Evidence for Immune Regulation as the Mechanism By Which Probiotic Strain OY15 (Vibrio alginolyticus) Can Improve Survival of Eastern Oyster Larvae (Crassostrea virginica)

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EVIDENCE FOR IMMUNE REGULATION AS THE MECHANISM BY WHICH PROBIOTIC STRAIN OY15 (*VIBRIO ALGINOLYTICUS*) CAN IMPROVE SURVIVAL OF EASTERN OYSTER LARVAE (*CRASSOSTREA VIRGINICA*)

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ABSTRACT Developing durable methods for controlling microbial pathogenesis in aquaculture with probiotic bacteria is becoming increasingly preferred over antibiotics. Recent research at the Milford Laboratory has shown that naturally occurring bacteria isolated from the digestive glands of adult oysters, *Crassostrea virginica* (Gmelin, 1791) could be potential probiotic candidates in oyster larviculture. Bacterial probiotic candidates were selected using the Kirby-Bauer disc diffusion method, wherein zones of inhibition suggest competitive exclusion of a pathogen by a probiotic candidate. Challenge studies reported previously indicated that addition of one probiotic candidate, isolate OY15 (*Vibrio alginolyticus*) [Miyamoto et al. 1961, Sakazaki 1968 (AL)] to oyster larvae challenged with a known shellfish-larval pathogen B183 (*Vibrio coralliilyticus*) (Ben-Haim et al.), significantly improved larval survival ($P < 0.0141$) compared with the pathogen alone. Mechanisms responsible for this effect were investigated with an *in vitro* study to determine the effects of probiotic strain OY15, or the pathogenic strain B183, upon oyster hemocytes and their immune functions. This study provides strong evidence that probiotic candidate OY15 stimulates oyster hemocyte immune functionality. In contrast, pathogen B183 caused immune suppression of hemocyte immune functions and significantly higher mortality of these cells. Based upon these results using hemocytes removed from adult oysters, upregulation of larval hemocyte immune activity is at least partially responsible for improved survival of larvae challenged with the pathogen B183.

KEY WORDS: probiotics, aquaculture, oyster immune functions, shellfish, flow cytometry, *Vibrio alginolyticus*, *Crassostrea virginica*

INTRODUCTION

Global aquaculture production of seafood for human consumption has grown to an all-time high of 136.16 million tons, with an estimated value of US $365.98 billion as of 2023 (FAO Fisheries & Aquaculture Department 2025). Aquaculture production is vulnerable to diseases caused by pathogenic microorganisms and adverse environmental conditions. Disease outbreaks resulting in partial or total loss of production runs can cause major financial losses for commercial growers. In recent years, disease outbreaks have caused major production losses affecting Atlantic salmon in Chile, oysters in Europe, and shrimp farming in Asia, South America, and Africa. Aquaculture in China, which currently accounts for more than 60% of global aquaculture production by volume (FAO Fisheries & Aquaculture Department 2025), suffered severe production losses in 2010, not only to disease but also to natural disasters and pollution (FAO Fisheries & Aquaculture Department 2020). Prophylactic use of chemical disinfectants and antimicrobial drugs for sanitation and growth enhancement used to control disease outbreaks have been overused (Van den Bogaard & Stobberingh 2000), leading to the emergence of antibiotic-resistant strains of bacteria capable of transferring their resistance genes to other bacterial strains via horizontal gene transfer (Schwarz et al. 2001, Akinbowale et al. 2006). Disease events caused by resistant bacterial strains are more difficult to treat by means of standard, aquaculture-approved methods (World Health Organization 2006), leading to considerable research interest in alternative management practices for controlling bacterial pathogenesis in aquaculture. Durable, sustainable methods, such as the use of probiotic bacteria, are becoming viable and sustainable alternatives in commercial hatcheries to use of chemical treatments. Fuller (1989) defined a probiotic bacterium as "a live microbial feed supplement which beneficially affects the host animal by improving its intestinal balance" (p. 366). According to Fuller, probiotic bacteria can enhance nutrition of bivalve veliger larvae by providing early colonization of microflora in the gut to aid digestion and enhance early immunity in developing oyster larvae. As filter feeders, shellfish filter bacteria from surrounding seawater, causing a natural interaction between ambient microbes and the gut microflora (Verschuere et al. 2000). Hence, Verschuere proposed a new definition of probiotic bacteria: "a live microbial adjunct which has a beneficial effect on the host by modifying the host-associated or ambient microbial community, by ensuring the improved use of the feed or enhancing its nutritional value, by enhancing the host response toward disease, or by improving the quality of its ambient environment: (p. 659)." These two definitions were adapted to summarize the concept of probiotics use in shellfish larviculture. More recently, Lazado and Caipang (2014a, 2014b) proposed a new definition of probiotics to reflect recent advances of probiotic research in aquaculture: "live or dead, or even a component of the microorganisms that act under different modes of action in conferring beneficial effects to the host or to its environment." These beneficial bacteria can be of host or nonhost origin (Lazado & Caipang 2014b, Lazado et al. 2015, Zorriehzahra et al. 2016). Lazado et al. (2015) found that host-associated microorganisms offer the best source of probiotics with diverse biochemical features.

Probiotics have several mechanisms to confer their beneficial effects to the host animal: probiotics can prevent damage to

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the host animal caused by bacterial pathogens by outcompeting the pathogen for nutrients, by improving the quality of the rearing environment, by producing antimicrobial substances that inhibit growth or attachment of pathogens, or by immune regulation (Moriarity 1998).

The Milford Laboratory has developed a naturally occurring probiotic bacterium, OY15, identified as a benign strain of *Vibrio alginolyticus* (Schreier & Schott 2014), isolated from the digestive glands of eastern oysters (*Crassostrea virginica*) that was found safe for use during coculture of oyster larvae and their microalgal feed T-ISO (*Tisochrysis lutea*) (Lim et al. 2011). In pilot-scale trials, supplementation of T-ISO with probiotic OY15 consistently conferred beneficial effects upon survival of veliger oyster larvae through metamorphosis (Kapareiko et al. 2011), the most critical phase in shellfish aquaculture when most mortalities often occur (Loosanoff & Davis 1963). Larval survival improved by 20%–35%, even when challenged with a known shellfish larval pathogen B183 (Kapareiko et al. 2011), identified as *Vibrio corallilyticus* by Schreier and Schott (2014).

Douillet and Langdon (1993) found that bacteria are readily ingested as food by oyster larvae. Development of a safe and effective probiotic strain is thought to be facilitated by the candidate probiotic being ingested by the host animal (Verschuere et al. 2000). A previous study at the Milford Laboratory confirmed ingestion of probiotic OY15 by oyster larvae (Kapareiko et al. 2011). Once within the larval digestive tract, OY15 may exert its probiotic effects by early colonization of the gut, resulting in improved digestion, growth, and survival either by competitive exclusion of the pathogen or by immune regulation. The objective of this study was to investigate immune regulation as a potential mechanism by which OY15 exerts its probiotic effects upon oyster larvae.

Bivalve molluscs rely upon an innate immune system to protect them against bacterial, viral, and parasitic diseases (Chong 2022). They have an open circulatory system wherein hemolymph circulates through a series of sinuses, providing oxygen and nutrients to the internal organs. Circulating white blood cells called hemocytes are responsible for oyster defense functions to protect against nonself particles that may be pathogens: recognition, aggregation, phagocytosis, and reactive oxygen species (ROS) release. Using flow cytometric techniques developed at the Milford Laboratory by Hégaret et al. (2003a, 2003b), hemolymph from adult oysters was used *in vitro* to determine effects of probiotic OY15 upon the innate immune cells, also present in oyster larvae (Elston 1980), targeting effects on hemocyte immune functions: viability, adhesion, phagocytosis, and ROS production.

MATERIALS AND METHODS

Hemolymph Extraction from Oysters

Hemolymph from 20 unselected, healthy, adult eastern oysters (*Crassostrea virginica*) held in ambient, nonfiltered seawater at the Milford Laboratory was extracted and pooled to perform total hemocyte counts and flow cytometric immune function analyses. A notch was snipped into the ventral edge of each oyster shell to provide space for insertion of a tuberculin, 1-mL needle and syringe for extraction of hemolymph from the adductor muscle of each oyster. Hemolymph from each oyster was examined by light microscopy to confirm that hemocytes were, indeed, present and the sample was not contaminated by other particles. Hemolymph was pooled and stored for a short time in a 50-mL conical-bottom Falcon centrifuge tube on ice to minimize cell clumping. Pooled hemolymph was vortexed and subsampled for total hemocyte counts both microscopically and by flow cytometry (Becton Dickinson BioSciences FACScan, San Diego, CA).

Bacterial Handling Protocols

Pure cultures of Milford probiotic strain OY15 and shellfish-larval pathogen B183 were revived from −80°C cryopreservation (REVCO Ultra-Low freezer), vortexed, inoculated into appropriate broth media (Marine Broth or OZR broth), and incubated for 18 h at 23°C in an AMBI-HI-LO incubator (Labline Instruments, Melrose Park, IL). Subsequent 18-h broth cultures were transferred aseptically into 15-mL Falcon centrifuge tubes and washed three times with sterile filtered seawater (SFSW), first by centrifugation at 4,000 × g for 20 min in a Beckman TJ-6 centrifuge to remove growth medium and any extracellular products (ECP) released by the bacterial isolates. Supernatant was removed by suction, and remaining bacterial pellets were resuspended in 1-mL SFSW. This suspension then was transferred aseptically into sterile 1.5-mL Eppendorf tubes for two more washes using a high-speed Tomy MTX-150 microcentrifuge for 2 min at 4,000 × g. After the final wash, pellets were resuspended (by vortexing) in a 1-mL volume of SFSW, resulting in a final bacterial concentration of 2×10^9 cfu/mL.

Protocols for Flow Cytometric Analysis of Hemocyte Immune Functions

Aliquots of pooled hemolymph from adult oysters were distributed into 5-mL flow cytometry tubes (Falcon; BD Biosciences, San Jose, CA) and mixed with SFSW for the following treatments: 10 control hemolymph tubes (no added bacteria), 10 tubes of hemolymph exposed to probiotic strain OY15, and 10 tubes of hemolymph exposed to pathogenic strain B183 (*Vibrio corallyliticus*) (Lim et al. 2011) at a ratio of 25 bacteria:1 hemocyte. This bacterium-to-hemocyte ratio was chosen to be high enough to activate immune function responses (Buggé et al. 2007). Oyster hemocyte immune functions were analyzed using methods developed by Hégaret et al. (2003a, 2003b, described briefly below). Flow cytometry data were analyzed using WINMDI 2.8 software.

Characterization of Oyster Hemocytes

A 100-µL subsample of pooled oyster hemolymph was distributed into each of 10 replicate, 5-mL flow cytometry tubes per treatment (three treatments: control with no added bacteria, probiotic OY15 added, and pathogen B183 added), and mixed with 300-µL SFSW to avoid osmotic stress on hemocytes during analysis. Flow cytometric analyses of these hemolymph samples occurred immediately after tube preparation to determine hemocyte size and complexity based upon Forward Scatter (hemocyte size)/Side Scatter (hemocyte complexity) plots to distinguish granular and agranular hemocytes.

Viability of Oyster Hemocytes

Flow cytometry, coupled with live/dead differential staining dyes SYBR Green I (SGI) and propidium iodide, was used to quantify the number of live versus dead oyster hemocytes.

SGI (Sigma Aldrich) is a nucleic acid stain that enters both live and dead cell membranes conferring green fluorescence detected by the flow cytometer FL1 channel at a wavelength of 520 nm. Propidium iodide (Sigma Aldrich) is membrane impermeable and enters only dead cells or cells with compromised membranes. Propidium iodide binds with double-stranded DNA in nonviable cells and fluoresces at wavelengths higher than 630 nm, that is, the flow cytometer FL2 channel. A 100-µL subsample of pooled oyster hemolymph was distributed into each of 10 replicate, 5-mL flow cytometry tubes per treatment (three treatments as above) and mixed with 300 µL of SFSW. To each tube, 4 µL of SGI (final concentration 10^3 mL^{-1}), and 4 µL of PI (final concentration 20 µg mL^{-1}) were added. Tubes were incubated in darkness at room temperature for 1 h and analyzed on the flow cytometer to determine the percentages of live and dead hemocytes.

Adhesion of Oyster Hemocytes

Using 24-well, flat-bottom, tissue culture microplates (Falcon, BD Biosciences), a 100-µL subsample of pooled oyster hemolymph was pipetted into each of 10 replicate wells per treatment (three treatments as above) and mixed with 100 µL of SFSW. Microplates were incubated in darkness at room temperature for 3 h, allowing time for hemocytes to settle and attach to well bottoms. After 3 h, any remaining unattached hemocytes in each well were resuspended by pipetting 150 µL of the hemolymph/SFSW mixture up and down several times and then transferring to corresponding 5-mL flow cytometry tubes containing 200 µL of 6% formalin and 4 µL of SGI. Flow cytometry tubes were vortexed and subsequently incubated in darkness at room temperature for one additional hour, and then analyzed on the flow cytometer to determine counts of nonadhered hemocytes. Percentages of adhered hemocytes were calculated as the difference between total hemocyte counts in characterization tubes and nonadhered hemocyte counts in adhesion tubes.

Phagocytosis of Fluorescent Beads by Oyster Hemocytes

A 150-µL subsample of pooled oyster hemolymph was distributed into each of 10 replicate, 5-mL flow cytometry tubes per treatment (three treatments as above) and mixed with 150 µL of SFSW and 30 µL of Fluoresbrite bead working stock suspension (Fluoresbrite YG Microspheres, 2.00 µm; Polysciences). Flow cytometry tubes were incubated in darkness at room temperature for 2 h and analyzed using the flow cytometer to determine counts of highly phagocytic hemocytes, that is, those with three or more fluorescent beads.

Reactive Oxygen Species Production of Oyster Hemocytes

Using flow cytometry, Bass et al. (1983) developed a quantitative assay for oxidative (respiratory) burst using 2′ 7′-dichlorofluorescein diacetate (DCFH-DA); DCFH-DA is a nonfluorescent fluorescein analogue that diffuses into blood cells and is then hydrolyzed into DCFH. Intracellular DCFH is oxidized to highly fluorescent 2′ 7′-dichlorofluorescein by ROS, for example, H_2O_2. In some applications, respiratory burst is induced by the addition of phorbol 1,2-meristate 1,3-acetate (PMA; Sigma Aldrich) to the hemocytes, achieving a quadrupling of fluorescence when compared with unstimulated hemocytes and measuring the ROS potential of the cells. Oyster hemocytes in this research were not stimulated; they were simply exposed to either the bacterial probiotic strain OY15 (*Vibrio alginolyticus*) or the shellfish larval pathogen B183 (*Vibrio coralliilyticus*). A 100-µL subsample of pooled oyster hemolymph was distributed into each of 10 replicate, 5-mL flow cytometry tubes per treatment (three treatments as above) and mixed with 300 µL of SFSW and 4 µL of dichlorofluorescein diacetate (DCFH-DA, Invitrogen). Flow cytometer tubes containing oyster hemocytes mixed in SFSW were used as hemocyte controls for background levels of respiratory burst without bacterial exposure. Flow cytometry tubes were incubated in darkness at room temperature for 2 h and flow cytometric analyses using the FACScan flow cytometer were conducted to determine ROS production by hemocytes in response to probiotic strain OY15 or pathogen B183.

STATISTICAL ANALYSIS

Hemocyte immune function data for characterization, viability, and phagocytosis percentages were presented as a square root of the percentage and arcsine-transformed to normalize variances (Zar 1996). Reactive oxygen species data were not transformed as these values are based upon fluorescence intensity. Analysis of variance (Statgraphics Plus 2001, Software Version 5.1; Statpoint Technologies, Warrenton, VA) was used to analyze nontransformed ROS data and transformed, normally distributed hemocyte immune function data (for granular and agranular hemocytes), for significant differences in comparison with control hemolymph (no bacteria added). Analysis was performed at the $P < 0.05$ level. All graphs are presented as untransformed percentage values to portray magnitudes of contrasts.

RESULTS

Characterization of Oyster Hemocytes

No significant differences were found in the percentages of granular or agranular hemocytes between control hemolymph (no bacteria, mean % granular hemocytes = 12.4, SE ± 0.33, mean % agranular hemocytes = 83.0, SE ± 0.29) or hemolymph exposed to probiotic OY15 (mean % granular hemocytes = 10.7, SE ± 0.37, mean % agranular hemocytes = 85.4, SE ± 0.50) (Fig. 1). In contrast, significant mortality of agranular hemocytes ($P < 0.0000$) was evident in hemolymph exposed to shellfish-larval pathogen B183; therefore, the percentage of granular hemocytes in pathogen-dosed hemolymph was shifted significantly upward ($P < 0.0000$) over that of the control (mean % granular hemocytes = 40.3, SE ± 2.04, mean % agranular hemocytes = 54.4, SE ± 2.01).

Viability of Oyster Hemocytes

No significant differences in mortality were found for granular ($P < 0.212$) or agranular ($P < 0.933$) hemocytes between control hemolymph (no bacteria, mean % granular hemocytes = 7.57, SE ± 1.18, mean % agranular hemocytes = 2.32, SE ± 0.20) and hemolymph dosed with probiotic OY15 (mean % granular hemocytes = 9.51, SE ± 1.01, mean % agranular

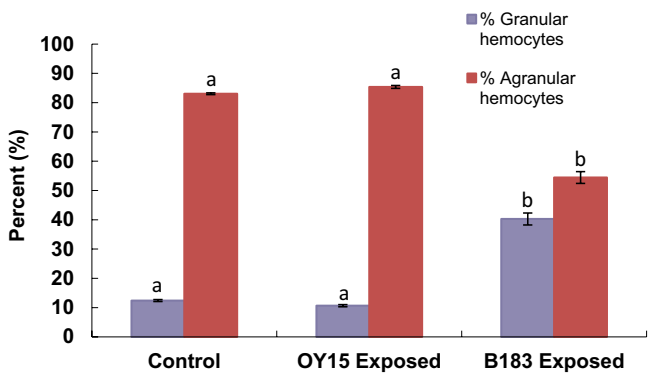

Figure 1. Characterization of immune cell types. No significant differences (ANOVA $P > 0.05$) were found in percentages of granular or agranular hemocytes between control hemolymph (no bacteria) and hemolymph dosed with probiotic OY15. The percentage of granular hemocytes in hemolymph dosed with shellfish-larval pathogen B183 significantly increased over that of the control (no bacteria) ($P < 0.0000$). This shift in percentage was the result of significant mortality of agranular hemocytes caused by pathogen B183.

hemocytes = 2.28, SE ± 0.16), confirming OY15 is safe as a probiotic candidate for use in oyster larviculture (Fig. 2). In contrast, exposing hemolymph to shellfish-larval pathogen B183 significantly increased mortality for both granular ($P < 0.024$) and agranular hemocytes ($P < 0.0037$) (mean % granular hemocytes = 11.6, SE ± 1.25, mean % agranular hemocytes = 4.12, SE ± 0.58) over that of control hemolymph (no bacteria).

Adhesion of Oyster Hemocytes

No significant differences in the percentage of adhered hemocytes were found for either granular ($P < 0.2904$) or agranular hemocytes ($P < 0.5078$) between control hemolymph (no bacteria, mean % granular hemocytes = 25.0, SE ± 3.26, mean % agranular hemocytes = 63.3, SE ± 4.05) and hemolymph dosed with probiotic OY15 (mean % granular hemocytes = 29.0, SE ± 2.35, mean % agranular hemocytes = 60.3, SE ± 2.39) (Fig. 3). Hemocyte exposure to pathogen B183 significantly decreased the percentage of adhered granular hemocytes by about 14% ($P < 0.0013$), (mean % granular hemocytes = 11.7, SE ± 1.75, mean % agranular hemocytes = 72.5, SE ± 2.12) when compared with control (no bacteria) hemolymph resulting from increased mortality of hemocytes from this pathogen. The percentage of adhered agranular hemocytes exhibited an unusual significant shift upward, although not statistically significant ($P < 0.0606$).

Phagocytosis of Oyster Hemocytes

Exposing hemolymph to probiotic OY15 significantly increased the percentage of highly phagocytic hemocytes by 7% ($P < 0.0335$) (mean % granular hemocytes = 14.4, SE ± 2.75), over that of the no-bacteria control (mean % granular hemocytes = 8.29, SE ± 0.76). Hemolymph exposure to pathogen B183 had no effect on phagocytosis ($P < 0.3405$) (mean % granular hemocytes = 7.08, SE ± 1.84) (Fig. 4).

Reactive Oxygen Species Release by Oyster Hemocytes

Hemolymph exposure to probiotic OY15 had no significant effects upon activation of ROS release by granular hemocytes (mean fluorescence of granular hemocytes = 13,100, SE ± 1,000) compared with the no-bacteria control (mean fluorescence of granular hemocytes = 10,340, SE ± 2,200). In contrast, hemolymph exposure to pathogen B183 significantly decreased ROS generation within granular hemocytes ($P < 0.0000$) (mean fluorescence of granular hemocytes = 6,950, SE ± 590) below that of the no-bacteria control, as well as hemolymph treated with OY15, indicating suppression of this important immune function. No ROS effects upon agranular hemocytes were evident for any treatment (Fig. 5).

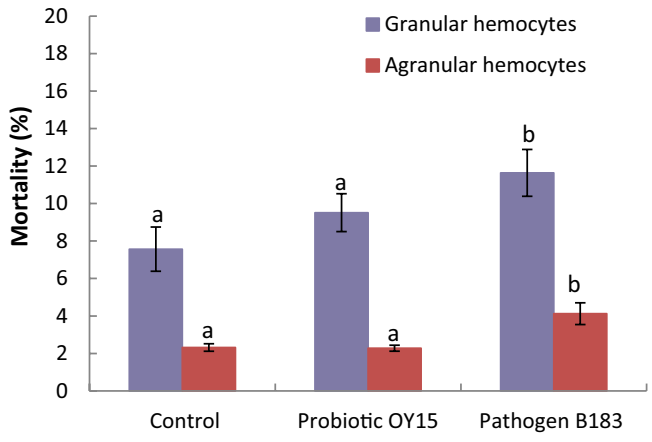

Figure 2. Viability of oyster hemocytes. No significant differences in percentage mortality were found for both granular ($P < 0.212$) and agranular ($P < 0.933$) hemocytes between control hemolymph (no bacteria) and hemolymph dosed with probiotic OY15, confirming OY15 is safe for use as a probiotic candidate for oyster larviculture. Conversely, hemolymph dosed with shellfish-larval pathogen B183 significantly increased mortalities for both granular and agranular hemocytes over that of control hemolymph (no bacteria).

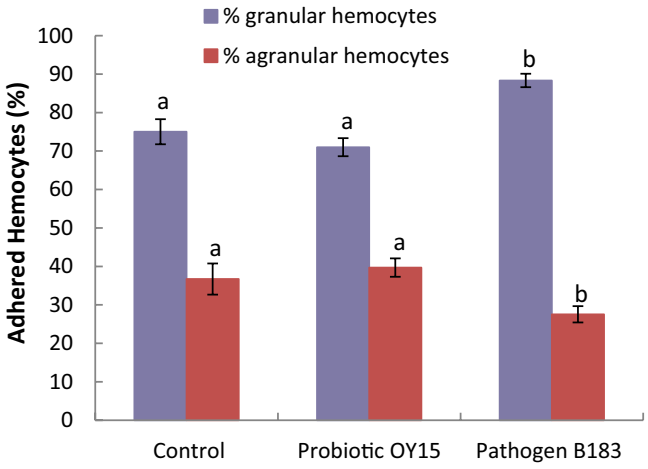

Figure 3. Percentage of adhered oyster hemocytes. No significant differences (ANOVA $P < 0.05$) in the percentage of adhered hemocytes were found for both granular ($P < 0.2904$) and agranular ($P < 0.5078$) hemocytes between control hemolymph (no bacteria) and hemolymph dosed with probiotic OY15. Exposure to pathogen B183 significantly stimulated adhesion of granular hemocytes by 14% and caused an unusual significant decrease in adhesion of agranular hemocytes.

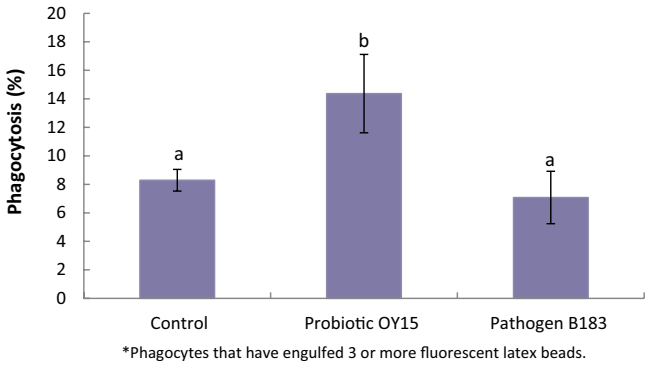

Figure 4. Percentage of highly phagocytic hemocytes. Hemocyte exposure to probiotic candidate OY15 significantly increased the percentage of highly phagocytic hemocytes (those that engulfed three or more fluorescent latex beads) by 7% over that of the no-bacteria control (ANOVA $P < 0.0335$). Hemolymph exposure to pathogen B183 appeared to have no effect on phagocytosis, possibly due to hemocyte mortality prior to phagocytosis.

DISCUSSION

This study provides strong evidence that immune stimulation is a mechanism by which probiotic strain OY15 exerts its positive effects upon eastern oyster larvae. Although adhesion of granular hemocytes, the preliminary step in oyster immune response before phagocytosis, was not affected by addition of OY15, phagocytosis was stimulated by this probiotic strain. Phagocytic hemocytes, whose functions are to engulf and kill potential pathogens, are primarily granular cells representing 6%–14% of the total population of circulating hemocytes in the adult eastern oyster (Wikfors & Alix 2014). These granular, phagocytic hemocytes are activated in response to foreign particles such as bacterial pathogens and parasites and are the primary line of defense for bivalve molluscs in foreign particle elimination. This study demonstrates the ability of probiotic OY15 to stimulate adult oyster hemocyte phagocytosis *in vitro*.

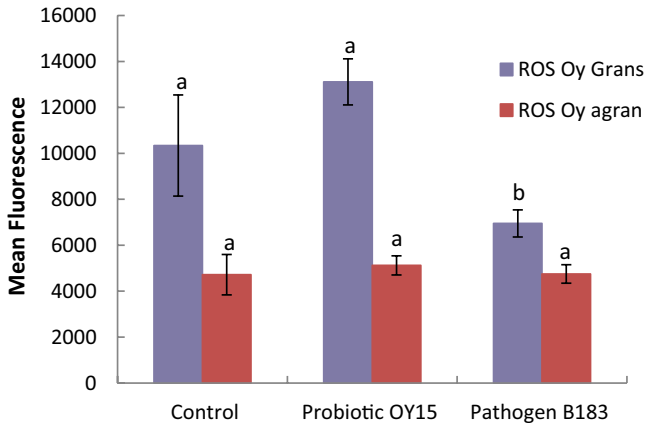

Figure 5. Hemolymph exposure to probiotic OY15 increased the mean fluorescence upon ROS release of granular hemocytes over that of the no-bacteria control, although not significantly. Hemolymph exposure to pathogen B183 significantly decreased ROS release of granular hemocytes (ANOVA $P < 0.0000$) below that of the no-bacteria control as well as hemolymph treated with OY15, indicating suppression of this immune function due to mortality of these cells. No ROS effects for agranular hemocytes were evident for any treatment.

Recognizing that hemocytes in oyster larvae have similar functions to those in adult oysters (Song et al. 2016), immunostimulation appears to be a mechanism by which OY15 improves survival of larval oysters when challenged with a pathogen (Kapareiko et al. 2011).

Several mechanisms of action by probiotics upon the host animal have been identified: (1) probiotic bacteria can modulate the growth of intestinal microbes (Giorgio et al. 2010) and suppress the proliferation of potential pathogens by production of ECP that have bactericidal or bacteriostatic effects, including antibiotic substances, bacteriocins, siderophores as well as lysozymes, and proteases (Bruno & Montville 1993, Pybus et al. 1994, Sugita et al. 1997); (2) competition with other microbial populations for available nutrients necessary for energy and survival; (3) competition for adhesion sites in the host gut tissue; and (4) upregulation of the host immune response (Moriarity 1998).

Initially, competitive exclusion was thought to be the mechanism by which OY15 exerted its probiotic effects on oyster larvae, thus improving survival. According to Gause's Law (Gause 1934), the principle of competitive exclusion states that two species competing for the same available energy and nutritional resources cannot coexist stably. One species will have an advantage that will bring about extinction of the inferior species. Probiotic bacteria can compete with pathogenic bacteria for nutrients, preventing them from multiplying and becoming established in the host digestive tract (Cummings & Macfarlane 1997). Probiotic bacteria also can exclude pathogenic bacteria by attaching to and colonizing the surface of the gut, thus "blocking" attachment and preventing colonization of pathogenic bacteria (Chichlowski et al. 2007). Attachment of probiotic bacteria to gut tissue benefits the host by enhancing the barrier integrity of the "tight junction" of intestinal epithelia, a biological barrier that prevents the entrance of pathogenic bacteria into the tissues of the host (Chichlowski et al. 2007, Ng et al. 2009).

The probiotic selection process was based upon the premise of competitive exclusion, which was based upon the effectiveness of probiotic OY15 in inhibiting pathogen B183 on solid agar in Kirby-Bauer Disc Diffusion studies (Lim et al. 2011). There is no evidence, however, that would indicate competitive exclusion as the mechanism by which OY15 exerts its probiotic effects upon oyster larvae. Denaturing gradient gel electrophoresis analysis of the microbial communities of both larvae and culture water sampled after supplementation with 10^3 cfu mL^{-1} of probiotic OY15 and challenged with 10^5 cfu mL^{-1} pathogen B183 during pilot-scale bioassays (Kapareiko et al. 2011) indicated a diverse microbial community associated with the larvae and the culture water. The 16S rRNA gene sequences for probiotic OY15 and pathogen B183, however, were not found in either the larvae or the culture water 2 days postdosing (Schreier et al., unpublished). This evidence suggests no colonization of larvae or culture water by either bacterium, which is inconsistent with the theory of competitive exclusion.

Successful hatchery production of molluscan shellfish seed requires stringent controls for providing good nutrition and disease prevention for growing, metamorphosing larvae. Pathogenic bacteria, commonly of the genus *Vibrio* (Estes et al. 2004) can cause disease outbreaks during shellfish larviculture, which can result in significant mortalities and financial losses for commercial growers. The most common mode of action

of these pathogens is to infect the ligament and soft tissue of larvae during metamorphosis, the most critical phase of shellfish larviculture (Elston et al. 2008). In 2006, the World Health Organization published warnings regarding overuse of chemical sanitation measures to prevent bacterial disease in shellfish hatcheries. Emergence of resistant strains of bacterial pathogens has increased, and resistant strains are increasingly difficult to treat with standard antibiotics approved for aquaculture (World Health Organization 2006). Hence, the use of probiotic bacteria as a viable, durable, alternative method for disease control during commercial shellfish larviculture has increased globally in recent years. Peraza-Gomez et al. (2009) confirmed that a probiotic strain of *Vibrio alginolyticus* improved survival of Ecuadorian-cultured white shrimp *Litopenaeus vannamei*, increasing production by 35%. Vieira et al. (2010) found that survival of marine shrimp fed a probiotic-supplemented diet was improved, even during challenge with *Vibrio harveyi*, a known shrimp pathogen. Mortality of juvenile Atlantic salmon (*Salmo salar*) challenged with pathogens *Aeromonas salmonicida*, *Vibrio anguillarum*, and *Vibrio ordalii* was significantly decreased using *V. alginolyticus* as a probiotic (Austin et al. 1995). Karim et al. (2013) identified two bacterial isolates, *Phaeobacter* sp. S4 and *Bacillus pumilus* R106-95, as probiotic agents that can improve survival of larval and juvenile eastern oysters (*Crassostrea virginica*) challenged with oyster pathogens *Vibrio tubiashii* and *Roseovarius crassostreae*. This broad, scientific evidence underscores the potential for probiotic isolates, including of *V. alginolyticus*, to be effective tools in aquaculture hatcheries.

The findings for this study demonstrating the ability of probiotic bacterium OY15 to stimulate hemocyte immune functions in hemolymph from adult eastern oysters are consistent with other published work regarding the ability of probiotic bacteria to stimulate the immune response in marine animals. Vaughan et al. (2002) found probiotic strains that stimulated the innate immune response against pathogenic bacteria in shellfish. Effects of probiotic bacteria and their ECP have been reported to stimulate ROS production in bivalves (Lambert et al. 2003, Buggé et al. 2007), the last step of the innate immune response of shellfish for elimination of pathogens. Probiotic bacteria have been shown to stimulate and improve immunity against known shrimp pathogens in the cultured shrimp *Litopenaeus vannamei* (Vieira et al. 2010, Partida-Arangure et al. 2013). Vieira et al. (2010) found probiotic supplementation improved resistance and survival of marine shrimp challenged with *Vibrio harveyi*, a known shrimp pathogen, by increasing total hemocyte counts and serum agglutination. Probiotic bacteria have been found to modulate immune responses in fish larvae (Olafsen & Hansen 1992, Balcazar et al. 2006), including carp (Stosik & Szenfeld 1996), and rainbow trout (Irianto & Austin 2002). Irianto and Austin (2002) confirmed probiotic supplementation with an inactivated culture of *Vibrio fluvialis* helped stimulate cellular immunity in rainbow trout, thus controlling furunculosis.

CONCLUSIONS

Greater investment in development of nonchemical methods, such as use of probiotics, to promote a healthy gut microbial community within oyster larvae, and thus improving survival and disease resistance, is needed to keep production of bivalve molluscs increasing efficiently and sustainably. This study has provided strong evidence that the mechanism of the probiotic effects of OY15 includes immune stimulation of the larval oyster innate immune system, thus improving the effectiveness of cellular pathogen-killing capability and improving larval survival by 20%–35%. Based upon the results of the present study, flow cytometric studies on shellfish hemocyte immune function responses to other bacterial isolates cultured from the digestive glands may be used as a screening method for selection and identification of future bacterial probiotic candidates.

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