DIETARY SUPPLEMENTATION OF *Debaryomyces hansenii* ENHANCED SURVIVAL, ANTIOXIDANT AND IMMUNE RESPONSE IN JUVENILE SHRIMP *Penaeus vannamei* CHALLENGED WITH *Vibrio Parahaemolyticus* †

[LA SUPLEMENTACIÓN DIETÉTICA CON *Debaryomyces hansenii* INCREMENTA LA SUPERVIVENCIA Y LA RESPUESTA ANTIOXIDANTE E INMUNE EN CAMARONES JUVENILES *Penaeus vannamei* RETADOS CON *Vibrio parahaemolyticus*]

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SUMMARY

Background: The excessive use of antibiotics in shrimp aquaculture cause severe ecological damage. Immunostimulant probiotics are an alternative prophylactic treatment to enhanceantioxidant 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*, 1×10^6 CFU g^{-1} feed; d) inulin, 2.5 mg g^{-1} feed; e) *D. hansenii*, 1×10^6 CFU g^{-1} feed + inulin, 2.5 mg g^{-1} feed; f) *D. hansenii*, 2×10^6 CFU g^{-1} feed + 1×10^6 CFU mL^{-1}; g) *D. hansenii*, 4×10^6 CFU g^{-1} feed + 1×10^6 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 1×10^6 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 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 10^6 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 lisozima (LYS), glutatión peroxidasa (GPX) y superóxido dismutasa (SOD) en músculo de camarón y hepatopáncreas antes 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 camaron cultivado e incrementar respuesta immune 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 × 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.

INTRODUCTION

Aquaculture is the fastest growing food production industry. In 2016 finfish production was 54 million t; 17.1 million t of mollusk and 7.9 million t of crustacean species. (FAO, 2018). The Pacific white shrimp *Penaeus vannamei* is the primary penaeid shrimp currently reared in Central and South America (Burge *et al*., 2007). Despite the explosive growth in world production of farmed shrimp, problems have also occurred related to deterioration of environmental conditions (Gyan *et al*., 2020) and economic losses due to diseases (Balcázar *et al*., 2007; Srinivas *et al*., 2016) caused mainly by *Vibrio* bacteria and viruses in a deteriorated pond environment (Lo *et al*., 2003). It is estimated that around 20% of disease losses in shrimp aquaculture have been caused by bacterial pathogens (Flegel, 2012; Tran *et al*., 2018). Generally, antibiotics and disinfectant agents are used in commercial shrimp hatcheries to control pathogenic microbes and improve shrimp growth (Gatesoupe, 1999; Balcázar *et al*., 2006). Unfortunately, antibiotic use leads to the emergence of antibiotic-resistant pathogenic bacteria. The use of some antibiotic agents has been prohibited in aquaculture in many countries due to their persistence, carcinogenic effects in some organisms, antibiotic residues in aquatic animals and surroundings, and the potential impact on human (Gatesoupe, 1999; Verschuere *et al*., 2000).

Probiotics are defined as live microbial or cultured product feed supplements, which benefit the host by producing inhibitory compounds, competing for chemicals and adhesion sites, modulating and stimulating the immune function, and improving the microbial balance (Fuller, 1989; Verschuere *et al*., 2000). They have been used in aquaculture as a means of disease control, supplementing or even in some cases replacing the use of antimicrobial compounds. A wide range of microalgae (*Tetraselmis*), yeasts (*Debaryomyces*, *Phaffia*, and *Saccharomyces*), and Gram-positive bacteria (*Bacillus*, *Carnobacterium*, *Enterococcus*, *Lactobacillus*, *Lactococcus*, *Micrococcus*, *Streptococcus*, and *Weissella*) and Gram-negative bacteria (*Aeromonas*, *Alteromonas*, *Photorbodobacterium*, *Pseudomonas*, and *Vibrio*) has been applied as probiotics in fish and shellfish cultures. Some studies have demonstrated that yeast culture is able to improve animal health by influencing the micro-ecological environment in the digestive system, improving nutrient digestion and absorption and stimulating immune responses (Tovar-Ramírez *et al*., 2002; Suphantharika *et al*., 2003; Marques *et al*., 2004; Haddad & Goussous, 2005). Some studies have reported that yeast could enhance growth, feed conversion ratio (FCR) and prevent some illness in aquaculture animals, such as shrimp *P. vannamei, Artemia franciscana, Penaeus chinesis* and fish *Oncorhynchus mykiss, Paralichthys olivaceus and Cyprinus carpio* L. (Burgents *et al*., 2004; Mai *et al*., 2004; Marques *et al*., 2006). The beneficial role of yeasts is in part for their cell wall structure composition, such as β-glucans, chitin, and also nucleotide content (Vecchiarelli, 2000). Besides

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*., 1991). One of these compounds is inulin and its derivates (oligofructose, fructooligosaccharides (FOS), which are basically constituted by linear fructose chains (Madrigal & 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, lysozymes are hemocyte-specific proteins that possess cell-wall lytic activity against a range of gram-positive and gram-negative bacterial species as *Vibrio* sp. Lysozyme 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 (CIIDIR-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 1×10^8 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 4ºC 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 1×10^8 CFU mL^{-1} for bacterial challenge. Before bioassay challenge, LC_{50} 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* 1×10^6 CFU g^{-1} feed; c) inulin 2.5 mg g^{-1} feed; d) *D. hansenii* 1×10^6 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* (1×10^6 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* 2×10^6 CFU g^{-1} feed + 1×10^6 CFU mL^{-1}; c) *D. hansenii* 4×10^6 CFU g^{-1} feed + 1×10^6 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* (1×10^6 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 $MgCl_2$, 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 $MgCl_2$, 1x GoTaq Flexi Buffer, 0.2 mM dNTP Mix (Promega), 1×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 $RQ = (1 + E^{(Cqmin - Cq)})$ (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.

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

Gene	Primer name	Sequence 5´- 3´	Efficiency	Fragment size (bp)
Ribosomal protein S12	S12-F	GTGGAAGGAGACGTTGGTGT	2.00	150 bp
	S12-R	AGAGCCTTGACCGCTTCAT		
Ubiquitin	UBI-F	GGGAAGACCATCACCCTTG	1.98	146 bp
	UBI-R	TCAGACAGAGTGCGACCATC		
Elongation factor	EF1a-F	TGATTGCCACACTGCTCAC	2.02	163 bp
	EF1a-R	GAAGGTCTCCACGCACATAG		
Ribosomal protein L8	L8-F	GCCTAAGGTGCGTGGTGT	2.00	181 bp
	L8-R	ATTCTGCCTTGGGTCCTTCT		
Manganese superoxide dismutase (MnSOD)	MnSOD-F	ATTGGGTGAGGAACGAGGTG	2.10	113 bp
	MnSOD-R	GGTGATGCTTTGTGTGGTGG		
Glutathione peroxidase	GPX-F	AGAAAGAAGATAAGAGAAGACC CG	2.28	146 bp
	GPX-R	TGGTTGGCGGTTGGAATG		
Lysozyme	Lyso-F	GAAGCGACTACGGCAAGAAC	1.86	216 bp
	Lyso-R	AACCGTGAGACCAGCACTCT		

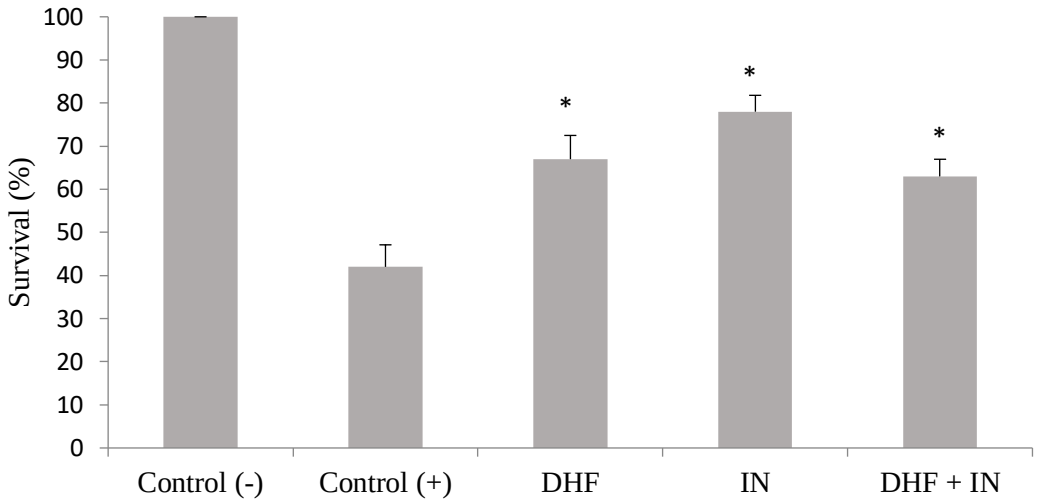

Figure 1. Survival of *Penaeus vannamei* juveniles exposed to feed additives for 10 days and after infected with *Vibrio parahaemolyticus*. Control (-): shrimp not fed with additives and non- infected; control (+): shrimp not fed with additives and infected; DHF: *Debaryomyces hansenii* 1×10^6 CFU g^{-1} feed; IN: inulin 2.5 mg g^{-1} feed; DHF+IN: *Debaryomyces hansenii* 1×10^6 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* 1×10^6 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.

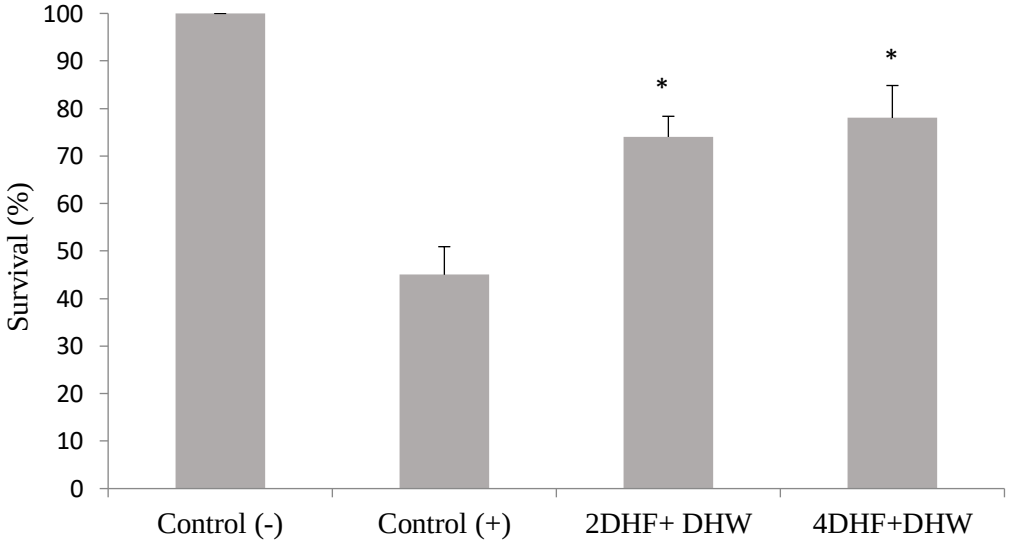

Figure 2. Survival of *Penaeus vannamei* juveniles exposed to *Debaryomyces hansenii* included in feed and in culture water for 10 days and post-infected with *Vibrio parahaemolyticus*. Control (-): shrimp not fed with additives and non- infected; control (+): shrimp not fed with additives and infected; 2DHF + DHW: *D. hansenii* 2×10^6 CFU g^{-1} feed + *D. hansenii* 1×10^6 CFU mL^{-1}; 4DHF + DHW: *D. hansenii* $4x10^6$ CFU g^{-1} feed + *D. hansenii* 1×10^6 CFU mL^{-1}. Error bars are mean ± SE (standard error). *Significant ($P < 0.05$) than positive control.

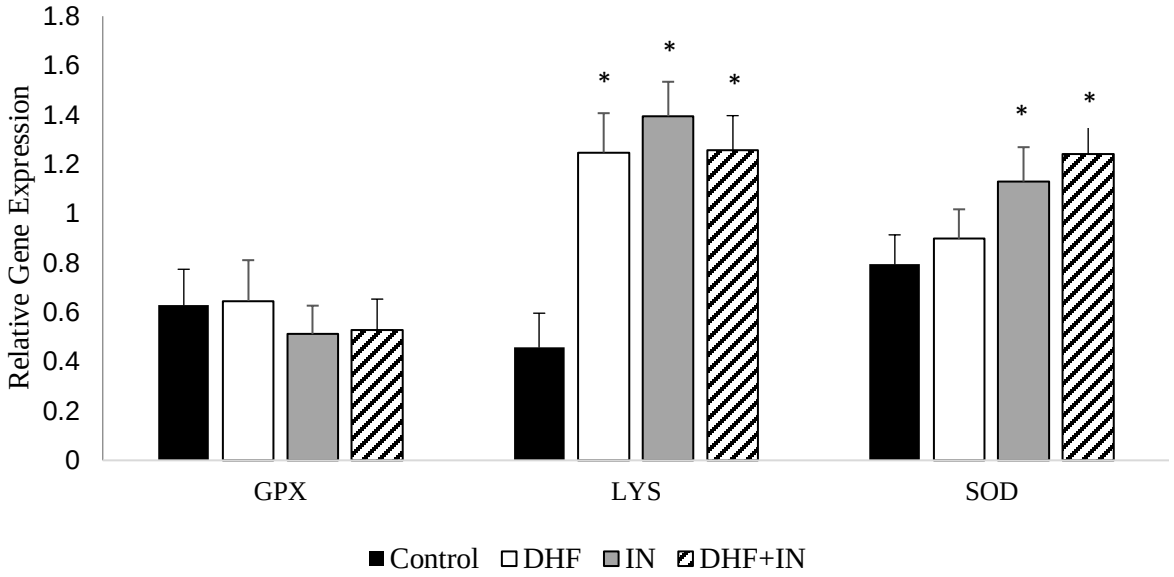

Figure 3. Relative gene expression in muscle of *Penaeus vannamei* fed for 10 days with different treatments: control (without treatment); (DHF) *Debaryomyces hansenii* (1×10^6 CFU g^{-1} feed); (IN) inulin (2.5 mg g^{-1} feed); (DHF+IN) *Debaryomyces hansenii* (1×10^6 CFU g^{-1} of feed) + inulin (2.5 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. parahaemolyticus*. Some studies have analyzed the effect of marine live yeasts strains as immunostimulants on fish (Tovar-Ramírez *et al*., 2010; Abdul-Rahman & Ahmed, 2016; Caruffo *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 Lt6) included in juvenile *L. 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 1×10^6 CFU g^{-1} feed, was sufficient to induce resistance significantly ($P < 0.05$) against experimental infection with *V. parahemolyticus*.

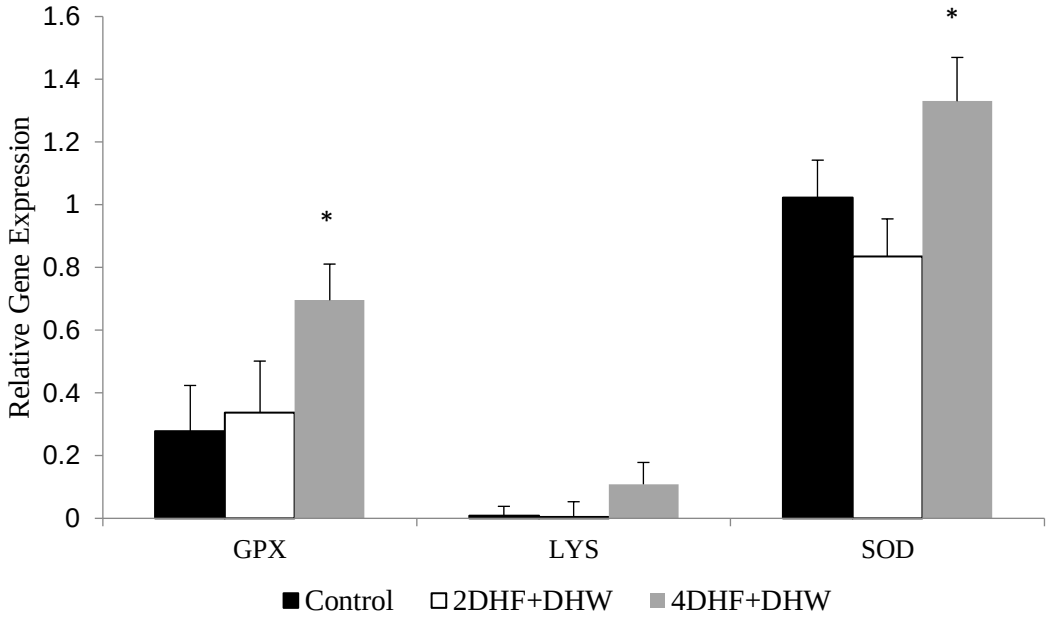

Figure 4. Relative gene expression in hepatopancreas of *Penaeus vannamei* fed for 10 days with *Debaryomyces hansenii* included in feed and in culture water: control (without treatment); (2DHF + DHW) *D. hansenii* 2×10^6 CFU g^{-1} feed + *D. hansenii* 1×10^6 CFU mL^{-1}; (4DHF + DHW) *D. hansenii* 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 ß-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. chiniensis* 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 derivates (carboxymethyl-glucans and sulfoethyl-glucans). Conversely, Wongsasak *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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Compliance with ethical standards. The research proposal was approved by the instititution ethical guidelines.

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

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