Dietary Supplementation of Debaryomyces Hansenii Enhanced Survival, Antioxidant and Immune Response in Juvenile Shrimp Penaeus Vannamei Challenged with Vibrio Parahaemolyticus

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SUMMARY

Background: The excessive use of antibiotics in shrimp aquaculture cause severe ecological damage. Immunosuppressant probiotics are an alternative prophylactic treatment to enhance antioxidant and immune response to reduce mortality induced by pathogenic microbes. Objective: This study investigated the effect of live yeast Debaryomyces Hansenii incorporated in diet and in culture water on survival and expression of the antioxidant and immune-related genes in Penaeus vannamei juvenile shrimp. Methodology: Shrimp were fed daily for 10 days with treatments of different doses and a post-infection with Vibrio parahaemolyticus as follows: a) control (-) without feed additives and non-infected; b) control (+) without feed additives and infected; c) D. hansenii, 1×10⁶ CFU g⁻¹ feed; d) inulin, 2.5 mg g⁻¹ feed; e) D. hansenii, 1×10⁶ CFU g⁻¹ feed + inulin, 2.5 mg g⁻¹ feed; f) D. hansenii, 2×10⁶ CFU g⁻¹ feed + 1×10⁶ CFU mL⁻¹; g) D. hansenii, 4×10⁶ CFU g⁻¹ feed + 1×10⁶ CFU mL⁻¹. Relative gene expression of lysozyme (LYS), glutathione peroxidase (GPX), and superoxide dismutase (SOD) were determined in shrimp muscle and hepatopancreas previous to challenge with V. parahaemolyticus. Results: Juvenile shrimp increased the resistance to V. parahaemolyticus infections significantly more than untreated shrimp after D. hansenii administration in feed and water, and mixed with inulin. Relative gene expressions of LYS and SOD increased significantly in shrimp muscle after treated with D. hansenii and D. hansenii + inulin, respectively. SOD and GPX were significantly expressed in shrimp hepatopancreas. Implications: The findings provide new insights to apply yeast immunostimulants in reared shrimp to increase immune response and survival against experimental bacterial infections. Conclusions: In this study, juvenile shrimp exposed to additive immunostimulants increased gene expression in shrimp tissues, muscle and hepatopancreas, and the dose of 1×10⁶ CFU g⁻¹ of D. hansenii in feed was sufficient to increase shrimp survival against V. parahaemolyticus infection.

Keywords: Penaeus vannamei; antioxidants; gene expression; Debaryomyces hansenii; immune response; immunostimulants.

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RESUMEN

Antecedentes: El uso excesivo de antibióticos en la acuicultura de camarón causa severos daños ecológicos. Los probióticos inmunoestimulantes son una alternativa para mejorar la respuesta inmune y reducir mortalidad inducida por microbios patógenos. Objetivo: Este estudio investigó el efecto de la levadura viva Debaryomyces hansenii incorporada en la dieta y en el agua de cultivo sobre la supervivencia y expresión de los genes antioxidantes y relacionados con el sistema inmunológico en camarones juveniles Penaeus vannamei. Metodología: Los camarones se alimentaron diariamente durante 10 días con tratamientos de diferentes dosis y una postinfección con Vibrio parahaemolyticus de la siguiente manera: a) control (-) sin aditivos alimentarios y no infectados; b) control (+) sin aditivos alimentarios e infectados; c) D. hansenii, 1 x 10^6 UFC g^-1 de alimento; d) inulina, 2,5 mg g^-1 de alimento; e) D. hansenii, 1 x 10^6 UFC g^-1 de alimento + inulina, 2,5 mg g^-1 de alimento; f) D. hansenii, 2 x 10^6 UFC g^-1 de alimento + 1 x 10^4 UFC mL^-1; g) D. hansenii, 4 x 10^6 UFC g^-1 de alimento + 1 x 10^6 UFC mL^-1. Se determinó la expresión genética relativa de lisoízima (LYS), glutación peroxidasa (GPX) y superoxído dismutasa (SOD) en músculo de camarón y hepatopáncreas antes y después de la exposición a V. parahaemolyticus. Resultados: Los camarones jóvenes aumentaron la resistencia a las infecciones por V. parahaemolyticus significativamente más que los camarones no tratados después de la administración de D. hansenii en el alimento y el agua, y mezclados con inulina. Las expresiones genéticas relativas de LYS y SOD aumentaron significativamente en el músculo del camarón después de ser tratado con D. hansenii y D. hansenii + inulina, respectivamente. SOD y GPX se expresaron significativamente en el hepatopáncreas de camarón. Implicaciones: Los resultados proveen nuevas perspectivas para aplicar levaduras inmunoestimulantes en camarón cultivado e incrementar respuesta inmune y supervivencia contra infecciones bacterianas experimentales. Conclusiones: En este estudio, los camarones juveniles expuestos a inmunoestimulantes aditivos aumentaron la expresión génica en los tejidos, músculos y hepatopáncreas del camarón, y la dosis de 1 x 10^6 UFC g^-1 de D. hansenii en el alimento fue suficiente para aumentar la supervivencia del camarón contra la infección por V. parahaemolyticus.

Palabras clave: Penaeus vannamei; antioxidantes; la expresión génica; Debaryomyces hansenii; respuesta inmune; inmunoestimulantes.
probiotics, a number of different biological and synthetic compounds have been used as immunostimulants to enhance the non-specific defense system in animals, including shrimp (Sung et al., 2001). One of these compounds is inulin and its derivates (oligofructose, fructooligosaccharides (FOS), which are basically constituted by linear fructose chains (Madriagil & Sangronis, 2007). Some authors have reported that diets supplemented with FOS have shown to improve immunity and growth rate of aquatic animals, such as soft-shell turtle, turbot larvae, and white shrimp (Mahious et al., 2006; Zhou et al., 2007).

The expression of immune-related genes is considered a potential marker of shrimp’s health status (Liu et al., 2007), as it provides relevant information about the activation and modulation of its immune system at molecular level (Qiao et al., 2013). In shrimp, lysoenzymes are hemocyte-specific proteins that possess cell-wall lytic activity against a range of gram-positive and gram-negative bacterial species as Vibrio sp. Lysoenzyme gene expression by reverse transcription polymerase chain reaction (RT-PCR) in hemocytes has been previously reported (De la Re-Vega et al., 2006; Burge et al., 2007; Qiao et al., 2013). Other important components in shrimp immune system are antioxidant molecules. They can prevent the deleterious effects of ROS (reactive oxygen species) playing a vital role in protecting cells against oxidative stress, preventing or repairing oxidative damage, and helping to maintain the levels of reducing agents, such as nicotinamide adenine dinucleotide (NAD+), nicotinamide adenine dinucleotide phosphate oxidase (NADPH), and glutathione (GSH) within cells (Downs et al., 2001). Antioxidant enzymes mainly include superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPX), glutathione reductase (GR) and glutathione-S-transferase GST (Zhang et al., 2005; Yang et al., 2010).

Therefore, the aim of this study was to evaluate the effect of dietary yeast D. hansenii on shrimp survival after challenge with V. parahaemolyticus and the immunological and antioxidant gene related indicators before infection.

MATERIALS AND METHODS

Juvenile shrimp

Shrimp were obtained from a commercial farm (Acuicola Cuate Machado, Guasave, Sinaloa, Mexico) and transferred to the laboratory at Centro Interdisciplinario de Investigación para el Desarrollo Integral Regional, Instituto Politécnico Nacional (CIDIR-IPN); they were acclimated for three days in a 1,000 L plastic tank provided with constant aeration and filtered (20 μm) seawater. Shrimp were fed ad libitum three times daily with commercial feed (40% crude protein; Animalnutri®), Los Mochis, Sinaloa, MX) and 50% water exchange was made daily. Water parameters (27°C; salinity of 30; dissolved oxygen 6 mg L⁻¹; pH 8.0) were maintained and daily recorded throughout the acclimation period.

Yeast preparation

Debaryomyces hansenii (CBS 8339) was provided by Dr. Dariel Tovar, preserved in 30% glycerol and stored at -80°C until use. The strain was inoculated in YPD (YPD, Sigma-Aldrich, U.S.A) broth medium containing 2.5% NaCl and incubated at 30°C for 48 h. Cell suspension was centrifuged at 1000 g at 4°C for 10 min, separating the pellet by decanting culture medium, resuspended in 2.5% sterile saline solution, and adjusted to an optical density of 1 at 620 nm. Cell density was adjusted to 1×10⁶ CFU mL⁻¹, which was finally confirmed by counting colony forming units (CFU) on plate serial dilutions of yeast cultures and incorporated in experimental diets at different concentrations.

Experimental diets

Yeast and inulin were incorporated at the basal diet (40% protein; Animalnutri®, Los Mochis, Sinaloa, MX) as described by Li et al. (2007). Briefly, live yeast cells were resuspended in 2.5% sterile saline solution and incorporated at the basal diet at different doses. Commercial inulin from blue agave (Agave tequiliana, IIDEAL, S.A. de C.V. Guadalajara, JAL, MX) was used as immunostimulant and included in juvenile shrimp diet (Luna-González et al., 2012). Dry Oil® (DO, Innovaciones Acuícolas, MX) was added to the feed as a binder to facilitate yeast and inulin incorporation. Pellets were made in a 3 mL plastic syringe without needle and then dried at room temperature for 18 h and stored at 4°C until use. Diets were prepared every other day according to the amount daily consumed for shrimp (5% of the shrimp biomass).

Vibrio parahaemolyticus preparation

Vibrio parahaemolyticus (strain IPNGS16) was isolated from shrimp farms during an acute hepatopancreatic necrosis disease (AHPND) outbreak in Mexico in 2014 by López-León et al. (2016). Briefly, bacteria were cultured in tryptic soy broth (TSB) (Difco, Le pont de Claix, FR) with 2.5% NaCl and incubated at 30°C for 18 h. Culture medium was removed by centrifugation (4000 g for 10 min); bacterial pellet was resuspended in saline buffer and adjusted to 1×10⁸ CFU mL⁻¹ for bacterial challenge. Before bioassay challenge, LC₅₀ was obtained as described in López-León et al. (2016).
Experimental design: Bioassay 1

Groups of 12 juvenile shrimp with an initial weight of 0.14 ± 0.02 g were distributed in 15 aquariums (6 L) with 4 L of filtered (20 μm) and aerated seawater in triplicate. Shrimp were evaluated for 10 days with feed additives as follows: a) Control (without feed additives); b) *D. hansenii* 1×10⁶ CFU g⁻¹ feed; c) inulin 2.5 mg g⁻¹ feed; d) *D. hansenii* 1×10⁶ CFU g⁻¹ feed + inulin 2.5 mg g⁻¹ feed. Juveniles were fed with diets twice a day at a rate of 5% at the total biomass per day. For the experimental tanks, seawater was set at 30% of salinity, average temperature of 28 ± 2°C, and 70% of water exchange was performed every other day. At the end of the feeding experiment, six shrimp per treatment were randomly sampled for gene expression analysis. After sampling, shrimp were infected with *V. parahaemolyticus* (1×10⁶ CFU mL⁻¹) by immersion method. Survival was determined at 48 h after infection.

Bioassay 2

From the results of bioassay 1, a second bioassay was designed, in which shrimp with an initial weight of 1.19 ± 0.02 g were distributed by triplicate in 42 L plastic tanks at a density of 10 juvenile tank⁻¹. Shrimp were fed daily with live *D. hansenii* in feed and directly into the rearing tanks as follows: a) Control (without feed additives); b) *D. hansenii* 2×10⁸ CFU g⁻¹ feed + 1×10⁶ CFU mL⁻¹; c) *D. hansenii* 4×10⁹ CFU g⁻¹ feed + 1×10⁶ CFU mL⁻¹. Juveniles were fed with diets twice a day at a rate of 5% at the total biomass per day. For the experimental tanks, filtered seawater (20 μm) was set at 30% of salinity, average temperature of 28 ± 2°C, and 70% of water exchange was performed every other day. After 10 days of culture, six shrimp per treatment were randomly sampled for gene expression analysis. After sampling, shrimp were infected with *V. parahaemolyticus* (1×10⁶ CFU mL⁻¹) by immersion method. Survival was determined at 72 h after infection.

Sampling

After 10 days of culture, muscle and hepatopancreas were extracted from each shrimp sampled for gene expression analysis. Six shrimp were taken per treatment for a total of 24 samples per bioassay, placed in Eppendorf tubes containing 400 μL of TRIzol™ reagent (Thermo Fisher Scientific, Waltham, MA USA), and kept at 2°C until use.

RNA extraction and cDNA synthesis

Total RNA was extracted from muscle and hepatopancreas of the shrimp sampled, using 800 μL TRIzol™ reagent following the manufacturer’s guidelines. The tissues were homogenized using 300-μM glass beads (Sigma-Aldrich, St. Louis, MO, USA) in a Fast Prep-24 Instrument (MP Biomedicals, Solon, OH, USA) using oscillatory pulses of 40 s at a speed of 6.0 m s⁻¹ at room temperature. RNA quantity was determined by spectrophotometry using a NanoDrop-1000 (ThermoScientific, Chicago, IL, USA). To visualize the integrity of the RNA obtained, 1 μg of total RNA from each sample was analyzed by electrophoresis technique in 1% non-denaturing agarose gels with 1X TAE buffer (Tris-Sodium acetate-EDTA), stained with GelRed (Biotium, Hayward, CA, USA). Total RNA was treated with RQ1 RNAse-free DNase (Promega, Madison, WI, USA) according to the manufacturer’s instructions. To verify the DNase treatment a non-amplification was done by polymerase chain reaction (PCR) using S12 primers (Table 1) in a 6 μL reaction containing 0.045 U GoTaq Flexi DNA Polymerase (Promega), 2.5 mM MgCl₂, 1X GoTaq Flexi Buffer, 0.3 mM dNTP Mix (Promega), 0.3 μM of each primer, and 1 μL of RNA-treatment. The PCR was performed using the following conditions: initial denaturation at 95°C for 4 min, 35 cycles at 95°C for 30 s, 60°C for 30 s, and 72°C for 30 s, and final extension at 72°C for 5 min. The non-amplification was verified with electrophoresis technique as described above. The cDNA synthesis was performed following the Improm II Reverse Transcriptase (Promega) protocol using 1 μg of RNA, with RNAsin Plus RNase inhibitor (Promega), and a mix of oligo (dT)₁₅ with Random Primers (Promega) in a volume of 20 μL. The synthesis of cDNA samples were confirmed with a PCR amplification using S12 primers (Table 1) with PCR conditions as mentioned above.

Gene expression analyses by qPCR

Muscle and hepatopancreas tissues were used to evaluate the expression of antioxidant and immune response related genes in juvenile shrimp after challenge with *V. parahaemolyticus*. The expression of the selected lysozyme (*LYS*), glutathione peroxidase (*GPX*), and superoxide dismutase (*SOD*) genes were analyzed by qPCR. The gene expression was performed in qPCR reactions in a total volume of 15 μL with 0.45 U of GoTaq Flexi DNA (Promega), 2.5 mM MgCl₂, 1x GoTaq Flexi Buffer, 0.2 mM dNTP Mix (Promega), 1x EvaGreen fluorescent dye (Biotium, Hayward, CA, USA), 0.2-0.48 μM each primer, and 5 μL cDNA (48 ng μL⁻¹). The qPCR conditions were as follows: denaturation at 95°C for 5 min; 40 cycles for 20 s at 95°C, 20 s at 60°C, 20 s at 72°C to acquire fluorescence. At the end of each PCR reaction, a melting curve analysis was performed to ensure the absence of artifacts, primer dimers, and verify the specificity of the PCR product. The samples were measured in triplicate, using a CFX96 Touch™ Real-Time PCR detection system (Bio-Rad Laboratories, Hercules, CA, USA). The primers for
reference and target genes are shown in Table 1. The selection of the most stable reference genes as endogenous controls was determined through four computational algorithms (ΔCt, NormFinder, BestKeeper, and GeNorm) that were integrated into the RefFinder software package, using the raw Cq values as input (Xie et al., 2012). Standard curves were made for each gene with six different dilutions (dilution factor: 1:5), in triplicate. The amplification efficiencies (E) were obtained from the log-linear function slopes of the dilution factor versus fluorescence, using the equation $E = (10^{(1/slope)} - 1)$. The relative expressions of the target genes were estimated from relative quantities for each target and reference genes, using the equation $RQ = (1 + E^{(C_{qmin} - C_{q})})$ (Hellemans et al., 2007). The relative expression value (RE) was calculated from the ratio of relative quantity (RQ) of each individual sample with the equation $RE = RQ_t / RQ_{nf}$, where $t$ is the target gene and $nf$ is the normalization factor obtained from the geometric mean calculated from the RQs of the most stable reference genes.

**Statistical analysis**

A one-way analysis of variance (ANOVA) was used to examine relative gene expression differences among treatments. The data were transformed to natural logarithms to correct heteroscedasticity, non-normality, and non-additivity (Zar, 1999). The relative gene expression is shown back transformed. If significant differences were found, Tukey's honestly significant difference (HSD) test was used to identify the source of these differences ($P < 0.05$). Data are shown as the means ± SE (standard error). Data obtained during the bioassays were analyzed by ANOVA followed by Tukey's HSD test using Statistica software (Version 6.0).

**RESULTS**

**Survival of Penaeus vannamei after infection with Vibrio parahaemolyticus**

After 10 days of culture with feed additives, juvenile shrimp were infected with a single exposure to *V. parahaemolyticus* (Figure 1). Shrimp treated with feed additives recorded significantly ($P < 0.05$) higher survival than the infected control group.

The use of different live-yeast administration at two concentrations in the culture of juvenile shrimp and a posterior challenge with *V. parahaemolyticus* is shown in Figure 2. Survival of shrimp treated for 10 days with live *D. hansenii* included in diet and directly into culture tanks increased significantly ($P < 0.05$) more than control (+) after challenge with the pathogen.

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**Table 1. Primer sequences used for gene expression analysis by quantitative real-time PCR (qPCR) after challenge with Vibrio parahaemolyticus.**

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer name</th>
<th>Sequence 5’- 3’</th>
<th>Efficiency</th>
<th>Fragment size (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ribosomal protein S12</td>
<td>S12-F</td>
<td>GTGAAGGAGACGTGGGTGT</td>
<td>2.00</td>
<td>150 bp</td>
</tr>
<tr>
<td></td>
<td>S12-R</td>
<td>AGAGCCTTGACCCTTCAT</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ubiquitin</td>
<td>UBI-F</td>
<td>GGAAAGACCATCACCCTTG</td>
<td>1.98</td>
<td>146 bp</td>
</tr>
<tr>
<td></td>
<td>UBI-R</td>
<td>TCAGACAGAGTGCAGACCATC</td>
<td>2.02</td>
<td>163 bp</td>
</tr>
<tr>
<td>Elongation factor</td>
<td>EF1a-F</td>
<td>TGATTGCCACACTGCTCAC</td>
<td>2.02</td>
<td>163 bp</td>
</tr>
<tr>
<td></td>
<td>EF1a-R</td>
<td>GAAGTGCTCACCACGCACATAG</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ribosomal protein L8</td>
<td>L8-F</td>
<td>GCCTAGTGGCTGGTG</td>
<td>2.00</td>
<td>181 bp</td>
</tr>
<tr>
<td></td>
<td>L8-R</td>
<td>ATCTGCTTGGGTCTCTTCT</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Manganese superoxide</td>
<td>MnSOD-F</td>
<td>ATTTGTTGAGGAACGAGGTG</td>
<td>2.10</td>
<td>113 bp</td>
</tr>
<tr>
<td>dismutase (MnSOD)</td>
<td>MnSOD-R</td>
<td>GGTGATGGCTTGGTGAGGAACGAGG</td>
<td>2.10</td>
<td>113 bp</td>
</tr>
<tr>
<td>Glutathione peroxidase</td>
<td>GPX-F</td>
<td>AGGGAAGAAGATAAGAGAACAGAC</td>
<td>2.28</td>
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<tr>
<td></td>
<td>GPX-R</td>
<td>TGTTGCGGCGGGATGAATG</td>
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<td></td>
</tr>
<tr>
<td>Lysozyme</td>
<td>Lyso-F</td>
<td>GAAGGCAGACTACGGCAAAGAC</td>
<td>1.86</td>
<td>216 bp</td>
</tr>
<tr>
<td></td>
<td>Lyso-R</td>
<td>AACCCTGAGACCAGCAGC</td>
<td></td>
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</tr>
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</table>
Figure 1. Survival of *Penaeus vannamei* juveniles exposed to feed additives for 10 days and after infected with *Vibrio parahaemolyticus*. Control (-): shrimp not fed with additives and non-infected; control (+): shrimp not fed with additives and infected; DHF: *Debaryomyces hansenii* 1×10⁶ CFU g⁻¹ feed; IN: inulin 2.5 mg g⁻¹ feed; DHF+IN: *Debaryomyces hansenii* 1×10⁶ CFU g⁻¹ feed + inulin 2.5 mg g⁻¹ feed. Error bars are mean ± SE (standard error). *Significant (P < 0.05) than positive control.

Gene expression

The relative gene expression of glutathione peroxidase (GPX), lysozyme (LYS), and superoxide dismutase (SOD) in shrimp muscle after exposed to feed additives are shown in Figure 3. The LYS gene expression increased significantly (P < 0.05) in juvenile muscle after fed for 10 days with all the additives and the SOD gene expression increased significantly with IN and DHF+IN (*D. hansenii* 1×10⁶ CFU g⁻¹ feed + inulin 2.5 mg g⁻¹ feed) additives. Lysozyme recorded the highest gene expression in shrimp muscle after 10 days of exposure to the treatments.

Figure 2. Survival of *Penaeus vannamei* juveniles exposed to *Debaryomyces hansenii* included in feed and in culture water for 10 days and post-infected with *Vibrio parahaemolyticus*. Control (-): shrimp not fed with additives and non-infected; control (+): shrimp not fed with additives and infected; 2DHF + DHW: *D. hansenii* 2×10⁶ CFU g⁻¹ feed + *D. hansenii* 1×10⁶ CFU mL⁻¹; 4DHF + DHW: *D. hansenii* 4×10⁶ CFU g⁻¹ feed + *D. hansenii* 1×10⁶ CFU mL⁻¹. Error bars are mean ± SE (standard error). *Significant (P < 0.05) than positive control.
Figure 3. Relative gene expression in muscle of *Penaeus vannamei* fed for 10 days with different treatments: control (without treatment); (DHF) *Debaryomyces hansenii* (1x10^6 CFU g^-1 feed); (IN) inulin (2.5 mg g^-1 feed); (DHF+IN) *Debaryomyces hansenii* (1x10^6 CFU g^-1 of feed) + inulin (2.5 mg g^-1 feed). Error bars are mean ± SE (standard error). *Significant (P < 0.05) than control.

The relative gene expressions of GPX, LYS, and SOD in hepatopancreas of juvenile *P. vannamei* exposed to live *D. hansenii* included in feed and culture water is shown in Figure 4. After exposure to the treatments for 10 days, the antioxidant enzymes showed higher expression than LYS. Shrimp exposed to higher concentration of yeast (4DHF + DHW) increased GPX and SOD expressions significantly (P < 0.05).

DISCUSSION

The use of natural immunostimulants derived from bacteria and plants is a promising area in aquaculture because they are safe for both environmental and human health (Abdul-Rahman & Ahmed, 2016). Yeasts are cheaper dietary supplements and easy to produce at industrial level because of their high *in vitro* growth rate (Abdul-Rahman & Ahmed, 2016); they are also rich nutritionally, which might have contributed to enhance health in reared organisms (Sarlin & Philip, 2011). Live yeast can also stimulate the immune response in shrimp and induce resistance to the pathogens (Sarlin & Phillip, 2011) because of their cell wall components, mainly β-glucans and also by their soluble factors (Abdul-Rahman & Ahmed, 2016; Gainza & Romero, 2017). Viral and bacterial diseases decrease shrimp production and cause serious economic losses. In recent years, probiotics (Wang et al., 2018) and antimicrobial agents (Mateus et al., 2014; Prabu et al., 2018; Rathnakumari et al., 2018; Tran et al., 2018; Soowannayan et al., 2019) have been used as an alternative to antibiotics against bacterial pathogens, such as *V. parahaemolyticus*, a causative agent of AHPND (Hong et al., 2016), *V. harveyi* (Zokaeifar et al., 2012), and *V. alginolyticus* (Kitikiew et al., 2013). Wang et al. (2018) reported a reduction of *Penaeus vannamei* mortality after being treated with two strains of *Pseudoalteromonas* spp. and challenged with *V. parahamolyticus*. Some studies have analyzed the effect of marine live yeasts strains as immunostimulants on fish (Tovar-Ramírez et al., 2010; Abdul-Rahman & Ahmed, 2016; Carufio et al., 2016) and relatively few studies on juvenile shrimp (Afsharnasab et al., 2016). Fierro-Coronado et al. (2019) studied the immunostimulant effect of the yeast *Candida parapsilosis* (strain L16) included in juvenile *P. vannamei* diet and found an increased survival after a viral infection with white spot syndrome virus (WSSV). In this study, juvenile shrimp fed with additive yeast at 1x10^6 CFU g^-1 feed, was sufficient to induce resistance significantly (P < 0.05) against experimental infection with *V. parahemolyticus*. 
Figure 4. Relative gene expression in hepatopancreas of *Penaeus vannamei* fed for 10 days with *Debaryomyces hansenii* included in feed and in culture water: control (without treatment); (2DHF + DHW) *D. hansenii* 2×10^6 CFU g^-1 feed + *D. hansenii* 1×10^6 CFU mL^-1; (4DHF + DHW) *D. hansenii* 4×10^6 CFU g^-1 feed + *D. hansenii* 1×10^6 CFU mL^-1. Error bars are mean ± SE (standard error). *Significant (P < 0.05) than control.

In aquaculture, prebiotics have received increased attention in feed utilization, growth performance, positive effect on digestive tract microbiota, immune system and disease resistance (Eshaghzadeh et al., 2014). Dong & Wang (2013) reported an increased resistance to *Aeromonas hydrophila* in juvenile crayfish *Procambarus clarkii* after fed with fructooligosaccharides (FOS) at 8 g kg^-1 for 30 days. Recently, Elshopakey et al. (2018) evaluated the prebiotic effect of β-1,4-mannobiose on the immune response of Kuruma shrimp (*Marsupenaeus japonicus*) to increase resistance against *V. parahaemolyticus* infection. Additionally, combinations of prebiotics and probiotics have been reported. Abdul-Rahman & Ahmed (2016) showed enhancement on immune response in common carp (*Cyprinus carpio*) after being exposed to one prebiotic (fructooligosacharide) and a yeast (*Saccharomyces cerevisiae*). In this study, juvenile shrimp fed with inulin at 2.5 mg g^-1 and combined with *D. hansenii* showed an increased resistance against *V. parahaemolyticus* compared with the control group.

The study of antioxidant mechanisms is an emerging field in aquaculture. However, few reports are available concerning the use of yeasts as feed additives and their antioxidant response in shrimp. In this study, the increased SOD gene expression in shrimp hepatopancreas were obtained after 10 days of daily treatment with *D. hansenii* at a concentration of 4×10^6 CFU g^-1 feed + *D. hansenii* 1×10^6 CFU mL^-1. According to this study, Bai et al. (2010) reported that the supplementation of 0.2% of β-glucans extracted from *Saccharomyces cerevisiae* increased the SOD activity in *L. vannamei*. In another study, Pacheco et al. (2012) observed that the yeast *D. hansenii* included in diet had a stimulating effect on the antioxidant response in *L. vannamei*. In a recent study, Afsharnasab et al. (2016) reported an increase in the SOD activity in *L. vannamei* as a result of *S. cerevisiae* β-glucan administration for 25 days. The SOD enzyme catalyzes the dismutation of superoxide anion resulting in oxygen and peroxide, which serve as substrate to GPX (Tovar-Ramírez et al., 2010). In our study, GPX increased the gene expression in shrimp hepatopancreas after treatment with the highest dose of *D. hansenii* for 10 days. Similarly, Castex et al. (2010) reported and increased in GPX and SOD responses in *L. stylirostris* treated with the probiotic *Pediococcus acidilactici* for 30 days. Thus, the increase in the antioxidant activity resulted from upregulated expression (Liu et al., 2007), and subsequently, this increase induced protection from oxidative stress and potential pathogen infections (Pacheco et al., 2012).

Non-specific immune genes may play important roles in preventing disease from infecting shrimp (Zhou et al., 2019). Lysozyme is a key component of innate immune system because of its anti-microbial and opsonization properties (Akhter et al., 2015). In
juvenile shrimp, few studies have demonstrated the effects of yeast administration in humoral lysozyme production (Yang et al., 2010; Flores-Miranda et al., 2011; Bai et al., 2014). Yao et al. (2008) reported higher lysozyme activity in F. chinhensis hemocytes after stimulated with laminarin at 3 h post-administration. On this regard, Bai et al. (2014) reported improvement of lysozyme activity in L. vannamei hemolymph treated with S. cerevisiae β-glucans and glucan derivatives (carboxymethyl-glucans and sulfoethyl-glucans). Conversely, Wongsa et al. (2015) did not find differences in lysozyme activity in L. vannamei hemolymph stimulated with dietary glucans. However, other dietary additives with immunostimulatory properties that have been evaluated in L. vannamei reported an increase of lysozyme activity in shrimp hemolymph by the use of Bacillus subtilis probiotic bacteria strains (Shen et al., 2010), dry powder extract of Spirulina sp. (Chen et al., 2016) and extract of Sargassum sp. (Yudiati et al., 2016). In this study, gene expression of lysozyme showed an increase in shrimp muscle after shrimp treated with D. hansenii, inulin, and the mix of yeast and prebiotic. In hepatopancreas, lysozyme gene expression increased only in shrimp treated with the highest concentration of D. hansenii. The upregulated lysozyme expression in muscle was similar to other studies that have reported higher lysozyme activity in shrimp tissues, such as hemolymph, intestine, gills, mucus, serum, and membrane eggs of marine animals (Qiao et al., 2013; Song et al., 2014).

CONCLUSION

The probiotic yeast D. hansenii (strain CBS 8339) evaluated in this study, showed the activation of immune and antioxidant gene expression in juvenile shrimp and enhanced survival against V. parahaemolyticus infection. Further experiments evaluating different bioindicators should be performed to broaden the understanding of immune and antioxidant response in marine organisms. Additionally, the use of appropriate probiotic strains and doses to induce resistance against bacterial and viral pathogens are necessary for enhancing quality and quantity of reared shrimp production.

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Data availability. Data are available with the correspondence author and may be provide upon reasonable request.

REFERENCES

Abdul-Rahman, N.M., Ahmed, V.V.M. 2016. Comparative effect of probiotic (Saccharomyces cerevisiae), prebiotic (fructooligosacharide FOS) and their combination on some differential white blood cells in young common carp (Cyprinus carpio L). Iraqi Journal of Veterinary Medicine, 40(1): 9-15. DOI: https://doi.org/10.30539/iraqijvm.v40i1.131

Afsharnasab, M., Kakoolaki, S., Mohammadiost, M. 2016. Immunity enhancement with administration of Gracilaria corticata and Saccharomyces cerevisiae compared to gamma irradiation in expose to WSSV in shrimp, in juvenile Litopenaeus vannamei: a comparative study. Fish & Shellfish Immunology, 56: 21-33. DOI: https://doi.org/10.1016/j.fsi.2016.06.052


Balcazar, J.L., Rojas-Luna, T., Cunningham, D.P. 2007. Effect of the addition of four potential probiotic strains on the survival of pacific white shrimp (Litopenaeus vannamei)
following immersion challenge with *Vibrio parahaemolyticus*. Journal of Invertebrate Pathology, 96: 147–150. DOI: https://doi.org/10.1016/j.jip.2007.04.008

Balcazar, J.L., de Blas, I., Ruiz-Zarzuela, I., Cunningham, D., Vendrell, D., Muzzquiz, J.L. 2006. The role of probiotics in aquaculture. Veterinary Microbiology, 114: 173-186. DOI: https://doi.org/10.1016/j.vetmic.2006.01.009


Marques, A., Dhont, J., Sorgeloos, P., Bossier, P. 2006. Immunostimulatory nature of β-glucans and baker’s yeast in gnotobiotic...
Artemia challenge tests. Fish & Shellfish Immunology, 20(5): 682-692. DOI: https://doi.org/10.1016/j.fsi.2005.08.008


Tran, C.K., Tran, M.P., Phan, T.V., Dalsgaard, A. 2018. Quality of antimicrobial products used in white leg shrimp (Litopenaeus vannamei)


