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Combined administration routes of marine yeasts enhanced immune-related genes and protection of white shrimp (*Penaeus vannamei*) against *Vibrio parahaemolyticus*

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ABSTRACT

Antibiotic usage to control infectious diseases in shrimp aquaculture has led to serious problems on antimicrobial resistance. An alternative to mitigate this issue is the use of probiotics, which can be easily administered by feed and water. This study examines immunomodulatory and protective effects of the marine yeasts *Debaryomyces hansenii* CBS8339 (Dh) and *Yarrowia lipolytica Yl*-N6 (Yl) -alone and mixed-in white shrimp *Penaeus vannamei* post-larvae. Administration routes (fed and water alone or in combination), supplementation frequency and time elapsed after the last dietary supplement were tested on growth and gene expression of penaeidin, lectin, lysozyme, superoxide dismutase, catalase, and peroxidase, as well as survival upon *Vibrio parahaemolyticus* IPNGS16 challenge. Penaeidin and lectin genes were upregulated in post-larvae fed orally with Yl or combined Dh + Yl. Higher growth and survival for yeast supplementation treatments were observed compared to the control group, mainly when yeasts (Dh + Yl) and administration routes (feed and water) were combined. In conclusion, mixed yeast and combined administration routes improved growth and immunity against *V. parahaemolyticus*.

1. Introduction

Aquaculture has become one of the fastest-growing food production sectors worldwide [1]. Several culture practices, such as intensification, induce stress and increase vulnerability to several diseases [2,3]. For example, the acute hepatopancreatic necrosis disease (AHPND) caused by a *Vibrio parahaemolyticus* strain that acquired the pVA1 plasmid carries PirAB-like genes, which code for a binary toxin [4], is responsible for severe economic losses in shrimp aquaculture [5–8]. To solve these problems, several eco-friendly alternative approaches to minimize the use of therapeutic chemicals have been proposed. Some of them include the use of beneficial microorganisms as prophylactic tools that enhance the host health [9,10]. These microbial approaches comprise the induction of the production of bacteriocines, lysozymes, proteases, and hydrogen peroxide. They also include competition for essential nutrients and adhesion sites, supply of essential nutrients and enzymes for enhanced nutrition of the cultured animals, direct uptake of dissolved organic material mediated by bacteria, and shrimp immune system modulation [11]. In this regard, yeast and their structural components of the cell wall (glucans) have demonstrated to increase shrimp defense response and protection against bacterial and viral infections [12–16]. Yeast and glucan administration activates shrimp innate immune system, promoting cellular functions, increasing multiple humoral parameters, and upregulating immune-relevant gene expression [12,13,

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17–19]. However, the optimum stimulation of shrimp immune system and protective effects by immunostimulants require to consider several aspects, such as dose, administration route, and frequency [20,21]. Although several immunostimulant products have been available, not enough information exists about the efficacy and application mode. The routes of administration may be an important factor in determining the success of immunostimulation in enhancing protective response [10]. Remarkably, immunostimulant dose and frequency have been critical to avoid immune system suppression and low disease resistance [22,23].

Among yeasts, *Debaryomyces hansenii* and *Yarrowia lipolytica* have shown an immunoprotective effect on aquatic animals [24–27]. For instance, both yeast improved immune parameters and survival of fish leucocytes upon *V. parahaemolyticus* challenge [26,27]. *In vivo* studies have demonstrated that *D. hansenii* and *Y. lipolytica* enhanced immune parameters, immune-associated gene expression and/or survival upon challenge in fish and shrimp [28,29]. Recently, Licona-Jain et al. [30] demonstrated *Y. lipolytica* Yl-N6 enhanced immune parameters in shrimp and antibacterial activity in plasma against *V. parahaemolyticus* (AHPND). Therefore, the purpose of this study is to evaluate the effects of yeast dose, administration route, and frequency of dietary supplement in *P. vannamei* on innate immune parameters, gene expression, and protection time against an experimental infection with AHPND.

2. Material and methods

2.1. Yeast culture

The marine yeasts *Debaryomyces hansenii* (CBS8339 strain) and *Yarrowia lipolytica* (*Yl*-N6 strain) were inoculated in YPD (yeast peptone dextrose; Sigma-Aldrich® St. Louis MO, USA) medium and incubated at 27 °C for 48 h. Yeast cells were centrifuged at 1000 *g* at 4 °C for 10 min, to separate yeast pellet, which was resuspended in saline solution (2.5% NaCl) and adjusted to a value of 1.0 at an optical density of 580 nm to obtain a concentration equivalent to 2 x 10^6 colony forming units (CFU) ml^{-1}.

2.2. Experimental diets

Commercial shrimp feed Camaronina 35% protein (Purina®, MO, USA) was pulverized and used as a base for producing the experimental diets. Immunostimulants (yeasts and glucans) were administered at different inclusion levels: 1% for yeast and 2% for commercial glucan (MacroGard ® Biorigin, Sao Paulo, BR) according to Yang et al. [31], Jin et al. [32], and Licona-Jain et al. [30]. Yeast diets were prepared with live yeast cells, resuspending yeast pellet in saline solution (2.5% NaCl) and incorporating them into the commercial shrimp diet. Glucan diets were similarly prepared by dissolving glucans in distilled water and incorporating them into the commercial shrimp diet. The food was pelleted using 4% of alginic acid (Sigma-Aldrich® St. Louis MO, USA) as a binding agent, and eventually, the pellets were dried at 25 °C for 24 h. To confirm the viable number of yeasts in the feed after the manufacturing process, a standard yeast plate count was performed.

2.3. Lethal concentration (LC_{50})

Lethal concentration (LC_{50}) determination was performed in pathogen-free shrimp post-larvae. Ten organisms were placed in glass aquariums of 3-L water capacity. The bioassay was conducted under the following conditions: 30 °C, 30‰ salinity, and pH of 8. For bacterial cultivation, trypticase soy broth (TSB) (Difco, Le Pont de Claix, FR) supplemented with 2.5% NaCl was used. *Vibrio parahaemolyticus* was cultivated at 30 °C for 24 h. Bacteria were separated from the culture medium by centrifugation (7000 *g* for 10 min), and the pellet obtained was suspended in saline solution (2.5% NaCl). The bacterial concentration was quantified by spectrophotometry, adjusting vibrio concentration by serial dilutions. Four experimental groups were established in which different *V. parahaemolyticus* IPNGS16-AHPND (10,000, 100,000, 500,000, and 1,000,000 CFU ml^{-1}) concentrations were inoculated. Similarly, a negative control group (uninfected shrimp) was considered. Mortality was monitored and recorded for 72 h. A visual inspection was performed to detect dead organisms and remove them from the experimental units every 12 h. The data obtained were analyzed using a Probit model to confirm the lethal concentration according to Finney [33].

2.4. Rearing conditions

Pathogen-free shrimp post-larvae were obtained from Acuícola Cuate Machado (Guasave, Sinaloa, MX) and kept in 600-L plastic tanks under acclimatization conditions with constant aeration for two weeks. White shrimp L. *vannamei* in post-larval stage were used for each experiment divided into three replicates per treatment, maintained in a glass aquarium (6-L) containing 4-L with 12 post-larvae per aquarium for each experimental group described below. In the aquariums the environmental parameters (27 °C; salinity 37‰; dissolved oxygen 6 mg L^{-1}; pH 8.0) were maintained for nine days throughout each trial. Shrimp were fed twice a day (09:00 and 17:00 h) at a total daily rate of 5% of their biomass. All shrimp were fed during infection period (72 h). The tests performed with the experimental organisms during this study were carried out in accordance with the legislation for the protection of animals used for scientific studies (Directive 2010/63 EU).

2.5. Experiment I. Administration route of immunostimulants

Eight yeast treatments were evaluated to determine the effect of administration route on resistance against *V. parahaemolyticus* IPNGS16 infection, causative agent of AHPND [4]. In the bioassay, *L. vannamei* post-larvae (weighing 50 mg) (pathogen-free) were used for the study. The immunostimulants were administered daily by two routes: in feed (oral-O) and dissolved in water (immersion-I). The experimental groups were assigned as follows: (**1**) control, commercial feed; (**2**) Glucan 2% in feed; (**3**) *D. hansenii* 1% in feed, Dh(O); (**4**) *Y. lipolytica* 1% in feed, Yl(O); (**5**) *D. hansenii* 0.5% in feed + *D. hansenii* 1×10^6 CFU ml^{-1} in water, Dh (O + I); (**6**) *Y. lipolytica* 0.5% in feed + *Y. lipolytica* 1×10^6 CFU ml^{-1} in water, Yl(O + I); (**7**) *D. hansenii* + *Y. lipolytica* (1%, proportion 1:1) in feed, Dh + Yl(O); (**8**) *D. hansenii* + *Y. lipolytica* (1%, proportion 1:1) in feed + *D. hansenii* + *Y. lipolytica* (1×10^6 CFU ml^{-1} in water, proportion 1:1), Dh + Yl(O + I).

At the end of the experiment, all organisms/experimental unit (n = 12) were obtained to evaluate final weight, weight gain, and 6 shrimp/treatment (2 shrimp per aquarium) for immune gene expression. The remaining live shrimp (10/aquarium, 30/treatment) were submitted to experimental infection with *V. parahaemolyticus*, and survival determined each 24 h for 72 h.

2.6. Experiment II. Frequency administration of immunostimulant

Based on data obtained from Experiment I, the diet corresponding to yeast mixture administered in two routes (oral + immersion) was selected for further study. *P. vannamei* postlarvae (mean body weight 100 mg) (pathogen-free) were stocked in 12 glass aquariums of 3-L water capacity (12 post-larvae/aquarium). Same larval rearing conditions were maintained for nine days as in Experiment I. Post-larvae were divided into four groups (three aquariums for each group), and each group was fed daily with treatments at different frequencies (F): (1) Control, commercial diet; (2) *D. hansenii* + *Y. lipolytica* (1%, proportion 1:1) in feed + *D. hansenii* + *Y. lipolytica* (1×10^6 CFU ml^{-1} in water, proportion 1:1) [Dh + Yl(O + I)], added daily (F1); (3) Dh + Yl(O + I), added every other day (F2); (4) Dh + Yl(O + I), added every third day. After the immunostimulation trial period, all groups received an infectious challenge with *V. parahaemolyticus* for 72 h.

2.7. Experiment III. Long-term yeast protection effects

Healthy *P. vannamei* post-larvae (mean body weight 100 mg) (pathogen-free) were stocked in 12 glass aquariums of 3-L water capacity (12 post-larvae/aquarium). Same larval rearing conditions were maintained throughout the stimulation trial period (9 days) as in the previous trials. Two experimental groups (three aquariums for each group) were considered and each group was fed daily with microbial immunostimulants: (1) Control, commercial diet; (**2**) *D. hansenii* + *Y. lipolytica* (1%, proportion 1:1) in feed + *D. hansenii* + *Y. lipolytica* (1×10^6 CFU ml^{-1} in water, proportion 1:1) [Dh + Yl(O + I)]. At the end of the trial, three bacterial infections were established at different times (1, 4, and 7 days) to verify the maximum protection time in post-larvae. Dead organisms were removed daily from the experimental units, recording mortality for 72 h.

2.8. Vibrio parahaemolyticus IPNGS16 infection

The experimental infection was performed at the previously established LC_{50}, corresponding to a concentration of 90,000 CFU ml^{-1} of *V. parahaemolyticus* IPNGS16. For the experimental infection, bacteria were cultured as previously described. The culture was adjusted to an optical density of 1.0–480 nm (equivalent to 1.6×10^7 CFU ml^{-1}). Then, the bacterial culture was diluted with saline solution (2.5% NaCl) to adjust concentration. The infection period was maintained for 72 h, and mortality was recorded continuously in each of the experimental units.

2.9. Growth

The initial weight was recorded during post-larvae distribution into the aquariums, and final weight for all shrimp was obtained after the immunostimulation period (9 days). For weight gain (WG), the following formula was used:

$$WG = w_2 - w_1$$

Where w_1 and w_2 are the initial and final weight of shrimp, respectively.

2.10. Immune-related gene expression

For gene expression, six whole post-larvae/treatment were kept on ice during sampling and subsequently transferred to 1.5 ml Eppendorf tubes previously filled with RNA-later. The relative mRNA expression of immune-related genes was analyzed using real-time polymerase chain reaction. In this study, the whole organism, was used for RNA extraction due to the limited tissue in post-larvae. Total RNA was performed using Trizol reagent (Sigma-Aldrich® St. Louis MO, USA) following the manufacturer's protocol. RNA was quantified and qualified by spectrophotometry at 260 and 280 nm. The synthesis of the complementary strand of DNA (cDNA) was performed following the Improm II (Promega®) protocol adjusting all samples at a concentration of 10 μg of RNA. Finally, the cDNA samples were stored at −20 °C.

The expression of seven selected genes (penaeidin 4 (Pen4), prophenoloxidase II, lysozyme, catalase, superoxide dismutase (MnSOD), glutathione peroxidase (GPX), and lectin (LecC)) was analyzed by Real-time PCR. Gene expression was performed with a CFX96 Real-Time PCR detection system (Bio-Rad, CA USA), using EvaGreen Sso-fast super mix reagent (Bio-Rad, CA USA), and gene-specific primers. The PCR program was performed as follows: denaturation at 95 °C for 5 min, 40 cycles at 95 °C for 20 s, 60 °C for 20 s, 72 °C for 20 s to acquire fluorescence. At the end of the PCR reaction, a melting curve analysis was performed to verify the specificity of the fragments, and the absence of artifacts.

The primers used for gene amplifications are shown in Table 1. Specific primers were designed for Lectin and Catalase gene using the software Primer 3 (http://bioin fo.ut.ee/prime r3-0.4.0/) from a sequence of *Litopenaeus vannamei* deposited in GenBank (accession number JX162772.1 and GU206552.1 respectively).

Relative expression (RE) of each immune gene was calculated from the ratio of relative quantities (RQ) of each 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 (ubiquitin and S12 genes). The relative quantities of each sample target and reference genes were calculated with the equation $RQ = (1 + E^{(Cq\ mean - Cq)})$ [34].

2.11. Statistical analyses

To determine significant differences, the results were analyzed using a one-way analysis of variance (ANOVA) and Tukey's honestly significant difference (HSD) multiple comparisons of the means using STATISTICA software (v. 6.0) (TIBCO Software, CA, USA). Differences were considered significant at $p < 0.05$.

3. Results

3.1. Growth performance

Shrimp growth performance is shown in Table 2. Significant ($p < 0.05$) higher final weight and weight gain were observed for shrimp treated with all immunostimulants compared to the control group

Table 1
Primer sequences used in the qPCR analysis.

Gene	Primer	Sequence	Reference
S12	S12–F	GTGGAAGGAGACGTTGGTGT	Ventura-López et al. [74]
	S12-R	AGAGCCTTGACCGCTTCAT	
Ubiquitin	UBI-F	GGGAAGACCATCACCCTTG	Álvarez-Ruiz et al. [75]
	UBI-R	TCAGACAGAGTGCGACCATC	
MnSOD	LvMnSOD_113F	ATTGGGTGAGGAACGAGGTG	Ceseña et al. [42]
	LvMnSOD_113R	GGTGATGCTTTGTGTGGTGG	
Penaeidin	Pen4-F	GCCCGTTACCCAAACCATC	Okumura [76]
	Pen4-R	CCGTATCTGAAGCAGCAAAGTC	
GPX	LvGPx_146F	AGAAAGAAGATAAGAGAAGACCCG	Ceseña et al. [42]
	LvGPx_146R	TGGTTGGCGGTTGGAATG	
Lysozyme	Liso_212F	GAAGCGACTACGGCAAGAAC	Wang et al. [68],
	Liso_212R	AACCGTGAGACCAGCACTCT	
Prophenoloxidase II	proPOII-F	GAGAGGCTGAACCGAGACTGA	Yeh et al. [77]
	proPOII-R	AAGAAAACGGCCCCCAATT	
Lectin	LvLecC_156F	CAGGGAAAGTAGAAGGGCGAG	Designed
	LvLecC_156R	CAACAAAGGTCACGAACAAGAGG	
Catalase	LvCatal_161F	ACTGTAGGAGGTGAGAGTGG	Designed
	LvCatal_161R	TGCGTGTGAATGAAGGATGG	

MnSOD = Manganese superoxide dismutase; GPX = Glutathione peroxidase.

Table 2
Growth of *Penaeus vannamei* post-larvae fed with single or combined yeasts administered by different feeding routes.

Treatments	Initial weight (mg)	Final weight (mg)	Weight gain (mg)
Control	49.9	85.2 ± 2^{a}	35.3 ± 2^{a}
Glucan	51.1	98.7 ± 6^{ab}	47.6 ± 6^{c}
Dh- O	49.3	112.5 ± 3^{bc}	63.2 ± 3^{d}
Yl - O	52.0	114.1 ± 2^{cd}	62.1 ± 2^{d}
Dh (O + I)	52.2	95 ± 2^{a}	42.8 ± 2^{ab}
Yl (O + I)	51.9	97.1 ± 7^{a}	45.2 ± 7^{bc}
Dh + Yl (O)	49.8	120.8 ± 1^{cd}	71 ± 1^{e}
Dh + Yl (O + I)	49.6	127.5 ± 5^{d}	77.9 ± 5^{f}

The data shown are expressed as mean and standard error (n = 6 shrimp/treatment). Different letters indicate significant differences ($p < 0.05$) between treatments. Dh (*Debaryomyces hansenii* CBS8339), Yl (*Yarrowia lipolytica Yl*-N6), O (oral administration), I (immersion).

(commercial feed). Shrimp that received a yeast mixture administered by two routes [Dh + Yl (O + I)] showed the highest growth compared to the other treatments and the control group.

3.2. Immune-related gene expression

The results showed that after nine days post-immunostimulation (Experiment I), a significant ($p < 0.05$) upregulation of penaeidin (Pen4) gene expression occurred in shrimp receiving orally both the yeast *Y. lipolytica Yl*-N6 strain [Yl(O)] and yeast mixture [Yl + Dh (O)] compared to the control and glucan groups with intermediate values for the rest of yeast treatments (Fig. 1a). The lectin gene expression was significantly ($p < 0.05$) higher in shrimp stimulated orally by administration of each yeast alone (Yl and Dh) or combined [(Yl + Dh (O)] with intermediate values for both yeast administered orally and immersed (Fig. 1b). No significant changes ($p > 0.05$) were detected in the superoxide dismutase, lysozyme, catalase, and glutathione peroxidase gene expressions (Table 3).

3.3. Administration route of immunostimulants influences survival against V. parahaemolyticus IPNGS16 infection

No mortality was observed during the immunostimulation trial (nine days) before *V. parahaemolyticus* IPNGS16 infection. After challenge, 43% of shrimp survival was observed for the control group (commercial feed). Survival increased, although not significantly up to 67% in shrimp with individual or combined oral yeast administration. Oral and immersion yeast administration and commercial glucan increased survival significantly ($p < 0.05$) compared to the control group. The highest survival (93%) was observed when yeast *D. hansenii* CBS8339 and *Y. lipolytica Yl*-N6 were administered simultaneously by both oral and immersion routes (Fig. 2).

3.4. Frequency administration of immunostimulants affects survival to Vibrio parahaemolyticus IPNGS16 infection

The combination of *D. hansenii* CBS8339 and *Y. lipolytica Yl*-N6 supplied in diet and water administered daily for nine days showed the highest survival (87%) of shrimp post-larvae after challenge with *V. parahaemolyticus* IPNGS16, compared to the control group (53%) (Fig. 3). However, significant differences ($p < 0.05$) were observed only for the treatments corresponding to the frequency of daily administration (F1) and every third-day administration (F3) compared to the control group.

3.5. Protective effect duration of yeast in shrimp against Vibrio parahaemolyticus IPNGS16 infection

Average survival in the control group was 55% with little variation in

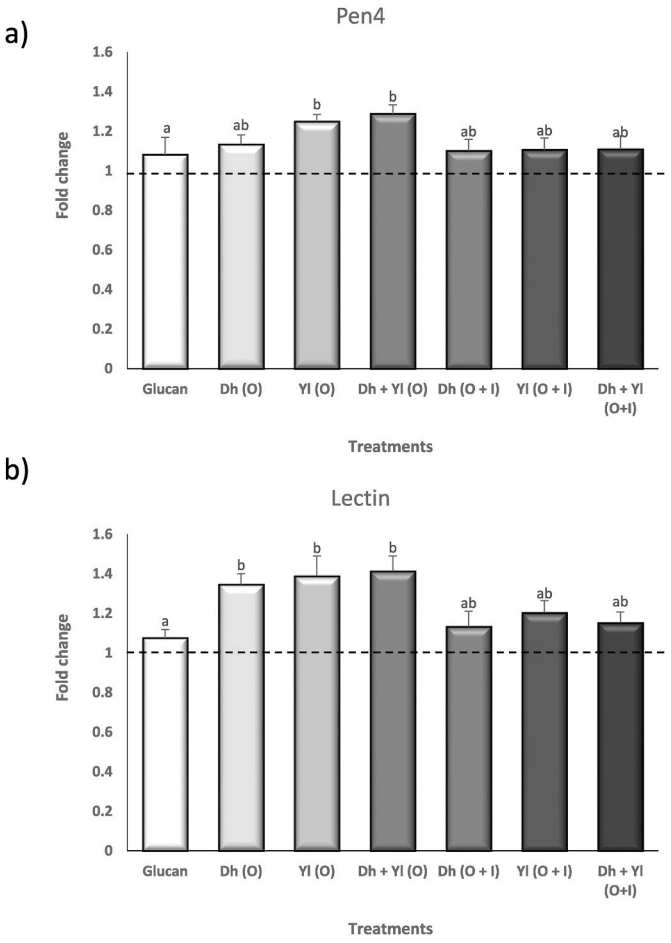

Fig. 1. Relative mRNA expression of Penaeidin (Pen4) and lectin gene in *Penaeus vannamei* post-larvae daily exposed to different dietary immunostimulants. Dh = *Debaryomyces hansenii* CBS8339; Yl = *Yarrowia lipolytica Yl*-N6; O = oral administration; I = immersion. Bars represent means ± standard error (SE) (n = 6 shrimp/treatment). Different letters denote significant differences between treatments ($p < 0.05$).

Table 3
Relative expression level in immune-related genes in shrimp *Penaeus vannamei post-larvae*.

Treatments	Lysozyme	MnSOD	Catalase	Peroxidase	Significance
Control	1.0 ± 0.19	0.95 ± 0.1	0.88 ± 0.14	0.81 ± 0.2	NS
Glucan	1.2 ± 0.06	0.93 ± 0.07	1.04 ± 0.07	0.92 ± 0.13	NS
Dh- O	1.0 ± 0.19	0.96 ± 0.07	1.02 ± 0.07	1.01 ± 0.05	NS
Yl - O	1.0 ± 0.21	0.98 ± 0.05	0.87 ± 0.06	1.09 ± 0.09	NS
Dh (O + I)	0.9 ± 0.16	0.94 ± 0.06	0.94 ± 0.03	1.07 ± 0.05	NS
Yl (O + I)	0.8 ± 0.15	1.04 ± 0.03	1.02 ± 0.03	1.1 ± 0.12	NS
Dh + Yl (O)	1.1 ± 0.13	1.08 ± 0.04	1.09 ± 0.04	0.93 ± 0.15	NS
Dh + Yl (O + I)	1.1 ± 0.14	1.13 ± 0.03	1.13 ± 0.05	1.12 ± 0.10	NS

The data shown are expressed as mean and standard error (n = 6 shrimp/treatment). NS Significant differences were not found between treatments. Dh (*Debaryomyces hansenii* CBS8339), Yl (*Yarrowia lipolytica Yl*-N6), O (oral administration), I (immersion); MnSOD = Manganese superoxidedismutase.

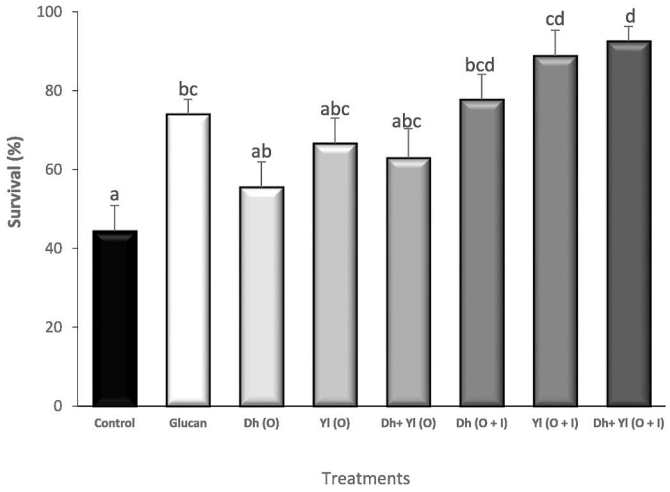

Fig. 2. Administration route effect of immunostimulants on survival in *Penaeus vannamei* post-larvae against *Vibrio parahaemolyticus* IPNGS16-AHPND (acute hepatopancreatic necrosis disease) infection. Dh = *Debaryomyces hansenii* CBS8339; Yl = *Yarrowia lipolytica Yl*-N6; O = oral administration; I = immersion. Bars represent means ± standard error (SE) (n = 10 shrimp/treatment). Different letters indicate significant differences between treatments ($p < 0.05$).

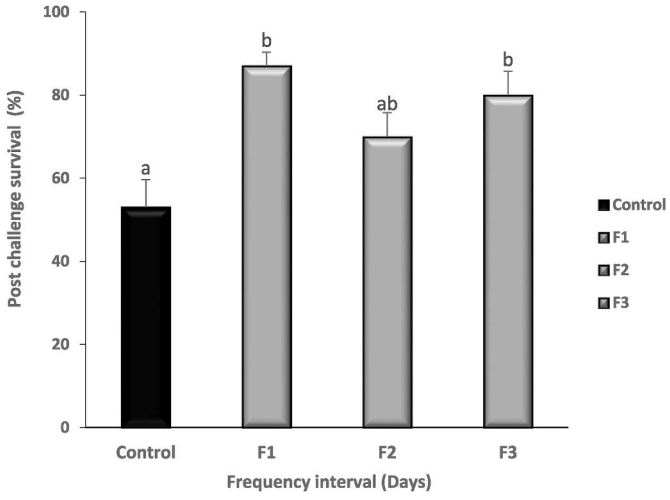

Fig. 3. Effect of administration frequency of immunostimulants in *Penaeus vannamei* post-larvae survival challenged with *Vibrio parahaemolyticus* IPNGS16-AHPND (acute hepatopancreatic necrosis disease). F1 = feed daily; F2 = feed every other day; F3 = feed every three day. Bars represent means ± standard error (SE) (n = 10 shrimp/treatment). Different letters indicate significant differences between treatments ($p < 0.05$).

relation to infection onset time. Survival of shrimp stimulated by oral and immersion route with a mixture of yeasts (Yl + Dh) decline over time from 91 to 77% when shrimp were challenged with *V. parahaemolyticus* IPNGS16 one to seven elapsed days upon last yeast administration, although survival was still significantly higher for all elapsed times compared to the control group (Fig. 4).

4. Discussion

This study evaluated the effects of yeast administration through oral and immersion routes on *P. vannamei* post-larvae growth, immune response by immune-related gene expression analysis, and disease resistance to *V. parahaemolyticus* IPNGS16 challenge.

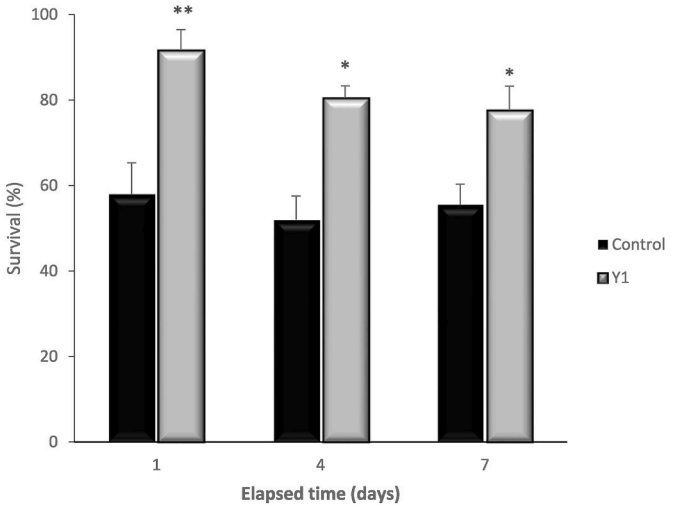

Fig. 4. Protective effect duration of daily yeast administration in *Penaeus vannamei* post-larvae against *Vibrio parahaemolyticus* IPNGS16-AHPND (acute hepatopancreatic necrosis disease) infection. Bars represent means ± standard error (SE) (n = 10 shrimp/treatment). Student's *t*-test indicates significant differences $p < 0.05$ (*) and highly significant $p < 0.01$ (**).

4.1. Growth

The efficacy of yeasts to increase growth in fish and crustaceans has been extensively studied in many aquaculture species [35–37]. Yeasts from industrial processes are used as emerging alternative feed ingredients in aquaculture because of their nutritional value [38]. Weight increase of shrimp fed with yeast has been associated with an increase in nutrient absorption in the intestine during the digestion process and digestive enzyme production by yeasts [39]. Some products, such as β-glucans and mannan-oligosaccharides from baker's yeast *Saccharomyces cerevisiae* have been used in shrimp diet with significant results on weight gain or specific growth rate [38,40,41]. Those products have been used as protein and amino acid source showing efficient effect on the use of dietary protein with a direct effect on shrimp growth [42]. An increase in shrimp growth associated with the use of immunostimulants is a goal pursued by researchers in the aquaculture industry [43]. In this study, a maximum weight gain (1.72 times) was recorded in the group of *P. vannamei* postlarvae stimulated with yeast *Y. lipolytica Yl*-N6 and *D. hansenii* CBS8339 via oral and immersion. Yang et al. [11] administered a live *Rhodosporidium paludigenum* yeast (1×10^8 CFU g^{-1}) and dry (1 g 100 g^{-1}) in the diet of *L. vannamei*, showing greater weight gain in shrimp fed with yeasts compared to the control group. Similarly, Nimrat et al. [44] reported higher final weight and weight gain in *L. vannamei* juveniles fed for 90 days with a combination of yeasts *D. hansenii* and *Rhodotorula* sp. compared to the control group. In a similar study, Apun-Molina et al. [45] evaluated dietary administration of four strains of lactic acid bacteria and a yeast strain (*Candida parapsilosis*) at a concentration of 5×10^5 CFU g^{-1}, finding greater growth in shrimp fed with a probiotic diet compared to shrimp from the control group. Additionally, the authors reported a greater weight gain when the treatment with bacteria and yeast was administered daily for a prolonged period (84 days) compared to the groups where they were administered with a frequency of every five and 10 days.

4.2. Immune-related gene expression

This study evaluated the immune-related gene expression in shrimp post-larvae because probiotic yeast strains have been widely evaluated in crustacean species for their ability to stimulate the immune system [16,46]. Remarkably, most of the studies focused on immune-related gene expression in shrimp have been performed in juvenile and adult

stages. For instance, scarce information is available regarding the innate immune system development in early shellfish stages [47,48].

The antimicrobial peptides (AMPs) play a critical role in protecting crustaceans against pathogenic bacteria and viruses [49,50]. In this study, penaeidin gene expression showed that oral administration of *D. hansenii* CBS8339 and *Y. lipolytica Yl*-N6 yeast upregulated the expression of this antimicrobial peptide in *L. vannamei* post-larvae. Similarly, an increase in the expression of the antimicrobial peptides crustin (crustin-1, crustin-2, and crustin-3) and penaeidin (Penaeidin-3 and penaeidin-5) has been reported in black tiger shrimp *Penaeus monodon* post-larvae fed with 10% of the *Candida aquaetextoris* yeast [12]. Jin et al. [32] reported a penaeidin gene upregulated expression in *L. vannamei* stimulated by oral route with 1% (diet) *S. cerevisiae*. In the same way, Miandare et al. [51] reported a higher penaeidin gene expression in shrimp post-larvae fed with a diet enriched with Prima-Lac® (1g kg^{-1}) a commercial probiotic multi-bacterial strain for eight weeks compared to the control group. In accordance with the results shown in this study, many authors have concluded that the administration of more than one probiotic strain potentiates the expression of antimicrobial peptides in shrimp [52,53].

Lectins are molecules associated with the antimicrobial response in crustaceans, since they enhance agglutination functions, recognition of specific carbohydrates on the surface of pathogens, and activation of the complement system [54]. In this study, a higher expression of lectins was observed in the treatments where yeast (individually or combined) was administered orally to post-larvae as compared to the control group. Ji et al. [55] evaluated the expression of lectins in *P. vannamei* juveniles injected intramuscularly with immunostimulants, recording a higher lectin expression in shrimp hemocytes stimulated with laminarin compared to the other immunostimulants and the control group. Chai et al. [56] observed that lectin type C gene expression increased in *L. vannamei* hemocytes by supplementing *Bacillus* sp. at higher concentrations of 1 x 10^7 and 1 × 10^9 CFU ml^{-1}. Similarly, Miao et al. [37] reported an increase in lectin gene expression in giant freshwater prawn *Macrobrachium rosenbergii* juveniles orally stimulated with a mixture of lactic acid bacteria and yeast *S. cerevisiae* at a concentration of 1 × 10^8 CFU g^{-1} for 60 days.

4.3. Administration route of immunostimulants on survival of shrimp post-larvae

A protective effect of yeasts and glucans administered by different routes is well known in shrimp post-larvae challenged with different pathogens [14,15,19], although the maximum efficiency of different administration routes was scarcely analyzed. This study obtained the highest protective effect (93% survival against *V. parahaemolyticus* IPNGS16 challenge) with the administration of two yeasts by oral and immersion routes. Oral administration of yeast offers beneficial effects, such as releasing digestive enzymes, biosynthesizing proteins, lipids, and nucleic acids, producing polyamines, immune-stimulating and enhancing microbiota community richness in the digestive tract [15,57–59]. For instance, Ceseña et al. [42] reported that *D. hansenni* (CBS 8339) administered by oral route enhanced immune response and survival against *V. parahaemolyticus* infection. Zheng et al. [59] observed that oral administration of 2% yeast for eight weeks increased growth performance and antioxidant response in shrimp *L. vannamei*. On the other hand, the incorporation of probiotic microorganisms in culture water enhances water quality, host health, and promotes biofilm formation inhibition of some pathogens because of the production of secondary metabolites with antibacterial properties [17,60,61]. For example, Pacheco et al. [62] observed enhanced antioxidant and immune response in juvenile shrimp *L. vannamei* exposed to *D. hansenii* by immersion route (1 × 10^6 CFU ml^{-1}) for 15 days. Nguyen et al. [63] reported the highest survival in *L. vannamei* juveniles exposed to probiotics by immersion route using a dosage from 1 x 10^5 to 1 × 10^6 CFU ml^{-1} and challenged with *V. parahaemolyticus*. Azad et al. [10] reported that administration by oral or immersion routes is the most preferred option in shrimp commercial farms. In this study, increase in survival rates by combined oral and immersion routes compared to oral administration alone can be associated with a possible synergistic effect between both administration routes. However, these results contrast with gene expression that was enhanced mainly by yeast oral administration alone. It seems likely that other immune effectors also enhanced by *Y. lipolytica* [30] and *D. hansenii* [64] are contributing to their protective effect.

4.4. Frequency immunostimulant administration

Parameters related to the use of immunostimulants, such as dose and frequency administration, are key aspects to achieve the correct immune system activation [23]. In this study, survival against *V. parahaemolyticus* IPNGS16 challenge among experimental groups was affected by the different frequencies of immunostimulant administration, obtaining the highest survival in the group of shrimp fed daily (F1) with the yeast (Yl + Dh) by oral and immersion routes. Sajeevan et al. [20] found that glucan administration at different feeding intervals in shrimp increased immune response and survival to the WSSV challenge. Interestingly, the authors demonstrated that a 0.2% glucan concentration supplied every seventh day for 21 days provided the highest shrimp survival upon viral challenge. In another study, Babu et al. [12] evaluated oral administration of 10% yeast *C. aquatextoris* in *P. monodon* at different feeding frequencies, obtaining the highest survival to WSSV infection in the shrimp group fed with a frequency of every seventh day. Similarly, Flores-Miranda et al. [65] evaluated the oral administration of a probiotic strain mixture of lactic acid bacteria and yeasts establishing three different feeding frequencies (1, 3, and 6 days) for 21 days. These authors reported an increase in survival to an experimental challenge with *V. sinaloensis* in juveniles of *L. vannamei* fed with immunostimulants at a feeding frequency of every third day. Fierro-Coronado et al. [66] determined that applying a mixture of probiotics with an administration frequency of every three days provided optimal survival in *P. vannamei* against *V. parahaemolyticus* infection. Similarly, Mameloco and Traifalgar [67] observed that oral administration of combined 0.2% mannan-oligosaccharide and β-glucan once every three days enhanced the immune response and resistance of *P. vannamei* to a *V. parahaemolyticus* infection.

The discontinuous oral administration of yeasts and glucans has been previously reported in penaeid shrimp, increasing survival against bacterial infections and viral challenges [68]. Conversely, the application of immunostimulants at different frequencies through immersion has been scarcely studied in shrimp. The correct stimulation of the defense system and maximum protection against pathogens can be achieved by implementing strategies in immunostimulants in shrimp farming, such as applying administration frequencies [20,69–71]. Several authors recommended avoiding continuous immunostimulant administration for extended periods (i.e., longer than 30 days) because it can cause immunosuppression, immunological fatigue, and decreased protection against pathogens [20,22,23].

4.5. Yeast administration confers extended protection against vibrio infection

Yeast and glucan incorporation in rearing increases the response capacity of the defense system in shrimp and survival against various pathogens [19,32,42]. However, most of the studies in which infection challenges are performed have a maximum duration of 72 h, making it difficult to have access to longer protection time achieved after immunostimulation. The results obtained in this study reveal a gradual loss of the protective effect in post-larvae concerning time, reaching maximum protection (77%) seven days after stimulation with a mixture of yeasts (Yl + Dh) administered by oral and immersion routes. Similarly, Pooljun et al. [72] evaluated in *L. vannamei* oral administration of a mixture of

bacteria (*L. acidophilus*) and yeast (*S. cerevisiae*), finding a 90% survival rate against infection with *V. parahaemolyticus*, 10 days after stimulation with a survival rate greater than 90%. Zokaeifar et al. [73] reported that stimulation with the probiotic *Bacillus subtilis* by immersion for eight weeks increases survival of shrimp challenged with *V. harveyi* with a protection of 63% that extends up to 10 days after the application of probiotics.

5. Conclusion

Yeast (*Debaryomyces hansenii* CBS8339 and *Yarrowia lipolytica Yl*-N6) supplementation in shrimp post-larvae significantly increased growth and immune response through the regulation of key genes and survival to experimental infection with *V. parahaemolyticus* IPNGS16. In addition, the most efficient way to stimulate shrimp -in terms of penaeidin and lectin gene expression-was through the oral route, either individually or combined yeast administration. However, the protective effect against *Vibrio* infection was enhanced by immersion in addition to oral administration of both yeast species. The continuous yeast supplementation for a nine-day period conferred a maximum survival against *Vibrio parahaemolyticus*, which provides high protection that extends at least up to seven days upon the last stimulation.

CRediT authorship contribution statement

Alan Licona-Jain: Conceptualization, Data curation, Formal analysis, Methodology, Investigation, Writing – original draft. **Ilie Racotta:** Investigation, Supervision, Writing – review & editing. **Carlos Angulo:** Investigation, Formal analysis, Writing – original draft, Writing – review & editing, Funding acquisition. **Antonio Luna-González:** Conceptualization, Supervision, Resources, Investigation. **Ruth Escamilla-Montes:** Investigation, Methodology, Formal analysis. **Edilmar Cortés-Jacinto:** Investigation, Validation. **Rosa M. Morelos-Castro:** Methodology, Writing – review & editing. **Ángel I. Campa-Córdova:** Conceptualization, Methodology, Supervision, Resources, Writing – review & editing, Visualization, Funding acquisition.

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