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*Research Article*

# Protector effect of beta-glucans from shrimp pond-related yeasts in *Penaeus vannamei* rearing under white spot syndrome virus presence

**Norma A. Ochoa-Álvarez[1,2], Ramón Casillas-Hernández[1], Francisco J. Magallón-Barajas[2]**
**Jesús M. Ramirez-Orozco[2] & Elizabeth Carvajal-Millan[3]**
[1]Instituto Tecnológico de Sonora, Departamento de Ciencias Agronómicas y Veterinarias
Área de Acuicultura, Obregón, Sonora, México
[2]Centro de Investigaciones Biológicas del Noroeste, La Paz, BCS, México
[3]Centro de Investigación en Alimentación y Desarrollo, Biopolímeros, Hermosillo, Sonora, México
Corresponding author: Norma Ochoa-Álvarez (nochoa04@cibnor.mx)

**ABSTRACT.** This research study tested the protective effect of prolonged exposure of shrimp food supplemented with glucans from shrimp-pond related yeasts on shrimp *Penaeus vannamei* reared under the presence of the white spot syndrome virus (WSSV). The glucans extracted and purified from isolated marine yeasts identified as *Debaryomyces hansenii*, *Candida tropicalis*, *Candida humilis*, *Candida glabrata*, *Pichia kudriavzevyi*, *Wickerhamomyces anomalus* and the terrestrial *Saccharomyces cerevisiae* yeast were characterized by the Fourier-transform infrared spectroscopy and proton nuclear magnetic resonance spectroscopy. The treatments were prepared with food enriched with the yeast beta-glucans and the control groups without beta-glucans. Shrimp were fed thrice a day and challenged orally with WSSV on days 31, 54, 66 and muscularly at day 70. The animals were assessed for the protective effect in terms of post-infection total hemocyte counts, and survival rate. The results indicated that marine yeasts possessed ß-1,3/1,6-glucans, and that *D. hansenii* was an excellent source yielding 30% of its dry biomass of pure glucans. For the positive control group where no glucans were added, WSSV challenges showed 100% survival when the virus was provided orally, and 40% when the virus was injected. These results also indicated that the shrimp line selected for this study was a resistant line for WSSV. Shrimp groups fed with glucans of the marine yeasts *D. hansenii* and *C. humillis* showed a significant protection, allowing shrimp survival of 66% while terrestrial yeast showed 57.14%. These results indicated that marine yeasts growing in the shrimp pond were an excellent source of beta-glucans that allowed extra protection against the mortality caused by this pathogenic virus.

**Keywords:** *Penaeus vannamei*; shrimp; WSSV; yeast; beta-glucan; rearing; aquaculture

## INTRODUCTION

The shrimp industry is one of the fastest-growing activities worldwide, both in the economic environment and its contribution to human nutrition (Castillo-Juárez et al. 2015), of which the white shrimp *Penaeus vannamei* is one of the most farmed and exploited species (Gong et al. 2012). However, this activity is not free of environmental and disease problems, mainly caused by pathogenic microorganisms (Perez-Enriquez et al. 2018, Flegel 2019). One of these diseases is the white spot syndrome virus (WSSV), which is not only important due to the high mortality rates but also for its capacity to infect a large number of crustaceans, leading to significant ecological risk (Witteveldt et al. 2004, Sarlin & Philip 2011, Parrilla-Taylor 2018).

One of the strategies to reverse WSSV infections has been to improve shrimp lines through the genetic selection to obtain resistance to viral infections and other diseases (Perez-Enriquez et al. 2009, Mohan et al. 2018) besides considering the nutritional factor as a source of additional protection. In the last strategy, countless efforts have been made to provide shrimp with the necessary elements to achieve optimal development and growth during the crop cycles and improve its resistance to various diseases. For example, adding molecules to shrimp feed to stimulate their defense system, such as glucans, have been used to con-

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trol some viral or bacterial diseases in crops, reducing mortalities and providing protection against resistant pathogens to drugs (Sajeevan et al. 2009, Bai et al. 2014).

Glucans are part of a group of compounds known as "biological response modifiers" and found in the cell wall of yeasts, fungi, and seaweeds (Uriza-Pinzón 2014, Vetvicka & Vetvickova 2016). Glucans interact with several receptors in microorganisms, invertebrates, and mammals, initiating several immune responses (Legentill et al. 2015). Research studies have reported the use of glucans obtained from yeasts, both of terrestrial and marine origin, indicating they are excellent sources for beta-glucans that confer protection against pathogenic microorganisms in Penaeid shrimp (Sukumaran et al. 2010, Flores-Miranda et al. 2011, Deng et al. 2013, Subramanian & Philip 2013, Bai et al. 2014). However, not all beta-glucans possess immunostimulatory properties; moreover, they vary depending on the source and even within the same source (Stier et al. 2014, Wang et al. 2016).

Additionally, yeast beta-glucans' leading source is the model yeast *Saccharomyces cerevisiae*, but very few studies have been performed in non-conventional yeasts, even less, with microorganisms coinhabiting with shrimp. Therefore, this study evaluated the prolonged exposure of beta-glucans extracted from yeasts associated with shrimp farming to determine their protective effect on shrimp rearing challenged with WSSV.

## MATERIALS AND METHODS

### Yeast isolation

The marine yeasts used in this study were obtained by microbiological isolation of water and bottom samples obtained from *Penaeus vannamei* ponds at Mahr Aquaculture Farm (Pichilingue, Baja California Sur, México). Yeast strains were obtained by sequential streaking on the Petri dish until pure colonies were obtained. Strains were preserved at 4°C on yeast-media plus antibiotics for short term use or at -80°C with glycerol as a cryogenic preservative for long term use. As a reference, terrestrial *Saccharomyces cerevisiae* strain was obtained as described from *S. cerevisiae* powder (Marcq-en-Baroeul, FR).

### Yeast identification

Identification was performed as described by Kurtzman & Robnett (1998) with some changes. Briefly, DNA was extracted using the Fast DNA Spin Kit for Soil, following the manufacturer's instructions (MP Biomedicals, Santa Ana, CA, USA). DNA quality and purity were assessed by agarose gels using GelRed and a photo documenter (BioDoc-IT UVP, Upland, CA, USA). DNA quantity was determined by NanoDrop determination (Thermo Scientific, Willmington, DE, USA). Primers used were NL-1 for forwarding string (5'-GCATATCAATAAGCGGAGGAAAAG) and NL-4 for reverse string (5'-GGTCCGTGTTTCAAGACGG), which amplified a variable region of the D1/D2 domains of the large ribosomal DNA (26s rDNA) subunit. DNA amplification was performed in a thermocycler (Verity Applied Biosystems, CA, USA) using the following thermal cycling program: one cycle at 95°C for one min; followed by 35 cycles at 94°C for 60 s, at 55°C for 60 s, and at 72°C for 30 s; one cycle 72°C for 7 min and a final step at 4°C for 10 min. Agarose gels and quantity assessed quality by NanoDrop determination. The obtained amplicons from polymerase chain reaction (PCR) were purified and sequenced by the MacroGen Company in Seoul, Korea. The obtained chromatograms were visually corrected using the BioEdit program (https://bioedit.software.informer.com). Consensus sequences were obtained using the ClustalW program. The nucleotides were resolved by visual inspection, genera, and species by sequence comparison with the National Center for Biotechnology Information (NCBI, USA) nucleotide data bank. The identification results were corroborated using a specialized yeast platform, named YeastIP (The International Center of Microbial Resources, French National Agronomical Research Institute, France).

### Biomass production

The marine yeast biomass was prepared using yeast-peptone-dextrose (YPD)-broth medium (DIFCO, Detroit, MI, USA) and maltha-broth for terrestrial yeast. Erlenmeyer flasks with yeast cultures were incubated at 30°C and 100 rpm for 24h. Biomass was recovered by centrifugation at 5000 g per 15 min at 4°C (Megafuge 16R, Thermo Scientific, Waltham, MA, USA) and dried to obtain lyophilized powder (Labconco Freeze Dryer, Kansas City, MO, USA).

### Beta-glucan extraction

Glucans were extracted from lyophilized biomass following Williams et al. (1991) with some modifications. Two grams of yeast biomass was resuspended in 40 mL of 3% NaOH solution and boiled for three h at 90°C. Samples were centrifuged at 5000 g per 30 min at 4°C. The precipitate was collected and treated thrice as described for the biomass. Fifteen milliliter of 0.5N acetic acid was added to the precipitate and boiled at 75°C for 6 h. The suspension was centrifuged at 5000 g per 30 min at 4°C and resuspended in 15 mL of ethanol at boiling temperature.

The suspension was centrifuged as previously performed, and the precipitate recovered. Steps from acetic acid to ethanol boiled addition were repeated thrice. Finally, the precipitate was washed with distilled water and centrifuged each time, as described before. The recovered pellet was lyophilized using the Freezer-Dryer described before and preserved at room temperature until use.

## Chemical characterization of extracted glucans

The obtained glucans were analyzed for ß-1,3/1,6 bond presence, glycogen content, Infra-Red Spectra, and Proton nuclear magnetic resonance (NMR) Spectrum. The glycogen determination was performed quantifying glucose through high-performance liquid chromatography (HPLC) (Waters Alliance e2695, Milford, MA, USA) after enzymatical hydrolysis with amylase/amyloglucosidase (Sigma-Aldrich). The determination of ß-1,3/1,6 bonds was performed through Congo Red Dye (Nitschke et al. 2011). The infrared spectrum was obtained in the Nicolet Fourier-transform infrared (FTIR) spectroscopy iS50 FT-IR (Waltham, MA, USA), with total attenuated reflection (ATR) by Fourier-transform infrared (ATR-FTIR) spectroscopy in a spectrum range from 400-4000 $cm^{-1}$. The proton nuclear magnetic resonance (NMR + H) spectrum was analyzed by dissolving 8 mg of sample in deuterated dimethyl sulfoxide in a Bruker Advance 600 Mhz, BBOF probe (Billerica, MA, USA)

## Shrimp feed production

Food was prepared at Centro de Investigaciones Biológicas del Noroeste (CIBNOR) food plant as follows. The formulation was carried out with the Nutrion® program (Guadalajara, México). Table 1 summarizes the nutrients added to prepare the basal shrimp feed for the control groups. This basal diet was added with the different purified glucans at a 0.2% proportion for the challenge experiments. Nine treatments were prepared, containing 27.52% crude protein and 7.03% lipids, of which seven treatments were supplemented with yeast beta-glucans. The other two treatments were used as control groups, one negative with no yeast beta-glucans and not virus, and the other positive treatment with WSSV but no beta-glucans. Before preparing the experimental feed, the macro ingredients: fishmeal, soybean-paste, and wheat-flour were ground and sieved through a 250 µm mesh sieve. The dry ingredients were thoroughly mixed in a food mixer, and oil fish and soybean were added. Pellets were produced through a meat mill using a 2 mm sieve. The produced pellets were dried in a convection oven at 60°C to obtain humidity from 8 to 10%.

**Table 1.** Composition of experimental feeds.

| Ingredients | Control feed (%) | Experimental glucan feed (%) |
|---|---|---|
| Fish meal | 6.5 | 6.5 |
| Soybean paste | 30.8 | 30.8 |
| Wheat flour | 48.4 | 48.4 |
| Fish oil | 4.1 | 3.9 |
| Soy lecithin | 2.2 | 2.2 |
| Alginic acid | 2.0 | 2.0 |
| Tryptophan | 0.2 | 0.2 |
| Methionine | 0.1 | 0.1 |
| Arginine | 0.1 | 0.1 |
| Vitamin and mineral mixture | 5.6 | 5.6 |
| Glucan yeast | 0 | 0.2 |

## Inclusion of glucans in the feed

Glucans were mixed with fish oil (41.1 g oil $kg^{-1}$ feed) and added to the dried pellets using a sprinkler. The final beta-glucans concentration was 0.2% of the feed. A different preparation was made for each of the beta-glucans of the assayed yeasts. As previously mentioned, the control groups were prepared without beta-glucans.

## Animal management

The authors confirm that this study adhered to ethical policies. Animal ethics approval was not required for crustacean species, but it was conducted following standard practices.

## Bioassays design

The shrimp used in this study were acclimatized in the Aquaculture Laboratory of the Instituto Tecnológico de Sonora (ITSON, Ciudad Obregón, SON, MX), as follows. Thirty juvenile of *P. vannamei* shrimp weighing 2 ± 0.67 g were selected. The absence of WSSV was determined by polymerase chain reaction (PCR) analysis. Shrimp were placed in acclimation units (200 L aquariums filled with 150 L water) with aerated water at room temperature and weekly 33-50% water exchange (no recirculating aquaculture system was used). Organisms were fed thrice a day for 25 days, using an average from 8-12% of feed relative to the organisms' weight. For the experimental units, eight shrimp were selected and placed in 40 L aquarium on day 26 and maintained under the same conditions as before. In total, 27 experimental units were used. Organisms were fed each day with the feed-BG preparation. Bioassays were developed by triplicate (three aquaria per assay). Eight organisms weighing 5.2 ± 1.0 g were confined to each aquarium. Three challenges were performed orally by feeding 1 g of WSSV shrimp-infected tissue per aquarium on days 31,

54, and 66. WSSV was determined by PCR using the IQ Real Kit (GeneReach Biotechnology Corp, Taiwan) with no quantification. Because the shrimp line turned out to be WSSV resistant, a fourth challenge was performed on day 70 by injecting shrimp 20 µL of a WSSV suspension per gram of shrimp weight with a 1 mL insulin syringe. The experiment was monitored for 120 h. Survival rates were recorded every 12 h.

### Hemolymph collection and total hemocyte count (THC)

On day three post-infection intramuscularly, four shrimp individuals were randomly selected from each treatment for THC determination. Shrimp hemolymph (100 µL) was collected with an insulin syringe pre-filled with 200 µL of EDTA-citrate anticoagulant (100 mM glucose, 30 mM sodium citrate, 510 mM NaCl, and 10 mM EDTA). Formaldehyde was added (400 µL) for fixing hemocytes to measure THC in a Neubauer chamber.

### Statistical analysis

One-way ANOVA determined the statistically significant difference between experimental feeding groups; differences in multiple comparisons among group means were determined using Tukey's test ($P < 0.05$).

## RESULTS

### Marine yeast isolation

A total of 21 strains of culturable marine yeasts were isolated from the shrimp ponds. The analyses of the D1/D2 domains of the 26s rDNA determined that the isolated yeasts corresponded to only seven species, which belonged to four different genera. Table 2 summarizes the different genera and species isolated, all of which were used to generate yeast biomass for glucan extraction.

### Beta-glucans extraction

The alkali-acid method allowed obtaining an extract consisting of glucan and glycogen, which was insoluble in water. With glycogen and glucans, the extract also had minimal cellular debris quantities, which were not considered for calculation. The amount and concentration of the extracted beta-glucans were obtained once the amount of glycogen was determined. The amount of glucan-glycogen obtained was variable and species-dependent, whose values ranged from 2.5 to 43.54% of the used biomass dry weight, which revealed that the maximum glucan extraction percentages were for the marine yeast *Debaryomyces hansenii* (Table 2).

Regarding the glucan-glycogen ratio in the obtained extract, on average, the relationship for the marine yeast was determined to be 64.16-35.84% (respectively), corresponding to the yeasts *D. hansenii* and *Candida glabrata* (70%) the highest glucan value and the lowest one (52%) for the yeast *Wickerhamomyces anomalus*. In comparison with the terrestrial yeast *Saccharomyces cerevisiae* (60%), the marine yeasts had better glucan content in the obtained extract (Table 2).

Glucan yield was calculated from the number of glucans obtained against the lyophilized yeast biomass used for the extraction. The results are summarized in Table 2, showing that the maximum glucan yield corresponded to the marine yeast *D. hansenii* (0.3), while the lowest one was for *W. anomalus* (0.013). As previously mentioned, the terrestrial yeast *S. cerevisiae* showed a low glucan yield value (0.0633).

### Glucan chemical characterization

#### Fourier-transform infrared (FTIR) spectroscopy

The infrared spectra obtained using the FTIR technique represented patterns unique for each molecule type, which turned out to be very useful for determining some characteristics of unknown samples. The purity of the glucans extracted by the alkali-acid chemical extraction method allowed obtaining spectra in which characteristic bands could be observed for all the analyzed samples (Fig. 1). Bands from the 850-1350 region were characteristic to polysaccharides; bands at the 892 region corresponded to ß-1,3 bonds; and those at 998 regions to ß-1,6 bonds; at 3,300 the hydroxyl group band was detected. In general, bands in region 1400 corresponded to beta-glucans.

#### Proton nuclear magnetic resonance (NMR) spectroscopy

Figure 2 shows the proton magnetic spectra obtained for the beta-glucans of the tested yeasts. They had very similar spectroscopic behavior because of the same number and type of peaks throughout the spectrum. The anomeric region could be seen from 4.3-4.6 ppm (anomeric proton). The spectrum from 4.7-4.8 ppm was characteristic for ß-1,3 bonds and for branched linkages bonds ß-1,6 were observed in the region from 4.4-4.5 ppm, which was characteristic of this type of bond. These results mean that the beta-glucans extracted from all the tested yeasts were linear ß-1,3 glucans with branches at position ß-1,6.

### Survival challenge

Figure 3 shows that when the organisms were challenged with WSSV through an oral application, no adverse effects were found (day 31) even for the shrimp in the control group fed with the basal diet (non-glucan diet). A second (day 54) and third (day 66) applications

**Table 2.** Characteristics of beta-glucans isolated from marine and terrestrial yeast. Amount and purity of beta-glucans obtained by the alkali-acid method. Values shown correspond to the average of three determinations.

| Scientific name (Strains) | Dry weight of biomass (mg) | Extract obtained (%) | Glycogen content (%) | Glucan content (%) | Glucans yield (biomass/glucans) | Link content β-1,3-1,6 (%) |
|---|---|---|---|---|---|---|
| *Debaryomyces hansenii* | 2000 | 43.5 (870 mg) | 30 (261 mg) | 70 (609 mg) | 0.3045 | 79.35 |
| *Candida tropicalis* | 2000 | 24.5 (490 mg) | 31 (151.9 mg) | 69 (338.1 mg) | 0.1690 | 74.57 |
| *Candida humilis* | 2000 | 9.25 (185 mg) | 36 (66.6 mg) | 64 (118.4 mg) | 0.0592 | 74.39 |
| *Candida glabrata* | 2000 | 5.3 (106 mg) | 30 (31.8 mg) | 70 (74.2 mg) | 0.0371 | 84.20 |
| *Picchia kudriavzevii* | 2000 | 7 (140 mg) | 40 (56 mg) | 60 (84 mg) | 0.0420 | 82.12 |
| *Wickerhamomyces anomalus* | 2000 | 2.5 (50 mg) | 48 (24 mg) | 52 (26 mg) | 0.0130 | 71.61 |
| *Saccharomyces cerevisiae* | 2000 | 10.55 (211 mg) | 40 (84.4 mg) | 60 (126.6 mg) | 0.0633 | 74.74 |

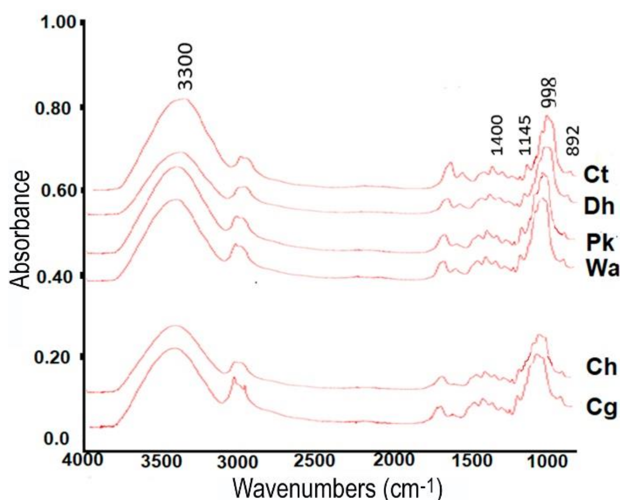


**Figure 1.** Fourier-transform infrared spectroscopy (FTIR). The spectrum of beta-glucans extracted from the cell wall of different yeasts by chemical methods. *Pichia kudriavzevii* (Pk), *Wickerhamomyces anomalus* (Wa), *Candida tropicalis* (Ct), *Debaryomyces hansenii* (Dh), *Candida humilis* (Ch), *Candida glabrata* (Cg).

of WSSV were necessary, obtaining similar results. At day 70, a WSSV application with a syringe was carried out to overcome the primary shrimp barrier, allowing the viral particles to be left directly in the shrimp tissue. Twelve hours after WSSV inoculation, the control (+) group, which was inoculated with WSSV but fed with no glucans, showed 50% of mortality, increasing to 60% at 24 h and remaining at this rate until the end of the experiment. Interestingly, the group fed with beta-glucans from the marine yeast *C. glabrata* showed a greater mortality rate than the control group (+), and *W. anomalus* showed no significant difference. The shrimp group fed with the terrestrial yeast *S. cerevisiae* glucans had a 57.14% survival rate, which was greater than those shown by the marine yeasts *W. anomalus*, *C. glabrata*, *C. tropicalis,* and *Pichia kudriavzevii* but significantly lower than those for the marine yeasts *C. humillis* and *D. hansenii*.

## Total hemocyte count (THC)

After the WSSV inoculum was applied intramuscularly, four shrimp were randomly selected from each treatment for THC determination at 72 h. No statistical differences ($P < 0.05$) were found for THC for *W. anomalus*, and the negative control group (no WSSV inoculum and no glucans added). Groups with glucans from the marine yeasts *C. tropicalis* and *C. humilis* showed a statistical difference ($P < 0.05$) with respect to the negative and positive control group (WSSV inoculum and no glucans added). Marine yeasts *P. kudriavzevii*, *D. hansenii,* and terrestrial yeast *S. cerevisiae* showed greater THC counts than the positive control group, but no statistical differences ($P < 0.05$) was found (Fig. 4).

## DISCUSSION

The attack of pathogenic microorganisms to shrimp ponds is one of the most pressing problems in the shrimp industry, of which the white spot virus is the main one. The use of immunostimulants that improve shrimp's ability to respond to external attacks has been proposed as an alternative, showing promising results. Beta-glucans are among the main proposed immunostimulants, and yeast beta-glucans have shown survival rates of up to 70% in the presence of WSSV (Wilson et

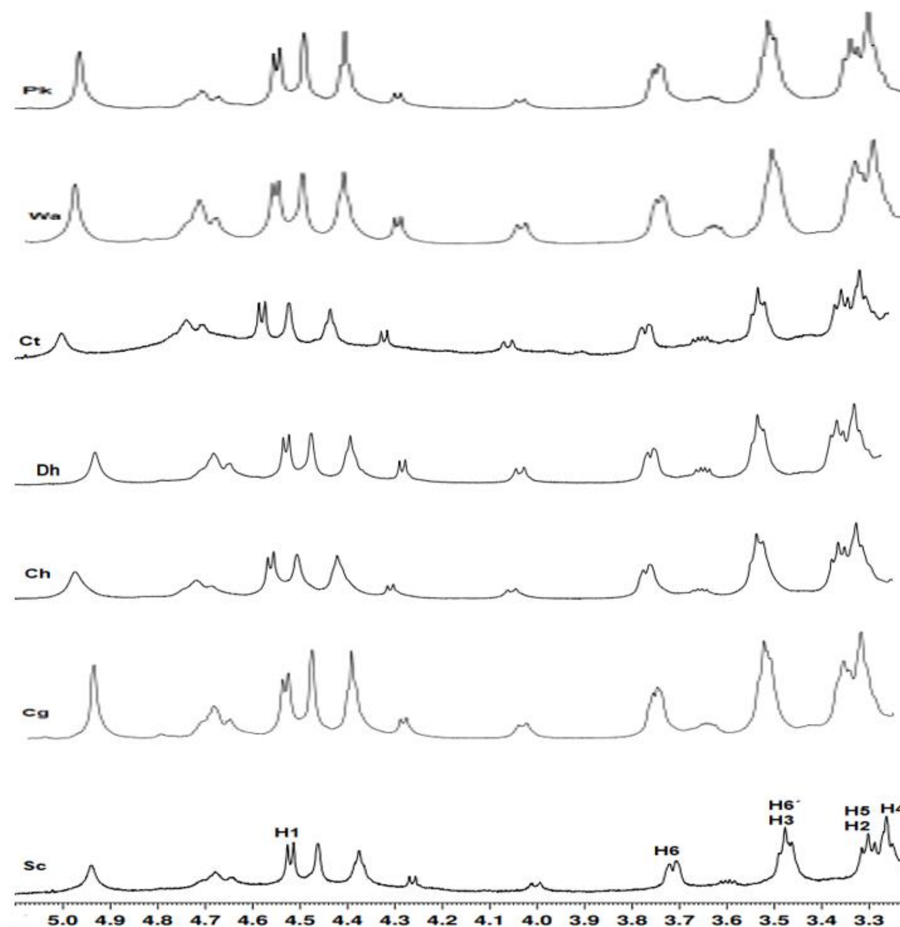


**Figure 2.** Proton nuclear magnetic resonance (NMR) spectrum of carbohydrate region of water-insoluble particulate beta-glucan from six different marine yeasts isolates. *Pichia kudriavzevii* (Pk), *Wickerhamomyces anomalus* (Wa), *Candida tropicalis* (Ct), *Debaryomyces hansenii* (Dh), *Candida humilis* (Ch), *Candida glabrata* (Cg), and one terrestrial yeast *Saccharomyces cerevisiae* (Sc).

al. 2015). This study assessed the efficacy of prolonged exposure of marine yeast glucans associated with shrimp ponds for protecting shrimp in the presence of the WSSV virus. Surprisingly, the shrimp line selected for this study was found to be resistant to the WSSV. Moreover, some isolated marine yeasts' glucans showed a greater protector effect than glucans for the terrestrial yeast *Saccharomyces cerevisiae*. These findings indicated that some marine yeasts that grow jointly with *Penaeus vannamei* might be a good source for biological compounds; beta-glucans could confer a better protector effect than those obtained from terrestrial sources, improving the survival rate even to resistant shrimp to the WSSV virus.

Some studies have reported that yeast beta-glucans improved the growth of several organisms, including shrimp (Shurson 2017, Jin et al. 2018); however, no study has been performed to our knowledge to date beta-glucans of the microbiota associated with shrimp farming ponds have been evaluated. The marine yeast strains obtained from the shrimp pond were characterized by analyzing the D1/D2 region of the large ribosomal DNA subunit (Kurtzman & Robnett 1998). These analyses allowed establishing the presence of four genera of culturable marine yeasts *Pichia*, *Wickerhamomyces*, *Candida*, and *Debaryomyces* coinhabiting the pond with *P. vannamei*. Although studies have already described marine yeasts from the genera *Candida* and *Debaryomyces* as potential sources for immunostimulants, either using the whole-cell, cell extract or its semi-purified or purified beta-glucans (Dalmo & Bogwald 2008, Sukumaran et al. 2010, Flores-Miranda et al. 2011, Deng et al. 2013), in the same manner, beta-glucans from the marine yeasts *Wickerhamomyces* and *Pichia* have been analyzed for the first time in this study.

Several methods have been reported for beta-glucans extraction (Ahmad et al. 2010, Sivamaruthi et al. 2016). Among those, chemical methods are the most used to extract glucans from cereals, yeasts, and

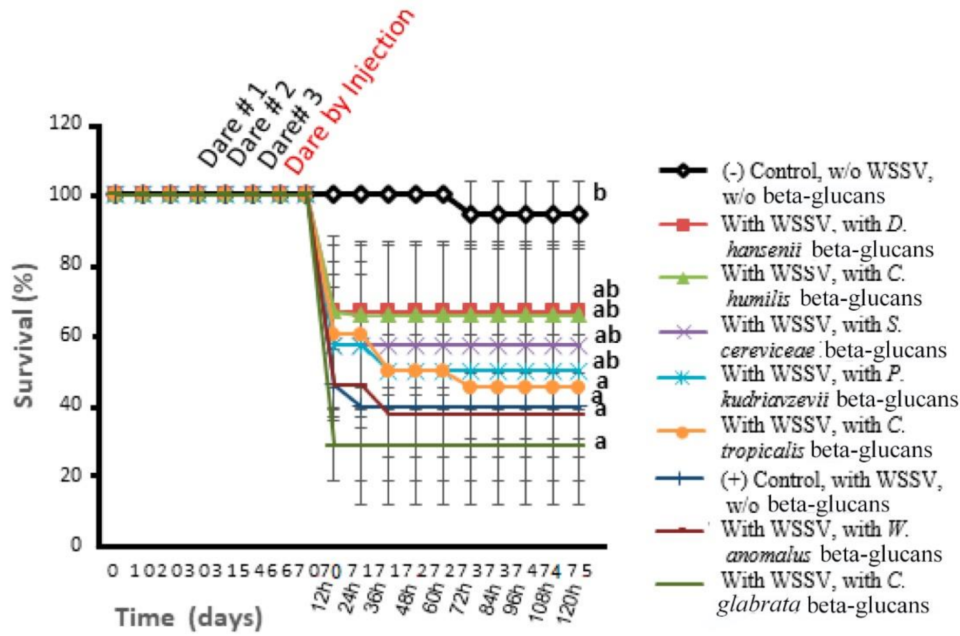


**Figure 3.** Post-challenge survival of *Penaeus vannamei* juveniles for 75 days fed with different marine yeast beta-glucans incorporated to diets and challenged with the white spot syndrome virus (WWSV). The line graphs with different letter were significantly different ($P < 0.05$).

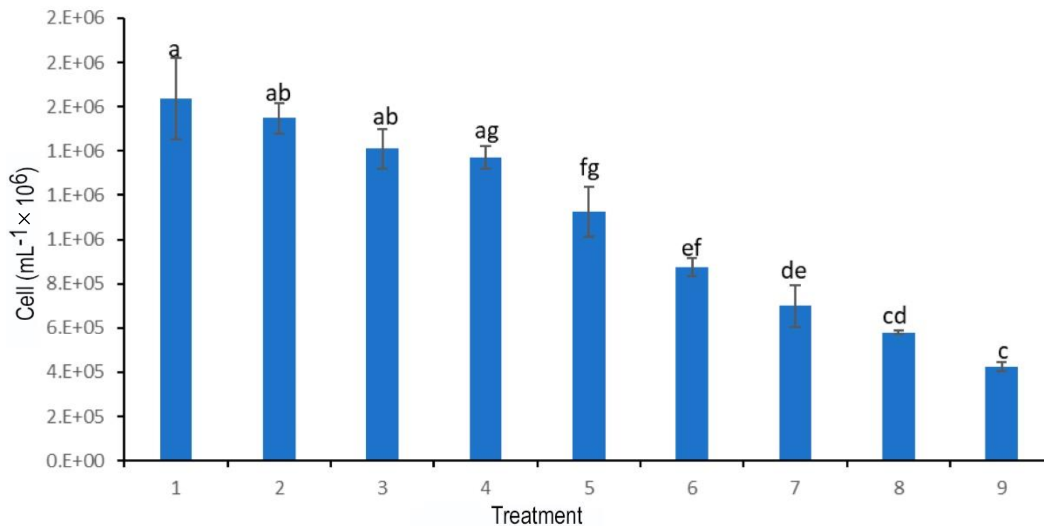


**Figure 4.** Total hemocyte count (THC). The number of hemocytes per milliliter of hemolymph in *Penaeus vannamei* shrimp groups fed with yeast beta-glucans, 72 h after white spot syndrome virus (WSSV) inoculum. 1) *Pichia kudriavzevii glucans*, 2) *Saccharomyces cerevisiae* glucans, 3) *Debaryomyces hansenii* glucans, 4) positive control group (with WSSV, without glucans), 5) *Candida glabrata* glucans, 6) *Candida humilis* glucans, 7) *Candida tropicalis* glucans, 8) *Wickerhamomyces anomalus* glucans, 9) negative control group (without WSSV, without BG). Different letters indicate significant differences ($P < 0.05$). Bars represented means with standard deviation (n = 3).

microalgae (Volman, 2007), but every extraction method affects the structure, quality, and functionality of beta-glucans. Using chemical methods, Sukumaran et al. (2010) found that the marine yeast *Debaryomyces hansenii* contributed the most beta-glucans with a 0.124 yield, which agrees with our results where this yeast produced the highest quantity of glucans with a 0.3 yield. Wilson et al. (2015) reported that out of eight strains of the marine yeasts evaluated, *D. fabryi* was one with the highest glucan yield with 0.275, while Angulo et al. (2018) reported a yield of 0.1482 for *D. hansenii*. In this study, the beta-glucans obtained from

*Wickerha-momyces anomalus* and *Candida glabrata* had the lowest yields with values of 0.013 and 0.037. In general, the yields obtained by the method here described were higher than those reported by the studies mentioned above, obtaining a 0.3 yield for the marine yeast *D. hansenii*, which showed the greatest amount of glucans extracted even in comparison with the terrestrial yeast *S. cerevisiae*.

The methods used to supply the beta-glucans to shrimp can be divided into direct or indirect methods. Direct methods are invasive, usually subcutaneous or intramuscular, and performed with a needle and syringe. In indirect application methods, beta-glucans are introduced to the shrimp by immersion, using a cannula, or by including them into shrimp feed (Flores-Miranda et al. 2011, Fonseca-Moreno et al. 2013). All methods mentioned, except for the latter, have the disadvantage of requiring direct handling of organisms, and thus less attractive for their use by the industry. Initially, due to the low systemic availability of oral preparations, beta-glucans applied parentally were considered the only way to modulate the immune system. However, various investigations *in vivo* and *in vitro* have revealed that orally applied beta-glucans also exerted these effects (Stier et al. 2014). One of the main problems with using beta-glucans is its hydrophobic nature due to the large number of -OH groups in their structure (Bai et al. 2014). various methods have been used to solve this problem, replacing the -OH group by sulfation, phosphorylation, or changing the medium pH to a very alkaline one. However, these modifications can strongly affect biological activity negatively (Zekovic et al. 2005). The insoluble nature of beta-glucans is also a problem when trying to integrate them into the feed. In this study, the methodology used to integrate beta-glucans in pelleted feeds was through a sprinkler, loaded with a fish oil suspension and yeast beta-glucans, which allowed to disperse the glucans evenly on the feed surface, and because of the oil, adhere to feed. An advantage of this procedure is to allow the molecules of the immunostimulants to be exposed directly to shrimp tissue once ingested, thus, accelerating the immunostimulatory effect.

The beta-glucans isolated from different sources have different immunomodulatory properties because of their size and different biochemical properties (Camilli et al. 2018). Differences in their structural characteristics include molecular mass, tertiary structure, polymer loading, conformation, branching degree, and solubility (Zechner-Krpan et al. 2010, Legentill et al. 2015, Yamamoto et al. 2019). Linear beta-glucans formed by 1,3 bonds possesses little or no immunomodulatory activity (Vetvicka & Vetvickova 2016), while beta-glucans possessing 1,3 bonds with ramifications at 1,6 position are the ones with the highest immunogenic activity and known as ß-1,3/1,6-glucans (Wang et al 2008, Subramanian & Philip 2013, Bai et al. 2014). In this study, the results indicated that *C. glabrata* showed the greatest number for ß-1,3/1,6 bonds, but it was the yeast with the lowest protective effect against WSSV. Sukumaran et al. (2010) found similar results when working with *P. monodon* finding low survival, so they concluded that a high number of ramifications affected the biological activity as an immunostimulant.

The periodicity and dose to which the beta-glucans are supplied are two important factors determining these molecules' protective effect. An overdose causes immunosuppression that leads to less protection and for animals to succumb to infection (Sajeevan et al. 2009). Thitamadee et al. (2014) found that if beta-glucans were given only once to the black tiger shrimp, the survival effect was 25-50% compared to the control group, but the second dose of beta-glucans led to a 100% of mortality. On the other hand, Bai et al. (2010) found that a continuous supply of beta-glucans ceased advantages over the control group while a discontinuous supply was the best option eliminating immune fatigue for *P. vannamei*. In this study, beta-glucans were ministered continuously at a concentration of 0.2% of the feed thrice a day for 75 days. The WSSV viral particles were introduced orally to shrimp on day 31, and the mortality rate was 0% even for the control group not fed with beta-glucans. Therefore, second and third doses of viral particles were provided, finding similar results (days 54 and 66, respectively). These results suggested that the selected shrimp line was WSSV resistant, which was confirmed by providing a fourth dose of viral particles but this time, intramuscularly (day 70). On this occasion, when the primary barrier (exoskeleton) was overcome by direct muscle invasion, mortality for the control group fed without beta-glucans was 60% during the first 24 h after viral inoculum, which indicated resistance to WSSV virus by the shrimp line used. It is noteworthy that 40% of the organisms survived even after 120 h after the inoculum was injected. Finding shrimp lines resistant to WSSV in Mexico is feasible because of a program initiated by the National Association of Shrimp Larvae Producers (NPLAC, for its acronym in Spanish) and by some independent aquaculture producers in 2013 who introduced shrimp lines resistant to WSSV that have been used by aquaculture in an attempt to counteract WSSV mortalities (Perez-Enriquez et al. 2018).

Regarding the protective effect of beta-glucans, they were applied continuously for 75 days, and some of the assayed beta-glucans caused the immune fatigue observed by Sajeevan et al. (2009) and Fonseca et al. (2013), causing rate mortality even greater than that

observed for the shrimp not fed with the viral particles. However, in some cases, the yeast beta-glucans protective effect was maintained, increasing survival of WSSV-resistant *P. vannamei* up to 26.66% higher than that shown by the shrimp in the positive control group, such was the case for the beta-glucans of *D. hansenii* and *C. humilis*. Sukumaran et al. (2010) reported that *S. cerevisiae* beta-glucans had a minimal protective effect (4%) than the marine yeast beta-glucans in studies with *P. monodon*. In this study, the results indicated that *S. cerevisiae* beta-glucans also provided a good protective effect to shrimp up to 17% more than the control group. On the other hand, glucans from the marine yeasts *C. humilis*, *C. glabrata*, *P. kudriavzevii*, *D. hansenii,* and terrestrial yeast *S. cerevisiae* showed a significantly higher ($P < 0.05$) value for THC than the negative control group, indicating no presence of immunogenic fatigue. These results also indicated that not all yeast beta-glucans prolongedly exposed to shrimp caused immunogenic fatigue, even with daily exposures up to 73 days.

The results in this study suggest that the immense variety of glucans offer a wide range of possibilities for application as protective molecules that help organisms survive from pathogen attacks.

## CONCLUSION

The results in this study suggest that the beta-glucans obtained from some marine yeasts that coinhabit the *Penaeus vannamei* culture ponds have a greater protective effect on white shrimp in the presence of WSSV viral particles compared to those of the terrestrial yeast *Saccharomyces cerevisiae*. Beta-glucans obtained from different yeasts have different protective effects; moreover, not all marine yeasts beta-glucans have such a protective effect. In some cases, immune fatigue is shown when beta-glucans are administered continuously, but some beta-glucans do not show this immune fatigue and could be safely used, incrementing the surveillance rate for shrimp. Further studies with a tendency to improve shrimp genetic lines are needed to make organisms more resistant to pathogens and diseases. Jointly, resistant shrimp lines and beta-glucans can avoid antibiotics, providing more robust organisms with high survival rates when attacked by pathogens.

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