent protein kinase catalytic subunit	
TPA_inf: DeltaNp63gamma	
epidermal growth factor receptor-like isoform X2	
Autophagy	
autophagy-related protein 9A-like isoform X1 *	
DNA damage-regulated autophagy modulator protein 1-like*	
phosphatidylinositol 4,5-bisphosphate 3-kinase catalytic subunit delta isoform-like isoform X1*	
toll-interacting protein-like isoform X3 (TOLLIP)	
next to BRCA1 gene 1 protein-like isoform X1	
Phagosome	
cation-dependent mannose-6-phosphate receptor-like *	
cytoplasmic dynein 2 light intermediate chain 1-like *	
Lysosome	
cation-dependent mannose-6-phosphate receptor-like *	
AP-1 complex subunit gamma-1-like isoform X2	
Endocytosis	
phosphatidylinositol-binding clathrin assembly protein LAP-like *	

Peroxisome	
D-aspartate oxidase-like isoform X1	
peroxisome proliferator-activated receptor delta-like isoform X1*	
peroxisome proliferator-activated receptor gamma coactivator 1-alpha-like	
prostaglandin E2 receptor EP4 subtype-like [Crassostrea virginica]	
Antioxidant enzymes	
maleylacetoacetate isomerase-like*	
gamma-glutamyltranspeptidase 1-like	
thioredoxin domain-containing protein 15-like	
thioredoxin domain-containing protein 3 homolog isoform X15	
thioredoxin-related transmembrane protein 2 homolog	
Acute phase proteins	
Heat shock 70 kDa protein 12A*	
heat shock 70 kDa protein 12A-like	
heat shock 70 kDa protein 12A-like isoform X1	
heat shock 70 kDa protein 12B-like	
heat shock 70 kDa protein 12B-like isoform X4 *	
Cholinergic immunomodulation	
Glutamate receptor *	
glutamate receptor ionotropic	
muscarinic acetylcholine receptor M3-like	
neuronal acetylcholine receptor subunit alpha-2-like	
neuronal acetylcholine receptor subunit alpha-5-like	
neuropeptide Y receptor type 2-like*	
RYamide receptor-like	
acetylcholinesterase-like isoform X1*	
Cytosletal reorganization	
septin-11-like isoform X2	
dynamin-1-like isoform X6	
PI3K-Akt signaling pathway	
PH domain leucine-rich repeat-containing protein phosphatase 2-like isoform X2 *	
phosphatidylinositol 4,5-bisphosphate 3-kinase catalytic subunit delta isoform-like isoform X1*	
phosphatidylinositol 3,4,5-trisphosphate 5-phosphatase 2A-like isoform X4	
RAC-gamma serine/threonine-protein kinase-like isoform X1	
Others	
multidrug resistance protein 1-like isoform X6	

glycine receptor subunit alpha-3-like isoform X5	
gamma-glutamyltranspeptidase 1-like	
1-phosphatidylinositol 4,5-bisphosphate phosphodiesterase gamma-1-like isoform X4	
Hemicentin-1	
Hemicentin-1 like	
Hemicentin-1 like isoform X2	
Hemicentin-1 like isoform X21*	
Hemicentin-1 like isoform X34*	
hemicentin-2-like isoform X2	
histamine H2 receptor-like	
oxidative stress-induced growth inhibitor 2-like	
cytochrome b5 reductase 4-like isoform X3 [Crassostrea virginica]	
cytochrome c oxidase subunit I *	
cytochrome c oxidase subunit III (mitochondrion) *	
cytochrome P450 27C1-like	
cytochrome P450 2C28-like isoform X2 *	
cytochrome P450 2F5-like *	
dual specificity protein phosphatase 18-like [Crassostrea virginica]	
dual specificity protein phosphatase 7-like [Crassostrea virginica]	
protein phosphatase 1 regulatory subunit 16A-like isoform X3	
protein phosphatase 1 regulatory subunit 37-like	
Tripartite motif-containing protein 2	
tripartite motif-containing protein 2-like	
tripartite motif-containing protein 2-like isoform X4	
tripartite motif-containing protein 3-like	
tripartite motif-containing protein 45-like	
cAMP-dependent protein kinase catalytic subunit	
PREDICTED: stress protein DDR48-like [Salmo salar]	
Biomineralization	
perlucin-like isoform X1 *	
perlucin-like protein isoform X1*	
Chitin synthase 3*	

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CHAPTER 4

IMMUNOLOGICAL RESPONSE OF *CRASSOSTREA VIRGINICA* LARVAE TO PROBIOTICS *BACILLUS PUMILUS* RI06-95 AND *PHAEOBACTER INHIBENS* S4.

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Abstract

The eastern oyster *Crassostrea virginica* is an ecologically and economically important species. Bacterial pathogens like vibrios cause heavy mortalities in oyster larvae in hatcheries. Probiotics are an inexpensive, practical, and natural method of disease control. Pretreatment of larval oysters with probiotics *Bacillus pumilus* RI06-95 and *Phaeobacter inhibens* S4 significantly decreases mortality caused by experimental challenge with the pathogen *Vibrio coralliilyticus* RE22. The aim of this study was to understand the oyster larval immune response to probiotics RI06-95 and S4 and the role it may play in protecting larvae from pathogen challenge. *C. virginica* larvae were exposed to each probiotic in two settings: controlled 6 and 24 hours laboratory exposures and 5 to 16 days exposure in a hatchery. Transcriptomes were sequenced using high throughput RNA sequencing and aligned to the *C. virginica* reference genome. Differential expression analysis compared probiotic treated transcriptomes to unexposed controls. Key features of the host immune response were shared despite the length of probiotic exposure, type of probiotic exposure and the type of environment in which exposures were conducted. Transcriptome analysis showed increased expression of genes for receptors involved in environmental sensing and detection of pathogens, immune signaling pathways, and immune effectors including serine protease inhibitor, mucins and perforin-2. In addition, patterns of differential gene expression suggest that inhibition of apoptosis, enhanced autophagy, and cytoskeletal reorganization may play a supplemental role in bacterial clearance. Thus, results from this study suggest that larval oysters show a robust and effective immune response to probiotic exposure, contributing to clearance of the probiotic within 24 hours after exposure. Activation of

antibacterial immune effectors by probiotics, when provided 6 – 24 hours prior to bacterial challenge, may play an important role in protecting larvae from mortality by *V. coralliilyticus* RE22. However, for continued effective protection, probiotics should be applied repeatedly and for at least 6 hours prior to RE22 challenge. This is the first time that immune responses of larval stages of *C. virginica* to bacteria are studied using a larval transcriptome. This research provides important new insights into host-microbe interactions in larval oysters that could be applied in the design of improved strategies for use of probiotic organisms for disease control in hatcheries.

1. Introduction

The eastern oyster *Crassostrea virginica* is an economically and ecologically important species (Newell 2004, NMFS 2014). Rearing of oyster larvae is a critical step to ensure a healthy and sufficient supply of seed for aquaculture industry. Bacterial diseases commonly described in larval stages are associated with high mortalities in hatcheries (Lauckner et al. 1983, Sinderman et al. 1990). Vibriosis is one such disease that leads to mortality in oyster larvae and juveniles (Tubiash et al, 1965). Bacteria of the genus *Vibrio* are ubiquitous within marine environments and detected in tissues of many marine organisms including abalones, bivalves, corals, fish, shrimp, sponges, squid, and zooplankton (Thompson et al. 2004). *Vibrio* can cause larval mass mortalities in hatcheries in a short period of time leaving few options for treatment (Helm and Lovatelli 2006). In order to eliminate *Vibrios* and sanitize the facility, hatcheries need to shut down for several days after a disease outbreak before production is resumed (Helm and Lovatelli 2006). In particular, *V. coralliilyticus* RE22 **(**previously *V. tubiashii* RE22) has caused high larval and juvenile mortality in hatcheries (Elston et al. 2008). Vibrios are known to produce potent exotoxins that affects larval motility in oysters. Incapacitated ciliary movement affects feeding, leading to death due to starvation (DiSalvo et al., 1978, Brown and Roland, 1984, Kennedy, 1996). The extracellular metalloprotease secreted by *V. coralliilyticus* is toxic and induces mortality in oyster larvae (Hasegawa et al., 2008).

Practices to reduce mortality due to bacterial disease include treatment with antibiotics and disinfection of seawater. Water treatment, however, is expensive and could be toxic to the larvae if not properly done, while antibiotic treatment can lead to bacterial

resistance. Treatment with antibiotics raises environmental and human health concerns as well (Prado et al. 2009, Akinbowale et al., 2016, Ho et al., 2000). Therefore, alternative methods need to be developed to manage good larval rearing environment and to control bacterial diseases in bivalve shellfish hatcheries.

Probiotics are defined as a live microbial food supplement that, when administered in a sufficient amount, confers a health benefit on the host (Food and Agricultural Organization of the United States 2006). Probiotics are known to benefit the host by a variety of means, including production of antimicrobials, improving water quality, enhancing the immune responses of host, and competing for space with pathogenic bacteria (Verschuere et al. 2000). There is growing evidence that probiotics show immunomodulatory effects in fish and shellfish (De et al., 2014, Newaj-Fyzul et al., 2015).

The benefits of probiotics have already been shown in Pacific oysters, *Crassostrea gigas* (Douillet and Langdon 1994) and the eastern oyster *C. virginica* (Karim et al. 2013). Pretreatment of larval and juvenile *C. virginica* with probiotic organisms *Phaeobacter inhibens* S4 (isolated from the inner shell of oysters) (referred to as S4) and *Bacillus pumilus* RI06-95 (isolated from a marine sponge from the Narrow River in Rhode Island) (referred to as RI) before exposure to the bacterial pathogens *Alliroseovarius crassostreae* and *Vibrio coralliilyticus* RE22 (referred to as RE22) improves oyster survival rate (Karim et al., 2013). Additionally, probiotics are not harmful to oysters in absence of pathogens (Karim et al., 2013).

S4 is a Gram-negative organism and production of the antibiotic tropodithietic acid (TDA) and biofilm formation are two mechanisms utilized by S4 for protecting oysters

from infection. Mutants of S4 unable to produce TDA and with decreased ability to produce biofilms, however, still provide some level of protection (Zhao et al. 2016), suggesting that other mechanisms are also potentially involved. RI is a Gram-positive organism and produces the antibiotic amicoumacin, but this antibiotic does not inhibit the growth of RE22 in an *in vitro* assay, indicating that other mechanisms of action are also likely involved in RI's protection of larvae against bacterial challenge (Karim et al., 2013). Probiotics are known to act as immunomodulators (Hardy et al., 2013, Mortha et al., 2014, Sanchez et al., 2015). For example, a strain of *B. pumilus* has been shown to improve immune responses of Orange-spotted grouper *Epinephelus coioides* (Sun et al., 2010), so immunomodulation may be one of the mechanisms involved in the probiotic activity of RI.

Transcriptomic analysis of *C. virginica* larval immune responses to pathogen *V. coralliilyticus* RE22 showed evidence of suppression of important immune signaling pathways and decreased expression of genes for immune effectors such as protease inhibitors, increased metabolic demand, and modulation of mucins in the early stages of infection (Modak et al, in prep; Chapter 3 of this dissertation). Immunosuppression as a pathogenesis strategy was also demonstrated in responses of coral *Pocillopora damicornis* to *V. coralliilyticus* YB1 (Vidal-Dupiol., et al., 2014). Similarly, immune response of soft-shell clams, *Mya arenaria*, to *V. splendidus* strain LGP32 showed an overall downregulation of immune genes such as ficolin, killer cell lectin-like receptor, natural resistance-associated macrophage protein 1 (Nramp-1), and mitogen-activated protein kinases (MAPK) (Araya et al., 2010). Our hypothesis is that pre-treatment of

oyster larvae with probiotics may cause an activated immune state in larvae that would serve to counteract the immunosuppressive effects of RE22.

Not much is known about the impact of friendly or beneficial bacteria on the immune system of oysters. The goal of this study is to determine the immunological response of *C. virginica* larvae to exposure to two probiotic bacterial species that differ in Gram character, in order to understand the potential role of immunomodulation as a potential mechanism of action of the probiotics in providing protection against *V. coralliilyticus* RE22.

2. Materials and methods

2.1 Probiotic Bacterial strains:

Probiotic isolates S4 and RI were maintained and stored in 50 % glycerol stocks at -80°C until use. Bacteria were cultured by plating out freezer stocks on yeast peptone with 3% NaCl (YP30) agar plates for 1 d then transferred to 5 mL of YP30 broth (5 g L^{-1} of peptone, 1 g L^{-1} of yeast extract, 30 g L^{-1} of ocean salt, Instant Ocean) incubated at 28°C on a shaker (134 rpm) for 2 d. Cultures were washed using Artificial Filtered Sterile Seawater (AFSW, 28 - 30 psu salinity) twice by centrifugation at 23,000 g for 10 min. The OD at 550 nm was measured and the stock was diluted to obtain a concentration of 5×10^4 colony forming units (CFU) mL^{-1} as previously described (Karim et al., 2013).

2.2 Oyster larvae:

C. virginica larvae were obtained from three shellfish hatcheries on the east coast of United States including Oyster Seed Holdings, VA, Virginia Institute of Marine Science, VA and Aeros Cultured Oyster Company, NY that served as three biological replicates. Larvae 6-10 days old were collected at the hatchery and shipped to the laboratory at the University of Rhode Island on a wet filter overnight. Upon arrival to the laboratory, larvae were washed with AFSW on top of a 40 μm pore size nylon filter to prepare a stock. The stock of larvae from each hatchery was used for probiotic exposures as described below. The same stock was also used for characterizing immune

response to pathogen *V. coralliilyticus* RE22 (Modak et al., in prep, Chapter 3 of this dissertation).

2.3 Effect of length of probiotic pretreatment on protection against bacterial challenge

Previous research on the effect of probiotics on protection against challenge with the bacterial pathogen *V. coralliilyticus* RE22 was performed using a 24 h pre-incubation period with the probiotics prior to bacterial challenge (Karim et al. 2013). In order to determine if a shorter pre-incubation period with probiotics would confer protection against bacterial challenge, ~100 larvae were placed in each well of a 6 well plate in 5 mL of AFSW and incubated with 10^4 CFU mL^{-1} of probiotics S4 or RI06-95 for 6 or 24h prior to bacterial challenge with 10^5 CFU mL^{-1} of RE22. Larval survival was determined 24 h after challenge using previously described methods (Karim et al. 2013). Survival rate was calculated as follows: *Survival rate (%) = 100 x (number of live larvae/total number of larvae)*. One-way analysis of variance (ANOVA) was used to determine significance between treatments and Tukey's multiple comparison tests were used for post-hoc pairwise comparisons ($p < 0.05$) (Sohn et al., 2016).

2.4. Effect of short-term exposure to probiotics on larval gene expression

2.4.1 Experimental set up for laboratory-scale experiments: For biological replicates, three independent experiments were performed using larvae from three different hatcheries. Larval density (larvae mL^{-1}) of the stock was determined using the Nikon E200 microscope. Two parallel exposures were performed with each set of larvae: *(i)*

a large-scale incubation for collection of larvae for transcriptome analysis, and *(ii)* a small-scale experiment in 6 well plates for evaluation of the effect of probiotic exposure on protection against bacterial challenge.

(i) Set up for transcriptome analysis: Larvae were distributed into tissue culture flasks (~10,000 per flask) in 500 mL AFSW based on the larval density (larvae/mL) and kept on a shaker with gentle shaking at ~50 rpm at room temperature. Larvae were acclimatized to the laboratory environment for 24 h. Each treatment was set up in duplicate as separate flasks to serve as technical replicates. There were five treatment groups in total viz. Control(0h), RI09-95(6 h), RI09-95(24 h), S4(6 h) and S4(24 h). Probiotics were applied at a concentration of 10^4 CFU mL^{-1}. Larvae were fed with instant algae Shellfish Diet 1800™ (20,000 cells/mL; Reed Mariculture Inc., San Jose, CA. USA) just prior to treatment in order to promote probiotic ingestion.

(ii) Set up for verification of protection by probiotics: Oyster larvae (~100) were placed in each well of a 6 well plate in 5 mL of AFSW and incubated with 10^4 CFU mL^{-1} of probiotics S4 or RI for 6 or 24 h prior to bacterial challenge with 10^5 CFU mL^{-1} of RE22, as described in section 2.3 above.

2.4.2 Larval Collection post treatments:

After incubation with probiotics, larvae from the flask set up for the transcriptome experiment were aspirated gently using a 100 mL serological pipette and filtered through a 40 μm sterile filter for collection. Since the dead larvae settle to the bottom, the last 25 mL of each flask was not collected to avoid bias in transcriptomic response. Larvae were washed with 2 mL of AFSW followed by 2 mL of RNAlater™ . Larvae

retained on the filter were aspirated with a pipette using 1.5 mL of RNAlater, placed in labeled 2 mL RNase free microfuge tubes, and held at 4°C for 24 h in RNAlater followed by storage at -20°C.

2.4.3 RNA extraction, cDNA prep and sequencing:

Tri-reagent™ (Sigma-Aldrich) was used for extracting total RNA from all the samples following manufacturer's instructions (TRI Reagent™ Protocol, Sigma-Aldrich). RNA extracts were DNase treated using the DNA-free™ DNA removal kit from Ambion and purity and concentration of RNA was checked using a Nanodrop 8000 spectrophotometer (Thermo Scientific). Technical replicates were pooled at equimolar concentration. The quality and quantity of the pools were assessed using Agilent 2100 Bioanalyzer and High Sensitivity D1000 ScreenTape®. RNA samples were selectively enriched for poly-A containing mRNA and cDNA libraries were prepared using the PrepX RNAseq library Prep Kit (Takara Bio USA, inc). Samples were sequenced on Illumina HiSeq platform with 2×125 reads and sequencing coverage of 20-30M per sample at the Harvard University, FAS Center for Systems Biology, MA.

2.5 Effect of exposure to probiotics in the hatchery on larval gene expression

2.5.1 Experimental set up of hatchery experiments:

Transcriptomes obtained from treatment of larvae with *B. pumilus* RI06-95 will be referred to as HT_RI. Adult eastern oysters were spawned at the Blount Shellfish Hatchery, Roger Williams University, RI. Each trial was initiated by adding 8-10 larvae

mL^{-1} (800,000 to 1,000,000 initial larvae) per tank 1 day post-fertilization. Larval oysters were distributed into 100 L conical tanks filled with filtered and UV treated seawater (20 – 24 C and 28 – 30 psu salinity) 1 day after fertilization and fed live microalgae daily from a microalgae production greenhouse. Water from Narragansett Bay, RI was filtered and UV treated and used for the larval tanks. Treatments included control and probiotic RI treated at a concentration of 10^4 CFU mL^{-1}. Each treatment was conducted in triplicate. Probiotics were added daily at the time of feeding.

2.5.2 Larval Collection post treatments:

Larvae for transcriptomes were collected at three time points: 5, 12 and 16 days post fertilization from probiotic-treated and control tanks. Larvae had been treated with probiotics daily starting 1 day after fertilization, as described in Sohn et al. (2016). Tanks were drained on a filter with suitable pore size (75 – 150 μm depending on the age of the larvae) at the time of collection. Using a serological pipette, larvae were aspirated gently and collected in RNase free microfuge tubes with RNAlater™ and stored at -80°C.

2.5.3 Verification of protection by probiotics: A subsample of larvae was collected from each treatment and control tanks on day 8 post-fertilization to determine the effect of exposure to the probiotics in the hatchery on protection against bacterial challenge. Levels of protection were determined using the methods described in 2.3. above, with the following modifications: larvae from each tank were placed in triplicate wells in 6-well plates with ~100 larvae per well *V. coralliilyticus* RE22 at 10^5 CFU mL^{-1} dose.

2.5.4 RNA extraction, cDNA prep and sequencing:

Larvae were processed for RNA extraction as described in 2.4.4 above. cDNA libraries were generated using random hexamer priming that were sequenced on Illumina HiSeq platform with 2×150 reads and sequencing coverage of 50-70M per sample at the McDonnell Genomics Institute, Washington University School of Medicine, MO.

2.6 Assembly, annotation and analysis

Raw reads obtained from sequencing were filtered, trimmed and adapters were removed using bbduk program in BBTools suite from Joint Genome Institute and viewed in FASTQC (Andrews, 2010). Processed reads were aligned to *C. virginica* reference genome (version 3.0) via HISAT2 2.1.0 (Kim et al., 2015) and assembly was performed using Stringtie (Pertea et al., 2016) with default parameters. To compare the depth of sequencing across all samples preseq package was used (Daley and Smith., 2013). Differential gene expression analysis between probiotic (RI or S4) treatment at each time point (6 or 24 h) and control (time 0, common to all treatments) was performed using DESeq2 (Love et al., 2014) and transcripts with Benjamini-Hochberg adjusted pvalue ≤ 0.05 and log fold change ≥ 2 or ≤ -2 were considered significantly differentially expressed. For hatchery transcriptomes, each of the days (5, 12 and 16) were considered as biological replicates and an overall comparison of treatment vs control was conducted. Transcript counts for each replicate were used to determine which DEGs are present in each replicate individually. This analysis design only allowed for the most conservative estimates and only showed differentially expressed genes

representing all the biological replicates. Annotation for differentially expressed genes (DEGs) was performed by mapping to NCBI protein non-redundant (NR) database using BLASTx (Altschul et al., 1997) with an e-value cutoff of $1e^{-3}$ and hit number threshold of 20. Mapping DEGs to GO terms was conducted using BLAST2GO v4.1.9 (Conesa et al., 2005) and functional enrichment was done using topGO (Alexa et al., 2006) with default parameters. ReviGO (Supek et al., 2011) was used to plot and visualize results obtained from topGO with default parameters (allowed similarity adjusted to medium). Significantly enriched GO terms were obtained by using Fishers exact test ($p \leq 0.01$). Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway annotations were also obtained using the KEGG Automatic Annotation Server (KAAS).

3. Results

3.1 Effect of length of probiotic pretreatment on protection against bacterial challenge

A short duration of S4 or RI pretreatment (6 h) showed variable levels of protection against bacterial challenge between technical replicates within experiments and between experiments, as reflected in the large standard deviations in the relative percent survival (RPS; Table 1). One out of three experiments showed no protection from probiotic treatment. The 24h probiotic pretreatment showed a more consistent level of protection against RE22 challenge (Table 1). In the hatchery trial, larvae treated daily with probiotics for 8 days in the hatchery showed an increase of 28 ± 6 % in relative percent survival as compared to untreated larvae after a laboratory challenge with *V. coralliilyticus* RE22.

3.2 Transcriptome completeness

Depth of sequencing for all the lab transcriptomes was comparable between samples ranging from 16– 25M paired end reads whereas HT_RI transcriptomes ranged from 50 - 70M reads (Table 2). Sequencing saturation curves for all transcriptomes were close to full saturation, indicating that all but the rarest (least abundant) transcripts would be represented (Figures 1a and b). The alignment rate to the *Crassostrea virginica* reference genome using HISAT2 ranged from 86 – 89% (Table 2).

3.3 Differential Expression Analysis

Probiotic treated larval transcriptomes (RI or S4) at each time point (6 or 24 h) were compared to control (0 h) transcriptome for normalization. S4 treated transcriptomes (both 6 h and 24 h) yielded more differentially expressed transcripts when compared to control (0 h) larvae than RI treated larval transcriptomes ($p \leq 0.05$) (Table 3). Larvae treated with probiotics for 24 h yielded more differentially expressed transcripts than larvae treated with probiotics for 6h (Table 3).

Comparison of the number of shared and unique differentially expressed genes across all treatments (Figure 2a) showed a dynamic response to each of the two probiotics. Overall, larvae treated with S4 for 6 or 24 h have a higher number of differentially expressed transcripts than larvae treated with RI at 6h or 24h. The percentage of DEGs shared between S4 and RI is the same (26%) at 6h or 24h suggesting pronounced effect of treatment as compared to time. Out of the total number of differentially expressed transcripts in response to S4 and RI at 6 and 24h, 50% transcripts were unique to S4 treatment and 21% were unique to RI treatment. Comparison of differentially expressed transcripts in hatchery transcriptomes (HT_RI) (Figure 2b) showed 43% transcripts

shared between RI treatments with only 8%, 3% and 8% unique transcripts in RI_5d, RI_12d and RI_16d respectively suggesting more of a treatment effect than time. Refer to supplementary data tables in appendix for descriptions and log fold change values for differentially expressed genes for all comparisons.

3.4 GO annotation

A Gene Ontology (GO) term enrichment analysis was performed on all the differentially expressed transcripts in response to probiotic treatment. S4 treatments at both time points shared terms related to recognition and signaling (Figure 3a, 3b). S4 treatment at 6h showed enrichment in "cellular response to stimulus" whereas at 24h it showed enrichment in processes related to activation of receptors and signaling pathways suggesting a progression of immune response to S4. Very few GO terms were significantly enriched among DEGs detected from comparison between the control and larvae exposed to RI in the laboratory and they were mostly related to larval development (not shown). The HT_RI transcriptomes shared enrichment of the term "cytoskeletal organization" (Figures 3c) with the S4 (24h) transcriptomes, but none with the RI laboratory transcriptomes.

3.5 KEGG annotation

Consistent with the results of the enrichment analysis, most of the KEGG pathways that were represented by differentially expressed *C. virginica* larval genes related to signal transduction, immune systems, and endocrine system (Table 4).

3.6 Differentially expressed immune genes shared between probiotics

An overview of the immune genes differentially expressed upon exposure to the probiotics is depicted in Figure 4. Transcripts corresponding to the genes for several

types of PRRs were modulated by probiotic treatment, out of which Toll-like receptors (TLRs), lectins, recognition protein, peptidoglycan receptor protein (PGRP) and leucine-rich repeat receptors (LRRs) were upregulated, with TLRs and lectins being most upregulated, while scavenger receptors, leucine rich repeat and fibronectin type III domain-containing proteins (LRFN), fibronectin domain containing proteins and C1-q proteins were downregulated. TLR 4, 6 and 13 were consistently upregulated in response to both probiotics with the exception of HT_RI transcriptome where TLR 13 is downregulated (Table 5).

Consistent with the observation that probiotic treatment led to differential expression of several TLR receptors, several transcripts involved in the TLR signaling pathway, including TNF receptor-associated factor 3-like (TRAF3) and mitogen-activated protein kinase kinase kinase 7-like (TAK1), were differentially expressed upon probiotic treatment (Table 6).

Moreover, DEG patterns suggested activation of the NF-kB and MAPK pathways by probiotic exposure. Activation of the NF-kB pathway was indicated by upregulation of activator B-cell lymphoma/leukemia 10-like (BCL10) and downregulation of inhibitor NF-kappa-B inhibitor alpha-like isoform X1 (IkB). Some of the key players of the MAPK pathway including dual specificity mitogen-activated protein kinase kinase 7-like (MAP2K7), TAK1, extracellular signal-regulated kinase 2-like (ERK2) were also upregulated in probiotic-treated larvae. Transcripts corresponding to a key molecule of the MAPK pathway, MAP2K7, were uniformly upregulated in almost all probiotic treatments (Table 6).

Probiotic treatment unanimously leads to modulation of three types of major effectors: serine protease inhibitor (SPI), mucin and macrophage-expressed gene 1 protein-like (Mpeg1/Perforin-2) (Table 7). Serine protease inhibitor Cvspi2 was highly upregulated in all probiotic treatments including HT_RI samples. Digestive cysteine proteinase 2 was highly upregulated in all treatments except HT_RI. Several different types of mucin genes were modulated in larvae due to probiotic treatment. Both secreted gel-forming mucins (MUC2, MUC5A, MUC5B and MUC19) and cell surface mucins (MUC3B, MUC4 and MUC12) were differentially expressed. MUC12 was highly upregulated in almost all probiotic treatments. MUC5AC was highly upregulated in probiotic treatments of 24h and MUC2 was upregulated at 6h. Perforin2 was highly upregulated in all probiotic treated larvae except in HT_RI samples.

Various molecules associated with cytoskeleton reorganization including actin, tubulin, integrin, myosin and septins (Table 8) as well as those related to phagosome, endocytosis, peroxisome and lysosome (Table 10) were differentially expressed in response to probiotics. Prostaglandin G/H synthase 2-like (PTGS2), important in inflammation reaction, was highly upregulated in all but S4(24h) treatment.

3.7 Differentially expressed immune genes unique to each probiotic

Transcripts of alpha-1–macroglobulin-like, integrins and antioxidant enzymes were downregulated in larvae exposed to S4 (Table 9). Transcripts corresponding to Tollo (TLR8) and E3 ubiquitin-protein ligase LRSAM1 were highly upregulated in RI(6h) alone. HT_RI transcriptomes showed upregulation of histone H2B-like and GTPase IMAP family member 7-like (GIMAP7) transcripts that were not seen in any other probiotic treatments.

3.8 Transcripts involved in antiviral responses

Surprisingly, several genes that are involved in antiviral pathways were also differentially expressed due to probiotic treatment. These included upregulation of recognition receptors (TLR3) for detecting intracellular nucleic acids and transcripts involved in the JAK-STAT and cGAS-STING pathways (Table 6). Stimulator of interferon genes protein-like (STING), an important part of the cGAS pathway, was upregulated in all probiotic treatments except HT_RI. Interferon induced protein 44 gene was upregulated in the HT_RI sample. E3 ubiquitin-protein ligase TRIM56 was heavily modulated in larvae from both probiotic treatments after a 24h exposure.

3.9 Cell death:

Autophagy related ATG9a was highly upregulated in all probiotic treatments except HT_RI (Table 9). Both initiator and executioner caspases in the apoptosis pathway were differentially expressed in probiotic treatments (Table 9). Transcripts for the initiator caspase 2 were upregulated in 6 h treatments while at least one of the executioner caspases 1,3,6 were upregulated in all treatments. Interestingly, caspases 1, 2, 7 and 8 were downregulated and only caspase-14 was upregulated in HT_RI. Several types of baculoviral IAP repeat-containing proteins were differentially expressed in response to probiotic treatments but the type of modulation and type of IAP differed between treatments. Inhibitor of apoptosis was highly up in all probiotic treatments except HT_RI, where GIMAP7 was highly upregulated.

4. Discussion

Exposure of larvae to probiotics S4 and RI induced the expression of a large variety of immune genes, suggesting a strong immune response comprising of heightened pathogen recognition, activation of immune signaling pathways and production of an arsenal of effectors. This probiotic mechanism of larval immunostimulation is consistent with previous observations that probiotics are cleared from the larvae within 12 - 24 h after treatment (Karim et al., 2013). These immune effectors activated in larvae upon probiotic exposure may also serve to provide protection against RE22 infection especially in light of the opposite effect of suppression of signaling pathways and lack of crucial effectors seen in response to RE22 challenge (Modak et al., in prep; Ch3 of this dissertation).

4.1 Mechanisms shared between probiotics

Overall, the immune response of larvae to each of the probiotics shared many features, including: (a) upregulation of a large variety of pathogen recognition receptors involved in environmental sensing and pathogen detection, followed by (b) activation of multiple signaling pathways; which ultimately led to the production of (c) an arsenal of effectors known to have a role in immune defenses against bacterial pathogens (Figures 4 & 5). Several probiotics are known to modulate (either activate or suppress) signaling pathways that benefit the host and protect them from pathogens (reviewed in Llewellyn et al., 2017). Usually, probiotics show a very strain specific response (Baarlen et al., 2011, Llewellyn et al., 2017). In this case however, despite the difference in Gram character between S4 and RI, many immune transcripts, especially effectors, were expressed in response to both probiotics.

Overall, differential expression analysis suggests activation of various immune signaling pathways like TLR, NF-kB and MAPK by both probiotics. The TLR pathway is crucial for bivalve innate immune systems. It recognizes a variety of damage-associated molecular patterns (DAMPs) and pathogen-associated molecular patterns (PAMPs) to activate NF-kB and MAPK pathway that protect the host from infection by producing cytokines, chemokines and other effectors (Gerdol et al., 2018). Our findings showing that TLR3, 4, 6, 8 and 13 were upregulated in response to probiotics are consistent with the important role of this pathway in bivalve immune responses, and indicate the potential of probiotics to provide protection against a broad spectrum of pathogens. Such PRR activation by probiotics due to shared cell envelope components like lipopolysaccharides, peptidoglycan, and ß-glucans with pathogens is well known (Pérez- Sánchez et al., 2014). Activation of TLR6 broadens the recognition spectrum to bacteria, fungi, LPS and peptidoglycan (PGN) (Wang et al., 2018). Subsequent activation of the MAPK pathway regulates several important cellular processes like cell proliferation, apoptosis, inflammatory response to pathogens and involved in the innate immunity of oysters (Wang et al., 2018). Activation of host MAPK and NF-kB and other signaling pathways by probiotics is seen in human gut associated probiotics (Thomas & Versalovic et al., 2010, Bermudez-Brito et al., 2012).

This transcriptome analysis also suggests that activation of these pathways leads to increased transcription of a variety of immune effectors. Larvae already equipped with effectors as a result of probiotic treatment can carry out expedited clearing of pathogen upon challenge. Some of these effectors have been shown in previous research to have the potential to be involved in protection against RE22.

Protease inhibitors: All probiotic treatments showed highly upregulated serine protease inhibitor Cvspi2. One of the important virulence factors of RE22 is production of proteases, most notably metalloproteases (Hasegawa et al., 2008), but also potentially serine proteases, which are encoded in the genome (Spinard et al., 2015, Richards et al., 2018). Presence of serine protease inhibitors might neutralize serine protease attack by RE22 in probiotic pretreated larvae thus playing a significant role in their survival from RE22 infection. cvSI-1 has been shown to play an important role in host defense against *Perkinsus marinus* by inhibiting proliferation of the parasite (LaPeyre et al., 2009, Yu et al., 2011, Nikapitiya et al., 2014) and is also upregulated in resistant oysters in response to challenge with the pathogen *Aliiroseovarious crassostreae* (McDowell et al., 2014) in *C. virginica*.

Mucins: Mucus is an important line of defense and plays multiple roles in the host-microbe interaction (Allam and Espinosa., 2016). Both secreted gel forming mucins and cell surface mucins modulated by both probiotics work in concert to clear infection (Linden et al., 2008). Both Gram negative and Gram-positive bacteria have been shown to upregulate mucins in humans (Dohrman et al., 1998) which explains how both probiotics could influence their production. Increased production of mucus could buffer action of proteases (Yan et al., 2017) used by pathogenic *Vibrio* spp. to penetrate mucus and spread infection (Silva et al., 2003). Probiotics modulate the mucus barrier to aid their adhesion thereby preventing invasion of pathogens (Tuomola et al., 1999, Allam and Espinosa, 2016). In addition, oysters can also benefit from presence of vast array of immune recognition and effector proteins in the mucus (Espinosa et al., 2016). Hence, modulation of mucins can have multiple advantages for probiotic pretreated larvae.

Perforin-2: Perforin-2/Mpeg1 was highly upregulated in all lab probiotic treated larvae. Perforin-2 is an important ancient innate immune system effector present in vertebrates as well as invertebrates that functions by forming pores in intracellular and extracellular pathogenic bacteria (McCormack and Podack, 2015). In invertebrates, LPS exposure significantly upregulated a homologue of perforin-2 in a sponge *Suberites domuncula* (Wiens et al., 2005) and in disk abalone *Haliotis discus discus* post *V. parahemolyticus* challenge (Bathige et al., 2014). In *C. gigas*, Cg-Mpeg1 showed significant antibacterial activity to both Gram-negative and positive bacteria and its transcription level was significantly up-regulated following infection with *V. alginolyticus* (He et al., 2011). Thus, elevated activation of perforin-2 in probiotic pretreated *C. virginica* larvae might act as an efficient effector against RE22 upon challenge.

Cytoskeletal organization: In addition, differential expression of actin, septin and dynamin 1 were shared by both probiotics suggesting a likelihood of their role in cytoskeletal reorganization (Pagliuso et al., 2016, Sirianni et al., 2016), possibly altering intracellular pathogenic invasion (Torraca and Mostowy, 2016, Mazon et al., 2017). Cytoskeletal rearrangements can help in bacterial sensing, compartmentalization of pathogens (Mostowy & Cossart, 2011), autophagy and apoptosis for host protection (Mostowy and Shenoy, 2015) as well as phagocytosis (Vicente-Manzanares and Sánchez-Madrid., 2004). PTGS2, which was upregulated in almost all probiotic treatments, is a key enzyme producing inflammatory prostaglandins and generation of inflammatory response activating the immune system in advance.

4.2 Mechanisms unique for each probiotic

Some unique aspects of the probiotic specific response are discussed below:

Specific response to S4:

In addition to protease inhibition, alpha-1–macroglobulin (which was downregulated only in S4) is also involved in complement and coagulation cascades (Xiao et al., 2000) suggesting possible modulation of complement cascades by S4. Integrins (also downregulated in S4(24h)) have been shown to be used by *V. splendidus* to enter hemocytes and evade immunity (Duperthuy, M, et al., 2011). Antioxidant enzymes were mostly downregulated with S4 treatment, suggesting that S4 treatment does not lead to oxidative damage, unlike pathogenic exposure (Lorgeril et al., 2008, McDowell et al., 2014).

Specific response to RI:

Tollo (TLR8, upregulated in response to RI) is related to larval innate immune response to Gram negative and positive bacteria and shown to regulate antimicrobial production in *Drosophila melanogaster* (Akhouayri et al., 2011). E3 ubiquitin-protein ligase LRSAM1 (highly upregulated in RI (6 h)) is a bacterial recognition protein and ubiquitin ligase that defends the cytoplasm from invasive pathogens. It is important for ubiquitin-dependent autophagy against invading intracellular bacterial pathogens (Huett et al., 2012).

Two unique aspects about HT_RI transcriptome were upregulated transcripts identified as histone H2B-like and GIMAP7. Histones show antimicrobial action against Gram negative bacteria like *Escherichia coli* (Kawasaki et al., 2008) and in *C. gigas* has been demonstrated to surround and engulf vibrios (Nikapitiya et al., 2013, Poirier et al., 2014). GIMAP7 is member of GTPase of the immune-associated proteins family that

acts an apoptosis regulator (Nitta and Takahama, 2007) and its upregulation suggests inhibition of apoptosis.

4.3 Unexpected responses to probiotics

Interestingly, multiple members of antiviral pathways were also modulated in response to probiotics. STING, an important part of the cGAS pathway, was highly upregulated in all lab probiotic treatments. A special STING homolog LvSTING was activated in shrimp in response to *V. parahaemolyticus* infections that participates in antimicrobial peptide production (Li et al., 2017). Thus, this pathway plays an essential role in host response to pathogen invasion including bacteria, owing to detection of cytosolic DNA recognition and type I IFN production (Tao et al., 2016). Activation of these pathway suggests that probiotic exposure may provide protection against viruses (Thomas et al. 2010, Bermudez-Brito et al., 2012).

4.4 Cell death

ATG9a was highly upregulated by all probiotic treatments suggesting activation of autophagy (He and Klionsky, 2009) consistently by both probiotics. Autophagy and septins together restrict cytosolic bacterial replication (Torraca and Mostowy, 2016) and maybe an additional mechanism of action against RE22 invasion.

Various apoptosis inhibitors were highly upregulated in response to both probiotic treatments suggesting overall inhibition of apoptosis in response to probiotics. However, patterns of expression of apoptotic genes vary across different environmental stressors in bivalves suggesting it is a very complex pathway that is still not completely understood (Gerdol et al., 2018). Surviving *C. gigas* also showed apoptosis inhibition in response to virulent *Vibrio* sp. (Lorgeril et al., 2011). One of the virulence factors of

RE22 is production of hemolysins (Spinard et al., 2015) showing toxic effects on hemocytes (Gómez-León et al., 2008). Inhibition of apoptosis by probiotic pretreatment might result in a higher number of hemocytes (Lee et al., 1993) that can potentially counter the effect of hemolysins secreted by RE22 upon challenge.

4.*5 Length of probiotic pretreatment for effective protection from challenge*

As seen in the results (Table 1), shorter probiotic pretreatment provides variable protection whereas longer pretreatment provides consistent protection from challenge. Comparison of 6h and 24h transcriptomes showed same key effector mechanisms activated at both time points viz upregulation of serine protease inhibitors, mucins and perforin-2. There are however subtle differences for example in types of PRRs, mucins and septins that are upregulated at 6 h compared to those at 24 h. Certain genes involved in biomineralization and larval development and growth were also upregulated at 24 h. This supports the observation that longer exposure provides better protection perhaps due to increased pathogen sensing, additional growth effects and longer time for all larvae to respond to probiotic pretreatment. Previous studies have also shown chronic exposure of probiotics work better (Llewellyn et al., 2017).

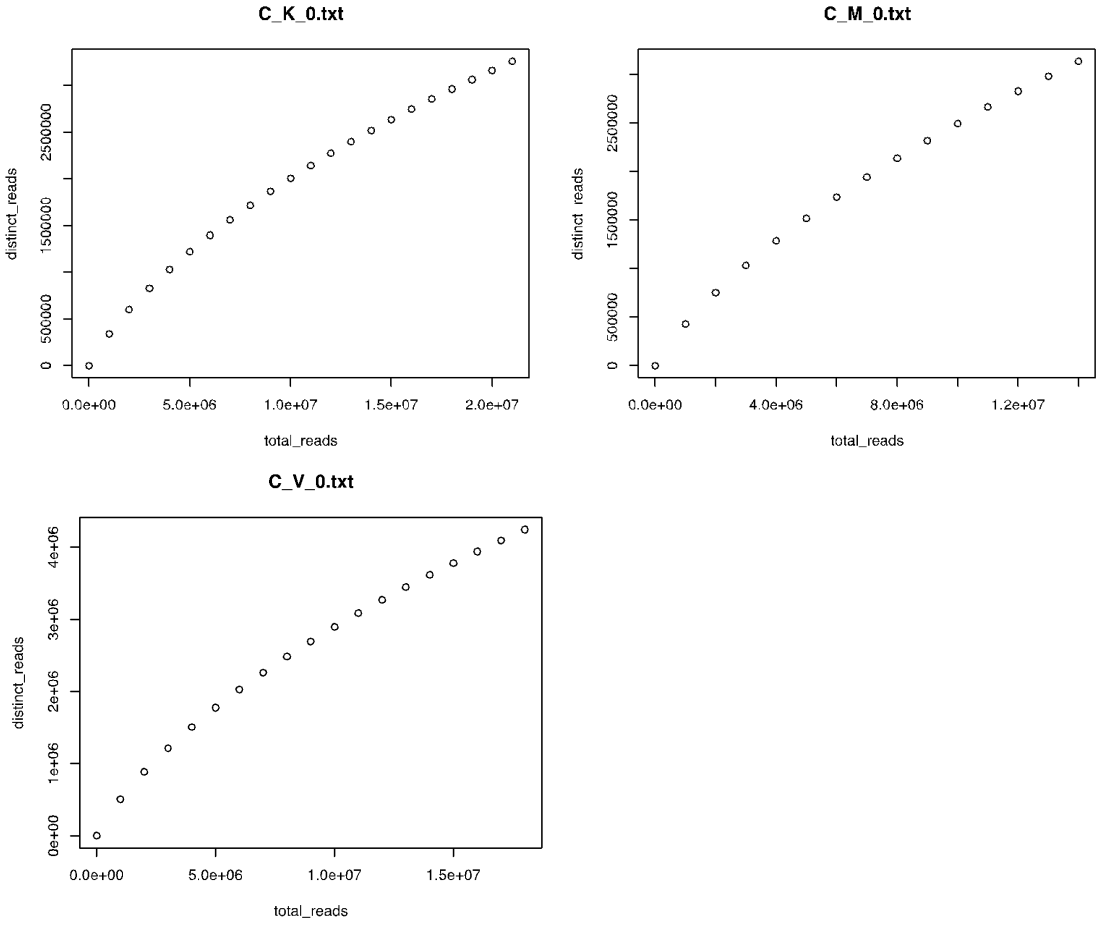
5. Conclusion

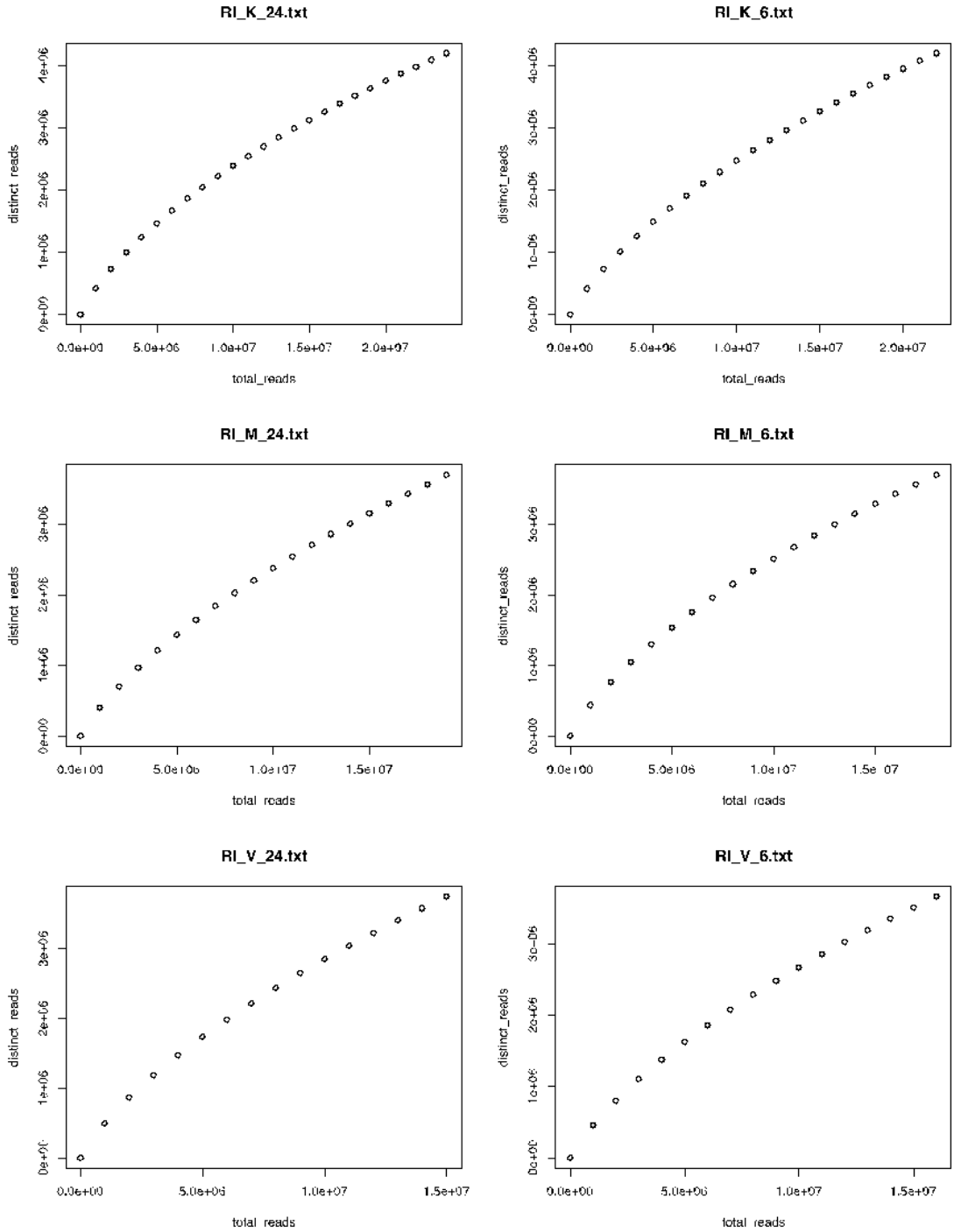
This study indicates that probiotics use immunomodulation as a mechanism of action that may play a role in the protection conferred against RE22 infection. Although 6 h of pretreatment with probiotics might suffice for some larvae to protect themselves from RE22 challenge, a 24 h pretreatment consistently allows majority of them to elicit the immune responses effective in providing protection. This knowledge might help in

designing better management strategies to control larval mortality in hatcheries by use of probiotics as a natural and environmental friendly solution. In the future, it would be beneficial to use this information to target the functional identification of effectors that serve in protecting larvae against RE22 infection.

Table 1: Effect of varying lengths of probiotic pretreatment on larval survival after experimental challenge with the pathogen *V. coralliilyticus* RE22. Results are expressed as the relative increase in percent survival +/- standard deviation (SD) of larvae pretreated with probiotics as compared to non-treated and challenged larvae. S4 + RE22: Larvae pretreated with *Phaeobacter inhibens* S4 and then challenged with RE22. RI + RE22: Larvae pretreated with *Bacillus pumilus* RI0-695 and then challenged with RE22. - Not Tested.

Treatment	RPS (average +/- SD)		
	6h	24h	8d
S4 + RE22	37 ± 26	41 ± 2	-
RI + RE22	30 ± 39	45 ± 5	28 ± 6




RI_K_24.txt
distinct reads
total_reads
RI_K_6.txt
distinct_reads
total_reads
RI_M_24.txt
distinct reads
total reads
RI_M_6.txt
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total reads
RI_V_24.txt
distinct reads
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RI_V_6.txt
distinct_reads
total reads

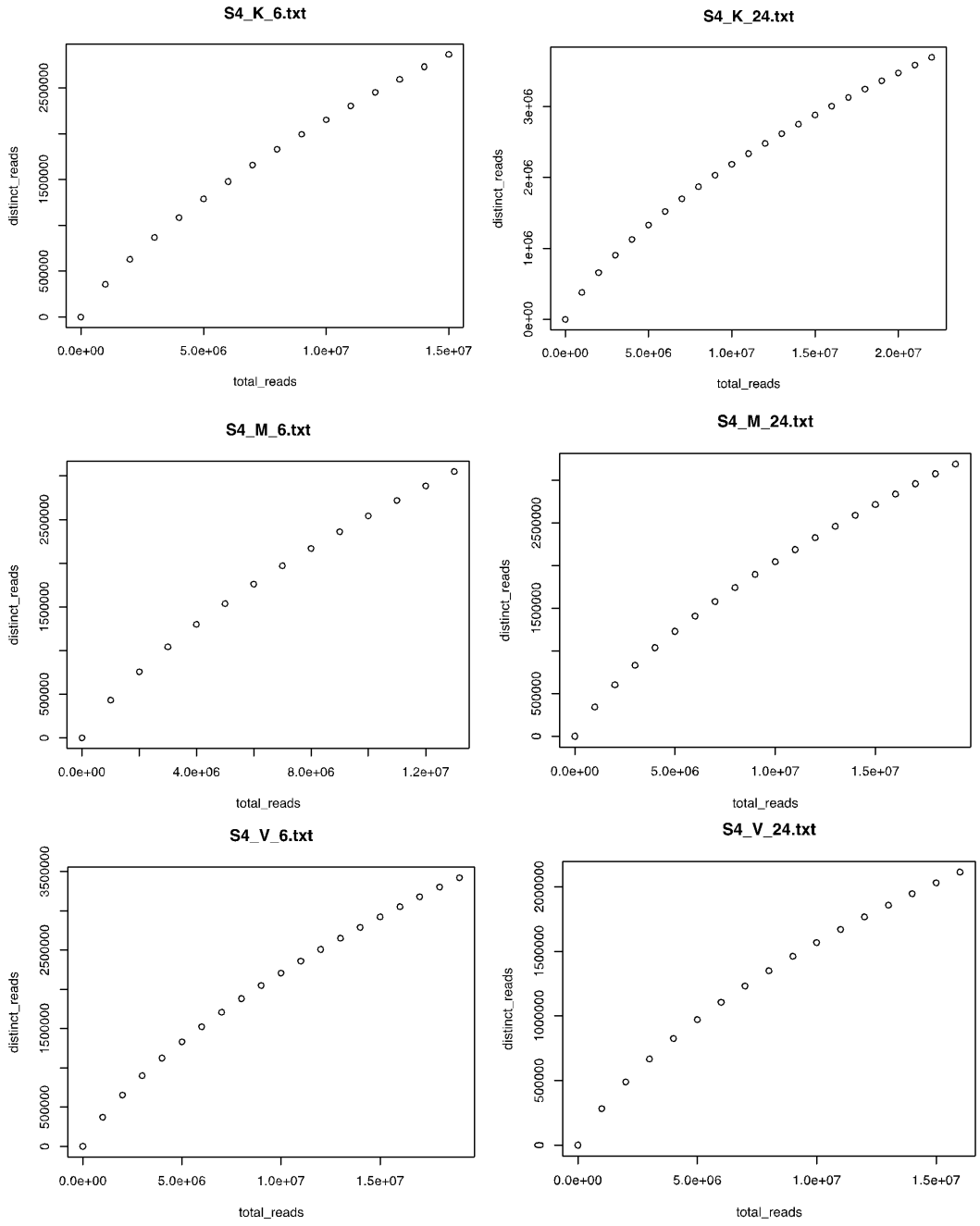

Figure 1a: Sequencing saturation curves for RNA-seq samples obtained in laboratory for control, RI treated 6h, RI treated 24h, S4 treated 6h, S4 treated 24h larval transcriptomes. Curves are provided for each experiment (biological replicates; K, M, V).

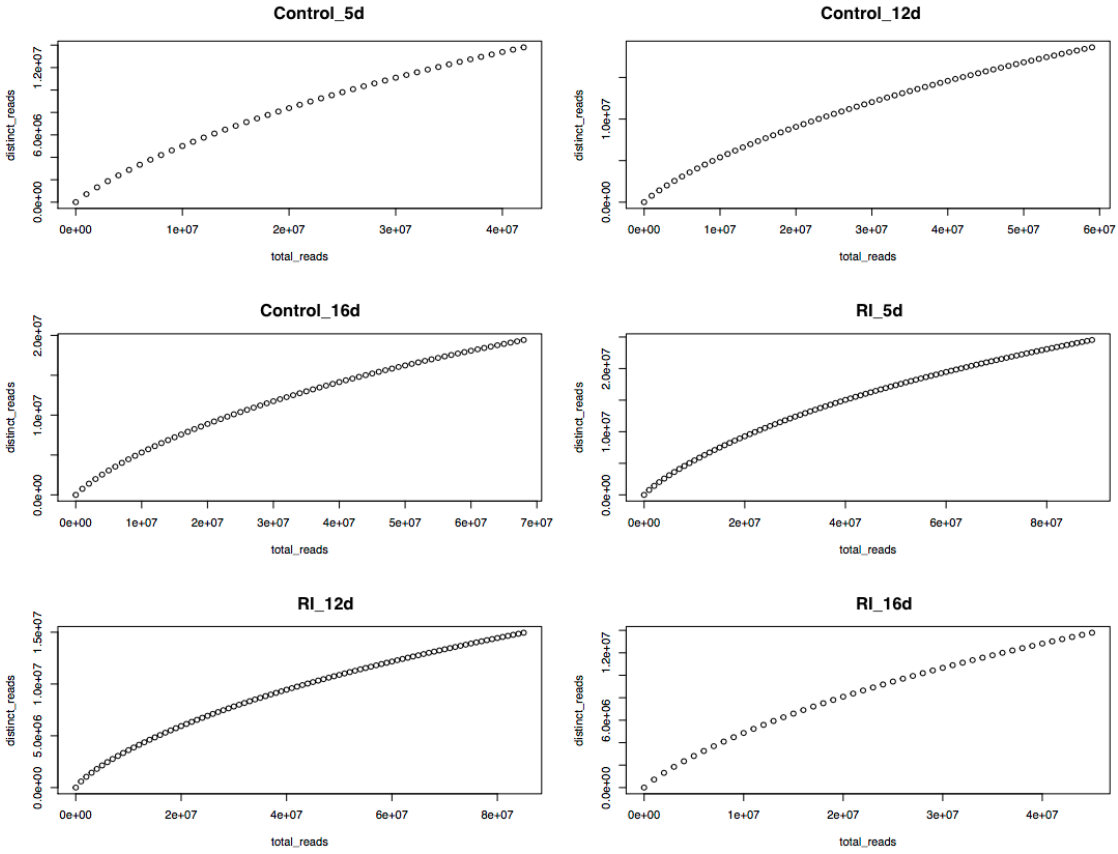

Figure 1b: Sequencing saturation curves for RNA-seq samples obtained in hatchery for control and *B. pumilis* RI06-95 treated oyster larval transcriptomes. Larvae were collected on day 5, 12 and 16 after fertilization, after being treated daily starting 1 day after fertilization.

Table 2: Oyster larval transcriptomes in response to probiotic treatment. Laboratory transcriptomes: Oyster larvae were treated with *B. pumilus* RI0-695 and *Phaeobacter inhibens* S4 for 6 or 24 h. Larvae for control (C) transcriptomes were collected at time 0h. Hatchery transcriptomes: Larvae were treated daily in the hatchery with RI06-95 (RI) or not-treated (Con), and collected 5, 12 or 16 d after fertilization. Three independent laboratory experiments (K, M, V) with two treatments (Control: C; RI treatment: RI, S4 treatment: S4) were performed in duplicate. Number of paired end reads per sample and % alignment rates to *Crassostrea virginica* reference genome using HISAT2 are shown.

Sample	# paired reads	% Alignment to *Crassostrea virginica* genome
Laboratory transcriptomes		
C_K_0	22,963,376	89
C_M_0	16,617,375	88

C_V_0	20,674,506	86
RI_K_24	27,507,148	86
RI_K_6	25,325,997	87
RI_M_24	22,339,707	86
RI_M_6	20,649,356	86
RI_V_24	18,412,447	83
RI_V_6	18,720,304	86
S4_K_24	25,285,770	87
S4_K_6	17,536,097	88
S4_M_24	21,950,812	87
S4_M_6	14,570,962	86
S4_V_24	17,840,669	89
S4_V_6	21,556,827	88
Hatchery transcriptomes		
Con_5d	73,690,654	53
Con_12d	60,768,394	93
Con_16d	70,771,125	64
RI_5d	61,710,678	94
RI_12d	59,865,884	94
RI_16d	50,226,597	61

Table 3: Number of differentially expressed genes per comparison ($p \leq 0.05$, upregulation: log fold change ≥ 2, downregulation: log fold change ≤ -2).

Comparison	# DEGs
Lab transcriptomes	
6h probiotic treatment	
RI vs Con	1,550
S4 vs Con	2,269
24h probiotic treatment	
RI vs Con	2,139
S4 vs Con	3,459
Hatchery transcriptome	
5, 12, 16d post fertilization	
RI vs Con	2,993

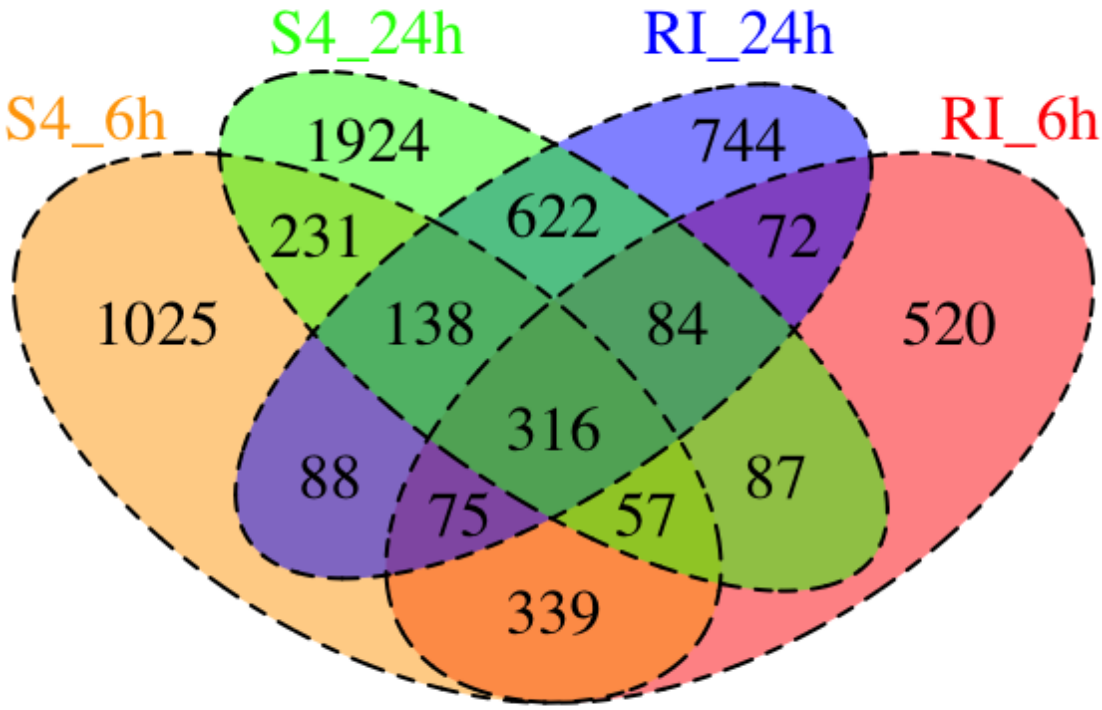

Figure 2a: Venn Diagram of shared and unique differentially expressed genes for each probiotic treatment (*B. pumilus* RI0-695 and *Phaeobacter inhibens* S4) at 6 h and 24 h in laboratory samples.

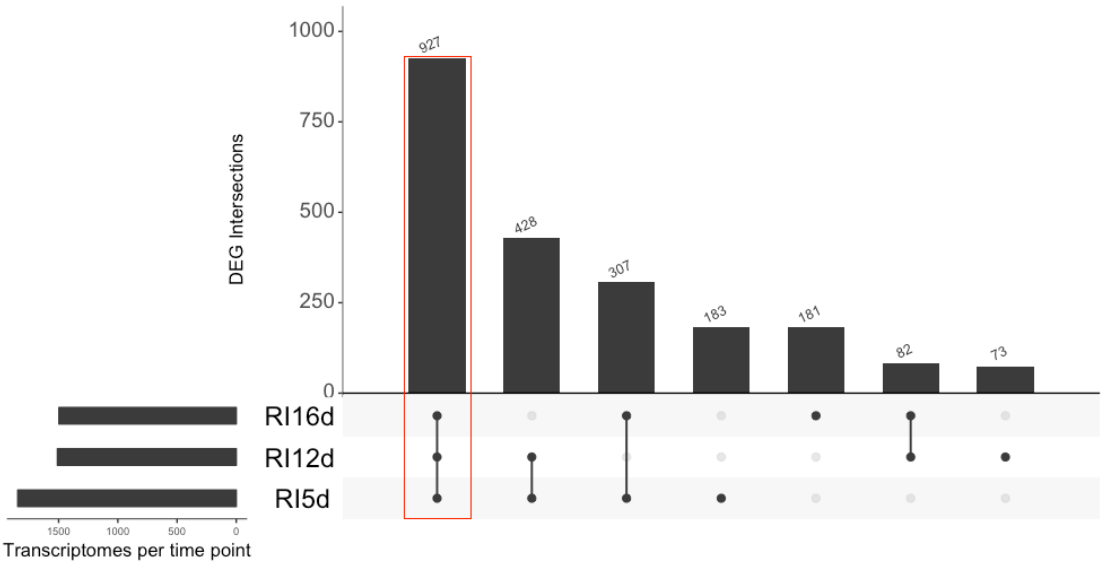

Figure 2b: Plot comparing number of differentially expressed genes in probiotic treatments at 5, 12 and 16 days in a hatchery. Numbers above the highlighted bar (boxed in red) show the number of differentially expressed genes shared in all probiotic treatments.

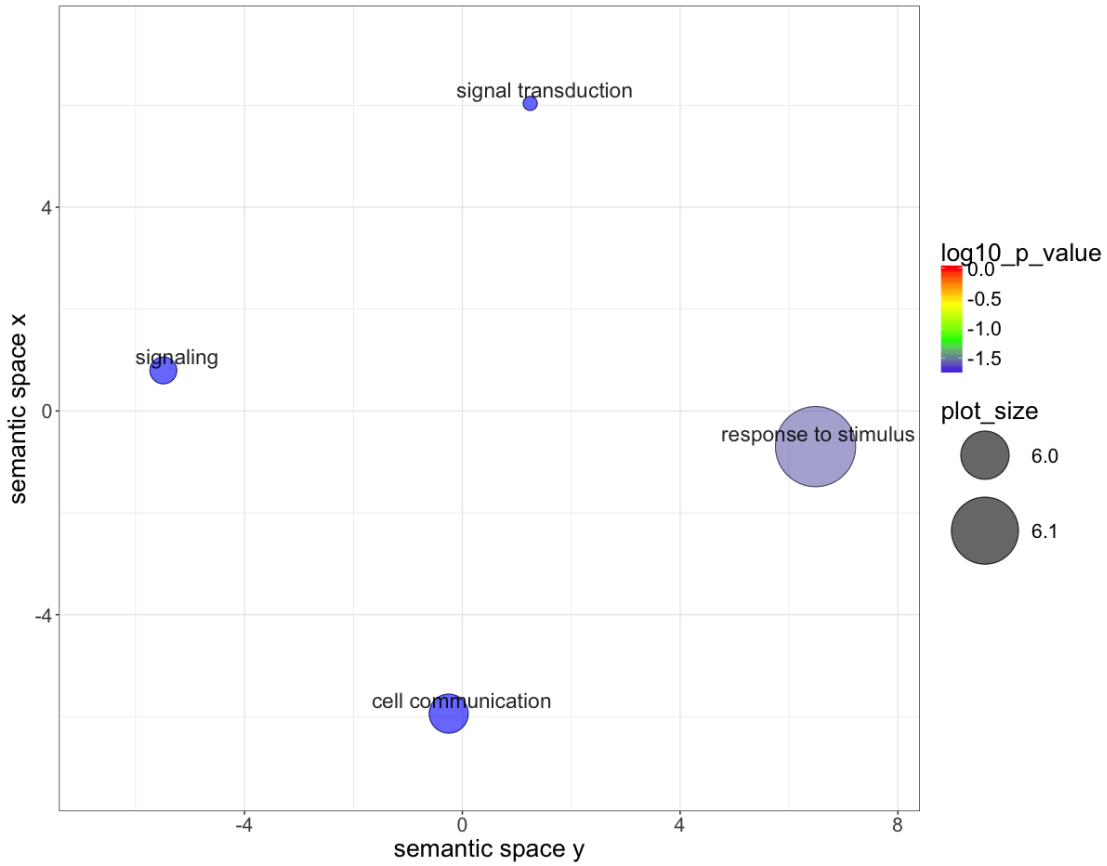

Figure 3a: Functional enrichment of differentially expressed transcripts in S4 (6h) using Gene Ontology terms in Biological Process. The color scale in the legend shows level of significance (warmer colors are less significant than cooler colors) and the size of the bubble corresponds to the number of significant transcripts mapped to the term.

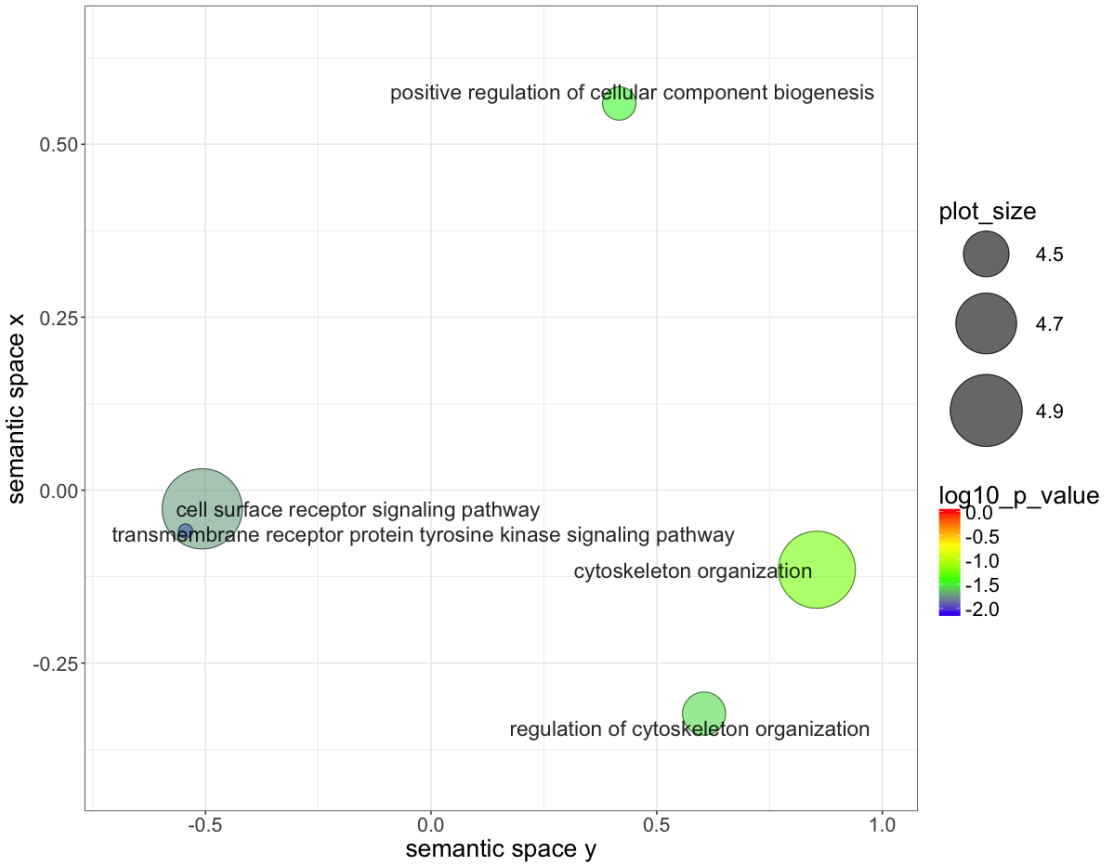

Figure 3b: Functional enrichment of differentially expressed transcripts in S4 (24h) using Gene Ontology terms in Biological Process. The color scale in the legend shows level of significance (warmer colors are less significant than cooler colors) and the size of the bubble corresponds to the number of significant transcripts mapped to the term.

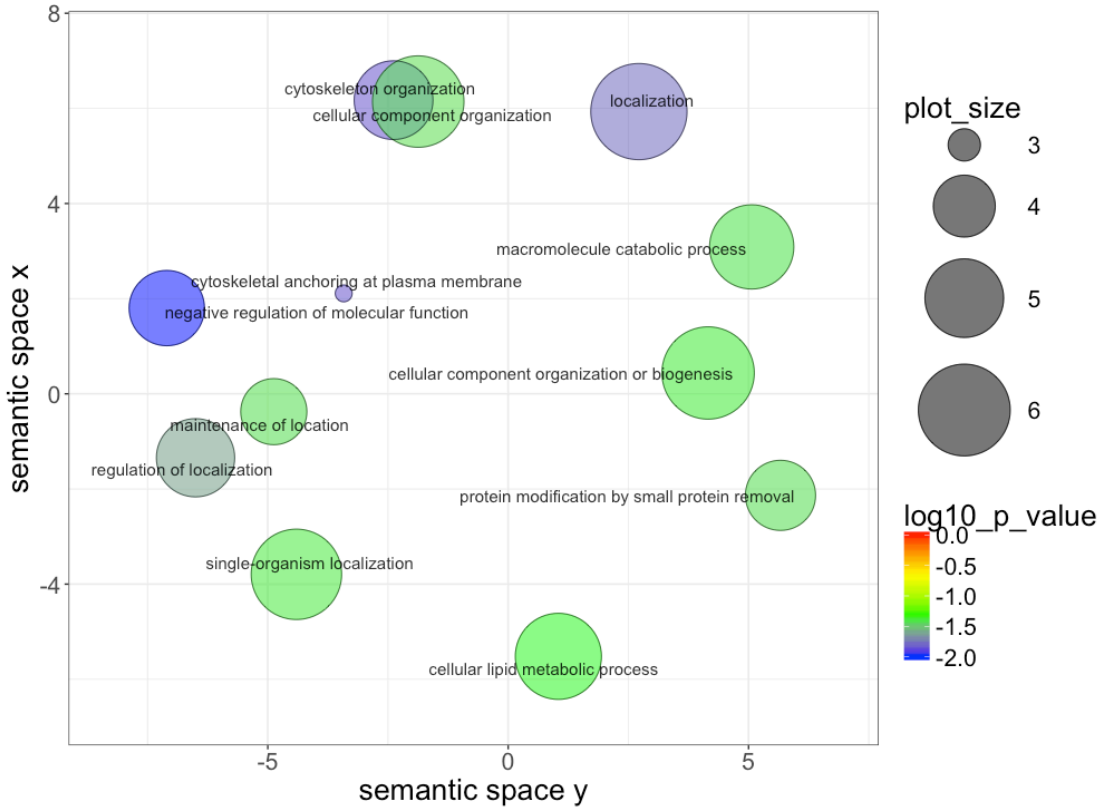

Figure 3c: Functional enrichment of differentially expressed transcripts in hatchery RI transcriptomes using Gene Ontology terms in Biological Process. The color scale in the legend shows level of significance (warmer colors are less significant than cooler colors) and the size of the bubble corresponds to the number of significant transcripts mapped to the term.

Table 4: KEGG annotation of differentially expressed genes

	RI(6h)	S4(6h)	RI(24h)	S4(24h)
Metabolism				
Carbohydrate metabolism	27	36	37	68
Energy metabolism	4	10	13	17
Lipid metabolism	25	29	40	48
Nucleotide metabolism	10	20	16	29
Amino acid metabolism	13	37	26	51
Metabolism of other amino acids	6	9	19	19
Glycan biosynthesis and metabolism	7	19	20	31
Metabolism of cofactors and vitamins	6	17	14	16
Metabolism of terpenoids and polyketids	3	3	4	5

Biosynthesis of other secondary metabolites	2	8	5	4
Xenobiotics and biodegradation metabolism	9	8	11	13
Genetic information processing				
Transcription	11	20	13	17
Translation	16	25	23	44
Folding sorting and degradation	16	23	25	43
Replication and repair	1	5	8	15
Environmental processing				
Membrane transport	1	3	2	5
Signal transduction	200	261	294	387
Signaling molecules and interaction	8	10	17	23
Cellular processes				
Transport and catabolism	45	58	64	87
Cell growth and death	38	51	64	106
Cellular community-eukaryotes	48	59	52	70
Cellular community-prokaryotes	1	2	0	1
Cell motility	8	9	12	17
Organismal systems				
Immune system	124	112	113	137
Endocrine system	133	114	171	210
Circulatory system	18	16	29	36
Digestive system	33	34	41	63
Excretory system	11	12	14	23
Nervous system	61	50	75	101
Sensory system	20	16	24	31
Development	22	22	23	31
Aging	20	20	19	22
Environmental adaptation	30	30	31	34

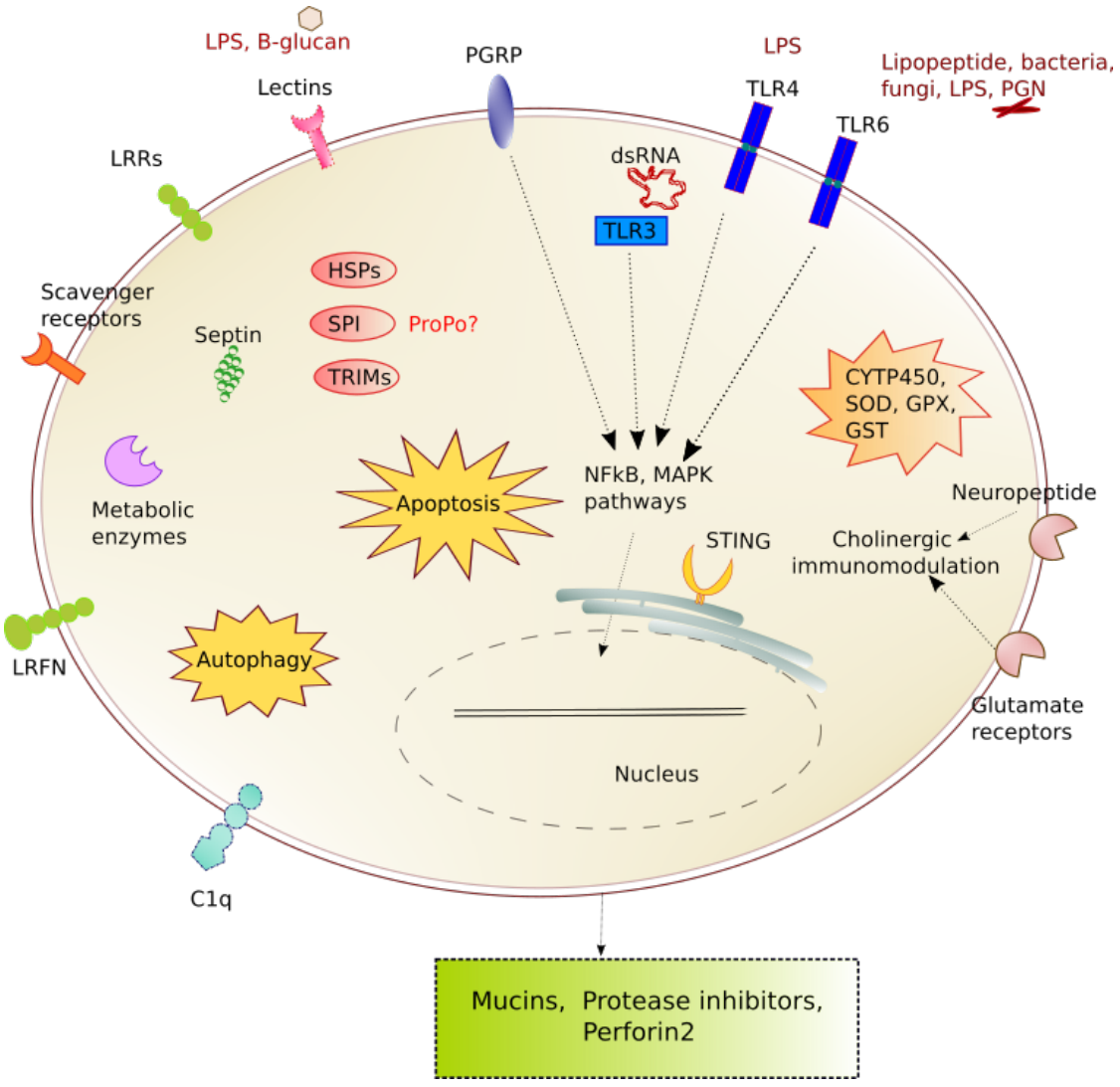

Figure 4: Overview of the immune responses induced in oyster larvae in response to treatment with probiotics S4 and RI, as measured through high-throughput analysis of differential gene expression. Overall, PRRs including TLRs, lectins, PGRPs and LRRs were upregulated while others were downregulated. Signaling pathways including TLR, NF-kB, MAPK and antiviral pathways including JAK-STAT, cGAS-STING were activated. Immune effectors were activated including mucins, protease inhibitor and perforin-2. Autophagy was activated and apoptosis was inhibited. Antioxidant enzymes were downregulated. Cytoskeleton related molecules including septins were modulated by both probiotics.

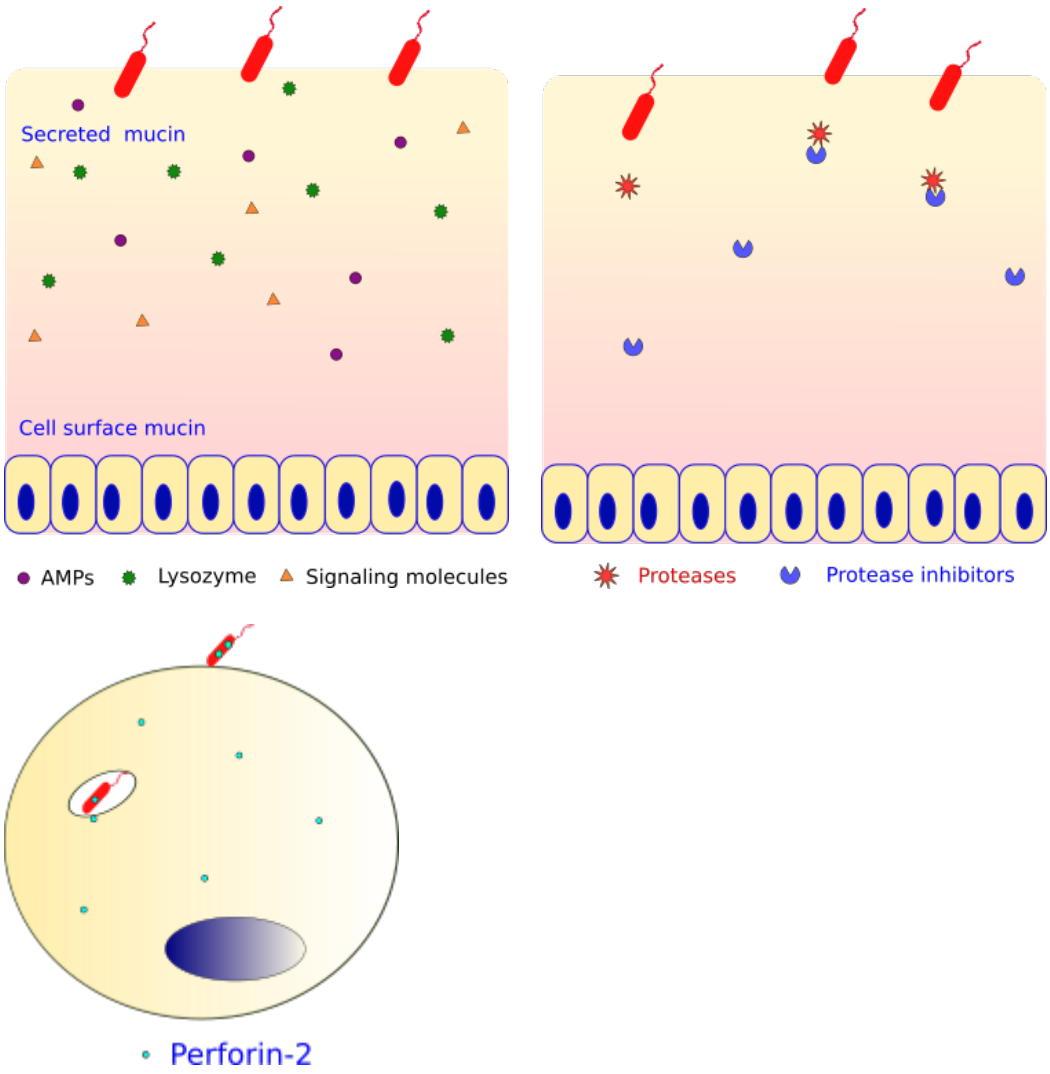

Figure 5: Hypothesized role of selected effectors of immunity whose expression was found to be upregulated in larval oysters in response to probiotic treatment on providing protection against challenge to *V. coralliilyticus* RE22. Mucin and protease inhibitors provide protection outside the oyster body and perforin-2 providing protection once the pathogen is within oyster tissues.

Table 5: Patterns of differential gene expression of immune receptors in oyster larvae in response to probiotic treatment ($p \leq 0.05$, upregulation: log fold change ≥ 2, downregulation: log fold change ≤ -2). Yellow denotes downregulation, orange denotes up and downregulation of transcripts mapped to the same gene, red denotes upregulation. HT-RI: larvae treated daily with probiotic *Bacillus pumilus* RI0695 (RI) for 5, 12 or 16 days; RI_6h: Larvae exposed to RI for 6h; RI_24h: Larvae exposed to RI for 24h; S4_6h: Larvae exposed to S4 for 6h; S4_24h: Larvae exposed to S4 for 24h.

	Probiotics				
	HT_RI	**RI_6h**	**RI_24h**	**S4_6h**	**S4_24h**
Receptors					
TLRs					
toll-like receptor 1					
TLR3 isoform X1					
TLR4					
TLR4 isoform X1					
toll-like receptor 6					
TLR6 isoform X1					
TLR13					
TLR13 isoform X1					
TLR Tollo isoform X2					
protein toll-like					
Lectins					
C-type lectin domain family 4 member E-like					
C-type lectin domain family 3 member A-like					
lectin-like *					
lectin BRA-3-like					
plectin-like isoform X4					

hepatic lectin like					
galactose-specific lectin nattectin-like					
fucolectin-like					
Scavenger receptors					
scavenger receptor class F member 2-like					
scavenger receptor class B member 1 isoform B					
scavenger receptor class B member 1-like isoform X1					
Scavenger receptor cysteine-rich type 1 protein M130					
scavenger receptor cysteine-rich type 1 protein M130-like isoform X1					
somatomedin-B and thrombospondin type-1 domain-containing protein-like					
proteoglycan 4-like isoform X4					
PGRP					
peptidoglycan-recognition protein SC2-like *					
LRFN					
leucine-rich repeat and fibronectin type III domain-containing protein 1-like protein					
leucine-rich repeat and fibronectin type-III domain-containing protein 5-like					
LRR					
leucine-rich repeat transmembrane neuronal protein 3-like isoform X1					
leucine-rich repeat transmembrane protein FLRT1-like					
leucine-rich repeat transmembrane protein FLRT3-like					
leucine-rich repeat-containing protein 24-like					
leucine-rich repeat-containing protein 27-like					
leucine-rich repeat-containing protein 28-like isoform X3					
leucine-rich repeat-containing protein 34-like isoform X2					
leucine-rich repeat-containing protein 40					

leucine-rich repeat-containing protein 45-like					
Leucine-rich repeat-containing protein 49					
leucine-rich repeat-containing protein 4C-like isoform X1					
leucine-rich repeat-containing protein 70-like					
leucine-rich repeat-containing protein 71-like isoform X21					
leucine-rich repeat-containing protein 71-like isoform X22					
leucine-rich repeat-containing protein 74A-like					
leucine-rich repeat-containing protein 74A-like isoform X2					
leucine-rich repeat-containing protein 74B-like					
leucine-rich repeat-containing protein 74B-like isoform X2					
leucine-rich repeat-containing protein 74B-like isoform X3					
leucine-rich repeat-containing protein 74B-like isoform X6					
leucine-rich repeat-containing protein 9-like isoform X2					
leucine-rich repeat and IQ domain-containing protein 1					
leucine zipper putative tumor suppressor 2 homolog					
Fibronectin type III domain					
fibronectin type III domain-containing protein 1-like					
fibronectin type III domain-containing protein 2-like isoform X3					
fibronectin type-III domain-containing protein 3A-like isoform X4					
ankyrin repeat and fibronectin type-III domain-containing protein 1-like					
C1q proteins					
C1q-related factor-like *					
complement C1q-like protein 2					
complement C1q tumor necrosis factor-related protein 2-like					
complement C1q tumor necrosis factor-related protein 4-like					

complement C1q tumor necrosis factor-related protein 4-like isoform X3					
alpha-1-macroglobulin-like					
alpha-1-macroglobulin-like isoform X2					
Macrophage mannose receptor 1					
Macrophage mannose receptor 1-like isoform X1*					
Metabolic Enzymes with New Role of Carbohydrate Binding					
Phosphoenolpyruvate carboxykinase					
hexokinase-2-like isoform X2					
B cell receptor					
dapp1 dual adaptor for phosphotirosine*					
Cholinergic immunomodulation					
Glutamate receptor *					
glutamate receptor 2-like					
glutamate receptor ionotropic					
dopamine receptor 2-like					
muscarinic acetylcholine receptor M1-like					
neuronal acetylcholine receptor subunit alpha-6-like					
neuronal acetylcholine receptor subunit alpha-9-like					
choline transporter-like protein 2					
apoptogenic protein 1					
anti-apoptotic protein NR13-like					
neuropeptide FF receptor 2-like					
neuropeptide SIFamide receptor-like					
neuropeptide Y receptor type 1-like					
neuropeptide Y receptor type 2-like*					

pro-neuropeptide Y-like isoform X1*					
metabotropic glutamate receptor 8-like isoform X1					
acetylcholinesterase-like isoform X1*					
acetylcholinesterase-like					

Table 6: Patterns of differential gene expression of immune signaling pathways in oyster larvae in response to probiotic treatment ($p \leq 0.05$, upregulation: log fold change ≥ 2, downregulation: log fold change ≤ -2). Yellow denotes downregulation, orange denotes up and downregulation of transcripts mapped to the same gene, red denotes upregulation. HT-RI: larvae treated daily with probiotic *Bacillus pumilus* RI0695 (RI) for 5, 12 or 16 days; RI_6h: Larvae exposed to RI for 6h; RI_24h: Larvae exposed to RI for 24h; S4_6h: Larvae exposed to S4 for 6h; S4_24h: Larvae exposed to S4 for 24h.

	Probiotics				
	HT_RI	**RI_6h**	**RI_24h**	**S4_6h**	**S4_24h**
Signaling pathways in Immune response					
TLR pathway					
TNF receptor-associated factor 3-like isoform X3 (TRAF3)					
tumor necrosis factor receptor superfamily member 1B-like isoform X1					
tumor necrosis factor receptor superfamily member					
mitogen-activated protein kinase kinase kinase 7-like isoform X3 (TAK1)					
JAK-STAT					
tyrosine-protein kinase JAK2-like (JAK)					
signal transducer and activator of transcription 3-like isoform X6 (STAT3)					
Suppressor of cytokine signaling 7 (SOCS)					
son of sevenless homolog 2-like * (SOS2)					
epidermal growth factor receptor-like (EGFR)					

epidermal growth factor receptor-like isoform X2					
epidermal growth factor receptor-like isoform X4					
tyrosine-protein phosphatase non-receptor type 11 isoform X3 (SHP2)					
tyrosine-protein phosphatase non-receptor type 4-like isoform X4 (PTPN4)					
tyrosine-protein phosphatase non-receptor type 9-like isoform X2 (PTPN9)					
tyrosine-protein phosphatase non-receptor type 23-like (PTPN23)					
NF-kB signaling pathway					
NF-kappa-B-activating protein-like (NKAP)					
NF-kappa-B inhibitor alpha-like isoform X1 (IkB)					
smad nuclear interacting protein 1-like (SNIP1)					
TNFAIP3-interacting protein 1-like * (TNIP1)					
B-cell lymphoma/leukemia 10-like (BCL10)					
ELKS/Rab6-interacting/CAST family member 1-like					
ELKS/Rab6-interacting/CAST family member 1-like isoform X3					
ELKS/Rab6-interacting/CAST family member 1-like isoform X5*					
TRAF-type zinc finger domain-containing protein 1-like *					
adapter protein CIKS-like isoform X4 (TRAF3IP2/Act1/CIKS)					
NF-kappa-B inhibitor-interacting Ras-like protein 1 isoform X8					
nuclear factor NF-kappa-B p105 subunit-like isoform X2					
lipopolysaccharide-induced tumor necrosis factor-alpha factor homolog					
Mitogen-Activated Protein Kinases (MAPK) pathway					
dual specificity mitogen-activated protein kinase kinase 1-like isoform X1					
mitogen-activated protein kinase-binding protein 1-like isoform X4 (MEKK1)					
mitogen-activated protein kinase kinase kinase kinase 3-like (MKK3)					
Mitogen-activated protein kinase kinase kinase 7 (MKK7)					

dual specificity mitogen-activated protein kinase kinase 7-like isoform X1 (MKK7)					
mitogen-activated protein kinase kinase kinase 7-like isoform X3 (TAK1)					
mitogen-activated protein kinase 11-like (MAPK11)					
mitogen-activated protein kinase kinase kinase 13-like isoform X2 (MAPK13)					
mitogen-activated protein kinase 14A-like (P38)					
transforming growth factor-beta, partial					
C-Jun-amino-terminal kinase-interacting protein 4-like *					
extracellular signal-regulated kinase 2-like isoform X4					
stress-activated protein kinase JNK-like isoform X1 (JNK)					
Regulator of G-protein signaling 3					
cGAS-STING pathway					
stimulator of interferon genes protein-like (STING)					
RIG-1 pathway related					
interferon regulatory factor 2-binding protein-like					
Signal transduction					
death domain-containing protein 1-like					
death domain-containing protein CRADD-like *					
integrin alpha-2-like isoform X5 *					
integrin alpha-4-like isoform X1 *					
integrin beta-3-like [Crassostrea virginica]					
ubiquitin carboxyl-terminal hydrolase 14-like					
ubiquitin carboxyl-terminal hydrolase 20-like isoform X2					
ubiquitin carboxyl-terminal hydrolase 22-like *					
ubiquitin carboxyl-terminal hydrolase 25-like isoform X3 * (USP25)					
cellular retinoic acid-binding protein 2-like					

1-phosphatidylinositol 4,5-bisphosphate phosphodiesterase gamma-1-like isoform X4 (PLCG1)					
epidermal growth factor receptor-like					
epidermal growth factor receptor-like isoform X2					
epidermal growth factor receptor-like isoform X4					
tyrosine-protein phosphatase non-receptor type 11 isoform X3					
basic leucine zipper transcriptional factor ATF-like 3					
nuclear factor of activated T-cells 5-like isoform X2					

Table 7: Patterns of differential gene expression of immune effectors in response to probiotic treatment ($p \leq 0.05$, upregulation: log fold change ≥ 2, downregulation: log fold change ≤ -2). Yellow denotes downregulation, orange denotes up and downregulation of transcripts mapped to the same gene, red denotes upregulation. HT-RI: larvae treated daily with probiotic *Bacillus pumilus* RI0695 (RI) for 5, 12 or 16 days; RI_6h: Larvae exposed to RI for 6h; RI_24h: Larvae exposed to RI for 24h; S4_6h: Larvae exposed to S4 for 6h; S4_24h: Larvae exposed to S4 for 24h.

			Probiotics		
	HT_RI	**RI_6h**	**RI_24h**	**S4_6h**	**S4_24h**
Effectors					
serine protease inhibitor Cvsi-2-like *					
serine protease inhibitor dipetalogastin-like *					
kunitz-type serine protease inhibitor conotoxin Cal9.1b-like					
digestive cysteine proteinase 2-like*					
serine protease 44-like					
interferon-induced protein 44-like isoform X2					
interleukin-17 receptor D-like					

Signaling mucin HKR1					
integumentary mucin C.1-like					
integumentary mucin C.1-like isoform X1					
integumentary mucin C.1-like isoform X3					
mucin-12-like *					
mucin-17-like isoform X2					
mucin-2-like					
mucin-2-like isoform X2					
mucin-3B-like isoform X4					
mucin-4 isoform X3					
mucin-4-like isoform X7					
mucin-4-like isoform X8					
mucin-5AC-like *					
mucin-5AC-like isoform X5					
mucin-5B-like					
mucin-19-like, partial					
mucin-19 isoform X2					
PREDICTED: mucin-19 isoform X7					
IgGFc-binding protein*					
septin-2-like					
septin-2-like isoform X1					
septin-2-like isoform X8					
septin-7 isoform X3					
septin-11-like isoform X2					
macrophage-expressed gene 1 protein-like * (Perforin-2/Mpeg1)					

antistasin-like					
SH3-domain binding protein 2					
alpha-1-macroglobulin-like					
alpha-1-macroglobulin-like isoform X2					
cystatin-A-like					

Table 8: Patterns of differential gene expression that are part of cytoskeletal reorganization in oyster larvae in response to probiotic treatment ($p \leq 0.05$, upregulation: log fold change ≥ 2, downregulation: log fold change ≤ -2). Yellow denotes downregulation, orange denotes up and downregulation of transcripts mapped to the same gene, red denotes upregulation. HT-RI: larvae treated daily with probiotic *Bacillus pumilus* RI0695 (RI) for 5, 12 or 16 days; RI_6h: Larvae exposed to RI for 6h; RI_24h: Larvae exposed to RI for 24h; S4_6h: Larvae exposed to S4 for 6h; S4_24h: Larvae exposed to S4 for 24h.

	Probiotics				
	HT_RI	**RI_6h**	**RI_24h**	**S4_6h**	**S4_24h**
Cytosletal reorganization					
Actin, cytoplasmic					
actin-like					
actin-3-like isoform X1					
septin-2-like					
septin-2-like isoform X1					
septin-2-like isoform X8					
septin-7 isoform X3					
septin-11-like isoform X2					
dynamin-1-like isoform X6					

Table 9: Patterns of differential gene expression that are part of apoptosis and autophagy in oyster larvae in response to probiotic treatment ($p \leq 0.05$, upregulation: log fold change ≥ 2, downregulation: log fold change ≤ -2). Yellow denotes downregulation, orange denotes up and downregulation of transcripts mapped to the same gene, red denotes upregulation. HT-RI: larvae treated daily with probiotic *Bacillus pumilus* RI0695 (RI) for 5, 12 or 16 days; RI_6h: Larvae exposed to RI for 6h; RI_24h: Larvae exposed to RI for 24h; S4_6h: Larvae exposed to S4 for 6h; S4_24h: Larvae exposed to S4 for 24h.

	Probiotics				
	HT_RI	**RI_6h**	**RI_24h**	**S4_6h**	**S4_24h**
Apoptosis					
Caspase 1					
Caspase 2					
Caspase 3					
caspase-3-like isoform X2					
Caspase 6					
Caspase 6 Isoform X2					
Caspase 7					
Caspase 7 Isoform X1					
Caspase 7 Isoform X3					
Caspase-8					
caspase-14-like isoform X2					
caspase recruitment domain-containing protein 14-like isoform X5					
baculoviral IAP repeat-containing protein 2-like					
baculoviral IAP repeat-containing protein 2-like isoform X2					
baculoviral IAP repeat-containing protein 2-like isoform X1					
baculoviral IAP repeat-containing protein 3-like					
baculoviral IAP repeat-containing protein 3-like isoform X1 *					

baculoviral IAP repeat-containing protein 6-like isoform X5					
baculoviral IAP repeat-containing protein 7A-like isoform X2					
baculoviral IAP repeat-containing protein 7-like isoform X3					
putative inhibitor of apoptosis*					
Apoptosis inhibitor IAP					
bifunctional apoptosis regulator-like isoform X1					
apoptogenic protein 1					
apoptosis-inducing factor 3-like					
protein kinase C iota type-like isoform X4					
multiple epidermal growth factor-like domains protein 10 isoform X2					
multiple epidermal growth factor-like domains protein 10 isoform X4					
multiple epidermal growth factor-like domains protein 6					
multiple epidermal growth factor-like domains protein 6 isoform X1					
death domain-containing protein CRADD-like *					
apoptotic chromatin condensation inducer in the nucleus-like					
cathepsin L-like isoform X2					
cathepsin L1-like					
cathepsin O-like					
programmed cell death protein 2-like isoform X1					
programmed cell death protein 6-like isoform X2					
programmed cell death 6-interacting protein-like isoform X3					
XK-related protein 8-like isoform X2 *					
XK-related protein 6, partial *					
XK-related protein 4					
cell death protein 3-like					

cell death abnormality protein 1-like					
cell death-inducing p53-target protein 1-like isoform X5					
cell death specification protein 2					
FAS-associated factor 1-like					
serine/threonine-protein kinase/endoribonuclease IRE1-like					
inositol 1,4,5-trisphosphate receptor type 1-like isoform X10*					
cAMP-dependent protein kinase catalytic subunit					
Actin, cytoplasmic					
actin-like					
actin-3-like isoform X1					
poly [ADP-ribose] polymerase 2-like isoform X2					
poly [ADP-ribose] polymerase 3-like					
epidermal growth factor receptor-like isoform X2					
epidermal growth factor receptor-like isoform X4					
basic immunoglobulin-like variable motif-containing protein isoform X5					
Oxidoreductase HTATIP2					
ATP-dependent zinc metalloprotease YME1L1-like					
GIMAP					
tax1-binding protein 1 homolog isoform X3*					
Autophagy					
autophagy-related protein 9A-like isoform X1 *					
transcription factor SPT20 homolog isoform X1					
vacuole membrane protein 1-like					
protein kinase C delta type					
DNA damage-regulated autophagy modulator protein 2-like					

DNA damage-regulated autophagy modulator protein 1-like*					
run domain Beclin-1-interacting and cysteine-rich domain-containing protein-like isoform X3					
phosphatidylinositol 4,5-bisphosphate 3-kinase catalytic subunit delta isoform-like isoform X1*					
TOLLIP (toll-interacting protein-like isoform X3)					
serine/threonine-protein kinase/endoribonuclease IRE1-like					
ubiquitin-like protein ATG12 isoform X1					
ubiquitin-like-conjugating enzyme ATG10 isoform X3					
inositol 1,4,5-trisphosphate receptor type 1-like isoform X10*					
protein kinase C delta type*					
insulin receptor substrate 1-B-like isoform X2 *					
Homeobox protein HD1*					
inositol 1,4,5-trisphosphate receptor type 1-like isoform X10*					
alpha-soluble NSF attachment protein-like					
endophilin-B1-like					
ras-related protein rab7					
hamartin-like isoform X2*					
ras-related protein M-Ras-like					
RAC-gamma serine/threonine-protein kinase-like isoform X1					
UV radiation resistance-associated gene protein-like					
next to BRCA1 gene 1 protein-like isoform X1					

Table 10: Patterns of differential gene expression that are part of phagosome, endosome, peroxisome, lysosome, antioxidant enzymes and acute phase proteins in oyster larvae in response to probiotic treatment ($p \leq 0.05$, upregulation: log fold change ≥ 2, downregulation: log fold change ≤ -2). Yellow denotes downregulation, orange denotes up and downregulation of transcripts mapped to the same gene, red denotes upregulation. HT-RI: larvae treated daily with probiotic *Bacillus pumilus* RI0695 (RI) for 5, 12 or 16

days; RI_6h: Larvae exposed to RI for 6h; RI_24h: Larvae exposed to RI for 24h; S4_6h: Larvae exposed to S4 for 6h; S4_24h: Larvae exposed to S4 for 24h.

	Probiotics				
	HT_RI	**RI_6h**	**RI_24h**	**S4_6h**	**S4_24h**
Phagosome					
Actin, cytoplasmic					
actin-like					
cathepsin L-like isoform X2					
cathepsin O-like					
cation-dependent mannose-6-phosphate receptor-like *					
Coagulation factor V *					
cytoplasmic dynein 1 heavy chain 1-like isoform X1 *					
cytoplasmic dynein 2 heavy chain 1-like isoform X4					
cytoplasmic dynein 1 light intermediate chain 2-like isoform X11 *					
cytoplasmic dynein 2 light intermediate chain 1-like *					
ras-related protein Rab-5B-like isoform X1					
macrophage mannose receptor 1-like isoform X1					
nitric oxide synthase brain-like isoform X2					
digestive cysteine proteinase 2-like*					
Lysosome					
lysosomal acid lipase/cholesteryl ester hydrolase-like					
lysosomal acid lipase/cholesteryl ester hydrolase-like isoform X2					
cation-dependent mannose-6-phosphate receptor-like *					
sulfatase-modifying factor 1-like					

lysosomal-associated transmembrane protein 4A-like					
ADP-ribosylation factor-binding protein GGA1-like *					
ADP-ribosylation factor-binding protein GGA1-like *					
AP-1 complex subunit gamma-1-like isoform X2					
AP-1 complex subunit sigma-2 isoform X4 *					
clathrin heavy chain 2 isoform X2					
lysosomal-trafficking regulator-like isoform X4					
lysosomal alpha-glucosidase-like isoform X1					
Endocytosis					
AP-2 complex subunit mu-1					
clathrin heavy chain 2 isoform X2					
tumor susceptibility gene 101 protein-like					
hepatocyte growth factor-regulated tyrosine kinase substrate-like					
syntaxin-7-like isoform X3					
phosphatidylinositol-binding clathrin assembly protein LAP-like *					
Peroxisome					
probable peroxisomal membrane protein PEX13					
D-aspartate oxidase-like *					
D-aspartate oxidase-like isoform X1					
phytanoyl-CoA dioxygenase, peroxisomal-like					
enoyl-CoA delta isomerase 2, mitochondrial-like isoform X2					
peroxisomal acyl-coenzyme A oxidase 1-like					
peroxisomal acyl-coenzyme A oxidase 1-like isoform X2					
peroxisome proliferator-activated receptor delta-like isoform X1*					
peroxisome proliferator-activated receptor gamma coactivator 1-alpha-like					

prostaglandin E synthase 2-like [Crassostrea virginica]					
prostaglandin E2 receptor EP4 subtype-like [Crassostrea virginica]					
prostaglandin G/H synthase 2-like isoform X2 [Crassostrea virginica] *					
prostaglandin reductase 1-like isoform X2 *					
peroxisomal carnitine O-octanoyltransferase-like					
Antioxidant enzymes					
glutathione peroxidase 7-like [Crassostrea virginica]					
glutathione S-transferase C-terminal domain-containing protein-like isoform X1 [Crassostrea virginica]					
glutathione S-transferase kappa 1-like [Crassostrea virginica]					
glutathione S-transferase omega-1-like					
glutathione S-transferase P 2-like					
glutathione S-transferase 3-like					
glutathione-independent glyoxalase HSP31-like					
glutathione S-transferase P 2-like					
maleylacetoacetate isomerase-like*					
gamma-glutamyltranspeptidase 1-like					
Superoxide dismutase [Cu-Zn]					
thioredoxin domain-containing protein 15-like					
thioredoxin domain-containing protein 3 homolog isoform X15					
thioredoxin domain-containing protein 5-like					
thioredoxin-like					
thioredoxin-like protein 1					
thioredoxin-related transmembrane protein 1-like isoform X1					
thioredoxin-related transmembrane protein 2 homolog					

Acute phase proteins					
heat shock 70 kDa protein 4*					
Heat shock 70 kDa protein 12A*					
heat shock 70 kDa protein 12A-like					
heat shock 70 kDa protein 12A-like isoform X1					
heat shock 70 kDa protein 12A-like isoform X3					
heat shock 70 kDa protein 12B-like					
heat shock 70 kDa protein 12B-like isoform X4 *					
heat shock factor protein-like					
heat shock protein 30C-like					
heat shock protein HSP 90-beta-like					
Stress response protein NhaX					

Table 11: Patterns of differential gene expression that are part of metabolism, biomineralization and other processes in oyster larvae in response to probiotic treatment ($p \leq 0.05$, upregulation: log fold change ≥ 2, downregulation: log fold change ≤ -2). Yellow denotes downregulation, orange denotes up and downregulation of transcripts mapped to the same gene, red denotes upregulation. HT-RI: larvae treated daily with probiotic *Bacillus pumilus* RI0695 (RI) for 5, 12 or 16 days; RI_6h: Larvae exposed to RI for 6h; RI_24h: Larvae exposed to RI for 24h; S4_6h: Larvae exposed to S4 for 6h; S4_24h: Larvae exposed to S4 for 24h.

	Probiotics				
	HT_RI	**RI_6h**	**RI_24h**	**S4_6h**	**S4_24h**
Others					
furin-like protease kpc-1 isoform X1					
multidrug resistance protein 1-like isoform X1					
multidrug resistance-associated protein 1-like isoform X3*					
multidrug resistance protein 1-like isoform X6					
multidrug resistance-associated protein 4-like					
multidrug resistance-associated protein 5-like					
multidrug resistance-associated protein 5-like isoform X2					
multidrug resistance-associated protein 7-like					
laccase-3-like					
laccase-like					
laccase-5-like					
peptidoglycan-recognition protein SC2-like					
glycine receptor subunit alpha-3-like isoform X5					
gamma-glutamyltranspeptidase 1-like					
1-phosphatidylinositol 4,5-bisphosphate phosphodiesterase gamma-1-like isoform X4					
cysteine proteinase inhibitor 8-like					
Hemicentin-1					
Hemicentin-1 like					
Hemicentin-1 like isoform X2					
Hemicentin-1 like isoform X3					
Hemicentin-1 like isoform X5					

Hemicentin-1 like isoform X6					
Hemicentin-1 like isoform X9					
Hemicentin-1 like isoform X21*					
Hemicentin-1 like isoform X34*					
Hemicentin-1 like isoform X40					
hemicentin-2-like isoform X2					
histamine H2 receptor-like					
oxidative stress-induced growth inhibitor 2-like					
cytochrome b [Crassostrea virginica]					
cytochrome b5 reductase 4-like isoform X3 [Crassostrea virginica]					
cytochrome c oxidase subunit I *					
cytochrome c oxidase subunit III (mitochondrion) *					
cytochrome P450 2C8					
cytochrome P450 2C42-like					
cytochrome P450 27C1-like					
cytochrome P450 2C28-like isoform X2 *					
cytochrome P450 2C42-like					
Cytochrome P450 2D14					
cytochrome P450 2F5-like *					
cytochrome P450 4F22-like					
cytochrome P450 2J5-like isoform X2*					
cytochrome P450 3A6-like					
Cytochrome P450 3A11					
cytochrome P450 3A24-like isoform X1					
cytochrome P450 3A29-like					

cytochrome P450 4A25-like					
cytochrome P450 4V2-like isoform X1					
cytochrome P450 4F22-like *					
dual specificity protein phosphatase 1-A-like [Crassostrea virginica]					
dual specificity protein phosphatase 14-like isoform X1 [Crassostrea virginica]					
dual specificity protein phosphatase 18-like [Crassostrea virginica]					
dual specificity protein phosphatase 19-like [Crassostrea virginica]					
dual specificity protein phosphatase 7-like [Crassostrea virginica]					
dual specificity tyrosine-phosphorylation-regulated kinase 4-like isoform X14 [Crassostrea virginica]					
protein phosphatase 1 regulatory subunit 12A-like isoform X2					
protein phosphatase 1 regulatory subunit 12A-like isoform X4					
protein phosphatase 1 regulatory subunit 16A-like isoform X3					
protein phosphatase 1 regulatory subunit 36-like isoform X1					
protein phosphatase 1 regulatory subunit 37-like					
protein phosphatase 1 regulatory subunit 42-like isoform X1					
Tripartite motif-containing protein 2					
tripartite motif-containing protein 2-like					
tripartite motif-containing protein 5-like					
tripartite motif-containing protein 5-like isoform X2					
tripartite motif-containing protein 2-like isoform X2					
tripartite motif-containing protein 2-like isoform X4					
tripartite motif-containing protein 2-like isoform X1					
tripartite motif-containing protein 3-like					
tripartite motif-containing protein 3-like isoform X1					
tripartite motif-containing protein 45-like					

tripartite motif-containing protein 55-like					
universal stress protein A-like protein isoform X5					
epididymal secretory protein E1-like *					
perilipin-2-like isoform X3*					
nitric oxide synthase brain-like isoform X2					
Ig-like and fibronectin type-III domain-containing protein 2 *					
macrophage migration inhibitory factor-like					
retinoic acid receptor RXR-gamma isoform X1					
NAD-dependent protein deacetylase sirtuin-1-like *					
cAMP-dependent protein kinase catalytic subunit					
PREDICTED: stress protein DDR48-like [Salmo salar]					
B-cell lymphoma 6 protein homolog isoform X3					
1-phosphatidylinositol 4,5-bisphosphate phosphodiesterase beta-1-like isoform X12					
cell wall integrity and stress response component 3-like isoform X3 [					
histone H2B-like					
Biomineralization					
perlucin-like					
perlucin-like isoform X1 *					
perlucin-like isoform X2					
perlucin-like protein					
perlucin-like protein isoform X1*					
Chitin synthase 3*					
Chitin synthase C					
putative carbonic anhydrase-like protein 1					

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CHAPTER 5

DISSERTATION SUMMARY: USE OF PROBIOTICS *BACILLUS PUMILUS* RI06-95 AND *PHAEOBACTER INHIBENS* S4 IN LARVICULTURE OF *CRASSOSTREA VIRGINICA* TO STIMULATE HOST IMMUNITY AND LIMIT IMPACT OF *VIBRIO CORALLIILYTICUS* RE22

This dissertation research confirms the benefits of use of probiotics *B. pumilus* RI06-95 and *P. inhibens* S4 as a natural and environmentally safe solution in disease management of *C. virginica* larviculture (Karim et al., 2013, Zhao et al., 2016, Sohn et al., 2016).

Use of suitably formulated probiotics can aid vibriosis management in hatchery larviculture of *Crassostrea virginica* preventing sudden and massive larval mortalities. This research demonstrates the successful formulation of a candidate probiotic strain, *Bacillus pumilus* RI06-95, that facilitates stable long-term storage and easy delivery in a hatchery setting. Daily treatment of oyster larvae with the spray dried formulation in pilot-scale hatchery trials provided significant protection against laboratory challenge with *Vibrio coralliilyticus* RE22 (RPS 43 ± 4 %). The results demonstrated that a sprayed-dried formulation for probiotic RI06-95 is a commercially viable product that can be safely and effectively used to limit negative impacts of vibriosis in shellfish hatcheries. Understanding host-microbe interactions between *C. virginica* larvae and pathogen or between larvae and probiotics would immensely help in designing protocols of probiotic use commercially.

This research showed the swift progression of disease both in terms of rapidly increasing mortality post 14h of exposure as well as impact on host immune system. Immunological responses of *C. virginica* larvae to pathogen *V. coralliilyticus* RE22, as measured through transcriptome analysis, suggest the ability of vibrio exposure to suppress immune-related pathway activation and immune effector production. The research also highlights the need and suitability of preventative measures like probiotics rather than treatment options to protect larvae from effects of *V. coralliilyticus* RE22.

This dissertation research on the immunological responses of *C. virginica* larvae to both probiotics *B. pumilus* RI06-95 and *P. inhibens* S4 shows that the immunosuppression by RE22 may be counteracted by probiotics 'priming' of the larval immune response. This research demonstrates the ability of both probiotics to activate pathogen recognition receptors (PRRs) that could aid in pathogen detection, activation of immune signaling pathways and production of immune effectors that could potentially aid in inactivation of RE22 and its virulence factors.

A hypothesized model based on the findings of this dissertation research and previously published work is proposed here (Fig 1). When *C. virginica* larvae are pretreated with probiotics, RI and S4 for 6 to 24 h, most larvae are protected from RE22 challenge (Fig1-1). A more prolonged 24 h pretreatment (versus 6 h) allows for more consistent elicitation of immune responses, and therefore more consistent levels of protection against RE22. Immune responses include activation of PRRs, immune signaling pathways and production of immune effectors like mucins, serine protease inhibitors and perforn-2 (Fig1-2). Oysters have a high basal rate of apoptosis that regulate hemocyte number (Sokolova 2009). Transcriptomic data suggests treatment with probiotics may inhibit hemocyte apoptosis, leading to increase in the number of hemocytes (Fig1-3). This immunostimulation likely contributes to clearing probiotics from the system (Karim et al., 2013), but also may contribute to counteracting RE22 virulence. When probiotic pretreated (and hence immunostimulated larvae) are challenged with RE22, a series of changes brought about by the probiotics in the host may assist the larvae in blocking RE22 (Fig1-4). Increased mucin production may enhance the epithelial barrier blocking penetration and prevent adhesion of pathogen.

Increased production of serine protease inhibitors may help to counter the effect of serine proteases potentially produced by RE22. This immunomodulation would complement other mechanisms of action of probiotics. Probiotic biofilm established during the pretreatment period may reduce colonization sites for RE22 competitively excluding them from colonizing the gut. Biofilm formation and competition assays between S4 and RE22 showed pretreatment with S4 excludes RE22 (Zhao et al., 2016). The draft genome of RI suggested its ability to form biofilms (Hamblin et al., 2015) but there is no experimental data to support it yet. Antibiotic tropodithietic acid (TDA) produced by S4 also aids in eliminating RE22 (Karim et al., 2013). S4 also secretes N-acyl homoserine lactones (AHLs) that quorum quench RE22 metalloprotease gene expression that are a crucial part of its virulence (Zhao et al., 2018). Increased production of perforin-2 due to probiotic pretreatment may also aid in neutralizing pathogens both intracellularly and extracellularly within oyster tissues. Increased number of hemocytes owing to apoptosis inhibition post probiotic treatment may increase phagocytic pressure on RE22 as well as buffer cytotoxic effects of hemolysins secreted by RE22 (Fig1-5) that diminish hemocyte survival (Gomez-Leon et al., 2008). All these effects probably work in concert to allow more probiotic pretreated *C. virginica* larvae to survive post RE22 challenge than those without probiotic pretreatment, by effectively reducing the infective dose of RE22 (Fig1-4) and providing larvae with mechanisms to further neutralize and kill RE22 within the oyster tissues (Fig1-5), leading to increased survival (Fig1-6). Due to effective clearing of probiotics within oysters due to the larval immune response, however, their protective effect

diminishes over time as also seen in experimental evidence (Karim et al., 2013) unless probiotics are applied repeatedly.

Immune effectors produced in response to probiotics, specifically highlighted in this study are highly suitable in blocking virulence factors and pathogenesis of RE22. However, application of probiotics and their overall immunostimulatory effect may likely help in protecting larvae from other bacterial and viral infections. Thus, this research advocates use of probiotic formulations in commercial shellfish aquaculture for their beneficial effects. In addition, it provides new insights in oyster immunity in response to non-pathogenic bacteria and the crosstalk between host and probiotics.

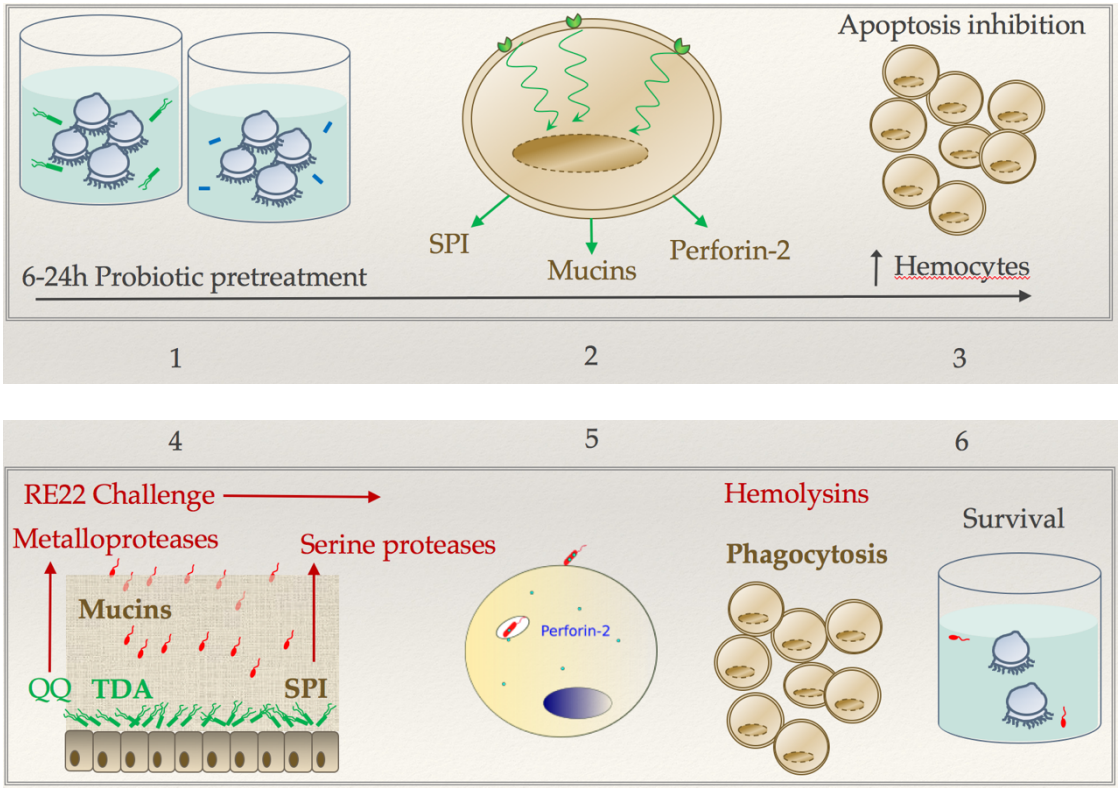

Figure 1: Hypothesized model showing in a series of steps (1-6) how effects of probiotic pretreatment on host immunity may complement other mechanisms of action of probiotics in providing protection from *V. coralliilyticus* RE22 challenge. QQ: quorum quenching, SPI: serine protease inhibitor, TDA: tropodithietic acid.

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APPENDIX

Table 1. Two-way ANOVA for the levels of *Vibrios* in water, tank surface, and oyster on each trial with RI formulations.

ANOVA table	SS	DF	MS	F (DFn, DFd)	P value
Trial I: oyster					
Interaction	9.009	2	4.505	F (2, 10) = 2.278	P = 0.1530
Time	1.689	2	0.8446	F (2, 10) = 0.4271	P = 0.6638
Treatment	0.03975	1	0.03975	F (1, 5) = 0.07956	P = 0.7892
Subjects (matching)	2.498	5	0.4996	F (5, 10) = 0.2526	P = 0.9289
Residual	19.78	10	1.978		
Trial I: water					
Interaction	2.834	2	1.417	F (2, 10) = 2.879	P = 0.1029
Time	4.138	2	2.069	F (2, 10) = 4.204	P = 0.0473
Treatment	0.05357	1	0.05357	F (1, 5) = 0.03503	P = 0.8589
Subjects (matching)	7.647	5	1.529	F (5, 10) = 3.107	P = 0.0599
Residual	4.922	10	0.4922		
Trial II: oyster					
Interaction	4.051	6	0.6752	F (6, 16) = 1.467	P = 0.2512
Time	46.39	2	23.19	F (2, 16) = 50.39	P < 0.0001
Treatment	8.178	3	2.726	F (3, 8) = 4.766	P = 0.0344
Subjects (matching)	4.576	8	0.572	F (8, 16) = 1.243	P = 0.3372
Residual	7.364	16	0.4603		
Trial II: tank surface					
Interaction	5.513	6	0.9188	F (6, 16) = 0.4252	P = 0.8515
Time	58.79	2	29.39	F (2, 16) = 13.60	P = 0.0004
Treatment	20.56	3	6.854	F (3, 8) = 4.529	P = 0.0389
Subjects (matching)	12.11	8	1.513	F (8, 16) = 0.7004	P = 0.6872
Residual	34.57	16	2.161		

Table 2: Differentially expressed genes with log fold change for probiotic or pathogen treatments when compared to control (Con 0 h) in laboratory transcriptomes ($p \leq 0.05$, upregulation: log fold change ≥ 2, downregulation: log fold change ≤ -2). RI_6h: Larvae exposed to RI for 6h; RI_24h: Larvae exposed to RI for 24h; S4_6h: Larvae exposed to S4 for 6h; S4_24h: Larvae exposed to S4 for 24h; RE22_6h: Larvae exposed to RE22 for 6h.

Log2FoldChange	**Hit_def**	**Treatment**
Recognition		
TLRs		
23.05478838	toll-like receptor 13 [Crassostrea virginica]	S4_6h
-22.74173118	toll-like receptor 13 [Crassostrea virginica]	S4_24h
20.83600237	toll-like receptor 13 [Crassostrea virginica]	S4_24h
21.32340853	toll-like receptor 13 [Crassostrea virginica]	RI_24h
22.15592205	toll-like receptor 13 [Crassostrea virginica]	RE22_6h
21.51071325	toll-like receptor 13 isoform X1 [Crassostrea virginica]	S4_24h

22.42155892	toll-like receptor 13 isoform X1 [Crassostrea virginica]	S4_24h
17.30506755	toll-like receptor 13 isoform X1 [Crassostrea virginica]	RI_24h
21.06459929	toll-like receptor 3 isoform X1 [Crassostrea virginica]	S4_24h
22.49716465	toll-like receptor 3 isoform X1 [Crassostrea virginica]	RI_24h
-22.48957053	toll-like receptor 4 [Crassostrea virginica]	S4_6h
-23.60095447	toll-like receptor 4 [Crassostrea virginica]	RI_24h
21.86007267	toll-like receptor 4 [Crassostrea virginica]	S4_6h
20.06293431	toll-like receptor 4 [Crassostrea virginica]	S4_24h
18.52915288	toll-like receptor 4 [Crassostrea virginica]	RI_6h
20.85337828	toll-like receptor 4 [Crassostrea virginica]	RE22_6h
-27.08359576	toll-like receptor 4 isoform X1 [Crassostrea virginica]	S4_6h
-23.23844181	toll-like receptor 4 isoform X1 [Crassostrea virginica]	S4_24h
-22.60452533	toll-like receptor 4 isoform X1 [Crassostrea virginica]	S4_24h
-21.22164677	toll-like receptor 4 isoform X1 [Crassostrea virginica]	S4_24h

-3.670100715	toll-like receptor 4 isoform X1 [Crassostrea virginica]	S4_24h
-21.77985815	toll-like receptor 4 isoform X1 [Crassostrea virginica]	RI_6h
-25.3837144	toll-like receptor 4 isoform X1 [Crassostrea virginica]	RE22_6h
-20.33473167	toll-like receptor 4 isoform X1 [Crassostrea virginica]	RE22_6h
-23.85245048	toll-like receptor 6 isoform X1 [Crassostrea virginica]	S4_6h
-24.52152638	toll-like receptor 6 isoform X1 [Crassostrea virginica]	RI_6h
6.948335986	toll-like receptor Tollo isoform X2 [Crassostrea virginica]	RI_24h
6.751339206	toll-like receptor Tollo isoform X2 [Crassostrea virginica]	RE22_6h
-18.59470318	toll-interacting protein-like isoform X3 [Crassostrea virginica]	RE22_6h
Lectins		
20.59470698	lectin BRA-3-like [Crassostrea virginica]	S4_24h
-21.67159	lectin BRA-3-like [Crassostrea virginica]	RI_6h
15.5998376	lectin BRA-3-like [Crassostrea virginica]	RI_24h
-21.98669883	hepatic lectin-like [Crassostrea virginica]	S4_6h

-28.16031439	complement C1q tumor necrosis factor-related protein 4-like isoform X3 [Crassostrea virginica]	RI_6h
-2.942969529	complement C1q-like protein 2 [Crassostrea virginica]	S4_6h
-2.720935883	complement C1q-like protein 2 [Crassostrea virginica]	RE22_6h
-4.990716982	complement C1q-like protein 4 [Crassostrea virginica]	RE22_6h
5.323119114	fucolectin-like [Crassostrea virginica]	S4_6h
5.500587362	fucolectin-like [Crassostrea virginica]	RI_6h
6.563854383	fucolectin-like [Crassostrea virginica]	RE22_6h
-21.98669883	hepatic lectin-like [Crassostrea virginica]	S4_6h
Scavenger receptors		
-24.64487832	scavenger receptor class B member 1 isoform B [Alligator mississippiensis]	RI_6h
-25.10222908	scavenger receptor class B member 1 isoform B [Alligator mississippiensis]	RE22_6h
-5.982694265	scavenger receptor class F member 2-like [Crassostrea virginica]	S4_6h
PGRP		

-5.798327381	peptidoglycan-recognition protein SC2-like [Crassostrea virginica]	S4_6h
LRRs		
-5.147401101	leucine-rich repeat and fibronectin type III domain-containing protein 1-like protein [Crassostrea virginica]	S4_24h
-6.212337034	leucine-rich repeat and fibronectin type III domain-containing protein 1-like protein [Crassostrea virginica]	RI_24h
-21.92443052	leucine-rich repeat and fibronectin type-III domain-containing protein 5-like isoform X1 [Crassostrea virginica]	RI_24h
9.844842922	leucine-rich repeat transmembrane neuronal protein 3-like isoform X1 [Crassostrea virginica]	RI_6h
20.97197566	leucine-rich repeat transmembrane neuronal protein 3-like isoform X1 [Crassostrea virginica]	RI_6h
20.96078701	leucine-rich repeat transmembrane neuronal protein 3-like isoform X1 [Crassostrea virginica]	RE22_6h

-23.84553133	leucine-rich repeat transmembrane protein FLRT1-like [Crassostrea virginica]	S4_6h
-23.84658918	leucine-rich repeat transmembrane protein FLRT1-like [Crassostrea virginica]	S4_24h
-23.04060289	leucine-rich repeat-containing protein 24-like [Crassostrea virginica]	S4_24h
-15.08871621	leucine-rich repeat-containing protein 24-like [Crassostrea virginica]	S4_24h
-8.156979152	leucine-rich repeat-containing protein 24-like [Crassostrea virginica]	S4_24h
20.08727174	leucine-rich repeat-containing protein 28-like isoform X3 [Crassostrea virginica]	S4_24h
6.821818096	leucine-rich repeat-containing protein 34-like isoform X2 [Crassostrea virginica]	S4_6h
-23.06503275	leucine-rich repeat-containing protein 45-like [Crassostrea virginica]	S4_24h
-23.72912188	leucine-rich repeat-containing protein 45-like [Crassostrea virginica]	RI_24h
22.71972652	leucine-rich repeat-containing protein 4C-like isoform X1 [Crassostrea virginica]	S4_6h
20.7202629	leucine-rich repeat-containing protein 4C-like isoform X1 [Crassostrea virginica]	RI_6h
22.88621537	leucine-rich repeat-containing protein 4C-like isoform X1 [Crassostrea virginica]	RI_24h
-24.40103127	leucine-rich repeat-containing protein 70-like [Crassostrea virginica]	S4_24h
-25.58565569	leucine-rich repeat-containing protein 71-like isoform X21 [Crassostrea virginica]	S4_24h

-11.00397714	leucine-rich repeat-containing protein 74A-like isoform X2 [Crassostrea virginica]	S4_6h
-11.92749456	leucine-rich repeat-containing protein 74A-like isoform X2 [Crassostrea virginica]	S4_6h
-22.72669381	leucine-rich repeat-containing protein 74B-like [Crassostrea virginica]	S4_24h
-23.52071418	leucine-rich repeat-containing protein 74B-like [Crassostrea virginica]	RI_24h
5.554434603	leucine-rich repeat-containing protein 74B-like isoform X6 [Crassostrea virginica]	S4_6h
7.401747777	leucine-rich repeat-containing protein 74B-like isoform X6 [Crassostrea virginica]	S4_24h
-23.01756736	leucine-rich repeat-containing protein 9-like isoform X2 [Crassostrea virginica]	RI_6h
-5.5609716	leucine-rich repeat-containing protein 9-like isoform X2 [Crassostrea virginica]	RI_6h
-5.297693704	leucine-rich repeat-containing protein 9-like isoform X2 [Crassostrea virginica]	RE22_6h
Fibronectin type III domain		
-4.785308683	fibronectin type III domain-containing protein 1-like [Crassostrea virginica]	S4_24h
-23.90865588	fibronectin type III domain-containing protein 2-like isoform X3 [Crassostrea virginica]	RI_24h
-22.69764157	fibronectin type III domain-containing protein 2-like isoform X3 [Crassostrea virginica]	RI_24h
-24.13260348	fibronectin type III domain-containing protein 2-like isoform X3 [Crassostrea virginica]	RE22_6h

C1q proteins		
-28.16031439	complement C1q tumor necrosis factor-related protein 4-like isoform X3 [Crassostrea virginica]	RI_6h
-2.942969529	complement C1q-like protein 2 [Crassostrea virginica]	S4_6h
-2.720935883	complement C1q-like protein 2 [Crassostrea virginica]	RE22_6h
-4.990716982	complement C1q-like protein 4 [Crassostrea virginica]	RE22_6h
-27.07147054	alpha-1-macroglobulin-like [Crassostrea virginica]	S4_6h
-27.09597681	alpha-1-macroglobulin-like [Crassostrea virginica]	S4_24h
-4.827641592	alpha-1-macroglobulin-like [Crassostrea virginica]	S4_24h
-16.82943987	Macrophage mannose receptor 1 [Crassostrea gigas]	S4_24h
-23.67745527	macrophage mannose receptor 1-like isoform X1 [Crassostrea virginica]	RI_24h
Metabolic Enzymes with New Role of Carbohydrate Binding		
-3.019898078	Phosphoenolpyruvate carboxykinase	S4_24h
20.50362782	hexokinase-2-like isoform X2 [Crassostrea virginica]	S4_6h

-27.8856754	hexokinase-2-like isoform X2 [Crassostrea virginica]	RI_6h
-24.29212676	hexokinase-2-like isoform X2 [Crassostrea virginica]	RI_6h
10.04796022	hexokinase-2-like isoform X2 [Crassostrea virginica]	RI_6h
20.45474609	hexokinase-2-like isoform X2 [Crassostrea virginica]	RI_6h
-27.93663561	hexokinase-2-like isoform X2 [Crassostrea virginica]	RI_24h
-28.20643409	hexokinase-2-like isoform X2 [Crassostrea virginica]	RE22_6h
-24.71581303	hexokinase-2-like isoform X2 [Crassostrea virginica]	RE22_6h
11.04164794	hexokinase-2-like isoform X2 [Crassostrea virginica]	RE22_6h
21.25153221	hexokinase-2-like isoform X2 [Crassostrea virginica]	RE22_6h
Cholinergic immunomodulation		
2.908686031	glutamate receptor 2-like [Crassostrea virginica]	RI_6h
-23.53192783	glutamate receptor ionotropic	S4_24h
-8.851813981	glutamate receptor ionotropic	S4_24h
-26.48442583	glutamate receptor ionotropic	RI_6h

-22.22604191	glutamate receptor ionotropic	RE22_6h
20.55673148	glutamate receptor-interacting protein 1-like isoform X4 [Crassostrea virginica]	RI_24h
7.766510402	muscarinic acetylcholine receptor M3-like [Crassostrea virginica]	RE22_6h
-23.96329241	neuronal acetylcholine receptor subunit alpha-2-like [Crassostrea virginica]	RE22_6h
10.39346971	neuronal acetylcholine receptor subunit alpha-5-like isoform X1 [Crassostrea virginica]	RE22_6h
21.77501036	neuronal acetylcholine receptor subunit alpha-9-like [Crassostrea virginica]	S4_24h
21.26581499	neuronal acetylcholine receptor subunit alpha-9-like [Crassostrea virginica]	RI_24h
-23.03542166	neuronal acetylcholine receptor subunit alpha-9-like [Crassostrea virginica]	S4_24h
-4.54262089	neuropeptide SIFamide receptor-like [Crassostrea virginica]	S4_6h
-23.60128855	neuropeptide Y receptor type 2-like [Crassostrea virginica]	RE22_6h
Signaling pathways in Immune response		
-11.1762089	TNF receptor-associated factor 3-like isoform X3 [Crassostrea virginica]	S4_6h
-9.894522176	TNF receptor-associated factor 4-like isoform X5 [Crassostrea virginica]	RE22_6h
24.64790366	LOW QUALITY PROTEIN: tyrosine-protein kinase JAK2-like [Crassostrea virginica]	S4_6h

-23.39582662	LOW QUALITY PROTEIN: tyrosine-protein kinase JAK2-like [Crassostrea virginica]	S4_24h
-4.478976796	LOW QUALITY PROTEIN: tyrosine-protein kinase JAK2-like [Crassostrea virginica]	S4_24h
-21.85010489	LOW QUALITY PROTEIN: tyrosine-protein kinase JAK2-like [Crassostrea virginica]	RI_6h
25.29177394	LOW QUALITY PROTEIN: tyrosine-protein kinase JAK2-like [Crassostrea virginica]	RI_24h
24.11940272	LOW QUALITY PROTEIN: tyrosine-protein kinase JAK2-like [Crassostrea virginica]	RE22_6h
-7.662488479	signal transducer and activator of transcription 3-like isoform X6 [Crassostrea virginica]	RI_24h
-26.82508167	LOW QUALITY PROTEIN: son of sevenless homolog 2-like [Crassostrea virginica]	RE22_6h
-20.08059767	LOW QUALITY PROTEIN: son of sevenless homolog 2-like [Crassostrea virginica]	S4_24h
-20.7042899	LOW QUALITY PROTEIN: son of sevenless homolog 2-like [Crassostrea virginica]	RI_24h
-21.98909234	epidermal growth factor receptor-like [Crassostrea virginica]	S4_24h
-21.85708608	epidermal growth factor receptor-like isoform X2 [Crassostrea virginica]	S4_6h
-22.21636099	epidermal growth factor receptor-like isoform X2 [Crassostrea virginica]	RI_24h
3.582236878	epidermal growth factor receptor-like isoform X2 [Crassostrea virginica]	S4_6h
-26.61502073	epidermal growth factor receptor-like isoform X4 [Crassostrea virginica]	S4_24h

4.440719006	epidermal growth factor receptor-like isoform X4 [Crassostrea virginica]	S4_24h
4.780146091	PREDICTED: tyrosine-protein phosphatase non-receptor type 11 isoform X3 [Crassostrea gigas]	S4_6h
-24.59389772	tyrosine-protein phosphatase non-receptor type 13-like isoform X3 [Crassostrea virginica]	S4_6h
-21.79438105	tyrosine-protein phosphatase non-receptor type 13-like isoform X3 [Crassostrea virginica]	S4_6h
-21.90808833	tyrosine-protein phosphatase non-receptor type 13-like isoform X3 [Crassostrea virginica]	S4_24h
-23.41598784	tyrosine-protein phosphatase non-receptor type 23-like isoform X1 [Crassostrea virginica]	S4_6h
20.99622685	tyrosine-protein phosphatase non-receptor type 23-like isoform X1 [Crassostrea virginica]	S4_6h

20.32249114	tyrosine-protein phosphatase non-receptor type 23-like isoform X1 [Crassostrea virginica]	S4_24h
21.72359644	tyrosine-protein phosphatase non-receptor type 23-like isoform X1 [Crassostrea virginica]	RI_6h
22.3115501	tyrosine-protein phosphatase non-receptor type 23-like isoform X1 [Crassostrea virginica]	RI_24h
20.18049393	tyrosine-protein phosphatase non-receptor type 23-like isoform X1 [Crassostrea virginica]	RE22_6h
19.62125156	tyrosine-protein phosphatase non-receptor type 4-like isoform X4 [Crassostrea virginica]	S4_6h
21.66755905	tyrosine-protein phosphatase non-receptor type 4-like isoform X4 [Crassostrea virginica]	S4_24h
23.55240592	tyrosine-protein phosphatase non-receptor type 4-like isoform X4 [Crassostrea virginica]	RI_6h

22.38124103	tyrosine-protein phosphatase non-receptor type 4-like isoform X4 [Crassostrea virginica]	RI_24h
23.05607193	tyrosine-protein phosphatase non-receptor type 4-like isoform X4 [Crassostrea virginica]	RE22_6h
-10.65160003	tyrosine-protein phosphatase non-receptor type 4-like isoform X6 [Crassostrea virginica]	S4_6h
-10.6365949	tyrosine-protein phosphatase non-receptor type 4-like isoform X6 [Crassostrea virginica]	S4_24h
-8.931960108	tyrosine-protein phosphatase non-receptor type 4-like isoform X6 [Crassostrea virginica]	RE22_6h
-24.13233234	tyrosine-protein phosphatase non-receptor type 9-like isoform X2 [Crassostrea virginica]	RI_24h
-23.73611409	NF-kappa-B inhibitor alpha-like isoform X1 [Crassostrea virginica]	S4_6h
-9.500032383	NF-kappa-B inhibitor alpha-like isoform X1 [Crassostrea virginica]	RI_6h

-9.657932705	NF-kappa-B inhibitor alpha-like isoform X1 [Crassostrea virginica]	RE22_6h
-24.81953437	NF-kappa-B-activating protein-like [Crassostrea virginica]	S4_6h
-24.69597209	NF-kappa-B-activating protein-like [Crassostrea virginica]	S4_24h
-24.42843374	NF-kappa-B-activating protein-like [Crassostrea virginica]	S4_24h
-23.65126954	NF-kappa-B-activating protein-like [Crassostrea virginica]	S4_24h
-24.77085054	NF-kappa-B-activating protein-like [Crassostrea virginica]	RI_6h
-25.40980564	NF-kappa-B-activating protein-like [Crassostrea virginica]	RI_24h
-24.86565855	NF-kappa-B-activating protein-like [Crassostrea virginica]	RI_24h
-25.18633897	NF-kappa-B-activating protein-like [Crassostrea virginica]	RE22_6h
-21.62114724	smad nuclear interacting protein 1-like [Crassostrea virginica]	S4_6h
-22.01002997	smad nuclear interacting protein 1-like [Crassostrea virginica]	RI_6h
22.23806571	TNFAIP3-interacting protein 1-like [Crassostrea virginica]	RI_6h
23.32946941	TNFAIP3-interacting protein 1-like [Crassostrea virginica]	RE22_6h
3.669180885	PREDICTED: B-cell lymphoma/leukemia 10-like [Crassostrea gigas]	S4_6h

5.125890801	PREDICTED: B-cell lymphoma/leukemia 10-like [Crassostrea gigas]	S4_24h
-24.48845992	ELKS/Rab6-interacting/CAST family member 1-like isoform X3 [Crassostrea virginica]	RI_6h
-24.52498266	ELKS/Rab6-interacting/CAST family member 1-like isoform X3 [Crassostrea virginica]	RI_24h
22.90392037	ELKS/Rab6-interacting/CAST family member 1-like isoform X5 [Crassostrea virginica]	S4_24h
21.99071309	ELKS/Rab6-interacting/CAST family member 1-like isoform X5 [Crassostrea virginica]	RI_24h
21.29621653	adapter protein CIKS-like [Crassostrea virginica]	S4_6h
21.89981234	adapter protein CIKS-like [Crassostrea virginica]	S4_24h
19.10179883	adapter protein CIKS-like [Crassostrea virginica]	RI_6h
21.9145228	adapter protein CIKS-like [Crassostrea virginica]	RI_24h
21.96174137	adapter protein CIKS-like [Crassostrea virginica]	RE22_6h
-21.68449844	adapter protein CIKS-like isoform X4 [Crassostrea virginica]	RI_24h
-5.87337841	PREDICTED: dual specificity mitogen-activated protein kinase kinase 1-like isoform X1 [Crassostrea gigas]	RI_24h
-25.72060852	MAP kinase-activated protein kinase 2-like isoform X2 [Crassostrea virginica]	RI_24h

-2.470755161	mitogen-activated protein kinase kinase kinase 13-like isoform X2 [Crassostrea virginica]	S4_24h
-24.25502986	mitogen-activated protein kinase kinase kinase 13-like isoform X2 [Crassostrea virginica]	RI_24h
-7.716909755	Mitogen-activated protein kinase kinase kinase 7 [Crassostrea gigas]	RI_6h
-21.70047837	mitogen-activated protein kinase kinase kinase 7-like isoform X3 [Crassostrea virginica]	S4_6h
-2.753446313	mitogen-activated protein kinase kinase kinase 7-like isoform X3 [Crassostrea virginica]	S4_6h
22.14143778	mitogen-activated protein kinase kinase kinase 7-like isoform X3 [Crassostrea virginica]	S4_6h
-21.62740093	mitogen-activated protein kinase kinase kinase 7-like isoform X3 [Crassostrea virginica]	S4_24h
21.84933712	mitogen-activated protein kinase kinase kinase 7-like isoform X3 [Crassostrea virginica]	RI_6h
-3.340398882	mitogen-activated protein kinase kinase kinase 7-like isoform X3 [Crassostrea virginica]	RI_24h
-22.1261532	mitogen-activated protein kinase kinase kinase 7-like isoform X3 [Crassostrea virginica]	RE22_6h
20.30696981	dual specificity mitogen-activated protein kinase kinase 7-like isoform X1 [Crassostrea virginica]	S4_6h

21.95392224	dual specificity mitogen-activated protein kinase kinase 7-like isoform X1 [Crassostrea virginica]	S4_24h
21.12567936	dual specificity mitogen-activated protein kinase kinase 7-like isoform X1 [Crassostrea virginica]	RI_6h
-18.51291804	dual specificity mitogen-activated protein kinase kinase 7-like isoform X1 [Crassostrea virginica]	RI_24h
22.55409809	dual specificity mitogen-activated protein kinase kinase 7-like isoform X1 [Crassostrea virginica]	RE22_6h
-10.42721287	LOW QUALITY PROTEIN: mitogen-activated protein kinase 14A-like [Crassostrea virginica]	RI_6h
-22.51874945	LOW QUALITY PROTEIN: mitogen-activated protein kinase kinase kinase kinase 3-like [Crassostrea virginica]	S4_24h
-23.17120745	LOW QUALITY PROTEIN: mitogen-activated protein kinase kinase kinase kinase 3-like [Crassostrea virginica]	RI_24h

7.880002894	transforming growth factor-beta, partial [Crassostrea ariakensis]	RI_24h
6.414756431	transforming growth factor-beta, partial [Crassostrea ariakensis]	S4_24h
-24.5406632	LOW QUALITY PROTEIN: C-Jun-amino-terminal kinase-interacting protein 4-like [Crassostrea virginica]	S4_24h
-23.28637433	LOW QUALITY PROTEIN: C-Jun-amino-terminal kinase-interacting protein 4-like [Crassostrea virginica]	S4_24h
8.201025972	extracellular signal-regulated kinase 2-like isoform X4 [Crassostrea virginica]	RI_24h
-22.50186319	stimulator of interferon genes protein-like [Crassostrea virginica]	S4_6h
20.17836595	stimulator of interferon genes protein-like [Crassostrea virginica]	S4_6h
-16.95226096	stimulator of interferon genes protein-like [Crassostrea virginica]	S4_24h
-7.478972473	stimulator of interferon genes protein-like [Crassostrea virginica]	RI_6h
17.42282629	stimulator of interferon genes protein-like [Crassostrea virginica]	RI_6h
20.17813132	stimulator of interferon genes protein-like [Crassostrea virginica]	RI_24h
20.42313211	stimulator of interferon genes protein-like [Crassostrea virginica]	RE22_6h

-6.713339842	death domain-containing protein 1-like [Crassostrea virginica]	S4_6h
-6.937781661	death domain-containing protein 1-like [Crassostrea virginica]	S4_24h
-4.130539001	death domain-containing protein 1-like [Crassostrea virginica]	RI_6h
-6.57016357	death domain-containing protein 1-like [Crassostrea virginica]	RE22_6h
21.29117555	death domain-containing protein CRADD-like [Crassostrea virginica]	S4_6h
19.33282647	death domain-containing protein CRADD-like [Crassostrea virginica]	RI_6h
21.07018009	death domain-containing protein CRADD-like [Crassostrea virginica]	RE22_6h
19.40341779	integrin alpha-4-like isoform X1 [Crassostrea virginica]	S4_24h
20.6821385	integrin alpha-4-like isoform X1 [Crassostrea virginica]	RI_24h
-2.282127264	integrin beta-3-like [Crassostrea virginica]	S4_24h
11.0047672	ubiquitin carboxyl-terminal hydrolase 14-like [Mizuhopecten yessoensis]	S4_6h
21.44939539	ubiquitin carboxyl-terminal hydrolase 14-like [Mizuhopecten yessoensis]	S4_24h
11.5782336	ubiquitin carboxyl-terminal hydrolase 14-like [Mizuhopecten yessoensis]	RI_6h
21.74942278	ubiquitin carboxyl-terminal hydrolase 14-like [Mizuhopecten yessoensis]	RI_24h

11.12138173	ubiquitin carboxyl-terminal hydrolase 14-like [Mizuhopecten yessoensis]	RE22_6h
-24.21434106	ubiquitin carboxyl-terminal hydrolase 20-like isoform X2 [Crassostrea virginica]	S4_24h
22.53788386	ubiquitin carboxyl-terminal hydrolase 25-like isoform X3 [Crassostrea virginica]	S4_6h
19.53896908	ubiquitin carboxyl-terminal hydrolase 25-like isoform X3 [Crassostrea virginica]	S4_24h
21.61706409	ubiquitin carboxyl-terminal hydrolase 25-like isoform X3 [Crassostrea virginica]	RI_6h
21.55529349	ubiquitin carboxyl-terminal hydrolase 25-like isoform X3 [Crassostrea virginica]	RI_24h
22.99322029	ubiquitin carboxyl-terminal hydrolase 25-like isoform X3 [Crassostrea virginica]	RE22_6h
	Effectors	
-7.636639706	serine protease 44-like [Crassostrea virginica]	S4_24h
21.50670849	serine protease inhibitor Cvsi-2-like [Crassostrea virginica]	S4_6h
12.83482328	serine protease inhibitor Cvsi-2-like [Crassostrea virginica]	S4_24h
21.11968406	serine protease inhibitor Cvsi-2-like [Crassostrea virginica]	RI_6h
20.26304644	serine protease inhibitor Cvsi-2-like [Crassostrea virginica]	RI_24h
28.4447526	serine protease inhibitor dipetalogastin-like [Crassostrea virginica]	S4_6h

22.31014239	serine protease inhibitor dipetalogastin-like [Crassostrea virginica]	RI_24h
15.3518296	serine protease inhibitor dipetalogastin-like [Crassostrea virginica]	S4_24h
-22.79409461	kunitz-type protease inhibitor 1-like [Crassostrea virginica]	S4_6h
20.0825116	LOW QUALITY PROTEIN: digestive cysteine proteinase 2-like [Crassostrea virginica]	S4_6h
20.26000192	LOW QUALITY PROTEIN: digestive cysteine proteinase 2-like [Crassostrea virginica]	S4_24h
21.99420619	LOW QUALITY PROTEIN: digestive cysteine proteinase 2-like [Crassostrea virginica]	RI_6h
19.1215388	LOW QUALITY PROTEIN: digestive cysteine proteinase 2-like [Crassostrea virginica]	RI_24h
-23.58012026	Signaling mucin HKR1 [Mizuhopecten yessoensis]	S4_6h
-23.80421719	Signaling mucin HKR1 [Mizuhopecten yessoensis]	S4_24h
-9.356963695	Signaling mucin HKR1 [Mizuhopecten yessoensis]	S4_24h
-24.38357841	Signaling mucin HKR1 [Mizuhopecten yessoensis]	RI_6h
-21.90572271	Signaling mucin HKR1 [Mizuhopecten yessoensis]	RI_6h
-16.19910691	Signaling mucin HKR1 [Mizuhopecten yessoensis]	RI_24h
-24.51441136	Signaling mucin HKR1 [Mizuhopecten yessoensis]	RE22_6h

-2.369460824	integumentary mucin C.1-like [Crassostrea virginica]	S4_24h
3.541415795	integumentary mucin C.1-like [Crassostrea virginica]	RI_24h
21.31111159	integumentary mucin C.1-like isoform X1 [Crassostrea virginica]	S4_24h
-15.40397187	integumentary mucin C.1-like isoform X1 [Crassostrea virginica]	RI_24h
-23.4017782	integumentary mucin C.1-like isoform X3 [Crassostrea virginica]	S4_24h
-24.11558579	integumentary mucin C.1-like isoform X3 [Crassostrea virginica]	RI_6h
15.84736053	LOW QUALITY PROTEIN: mucin-12-like [Crassostrea virginica]	S4_6h
19.8605114	LOW QUALITY PROTEIN: mucin-12-like [Crassostrea virginica]	S4_24h
21.24020302	LOW QUALITY PROTEIN: mucin-12-like [Crassostrea virginica]	RI_6h
20.3172585	LOW QUALITY PROTEIN: mucin-12-like [Crassostrea virginica]	RI_24h
20.50006596	LOW QUALITY PROTEIN: mucin-12-like [Crassostrea virginica]	RE22_6h
-25.58549501	mucin-17-like isoform X2 [Crassostrea virginica]	S4_6h
-22.60499384	mucin-17-like isoform X2 [Crassostrea virginica]	S4_24h
-23.3302576	mucin-17-like isoform X2 [Crassostrea virginica]	RI_24h

-12.40357314	mucin-2-like [Crassostrea virginica]	RE22_6h
3.74775207	mucin-3B-like isoform X4 [Crassostrea virginica]	S4_6h
4.020665081	mucin-3B-like isoform X4 [Crassostrea virginica]	S4_6h
-22.20357216	mucin-3B-like isoform X4 [Crassostrea virginica]	S4_24h
3.986726111	mucin-3B-like isoform X4 [Crassostrea virginica]	RI_6h
4.460044136	mucin-3B-like isoform X4 [Crassostrea virginica]	RI_24h
-10.73484035	mucin-3B-like isoform X4 [Crassostrea virginica]	S4_24h
-23.44462362	mucin-4-like isoform X7 [Crassostrea virginica]	S4_24h
-6.728200181	mucin-4-like isoform X8 [Crassostrea virginica]	RI_6h
19.8087086	mucin-5AC-like [Crassostrea virginica]	S4_24h
24.20835013	mucin-5AC-like [Crassostrea virginica]	RI_24h
-23.63449535	mucin-5AC-like isoform X2 [Crassostrea virginica]	S4_24h
-25.71724187	mucin-5AC-like isoform X5 [Crassostrea virginica]	S4_24h
-25.5565658	mucin-5AC-like isoform X5 [Crassostrea virginica]	S4_24h

-25.02710707	mucin-5AC-like isoform X5 [Crassostrea virginica]	S4_24h
-24.9869002	mucin-5AC-like isoform X5 [Crassostrea virginica]	S4_24h
-24.55559512	mucin-5AC-like isoform X5 [Crassostrea virginica]	S4_24h
20.15216503	macrophage-expressed gene 1 protein-like [Crassostrea virginica]	S4_6h
-23.12210565	macrophage-expressed gene 1 protein-like [Crassostrea virginica]	S4_24h
21.2588995	macrophage-expressed gene 1 protein-like [Crassostrea virginica]	S4_24h
21.13829677	macrophage-expressed gene 1 protein-like [Crassostrea virginica]	RI_6h
19.19639966	macrophage-expressed gene 1 protein-like [Crassostrea virginica]	RI_24h
-25.14528973	antistasin-like [Crassostrea virginica]	RI_6h
-25.22546326	antistasin-like [Crassostrea virginica]	RI_24h
-8.191296146	cystatin-A-like [Crassostrea virginica]	RI_24h
	Apoptosis	
-6.21042132	caspase-1-like [Crassostrea virginica]	S4_24h
20.95052586	caspase-1-like [Crassostrea virginica]	S4_6h

20.96735265	caspase-1-like [Crassostrea virginica]	S4_24h
21.36960577	caspase-1-like [Crassostrea virginica]	RI_6h
21.94629746	caspase-1-like [Crassostrea virginica]	RI_24h
20.13240973	caspase-1-like [Crassostrea virginica]	RE22_6h
8.059692737	caspase-3-like isoform X2 [Crassostrea virginica]	S4_6h
-22.07884074	caspase-3-like isoform X2 [Crassostrea virginica]	S4_24h
19.83057052	caspase-3-like isoform X2 [Crassostrea virginica]	S4_24h
-8.724115052	caspase-3-like isoform X2 [Crassostrea virginica]	RI_24h
18.89735984	caspase-3-like isoform X2 [Crassostrea virginica]	RI_24h
5.865260258	caspase-6-like [Crassostrea virginica]	RI_6h
6.332228555	caspase-6-like [Crassostrea virginica]	RE22_6h
-11.6773146	caspase-6-like isoform X2 [Crassostrea virginica]	S4_24h
-8.871803044	caspase-6-like isoform X2 [Crassostrea virginica]	S4_24h
3.340083172	caspase-6-like isoform X2 [Crassostrea virginica]	S4_24h

-12.17173082	caspase-6-like isoform X2 [Crassostrea virginica]	RI_24h
-8.404424419	caspase-6-like isoform X2 [Crassostrea virginica]	RI_24h
3.27853441	caspase-6-like isoform X2 [Crassostrea virginica]	RI_24h
18.19169296	caspase-7-like [Crassostrea virginica]	S4_6h
19.24463486	caspase-7-like [Crassostrea virginica]	RI_6h
19.8989936	caspase-7-like [Crassostrea virginica]	RE22_6h
-7.554426297	caspase-7-like isoform X1 [Crassostrea virginica]	S4_6h
-6.02479199	caspase-7-like isoform X1 [Crassostrea virginica]	S4_24h
-6.826149987	caspase-7-like isoform X1 [Crassostrea virginica]	RI_24h
-6.880726858	caspase-7-like isoform X1 [Crassostrea virginica]	RE22_6h
-21.01107236	caspase-7-like isoform X3 [Crassostrea virginica]	RI_24h
-21.23808674	caspase-7-like isoform X3 [Crassostrea virginica]	RE22_6h
-25.94532375	baculoviral IAP repeat-containing protein 2-like [Crassostrea virginica]	S4_24h
-26.32635634	baculoviral IAP repeat-containing protein 2-like [Crassostrea virginica]	RE22_6h

-24.35012269	baculoviral IAP repeat-containing protein 3-like [Crassostrea virginica]	S4_24h
19.47455056	baculoviral IAP repeat-containing protein 3-like isoform X1 [Crassostrea virginica]	S4_6h
19.10971772	baculoviral IAP repeat-containing protein 3-like isoform X1 [Crassostrea virginica]	RI_6h
21.20706554	baculoviral IAP repeat-containing protein 3-like isoform X1 [Crassostrea virginica]	RE22_6h
-11.87771995	baculoviral IAP repeat-containing protein 6-like isoform X5 [Crassostrea virginica]	S4_6h
-6.7526098	baculoviral IAP repeat-containing protein 6-like isoform X5 [Crassostrea virginica]	S4_6h
-23.00355415	baculoviral IAP repeat-containing protein 7-A-like isoform X2 [Crassostrea virginica]	RI_24h
-23.45580618	baculoviral IAP repeat-containing protein 7-A-like isoform X2 [Crassostrea virginica]	RI_24h
18.88003432	putative inhibitor of apoptosis [Crassostrea virginica]	RE22_6h
20.38877928	putative inhibitor of apoptosis [Crassostrea virginica]	RI_6h
20.544862	putative inhibitor of apoptosis [Crassostrea virginica]	S4_6h
20.82027693	putative inhibitor of apoptosis [Crassostrea virginica]	RI_24h
21.23594337	putative inhibitor of apoptosis [Crassostrea virginica]	S4_24h
-21.79877969	bifunctional apoptosis regulator-like isoform X1 [Crassostrea virginica]	S4_6h

-21.84056799	bifunctional apoptosis regulator-like isoform X1 [Crassostrea virginica]	S4_24h
-4.862913449	apoptogenic protein 1	RI_6h
-22.71920742	cathepsin L-like isoform X2 [Crassostrea virginica]	S4_24h
-25.24123094	cathepsin L-like isoform X2 [Crassostrea virginica]	RI_6h
-22.92152325	cathepsin O-like [Crassostrea virginica]	S4_6h
-24.09391392	programmed cell death 6-interacting protein-like isoform X3 [Crassostrea virginica]	S4_24h
-7.976483879	programmed cell death protein 6-like isoform X2 [Crassostrea virginica]	S4_6h
23.29208898	XK-related protein 6-like [Crassostrea virginica]	RI_6h
24.85645254	XK-related protein 6, partial [Stegodyphus mimosarum]	RE22_6h
8.288290515	XK-related protein 6, partial [Stegodyphus mimosarum]	RI_6h
20.4704348	XK-related protein 6, partial [Stegodyphus mimosarum]	S4_6h
24.25694523	XK-related protein 6, partial [Stegodyphus mimosarum]	RI_24h
23.1394026	XK-related protein 6, partial [Stegodyphus mimosarum]	S4_24h
19.26657006	XK-related protein 8-like isoform X2 [Crassostrea virginica]	RE22_6h

20.93957528	XK-related protein 8-like isoform X2 [Crassostrea virginica]	RI_6h
19.66346021	XK-related protein 8-like isoform X2 [Crassostrea virginica]	S4_6h
4.704739877	cell death-inducing p53-target protein 1-like isoform X5 [Crassostrea virginica]	S4_6h
7.038385691	cell death-inducing p53-target protein 1-like isoform X5 [Crassostrea virginica]	S4_24h
	Autophagy	
19.68063935	autophagy-related protein 9A-like isoform X1 [Crassostrea virginica]	S4_6h
22.23975139	autophagy-related protein 9A-like isoform X1 [Crassostrea virginica]	S4_6h
19.00211451	autophagy-related protein 9A-like isoform X1 [Crassostrea virginica]	S4_24h
20.33463466	autophagy-related protein 9A-like isoform X1 [Crassostrea virginica]	S4_24h
20.2328524	autophagy-related protein 9A-like isoform X1 [Crassostrea virginica]	RI_6h
22.04434718	autophagy-related protein 9A-like isoform X1 [Crassostrea virginica]	RI_6h
17.69258697	autophagy-related protein 9A-like isoform X1 [Crassostrea virginica]	RI_24h
19.97079557	autophagy-related protein 9A-like isoform X1 [Crassostrea virginica]	RI_24h
17.464467	autophagy-related protein 9A-like isoform X1 [Crassostrea virginica]	RE22_6h

20.11113826	autophagy-related protein 9A-like isoform X1 [Crassostrea virginica]	RE22_6h
-22.38835903	transcription factor SPT20 homolog isoform X1 [Crassostrea virginica]	S4_6h
-22.81940536	transcription factor SPT20 homolog isoform X1 [Crassostrea virginica]	RI_6h
-23.79270551	vacuole membrane protein 1-like [Crassostrea virginica]	S4_6h
-24.49304386	vacuole membrane protein 1-like [Crassostrea virginica]	RI_6h
-22.8200889	PREDICTED: protein kinase C delta type [Crassostrea gigas]	S4_24h
-23.97489076	PREDICTED: protein kinase C delta type [Crassostrea gigas]	RI_24h
20.26349909	DNA damage-regulated autophagy modulator protein 1-like [Crassostrea virginica]	S4_6h
20.91174474	DNA damage-regulated autophagy modulator protein 1-like [Crassostrea virginica]	S4_24h
20.84051785	DNA damage-regulated autophagy modulator protein 1-like [Crassostrea virginica]	RE22_6h
-6.547974505	run domain Beclin-1-interacting and cysteine-rich domain-containing protein-like isoform X3 [Crassostrea virginica]	S4_24h
20.7845565	phosphatidylinositol 4,5-bisphosphate 3-kinase catalytic subunit delta isoform-like isoform X1 [Crassostrea virginica]	S4_6h

21.39532399	phosphatidylinositol 4,5-bisphosphate 3-kinase catalytic subunit delta isoform-like isoform X1 [Crassostrea virginica]	S4_24h
22.58624048	phosphatidylinositol 4,5-bisphosphate 3-kinase catalytic subunit delta isoform-like isoform X1 [Crassostrea virginica]	RI_6h
21.22674556	phosphatidylinositol 4,5-bisphosphate 3-kinase catalytic subunit delta isoform-like isoform X1 [Crassostrea virginica]	RI_24h
22.60384771	phosphatidylinositol 4,5-bisphosphate 3-kinase catalytic subunit delta isoform-like isoform X1 [Crassostrea virginica]	RE22_6h
	Phagosome	
-11.04839433	Actin, cytoplasmic [Crassostrea gigas]	S4_6h
-22.15040686	actin-3-like isoform X1 [Crassostrea virginica]	S4_6h
-23.28807439	actin-3-like isoform X1 [Crassostrea virginica]	RI_6h
-2.009714808	actin-like [Crassostrea virginica]	S4_24h
-25.67615427	cytoplasmic dynein 1 heavy chain 1-like isoform X1 [Crassostrea virginica]	S4_6h

-24.78846969	cytoplasmic dynein 1 heavy chain 1-like isoform X1 [Crassostrea virginica]	S4_24h
-18.35355926	cytoplasmic dynein 1 light intermediate chain 2-like isoform X11 [Crassostrea virginica]	S4_6h
-23.99337051	cytoplasmic dynein 1 light intermediate chain 2-like isoform X11 [Crassostrea virginica]	S4_24h
20.46400243	cytoplasmic dynein 2 light intermediate chain 1-like [Crassostrea virginica]	S4_6h
19.94858466	cytoplasmic dynein 2 light intermediate chain 1-like [Crassostrea virginica]	S4_24h
19.68213706	cytoplasmic dynein 2 light intermediate chain 1-like [Crassostrea virginica]	RI_6h
20.2666716	cytoplasmic dynein 2 light intermediate chain 1-like [Crassostrea virginica]	RI_24h
19.56136995	cytoplasmic dynein 2 light intermediate chain 1-like [Crassostrea virginica]	RE22_6h
22.04398056	cation-dependent mannose-6-phosphate receptor-like [Crassostrea virginica]	S4_6h
-6.510608147	cation-dependent mannose-6-phosphate receptor-like [Crassostrea virginica]	RI_6h
22.65257645	cation-dependent mannose-6-phosphate receptor-like [Crassostrea virginica]	RI_6h
22.2320337	cation-dependent mannose-6-phosphate receptor-like [Crassostrea virginica]	RI_24h

21.24015816	cation-dependent mannose-6-phosphate receptor-like [Crassostrea virginica]	RE22_6h
Lysosome		
-26.35735397	lysosomal-associated transmembrane protein 4A-like [Crassostrea virginica]	S4_6h
-27.83132813	lysosomal-associated transmembrane protein 4A-like [Crassostrea virginica]	RI_24h
-5.595601397	lysosomal acid lipase/cholesteryl ester hydrolase-like [Crassostrea virginica]	RI_6h
Endocytosis & Peroxisome		
7.204505845	AP-2 complex subunit mu-1 [Crassostrea gigas]	RI_24h
8.234098827	probable peroxisomal membrane protein PEX13 [Crassostrea virginica]	S4_6h
7.151949067	probable peroxisomal membrane protein PEX13 [Crassostrea virginica]	S4_24h
-24.32891036	peroxisomal acyl-coenzyme A oxidase 1-like [Crassostrea virginica]	RI_24h
-23.1387346	peroxisomal acyl-coenzyme A oxidase 1-like [Crassostrea virginica]	S4_24h
-23.64616222	peroxisomal acyl-coenzyme A oxidase 1-like [Crassostrea virginica]	RI_24h
-6.184641444	peroxisomal acyl-coenzyme A oxidase 1-like isoform X2 [Crassostrea virginica]	S4_24h
Antioxidant enzymes		

-3.86662093	glutathione peroxidase 7-like [Crassostrea virginica]	S4_24h
-21.14341297	glutathione S-transferase C-terminal domain-containing protein-like isoform X1 [Crassostrea virginica]	S4_24h
4.340577781	glutathione S-transferase kappa 1-like [Crassostrea virginica]	RI_6h
-21.74743898	maleylacetoacetate isomerase-like [Crassostrea virginica]	S4_6h
-22.28036183	maleylacetoacetate isomerase-like [Crassostrea virginica]	RI_24h
-22.57607848	maleylacetoacetate isomerase-like [Crassostrea virginica]	RE22_6h
-9.092994732	Superoxide dismutase [Cu-Zn] [Crassostrea gigas]	S4_24h
24.656834	thioredoxin domain-containing protein 15-like [Crassostrea virginica]	S4_6h
22.08653805	thioredoxin domain-containing protein 15-like [Crassostrea virginica]	RE22_6h
-22.17861058	thioredoxin domain-containing protein 3 homolog isoform X15 [Crassostrea virginica]	S4_6h
-5.275085802	thioredoxin domain-containing protein 3 homolog isoform X15 [Crassostrea virginica]	S4_6h
-24.08250505	thioredoxin domain-containing protein 3 homolog isoform X15 [Crassostrea virginica]	S4_24h
-22.68468309	thioredoxin domain-containing protein 3 homolog isoform X15 [Crassostrea virginica]	S4_24h

-15.3248519	thioredoxin domain-containing protein 3 homolog isoform X15 [Crassostrea virginica]	S4_24h
9.959973369	thioredoxin domain-containing protein 3 homolog isoform X15 [Crassostrea virginica]	S4_24h
19.68049721	thioredoxin domain-containing protein 3 homolog isoform X15 [Crassostrea virginica]	S4_24h
-22.94170493	thioredoxin domain-containing protein 3 homolog isoform X15 [Crassostrea virginica]	RI_6h
-24.96201578	thioredoxin domain-containing protein 3 homolog isoform X15 [Crassostrea virginica]	RI_24h
-24.87983434	thioredoxin domain-containing protein 3 homolog isoform X15 [Crassostrea virginica]	RI_24h
-22.95218203	thioredoxin domain-containing protein 3 homolog isoform X15 [Crassostrea virginica]	RI_24h
7.365024046	thioredoxin domain-containing protein 3 homolog isoform X15 [Crassostrea virginica]	RI_24h
18.02755971	thioredoxin domain-containing protein 3 homolog isoform X15 [Crassostrea virginica]	RI_24h
23.85507502	thioredoxin domain-containing protein 3 homolog isoform X15 [Crassostrea virginica]	RI_24h
-23.27838106	thioredoxin domain-containing protein 3 homolog isoform X15 [Crassostrea virginica]	RE22_6h
-2.096359322	thioredoxin domain-containing protein 5-like [Crassostrea virginica]	S4_24h
-25.88062202	thioredoxin-like [Crassostrea virginica]	S4_24h
-10.64240601	thioredoxin-like [Crassostrea virginica]	RI_6h

-26.51486203	thioredoxin-like [Crassostrea virginica]	RI_24h
-3.729163339	thioredoxin-like protein 1 [Crassostrea virginica]	RI_24h
3.293281563	thioredoxin-related transmembrane protein 1-like isoform X1 [Crassostrea virginica]	RI_6h
3.156411653	thioredoxin-related transmembrane protein 1-like isoform X1 [Crassostrea virginica]	RI_24h
-24.70491532	thioredoxin-related transmembrane protein 2 homolog [Crassostrea virginica]	RI_24h
-24.9360089	thioredoxin-related transmembrane protein 2 homolog [Crassostrea virginica]	RE22_6h
Acute phase proteins		
-9.723127722	Heat shock 70 kDa protein 12A [Crassostrea gigas]	S4_24h
5.535364462	heat shock 70 kDa protein 12A-like [Crassostrea virginica]	S4_24h
5.686282343	heat shock 70 kDa protein 12A-like [Crassostrea virginica]	RI_24h
-22.26223254	heat shock 70 kDa protein 12A-like [Crassostrea virginica]	RE22_6h
-23.79702313	heat shock 70 kDa protein 12A-like isoform X1 [Crassostrea virginica]	S4_6h
6.658680636	heat shock 70 kDa protein 12A-like isoform X1 [Crassostrea virginica]	S4_6h
5.254504823	heat shock 70 kDa protein 12A-like isoform X1 [Crassostrea virginica]	RI_6h

5.250678277	heat shock 70 kDa protein 12A-like isoform X1 [Crassostrea virginica]	RE22_6h
-4.162351016	heat shock 70 kDa protein 12B-like [Crassostrea virginica]	RE22_6h
-23.06134796	heat shock 70 kDa protein 12B-like [Crassostrea virginica]	S4_24h
19.64075977	heat shock 70 kDa protein 12B-like isoform X4 [Crassostrea virginica]	S4_6h
19.49655284	heat shock 70 kDa protein 12B-like isoform X4 [Crassostrea virginica]	RI_6h
18.54456763	heat shock 70 kDa protein 12B-like isoform X4 [Crassostrea virginica]	RE22_6h
-22.43374437	heat shock factor protein-like [Crassostrea virginica]	RI_24h
-18.35638131	heat shock protein 30C-like [Crassostrea virginica]	S4_24h
3.254119893	heat shock protein HSP 90-beta-like [Crassostrea virginica]	S4_6h
Cytoskeletal organization		
-24.67660059	PREDICTED: dynamin-1 isoform X2 [Crassostrea gigas]	S4_6h
-11.01417755	dynamin-1-like isoform X6 [Crassostrea virginica]	S4_6h
23.58398582	dynamin-1-like isoform X6 [Crassostrea virginica]	S4_6h
-21.25056055	dynamin-1-like isoform X6 [Crassostrea virginica]	S4_24h

-23.67694566	dynamin-1-like isoform X6 [Crassostrea virginica]	RI_6h
23.2104963	dynamin-1-like isoform X6 [Crassostrea virginica]	RI_6h
-13.1405264	dynamin-1-like isoform X6 [Crassostrea virginica]	RI_24h
18.62495089	dynamin-1-like isoform X6 [Crassostrea virginica]	RE22_6h
9.249110026	PREDICTED: septin-7 isoform X3 [Crassostrea gigas]	S4_6h
9.161299284	PREDICTED: septin-7 isoform X3 [Crassostrea gigas]	RI_6h
-21.6896377	septin-11-like isoform X2 [Crassostrea virginica]	RE22_6h
-22.00129053	septin-2-like isoform X1 [Crassostrea virginica]	S4_6h
7.044855946	septin-2-like isoform X1 [Crassostrea virginica]	S4_6h
-21.88584245	septin-2-like isoform X1 [Crassostrea virginica]	S4_24h
-22.73608544	septin-2-like isoform X1 [Crassostrea virginica]	RI_24h
-23.27740458	septin-2-like isoform X1 [Crassostrea virginica]	S4_24h
-10.38732509	septin-2-like isoform X8 [Crassostrea virginica]	RI_6h
-7.952386425	septin-2-like isoform X8 [Crassostrea virginica]	RI_6h

Others		
-23.59339061	multidrug resistance protein 1-like isoform X1 [Crassostrea virginica]	S4_6h
-14.96548899	multidrug resistance protein 1-like isoform X6 [Crassostrea virginica]	S4_24h
21.14757041	multidrug resistance protein 1-like isoform X6 [Crassostrea virginica]	RI_6h
22.22331368	multidrug resistance protein 1-like isoform X6 [Crassostrea virginica]	RI_24h
21.3339868	multidrug resistance protein 1-like isoform X6 [Crassostrea virginica]	RE22_6h
-25.28253245	multidrug resistance-associated protein 4-like [Crassostrea virginica]	S4_6h
-11.35931265	multidrug resistance-associated protein 4-like [Crassostrea virginica]	S4_24h
-2.996574031	multidrug resistance-associated protein 5-like isoform X2 [Crassostrea virginica]	S4_24h
-22.29106079	multidrug resistance-associated protein 7-like [Crassostrea virginica]	S4_24h
-2.512272237	laccase-3-like [Crassostrea virginica]	RI_24h
-4.990554678	laccase-like [Crassostrea virginica]	RI_6h
-23.49586873	Hemicentin-1, partial [Crassostrea gigas]	S4_6h
-21.69620493	Hemicentin-1	S4_6h

-8.779619013	Hemicentin-1	S4_6h
-26.12075367	Hemicentin-1	S4_24h
-22.82446184	Hemicentin-1	RE22_6h
4.970041769	hemicentin-1-like [Crassostrea virginica]	S4_6h
-23.57120259	hemicentin-1-like [Crassostrea virginica]	S4_6h
7.211995212	hemicentin-1-like [Crassostrea virginica]	S4_6h
-24.43651737	hemicentin-1-like [Crassostrea virginica]	RE22_6h
-22.74745907	hemicentin-1-like isoform X2 [Crassostrea virginica]	S4_24h
-22.04213544	hemicentin-1-like isoform X2 [Crassostrea virginica]	S4_24h
-23.91446285	hemicentin-1-like isoform X2 [Crassostrea virginica]	RE22_6h
-6.483050928	hemicentin-1-like isoform X2 [Crassostrea virginica]	RI_6h
-20.92100668	hemicentin-1-like isoform X21 [Crassostrea virginica]	S4_6h
-21.90630905	hemicentin-1-like isoform X21 [Crassostrea virginica]	RI_6h
-21.71429592	hemicentin-1-like isoform X21 [Crassostrea virginica]	RI_24h

-22.25262628	hemicentin-1-like isoform X21 [Crassostrea virginica]	RE22_6h
-21.95606244	hemicentin-1-like isoform X21 [Crassostrea virginica]	RE22_6h
-25.24784795	hemicentin-1-like isoform X3 [Crassostrea virginica]	S4_24h
15.22210309	hemicentin-1-like isoform X34 [Crassostrea virginica]	S4_6h
19.54287658	hemicentin-1-like isoform X34 [Crassostrea virginica]	S4_24h
19.38940516	hemicentin-1-like isoform X34 [Crassostrea virginica]	RI_6h
19.36636019	hemicentin-1-like isoform X34 [Crassostrea virginica]	RI_24h
19.77553284	hemicentin-1-like isoform X34 [Crassostrea virginica]	RE22_6h
-21.26298714	hemicentin-1-like isoform X40 [Crassostrea virginica]	S4_6h
-22.32330998	hemicentin-1-like isoform X40 [Crassostrea virginica]	S4_24h
7.289148836	hemicentin-1-like isoform X5 [Crassostrea virginica]	S4_6h
17.82784086	hemicentin-1-like isoform X5 [Crassostrea virginica]	S4_24h
20.84952352	hemicentin-1-like isoform X5 [Crassostrea virginica]	RI_24h
-24.07441428	hemicentin-1-like isoform X6 [Crassostrea virginica]	S4_6h

-22.70945451	hemicentin-1-like isoform X9 [Crassostrea virginica]	S4_24h
-25.40188462	hemicentin-1-like isoform X9 [Crassostrea virginica]	RI_6h
-29.99115794	hemicentin-1-like isoform X9 [Crassostrea virginica]	RI_24h
9.38130971	hemicentin-2-like isoform X2 [Crassostrea virginica]	S4_6h
20.95642279	hemicentin-2-like isoform X2 [Crassostrea virginica]	S4_24h
9.373853638	hemicentin-2-like isoform X2 [Crassostrea virginica]	RI_6h
20.55310454	hemicentin-2-like isoform X2 [Crassostrea virginica]	RI_24h
9.777286784	hemicentin-2-like isoform X2 [Crassostrea virginica]	RE22_6h
-8.922154094	histamine H2 receptor-like [Crassostrea virginica]	S4_24h
-22.19114574	histamine H2 receptor-like [Crassostrea virginica]	S4_24h
-22.75801453	histamine H2 receptor-like [Crassostrea virginica]	RI_24h
-22.90867354	histamine H2 receptor-like [Crassostrea virginica]	RE22_6h
-5.471576144	oxidative stress-induced growth inhibitor 2-like [Crassostrea virginica]	S4_6h
-9.718337318	oxidative stress-induced growth inhibitor 2-like [Crassostrea virginica]	RI_6h

-6.452016421	oxidative stress-induced growth inhibitor 2-like [Crassostrea virginica]	RE22_6h
-2.071397009	cytochrome b [Crassostrea virginica]	RI_6h
20.23697806	cytochrome b5 reductase 4-like isoform X3 [Crassostrea virginica]	S4_24h
22.07024714	cytochrome b5 reductase 4-like isoform X3 [Crassostrea virginica]	RI_6h
17.91802039	cytochrome b5 reductase 4-like isoform X3 [Crassostrea virginica]	RI_24h
21.0441541	cytochrome b5 reductase 4-like isoform X3 [Crassostrea virginica]	RE22_6h
-30	cytochrome c oxidase subunit I [Crassostrea virginica]	S4_6h
-12.21152488	cytochrome c oxidase subunit I [Crassostrea virginica]	S4_6h
13.83136184	cytochrome c oxidase subunit I [Crassostrea virginica]	S4_6h
-12.7345641	cytochrome c oxidase subunit I [Crassostrea virginica]	S4_24h
23.04305396	cytochrome c oxidase subunit I [Crassostrea virginica]	S4_24h
27.42296011	cytochrome c oxidase subunit I [Crassostrea virginica]	S4_24h
29.20729823	cytochrome c oxidase subunit I [Crassostrea virginica]	S4_24h
-24.69050988	cytochrome c oxidase subunit I [Crassostrea virginica]	RI_6h

-24.66864706	cytochrome c oxidase subunit I [Crassostrea virginica]	RI_6h
29.16889206	cytochrome c oxidase subunit I [Crassostrea virginica]	RI_6h
-30	cytochrome c oxidase subunit I [Crassostrea virginica]	RI_24h
-13.01827661	cytochrome c oxidase subunit I [Crassostrea virginica]	RI_24h
20.69401443	cytochrome c oxidase subunit I [Crassostrea virginica]	RI_24h
30	cytochrome c oxidase subunit I [Crassostrea virginica]	RI_24h
-30	cytochrome c oxidase subunit I [Crassostrea virginica]	RE22_6h
-11.365341	cytochrome c oxidase subunit I [Crassostrea virginica]	RE22_6h
-10.31599264	cytochrome c oxidase subunit I [Crassostrea virginica]	RE22_6h
30	cytochrome c oxidase subunit I [Crassostrea virginica]	RE22_6h
-12.33130181	cytochrome c oxidase subunit III (mitochondrion) [Crassostrea virginica]	S4_24h
-26.50197006	cytochrome c oxidase subunit III (mitochondrion) [Crassostrea virginica]	RI_6h
-17.04367675	cytochrome c oxidase subunit III (mitochondrion) [Crassostrea virginica]	RI_24h
11.0050444	cytochrome c oxidase subunit III (mitochondrion) [Crassostrea virginica]	S4_6h

15.84125515	cytochrome c oxidase subunit III (mitochondrion) [Crassostrea virginica]	S4_6h
26.82143733	cytochrome c oxidase subunit III (mitochondrion) [Crassostrea virginica]	S4_6h
28.26611263	cytochrome c oxidase subunit III (mitochondrion) [Crassostrea virginica]	S4_6h
10.86124348	cytochrome c oxidase subunit III (mitochondrion) [Crassostrea virginica]	S4_24h
11.67321719	cytochrome c oxidase subunit III (mitochondrion) [Crassostrea virginica]	S4_24h
30	cytochrome c oxidase subunit III (mitochondrion) [Crassostrea virginica]	S4_24h
30	cytochrome c oxidase subunit III (mitochondrion) [Crassostrea virginica]	S4_24h
12.78997263	cytochrome c oxidase subunit III (mitochondrion) [Crassostrea virginica]	RI_6h
30	cytochrome c oxidase subunit III (mitochondrion) [Crassostrea virginica]	RI_6h
30	cytochrome c oxidase subunit III (mitochondrion) [Crassostrea virginica]	RI_6h
14.9301273	cytochrome c oxidase subunit III (mitochondrion) [Crassostrea virginica]	RI_24h
25.83550682	cytochrome c oxidase subunit III (mitochondrion) [Crassostrea virginica]	RI_24h
27.94606978	cytochrome c oxidase subunit III (mitochondrion) [Crassostrea virginica]	RI_24h
15.68484216	cytochrome c oxidase subunit III (mitochondrion) [Crassostrea virginica]	RE22_6h

29.89148054	cytochrome c oxidase subunit III (mitochondrion) [Crassostrea virginica]	RE22_6h
7.51088163	cytochrome P450 27C1-like [Crassostrea virginica]	S4_6h
8.171393189	cytochrome P450 27C1-like [Crassostrea virginica]	S4_24h
-24.81197395	cytochrome P450 27C1-like [Crassostrea virginica]	RI_6h
9.022848188	cytochrome P450 27C1-like [Crassostrea virginica]	RI_6h
7.477144414	cytochrome P450 27C1-like [Crassostrea virginica]	RI_24h
6.794994273	cytochrome P450 27C1-like [Crassostrea virginica]	RE22_6h
21.27504686	cytochrome P450 2C28-like isoform X2 [Crassostrea virginica]	S4_6h
18.70986303	cytochrome P450 2C28-like isoform X2 [Crassostrea virginica]	S4_24h
-22.33591302	cytochrome P450 2C28-like isoform X2 [Crassostrea virginica]	RI_6h
20.28813849	cytochrome P450 2C28-like isoform X2 [Crassostrea virginica]	RI_24h
20.46500703	cytochrome P450 2C28-like isoform X2 [Crassostrea virginica]	RE22_6h
6.238313253	Cytochrome P450 2D14 [Crassostrea gigas]	RI_6h
-21.35382089	cytochrome P450 2F5-like [Crassostrea virginica]	S4_6h

-21.63670878	cytochrome P450 2F5-like [Crassostrea virginica]	S4_24h
-22.27759983	cytochrome P450 2F5-like [Crassostrea virginica]	RI_24h
-22.65584492	cytochrome P450 2F5-like [Crassostrea virginica]	RE22_6h
-21.3071929	cytochrome P450 2J5-like isoform X2 [Crassostrea virginica]	S4_24h
-22.51502577	cytochrome P450 3A6-like [Crassostrea virginica]	S4_24h
-9.01112453	cytochrome P450 4A25-like [Crassostrea virginica]	S4_6h
-10.01277913	cytochrome P450 4V2-like isoform X1 [Crassostrea virginica]	S4_6h
-20.02751343	cytochrome P450 4V2-like isoform X1 [Crassostrea virginica]	RI_24h
-8.791304071	dual specificity protein phosphatase 1-A-like [Crassostrea virginica]	RI_24h
4.381729845	dual specificity protein phosphatase 14-like isoform X1 [Crassostrea virginica]	RI_24h
-23.83873688	dual specificity protein phosphatase 18-like [Crassostrea virginica]	RI_24h
-24.07000154	dual specificity protein phosphatase 18-like [Crassostrea virginica]	RE22_6h
9.288349328	dual specificity protein phosphatase 19-like [Crassostrea virginica]	S4_24h
8.034347824	dual specificity protein phosphatase 19-like [Crassostrea virginica]	RI_24h

-24.95777889	dual specificity protein phosphatase 7-like [Crassostrea virginica]	RI_6h
8.541240553	dual specificity protein phosphatase 7-like [Crassostrea virginica]	RI_24h
-25.38312764	dual specificity protein phosphatase 7-like [Crassostrea virginica]	RE22_6h
-8.245775085	protein phosphatase 1 regulatory subunit 12A-like isoform X4 [Crassostrea virginica]	S4_24h
-24.53980697	protein phosphatase 1 regulatory subunit 16A-like isoform X3 [Crassostrea virginica]	S4_6h
10.20776883	protein phosphatase 1 regulatory subunit 16A-like isoform X3 [Crassostrea virginica]	S4_6h
9.225134929	protein phosphatase 1 regulatory subunit 16A-like isoform X3 [Crassostrea virginica]	S4_24h
9.587762441	protein phosphatase 1 regulatory subunit 16A-like isoform X3 [Crassostrea virginica]	RI_6h
8.067829643	protein phosphatase 1 regulatory subunit 16A-like isoform X3 [Crassostrea virginica]	RI_24h
8.666364977	protein phosphatase 1 regulatory subunit 16A-like isoform X3 [Crassostrea virginica]	RE22_6h
8.659545152	protein phosphatase 1 regulatory subunit 36-like isoform X1 [Crassostrea virginica]	RI_6h
-7.364558291	protein phosphatase 1 regulatory subunit 37-like [Crassostrea virginica]	S4_6h
-7.980896037	protein phosphatase 1 regulatory subunit 37-like [Crassostrea virginica]	RE22_6h
7.015542075	protein phosphatase 1 regulatory subunit 42-like isoform X1 [Crassostrea virginica]	RI_24h

-24.30599943	protein phosphatase 1 regulatory subunit 42-like isoform X5 [Crassostrea virginica]	S4_24h
20.18059945	Tripartite motif-containing protein 2 [Crassostrea gigas]	S4_6h
23.03367057	Tripartite motif-containing protein 2 [Crassostrea gigas]	S4_6h
-7.262763056	Tripartite motif-containing protein 2 [Crassostrea gigas]	S4_24h
21.37325729	Tripartite motif-containing protein 2 [Crassostrea gigas]	S4_24h
-22.62992934	Tripartite motif-containing protein 2 [Crassostrea gigas]	RI_6h
-11.07407202	Tripartite motif-containing protein 2 [Crassostrea gigas]	RI_6h
20.48455649	Tripartite motif-containing protein 2 [Crassostrea gigas]	RI_6h
20.56883143	Tripartite motif-containing protein 2 [Crassostrea gigas]	RI_6h
-22.63181448	Tripartite motif-containing protein 2 [Crassostrea gigas]	RI_24h
21.63871709	Tripartite motif-containing protein 2 [Crassostrea gigas]	RI_24h
19.01092158	Tripartite motif-containing protein 2 [Crassostrea gigas]	RE22_6h
21.96955011	Tripartite motif-containing protein 2 [Crassostrea gigas]	RE22_6h
-9.107874381	Tripartite motif-containing protein 2 [Crassostrea gigas]	S4_24h

-24.94189095	tripartite motif-containing protein 2-like [Crassostrea virginica]	S4_6h
-21.21518109	tripartite motif-containing protein 2-like [Crassostrea virginica]	S4_6h
-24.99785172	tripartite motif-containing protein 2-like [Crassostrea virginica]	S4_24h
-22.97055523	tripartite motif-containing protein 2-like [Crassostrea virginica]	S4_24h
-22.56316651	tripartite motif-containing protein 2-like [Crassostrea virginica]	S4_24h
-21.63143212	tripartite motif-containing protein 2-like [Crassostrea virginica]	S4_24h
-21.555795	tripartite motif-containing protein 2-like [Crassostrea virginica]	S4_24h
-21.48502657	tripartite motif-containing protein 2-like [Crassostrea virginica]	S4_24h
2.440722296	tripartite motif-containing protein 2-like [Crassostrea virginica]	S4_24h
-23.52232576	tripartite motif-containing protein 2-like [Crassostrea virginica]	RI_24h
-22.27754888	tripartite motif-containing protein 2-like [Crassostrea virginica]	RI_24h
-6.664288475	tripartite motif-containing protein 2-like [Crassostrea virginica]	RI_24h
-23.67795281	tripartite motif-containing protein 2-like [Crassostrea virginica]	RE22_6h
-22.94845541	tripartite motif-containing protein 2-like [Crassostrea virginica]	RE22_6h

20.60131168	tripartite motif-containing protein 2-like [Crassostrea virginica]	S4_6h
20.75582702	tripartite motif-containing protein 2-like [Crassostrea virginica]	S4_24h
20.51645227	tripartite motif-containing protein 2-like [Crassostrea virginica]	RI_6h
-23.97927626	tripartite motif-containing protein 2-like [Crassostrea virginica]	RI_24h
20.6240354	tripartite motif-containing protein 2-like [Crassostrea virginica]	RI_24h
20.61455877	tripartite motif-containing protein 2-like [Crassostrea virginica]	RE22_6h
-21.16657646	tripartite motif-containing protein 2-like isoform X2 [Crassostrea virginica]	S4_6h
9.572758843	tripartite motif-containing protein 2-like isoform X2 [Crassostrea virginica]	S4_6h
-21.69698695	tripartite motif-containing protein 2-like isoform X2 [Crassostrea virginica]	RI_6h
7.793596061	tripartite motif-containing protein 2-like isoform X2 [Crassostrea virginica]	RI_6h
22.30459712	tripartite motif-containing protein 2-like isoform X4 [Crassostrea virginica]	S4_6h
21.31432232	tripartite motif-containing protein 2-like isoform X4 [Crassostrea virginica]	RE22_6h
-24.62951513	tripartite motif-containing protein 3-like [Crassostrea virginica]	S4_24h
-25.52121202	tripartite motif-containing protein 3-like [Crassostrea virginica]	RI_24h

-25.70706555	tripartite motif-containing protein 3-like [Crassostrea virginica]	RE22_6h
-6.057275712	tripartite motif-containing protein 3-like [Crassostrea virginica]	RE22_6h
-24.1355023	tripartite motif-containing protein 45-like [Crassostrea virginica]	RE22_6h
-21.78539178	tripartite motif-containing protein 5-like isoform X2 [Crassostrea virginica]	RI_24h
Biomineralization		
-21.98439629	perlucin-like [Crassostrea virginica]	S4_6h
-23.38363804	perlucin-like [Crassostrea virginica]	S4_24h
-21.96732063	perlucin-like [Crassostrea virginica]	S4_24h
-8.094282488	perlucin-like [Crassostrea virginica]	S4_24h
-23.03677587	perlucin-like isoform X1 [Crassostrea virginica]	S4_24h
-22.45971194	perlucin-like isoform X1 [Crassostrea virginica]	RE22_6h
18.507209	perlucin-like isoform X2 [Crassostrea virginica]	S4_24h
15.20165795	perlucin-like isoform X2 [Crassostrea virginica]	RI_24h
-23.43817576	perlucin-like protein [Crassostrea virginica]	S4_24h

-6.765931252	perlucin-like protein [Crassostrea virginica]	RI_24h
21.94929471	perlucin-like protein isoform X1 [Crassostrea virginica]	S4_6h
21.68188164	perlucin-like protein isoform X1 [Crassostrea virginica]	RE22_6h
25.09518802	Chitin synthase 3 [Crassostrea gigas]	RE22_6h
24.46274385	Chitin synthase 3 [Crassostrea gigas]	RI_6h
21.58959401	Chitin synthase 3 [Crassostrea gigas]	S4_6h
-7.045235918	Chitin synthase C [Crassostrea gigas]	S4_24h

Table 3: Differentially expressed genes with log fold change for probiotic treatment when compared to control in hatchery transcriptomes ($p \leq 0.05$, upregulation: log fold change ≥ 2, downregulation: log fold change ≤ -2). HT-RI: larvae treated daily with probiotic *Bacillus pumilus* RI0695 (RI) for 5, 12 or 16 days

Log2FoldChange	**Hit_def**	**Treatment**

Recognition		
TLRs		
-6.333302299	toll-like receptor 1 [Crassostrea virginica]	HT_RI
-5.117571098	toll-like receptor 6 [Crassostrea virginica]	HT_RI
10.50152784	toll-like receptor 6 [Crassostrea virginica]	HT_RI
-7.016997176	toll-like receptor 4 isoform X1 [Crassostrea virginica]	HT_RI
5.181661209	toll-like receptor 4 isoform X1 [Crassostrea virginica]	HT_RI
-13.50652647	toll-like receptor 4 isoform X1 [Crassostrea virginica]	HT_RI
-28.195436	toll-like receptor 4 isoform X1 [Crassostrea virginica]	HT_RI
-10.52830632	toll-like receptor 13 [Crassostrea virginica]	HT_RI
Lectin		
-7.548517655	C-type lectin domain family 4 member E-like [Crassostrea virginica]	HT_RI
-9.313083618	C-type lectin domain family 3 member A-like [Crassostrea virginica]	HT_RI
Scavenger receptors		

-8.035179262	scavenger receptor class B member 1-like isoform X1 [Crassostrea virginica]	HT_RI
-8.089778373	scavenger receptor class B member 1-like isoform X1 [Crassostrea virginica]	HT_RI
-3.505324316	scavenger receptor cysteine-rich type 1 protein M130-like isoform X1 [Crassostrea virginica]	HT_RI
-5.113872743	scavenger receptor cysteine-rich type 1 protein M130-like isoform X1 [Crassostrea virginica]	HT_RI
LRFN		
-7.614151619	leucine-rich repeat and fibronectin type III domain-containing protein 1-like protein [Crassostrea virginica]	HT_RI
-10.54947853	leucine-rich repeat and fibronectin type-III domain-containing protein 5-like [Crassostrea virginica]	HT_RI
LRRs		
7.538001578	leucine-rich repeat and IQ domain-containing protein 1-like [Crassostrea virginica]	HT_RI

7.603603801	leucine-rich repeat neuronal protein 3-like [Crassostrea virginica]	HT_RI
-7.290369986	leucine-rich repeat neuronal protein 3-like [Crassostrea virginica]	HT_RI
-3.910728944	leucine-rich repeat transmembrane protein FLRT3-like [Crassostrea virginica]	HT_RI
8.884936086	leucine-rich repeat-containing G-protein coupled receptor 4-like [Crassostrea virginica]	HT_RI
-5.222764772	leucine-rich repeat-containing G-protein coupled receptor 4-like [Crassostrea virginica]	HT_RI
6.417892215	leucine-rich repeat-containing protein 24-like [Crassostrea virginica]	HT_RI
-5.851688629	leucine-rich repeat-containing protein 24-like [Crassostrea virginica]	HT_RI
-6.888253251	leucine-rich repeat-containing protein 24-like [Crassostrea virginica]	HT_RI
-10.67681921	leucine-rich repeat-containing protein 71-like isoform X21 [Crassostrea virginica]	HT_RI
11.13843633	leucine-rich repeat-containing protein 71-like isoform X22 [Crassostrea virginica]	HT_RI

-12.72995479	leucine-rich repeat-containing protein 74A-like isoform X2 [Crassostrea virginica]	HT_RI
-9.675716266	leucine-rich repeat-containing protein 74B-like isoform X3 [Crassostrea virginica]	HT_RI
Fibronectin domain containing		
3.471877714	fibronectin type-III domain-containing protein 3A-like isoform X4 [Crassostrea virginica]	HT_RI
-7.597144145	fibronectin type-III domain-containing protein 3A-like isoform X4 [Crassostrea virginica]	HT_RI
C1q proteins		
7.068747239	complement C1q tumor necrosis factor-related protein 2-like [Crassostrea virginica]	HT_RI
-8.687727906	complement C1q tumor necrosis factor-related protein 4-like isoform X3 [Crassostrea virginica]	HT_RI

Others		
-5.683456288	Macrophage mannose receptor 1 [Crassostrea gigas]	HT_RI
-9.358422983	macrophage mannose receptor 1-like [Crassostrea virginica]	HT_RI
7.683007501	macrophage migration inhibitory factor-like [Crassostrea virginica]	HT_RI
Cholinergic immunomodulation		
-6.155933795	muscarinic acetylcholine receptor M1-like [Crassostrea virginica]	HT_RI
-10.64631968	muscarinic acetylcholine receptor M1-like [Crassostrea virginica]	HT_RI
-10.62270936	glutamate receptor ionotropic	HT_RI
-10.76881678	glutamate receptor ionotropic	HT_RI
-9.746999757	neuronal acetylcholine receptor subunit alpha-10-like isoform X2 [Crassostrea virginica]	HT_RI
-10.55585177	neuronal acetylcholine receptor subunit alpha-10-like isoform X2 [Crassostrea virginica]	HT_RI

-10.41167226	neuronal acetylcholine receptor subunit alpha-10-like isoform X3 [Crassostrea virginica]	HT_RI
2.6604451	neuronal acetylcholine receptor subunit alpha-6-like [Crassostrea virginica]	HT_RI
11.8076763	neuronal acetylcholine receptor subunit alpha-9-like [Crassostrea virginica]	HT_RI
-7.276192743	neuronal acetylcholine receptor subunit alpha-9-like [Crassostrea virginica]	HT_RI
-12.41663085	neuronal acetylcholine receptor subunit alpha-9-like [Crassostrea virginica]	HT_RI
10.57507598	neuropeptide FF receptor 2-like [Crassostrea virginica]	HT_RI
19.88309004	neuropeptide Y receptor type 1-like [Crassostrea virginica]	HT_RI
Signaling pathways		
-11.49570393	TNF receptor-associated factor 3-like isoform X3 [Crassostrea virginica]	HT_RI
8.268728895	tumor necrosis factor receptor superfamily member 1B-like isoform X1 [Crassostrea virginica]	HT_RI
4.135043574	NF-kappa-B inhibitor-interacting Ras-like protein 1 isoform X8 [Crassostrea virginica]	HT_RI

3.572468448	NF-kappa-B inhibitor-interacting Ras-like protein 1 isoform X8 [Crassostrea virginica]	HT_RI
-6.180435424	nuclear factor NF-kappa-B p105 subunit-like isoform X2 [Crassostrea virginica]	HT_RI
6.794484856	C-Jun-amino-terminal kinase-interacting protein 4-like [Crassostrea virginica]	HT_RI
11.83894875	stress-activated protein kinase JNK-like isoform X1 [Crassostrea virginica]	HT_RI
11.81769312	stress-activated protein kinase JNK-like isoform X1 [Crassostrea virginica]	HT_RI
5.027413466	stress-activated protein kinase JNK-like isoform X1 [Crassostrea virginica]	HT_RI
-7.126718787	mitogen-activated protein kinase 11-like [Crassostrea virginica]	HT_RI
-9.730631672	mitogen-activated protein kinase 11-like [Crassostrea virginica]	HT_RI
-13.9275785	mitogen-activated protein kinase 11-like [Crassostrea virginica]	HT_RI
-10.23892565	mitogen-activated protein kinase 7-like isoform X2 [Crassostrea virginica]	HT_RI
-11.91394746	interferon regulatory factor 2-binding protein-like [Crassostrea virginica]	HT_RI
-10.43886642	integrin alpha-2-like isoform X10 [Crassostrea virginica]	HT_RI
11.26277301	integrin alpha-2-like isoform X5 [Crassostrea virginica]	HT_RI

12.22522579	integrin beta-like protein C isoform X1 [Crassostrea virginica]	HT_RI
9.093480693	nuclear factor of activated T-cells 5-like isoform X2 [Crassostrea virginica]	HT_RI
3.339268822	nuclear factor of activated T-cells 5-like isoform X2 [Crassostrea virginica]	HT_RI
Effectors		
5.805191913	serine protease inhibitor Cvsi-2-like [Crassostrea virginica]	HT_RI
-2.31732885	kunitz-type serine protease inhibitor conotoxin Cal9.1b-like [Crassostrea virginica]	HT_RI
-11.91394746	interferon regulatory factor 2-binding protein-like [Crassostrea virginica]	HT_RI
10.23389419	interferon-induced protein 44-like isoform X2 [Crassostrea virginica]	HT_RI
4.93870882	interferon-induced protein 44-like isoform X2 [Crassostrea virginica]	HT_RI
10.86738137	Signaling mucin HKR1 [Mizuhopecten yessoensis]	HT_RI
-9.926277007	Signaling mucin HKR1 [Mizuhopecten yessoensis]	HT_RI
-11.19554614	Signaling mucin HKR1 [Mizuhopecten yessoensis]	HT_RI
-7.627259354	integumentary mucin C.1-like isoform X3 [Crassostrea virginica]	HT_RI

5.366202536	mucin-17-like isoform X2 [Crassostrea virginica]	HT_RI
-5.772730703	mucin-17-like isoform X2 [Crassostrea virginica]	HT_RI
-13.12905042	mucin-2-like [Crassostrea virginica]	HT_RI
-5.905452651	mucin-5AC-like [Crassostrea virginica]	HT_RI
-8.77598416	mucin-5AC-like isoform X5 [Crassostrea virginica]	HT_RI
Apoptosis		
-7.924485961	caspase-1-like [Crassostrea virginica]	HT_RI
-9.46806142	caspase-1-like [Crassostrea virginica]	HT_RI
10.97406116	caspase-14-like isoform X2 [Crassostrea virginica]	HT_RI
-3.233698531	caspase-7-like [Crassostrea virginica]	HT_RI
-10.00654536	caspase-7-like [Crassostrea virginica]	HT_RI
-3.686137851	caspase recruitment domain-containing protein 14-like isoform X5 [Crassostrea virginica]	HT_RI

6.279723333	baculoviral IAP repeat-containing protein 2-like isoform X1 [Crassostrea virginica]	HT_RI
10.35840243	baculoviral IAP repeat-containing protein 2-like isoform X2 [Crassostrea virginica]	HT_RI
-9.552992158	baculoviral IAP repeat-containing protein 3-like [Crassostrea virginica]	HT_RI
-10.01313701	baculoviral IAP repeat-containing protein 3-like [Crassostrea virginica]	HT_RI
-7.12622932	baculoviral IAP repeat-containing protein 7-like isoform X3 [Crassostrea virginica]	HT_RI
11.86479373	Apoptosis inhibitor IAP [Crassostrea gigas]	HT_RI
5.960093895	LOW QUALITY PROTEIN: apoptosis-inducing factor 3-like [Crassostrea virginica]	HT_RI
2.788401176	protein kinase C iota type-like isoform X4 [Crassostrea virginica]	HT_RI
20.80442465	multiple epidermal growth factor-like domains protein 10 [Crassostrea virginica]	HT_RI
-6.43860015	multiple epidermal growth factor-like domains protein 10 [Crassostrea virginica]	HT_RI

-7.224748576	multiple epidermal growth factor-like domains protein 10 [Crassostrea virginica]	HT_RI
10.58406136	multiple epidermal growth factor-like domains protein 10 isoform X2 [Crassostrea virginica]	HT_RI
7.215833061	multiple epidermal growth factor-like domains protein 10 isoform X2 [Crassostrea virginica]	HT_RI
5.064940886	multiple epidermal growth factor-like domains protein 10 isoform X2 [Crassostrea virginica]	HT_RI
2.789935488	multiple epidermal growth factor-like domains protein 10 isoform X2 [Crassostrea virginica]	HT_RI
8.594189834	multiple epidermal growth factor-like domains protein 6 [Crassostrea virginica]	HT_RI
-10.50534165	multiple epidermal growth factor-like domains protein 6 [Crassostrea virginica]	HT_RI
10.20740385	multiple epidermal growth factor-like domains protein 6 isoform X1 [Crassostrea virginica]	HT_RI

10.20312945	multiple epidermal growth factor-like domains protein 6 isoform X1 [Crassostrea virginica]	HT_RI
7.471679862	multiple epidermal growth factor-like domains protein 6 isoform X1 [Crassostrea virginica]	HT_RI
8.053324948	programmed cell death protein 2-like isoform X1 [Crassostrea virginica]	HT_RI
11.33719413	cell death abnormality protein 1-like [Crassostrea virginica]	HT_RI
11.40372414	GTPase IMAP family member 7-like isoform X1 [Crassostrea virginica]	HT_RI
-10.0861395	DNA damage-regulated autophagy modulator protein 2-like [Crassostrea virginica]	HT_RI
Cytoskeletal reorganization		
9.029226712	LOW QUALITY PROTEIN: septin-2-like [Crassostrea virginica]	HT_RI
5.82670463	LOW QUALITY PROTEIN: septin-2-like [Crassostrea virginica]	HT_RI
4.705387778	LOW QUALITY PROTEIN: septin-2-like [Crassostrea virginica]	HT_RI
Others		

-9.657810466	lysosomal acid lipase/cholesteryl ester hydrolase-like [Crassostrea virginica]	HT_RI
14.2444413	lysosomal alpha-glucosidase-like isoform X1 [Crassostrea virginica]	HT_RI
-10.73769432	lysosomal-trafficking regulator-like isoform X4 [Crassostrea virginica]	HT_RI
10.97945875	glutathione S-transferase C-terminal domain-containing protein-like isoform X1 [Crassostrea virginica]	HT_RI
-6.354844406	glutathione-independent glyoxalase HSP31-like [Crassostrea virginica]	HT_RI
4.264547363	maleylacetoacetate isomerase-like [Crassostrea virginica]	HT_RI
11.70064297	Heat shock 70 kDa protein 12A [Crassostrea gigas]	HT_RI
-6.944842925	Heat shock 70 kDa protein 12A [Crassostrea gigas]	HT_RI
-6.695090302	heat shock 70 kDa protein 12A-like [Crassostrea virginica]	HT_RI
-10.35513436	heat shock 70 kDa protein 12A-like [Crassostrea virginica]	HT_RI
-10.53471861	heat shock 70 kDa protein 12A-like [Crassostrea virginica]	HT_RI
-10.66480031	heat shock 70 kDa protein 12A-like [Crassostrea virginica]	HT_RI
10.32935057	heat shock 70 kDa protein 12A-like isoform X1 [Crassostrea virginica]	HT_RI

5.822952857	heat shock 70 kDa protein 12A-like isoform X1 [Crassostrea virginica]	HT_RI
3.278219628	actin	HT_RI
11.26723475	actin-3-like isoform X1 [Crassostrea virginica]	HT_RI
-3.288605929	tripartite motif-containing protein 2-like [Crassostrea virginica]	HT_RI
-28.23091398	tripartite motif-containing protein 2-like [Crassostrea virginica]	HT_RI
11.97821512	tripartite motif-containing protein 2-like isoform X1 [Crassostrea virginica]	HT_RI
6.138377301	tripartite motif-containing protein 3-like [Crassostrea virginica]	HT_RI
4.616161652	tripartite motif-containing protein 3-like [Crassostrea virginica]	HT_RI
-5.355761746	tripartite motif-containing protein 3-like [Crassostrea virginica]	HT_RI
20.95857011	tripartite motif-containing protein 3-like isoform X1 [Crassostrea virginica]	HT_RI
-6.051600924	tripartite motif-containing protein 55-like [Crassostrea virginica]	HT_RI
-12.53777229	protein phosphatase 1 regulatory subunit 11-like [Crassostrea virginica]	HT_RI
7.873357051	protein phosphatase 1 regulatory subunit 12B-like isoform X13 [Crassostrea virginica]	HT_RI

7.282656314	protein phosphatase 1 regulatory subunit 12B-like isoform X13 [Crassostrea virginica]	HT_RI
-12.67858882	protein phosphatase 1 regulatory subunit 12B-like isoform X13 [Crassostrea virginica]	HT_RI
14.50001928	protein phosphatase 1 regulatory subunit 37-like [Crassostrea virginica]	HT_RI
-3.004388061	protein phosphatase 1 regulatory subunit 37-like [Crassostrea virginica]	HT_RI
-6.650729756	protein phosphatase 1 regulatory subunit 42-like isoform X5 [Crassostrea virginica]	HT_RI
6.602761261	multidrug resistance-associated protein 5-like isoform X2 [Crassostrea virginica]	HT_RI
-10.91470969	multidrug resistance-associated protein 5-like isoform X2 [Crassostrea virginica]	HT_RI
-13.24809012	multidrug resistance-associated protein 5-like isoform X2 [Crassostrea virginica]	HT_RI
9.2023305	cytochrome b5 reductase 4-like isoform X3 [Crassostrea virginica]	HT_RI
16.35993502	cytochrome c oxidase subunit III (mitochondrion) [Crassostrea virginica]	HT_RI
-12.35764438	cytochrome P450 27C1-like [Crassostrea virginica]	HT_RI

-10.85697882	cytochrome P450 2C42-like [Crassostrea virginica]	HT_RI
2.675102652	Cytochrome P450 3A11 [Crassostrea gigas]	HT_RI
-4.440107252	cytochrome P450 3A29-like [Crassostrea virginica]	HT_RI
-7.518489118	cytochrome P450 4V2-like isoform X1 [Crassostrea virginica]	HT_RI
-12.02720282	Ig-like and fibronectin type-III domain-containing protein 2 [Crassostrea virginica]	HT_RI
7.683007501	macrophage migration inhibitory factor-like [Crassostrea virginica]	HT_RI
2.309585304	histone H2B-like [Crassostrea virginica]	HT_RI
-9.231433964	perlucin-like isoform X2 [Crassostrea virginica]	HT_RI
9.231041006	perlucin-like protein [Crassostrea virginica]	HT_RI
9.062890569	perlucin-like protein [Crassostrea virginica]	HT_RI
-4.500074021	perlucin-like protein [Crassostrea virginica]	HT_RI
-6.333143999	Chitin synthase C [Crassostrea gigas]	HT_RI