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Enhancement of vibriosis resistance in juvenile *Penaeus vannamei* by supplementation of diets with different yeast products

U. Scholz [a], G. Garcia Diaz [b], D. Ricque [b], L.E. Cruz Suarez [b,*], F. Vargas Albores [c], J. Latchford [a]

[a] *University of Wales, Bangor, School of Ocean Sciences, Menai Bridge, Gwynedd, LL59 5AD, UK*
[b] *Programa Maricultura, Facultad de Ciencias Biológicas, Universidad Autónoma de Nuevo León, Apdo. Post. F-56, San Nicolas de los Garza, Nuevo Leon 66450, Mexico*
[c] *Centro de Investigación en Alimentación y Desarrollo, A.C. Km. 0.6 Carretera a la Victoria, Apdo. Post. 1735, Hermosillo, Sonora 83000, Mexico*

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Abstract

Penaeus vannamei were reared on five different experimental diets containing: (1) *Saccharomyces cerevisiae* at 1%; (2) β-glucan, extracted from *S. cerevisiae*, at 0.1%; (3) *Phaffia rhodozyma* at 1%; (4) experimental yeast (HPPR1) at 1% and; (5) a control diet. Wet weight, survival (5) and feed conversion ratio were monitored over a 7-week period. Results indicate that, while there was no significant difference in weight gain on the different diets ($P = 0.196$), survival of animals reared on the diets containing *S. cerevisiae*, *P. rhodozyma* and the experimental yeast was significantly higher ($P = 0.006$) than the animals reared on the diet containing β-glucan, and higher, though not significantly, than those reared on the control diet. Biomass was found to be statistically different ($P = 0.042$), the animals reared on the *P. rhodozyma* diet displaying greater biomass than the animals on the β-glucan diet. Feed conversion ratio was not different for any of the diets ($P = 0.233$). At the end of the growth trial, the ability of the shrimp to clear bacteria from the hemolymph was tested by immersing the animals in a viable cell suspension of *Vibrio harveyi* strain BP05. Twenty-seven hours post immersion, the animals fed the *S. cerevisiae*, *P. rhodozyma* HPPR1 and control diet had effectively cleared the bacteria from the hemolymph and were not statistically different from nonchallenged control shrimp, while the animals fed the glucan diet still displayed elevated numbers in the order of 10^3 CFU/ml ($P = 0.002$). Additionally, mortalities were noted for these animals during the immersion challenge, unlike for the animals on the other treatments. Determination of phenoloxidase activity of

* Corresponding author. Telefax: +52-8-3526380; E-mail: lucruz@ccr.dsi.uanl.mx

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the animals showed a significant difference among the five treatments with phenoloxidase activity for the *Phaffia*-treated animals being significantly lower ($P = 0.003$) than any of the other diets except the β-glucan diet. These results indicate that even though no clear immunostimulatory effect could be found for the different treatments, it appears that all three yeasts, and especially the *Phaffia* diet, had a positive effect on the animals, leading to better survival. The animals on the glucan diet showed poor performance overall, indicating that not only did this treatment not aid in increasing resistance of the animals to infection, but on the contrary, these animals showed reduced performance when compared to the animals fed the control diet.

Keywords: Penaeid shrimp; Immunostimulation; Yeast; Glucan; Pigment

1. Introduction

Bacterial and viral diseases are known to be the major constraint in the further progress of semi-intensive and intensive shrimp culture throughout the world. Major disease outbreaks due to both *Vibrio* spp. and viruses, have been detrimental to the industry in recent years.

Viruses constitute the most serious stress factor, affecting pond reared penaeids. Often, viruses will remain latent and will only cause disease when the animals are stressed further, but bacterial septicaemia has been identified as the major cause of mortalities in shrimp culture, causing secondary disease in shrimp exposed to relatively long term stress and thus reduced immune defences (Nash, 1990).

Vibrio spp. constitute the majority of bacteria associated with the gut, gills and cuticles of wild and cultured shrimp (Lightner et al., 1992). *Vibrio* spp. have also been isolated in low numbers from the hemolymph of apparently healthy shrimp, probably introduced as a result of stress. This is often visible on the shrimp as dark localised lesions due to melanin produced by host haemocytes involved in the inflammatory process, necrosis of the appendage tips and rostrum, and tail rot (Boonyaratpalin, 1990).

Shrimp have been shown to possess a primitive immune system that relies mainly on phagocytosis, encapsulation, agglutination and the lysis activity of the haemocytes (Smith and Söderhäll, 1986). Central to any active cellular or humoral response to microbial or parasitic invasion is the initial recognition of foreignness by the host. Crustaceans accomplish this through a complex cascade of serine proteases and other factors in the haemocytes that are specifically triggered by foreign molecules. This is known as the prophenoloxidase system or pro PO system, and is confined to the semigranular and granular cells (Smith and Söderhäll, 1986). The pro PO activating cascade serves as the receptor for nonself signals, released from the surface of microorganisms or parasites (Söderhäll, 1982) and terminates in the conversion of proenzyme to activate phenoloxidase which is needed to synthesise bactericidal melanin. It is activated by minute amounts of β-1,3-glucans, lipopolysaccharides or spontaneously at low Ca^{2+} concentrations (Smith and Söderhäll, 1991).

The need for alternative methods for regulating the number of pathogenic bacteria but also the detrimental effects of viruses in aquaculture, has led researchers to turn to different methods of treatment such as probiotics and immunostimulation. Probiotics aim

at improving the intestinal microbial balance of the host animal with the objective of having beneficial microorganisms dominate the harmful bacteria that cause disease (Ewing and Haresign, 1989). Immunostimulants on the other hand aim at enhancing the nonspecific defence mechanisms in animals. A number of different biological and synthetic compounds have been found to enhance the nonspecific defence system in animals, including shrimp (Song and Sung, 1990; Sung et al., 1991). They have shown to increase the barrier of infection against a series of pathogens simultaneously, both specific and opportunistic ones (Raa et al., 1992). β-glucans, some of the most important structural components of fungal cell walls, have been successfully used to enhance the nonspecific defence system of a wide range of animals. In crustaceans, glucans have been shown to activate polyphenoloxidase in the hemolymph (Unestam and Söderhäll, 1977; Söderhäll et al., 1990). It is a β-1,3 linked glucose polymer with some β-1,6 linkages (Anonymous, 1986), found on the inner surface of the cell wall (Coutteau et al., 1990).

Baker's yeast, *Saccharomyces cerevisiae*, a single-celled eucaryotic fungi used primarily in the production of bread and beer, has been suggested for use in aquaculture because it is readily available in large quantities and has the capacity to adapt to high salt concentrations (Adler, 1994), making it suitable for mariculture. About 90% of the cell wall of *S. cerevisiae* consists of glucan, mannoproteins and chitin. Reports of enhanced survival and evidence of immunostimulation due to purified β-glucan, are more numerous than for yeast both for terrestrial and aquatic organisms (Itami et al., 1993) thus encouraging further research.

The present paper describes the results of a study, testing three different yeast products and one β-1,3 glucan, included in the diet, to determine if any of the products positively effect growth, survival, feed conversion ratio or the immune response of *Penaeus vannamei* juveniles.

2. Materials and methods

2.1. Experimental diets

An experimental basic diet was formulated, based on a protein content of 30% and taking the nutritional requirements of *P. vannamei*, as recommended by Akiyama et al. (1993), into account. The basic diet was prepared using the ingredients at the percentage outlined in Table 1.

All ingredients, except the fish oil, were mixed well, 270 ml water/kg of diet was added and pellets of 2 mm diameter were produced by passing the mixed product through a meat grinder at 70°–76°C. After drying in the sun for 8 h, the different yeast and glucan products to be tested were mixed with the fish oil and applied to the diets as a 'top-dressing' with a brush and the diets dried again. Five different treatments were prepared: (1) basic diet with fish oil only (control), (2) basic diet + *S. cerevisiae*-Procreatin 7 (Safmex, Toluca, Mexico) at 1%, (3) basic diet + experimental live yeast consisting of *Saccharomyces exiguus* containing the pigment xeaxanthin (HPPR1) at 1%, (4) basic diet + *Phaffia rhodozyma* (Merck) at 1% and (5) basic diet + β-1,3 glucan extracted from *S. cerevisiae* at LeSaffe, France, included at 0.1%.

Table 1
Composition of the basic diet (as percentage dry weight)

Ingredient	Percentage
Fish meal	17.00
Antarctic krill meal	3.00
Wheat meal	56.18
Soy paste	7.57
Wheat gluten	8.00
Sodium monophosphate	1.79
Choline	0.03
Inositol	0.08
Vitamin C	0.03
Vitamin pre-mix	0.02
L-Methionine	0.02
Cholesterol	0.17
Soy lecithin	3.29
Menhaden fish oil	2.70

The β-1,3 glucan was prepared by rupturing the cells of *S. cerevisiae* with enzymes, separating the soluble from the insoluble fraction, washing in ethanol, acidification and drying. The resulting extract consisted of 48.2% total carbohydrates, 26.5% glucans at a ratio of 90:10 β-1,3 glucan and β-1,6 glucan, and other components (Auclair, personal communication, 1997, Marcq-En-Barœul Cedex, France).

All diets were left to dry overnight on trays and stored in sealed, plastic containers at 4°C.

2.2. Feeding trial

P. vannamei juveniles were obtained from a hatchery in La Paz, Mexico and kept in an acclimation tank of the closed recirculating, artificial seawater system of the Programa Maricultura, Faculty of Biological Sciences, Universidad Autonoma de Nuevo Leon (UANL), Mexico, prior to the experiment. Water quality parameters remained constant throughout the acclimation and feeding trial period: temperature, 28°C; salinity, 3.3%; dissolved oxygen, 6 mg/l; NH_3, 0.01 mg/l; NO^{3-}, 15 mg/l; NO^{2-}, 0.49 mg/l; pH, 8.2.

Ten animals each were stocked to 60 L fiber glass tanks (initial weight: 450 mg) with four to eight replicate tanks per treatment in a random block design. The animals were fed ad libitum, once daily at 1700 h throughout the 7-week feeding trial. Feed consumption and survival was estimated visually each morning by the amount of uneaten food left in each tank and the number of animals present in each tank, respectively. Wet body weight of all animals was determined weekly by removing excess water with a cloth and weighing the individual animals on a digital balance. Feed conversion ratio (FCR) was calculated as:

$$\mathrm{FCR} = \frac{\text{total amount of feed consumed per individual animal over time } X \text{ in grams}}{\text{average increase in weight of individuals over time } X \text{ in grams}}$$

2.3. Phenoloxidase activity

All shrimp from the five different treatments remaining at the end of the feeding trial were transported live to the Centro de Investigacion en Alimentacion y Desarollo (CIAD), Hermosillo, Mexico. Twenty microliters of hemolymph were drawn from the ventral sinus via the pleopod base of the first abdominal segment from each shrimp with micropipettes and rinsed into microwells, containing 50 μl of anticoagulant solution (450 mM NaCl, 10 mM KCl, 10 mM EDTA · Na_2, 10 mM HEPES, pH 7.3, 850 mosM/kg). To determine the exact amount of hemolymph drawn, the microwells were weighed before and after the addition of hemolymph.

2.3.1. Protein determination

Total protein in the hemolymph was determined according to Bradford (1976) using bovine serum albumin (BSA) as standard.

2.3.2. Determination of phenoloxidase activity

Trypsin solution (55.6 μl; 90 μg/ml) was added to each microwell and left to react at 28°C for 10 min. L-DOPA solution (64.6 μl; 2.32 μg/ml) was then added and left to react for exactly 10 min at 28°C, after which 850 μl of PBS buffer (10 mM Na_2HPO_4, 1.7 mM KH_2PO_4, 137 mM NaCl, 2.7 mM KCl, pH 7.2) was added and absorbance read at 490 nm. The results were calculated as ΔAbs min^{-1} mg $protein^{-1}$ and were expressed in units. One unit equals a change in absorbance of 0.001 min^{-1} mg $protein^{-1}$.

2.4. Bacterial clearance

At the end of the 7-week feeding trial, three replicates of 10 animals each from each treatment were transferred to 10-l fiberglass tanks containing 5 l of seawater. A further three tanks with 10 animals each was set up as untreated control. Each tank except for the untreated control, was inoculated with a viable cell suspension of 10^7 CFU/ml of a pathogenic *Vibrio harveyi* (strain BP05), obtained from INVE Technologies, Belgium. Bacterial clearance from the haemolymph of shrimp was determined by taking 10-μl samples of haemolymph from the ventral sinus via the pleopod base of the first abdominal segment from three to six shrimp per treatment with micropipettes, 3, 6, 9, 15, and 27 h post immersion. The hemolymph was immediately transferred to TCBS agar plates and colony-forming units were counted after 24 h incubation at 26°C. The results were analysed by pooling the data of each treatment.

2.5. Statistical analysis

To determine if significant differences exist between the different treatments and the parameters tested, all results were analysed using one-way analysis of variance (ANOVA) and Duncan's multiple comparison of the means. Significant differences were indicated at $P < 0.05$.

3. Results and discussion

3.1. Weight, survival and feed conversion ratio

The results of the feeding trial are presented in Figs. 1–4. Fig. 1 shows the mean weight of the animals throughout the 7-week feeding trial. Statistical analysis displayed no significant difference ($P = 0.196$) in the weight of the animals after 7 weeks on the different treatments. Total biomass on the other hand, shown in Fig. 3, was significantly affected ($P = 0.043$), the animals on the *P. rhodozyma* treatment displaying higher biomass after 7 weeks of the feeding trial than the shrimp on the β-glucan treatment but not when compared to the control or the other two treatments. No significant difference ($P = 0.233$) was found between any of the diets in terms of feed conversion ratio. The results are presented in Fig. 4.

Survival (Fig. 2) was significantly affected ($P = 0.006$). *P. vannamei*, fed the diets containing *S. cerevisiae*, *P. rhodozyma* and the experimental yeast, had significantly higher survival (71.25%, 77.5% and 76.7%, respectively) than the shrimp fed the diet containing β-glucan (47.5%), but not than the animals fed the control diet (56.25%). β-1,3 glucan, found in the cell walls of bacteria and fungi, has been shown to trigger melanisation of the haemolymph by activating prophenoloxidase within the haemocytes of crustaceans (Söderäll, 1981), serving as signals for the presence of potentially pathogenic bacteria and fungi (Söderäll et al., 1985) which then results in a highly specific reaction. Fungal and bacterial β-1,3 glucan binds to the glucan-binding protein in the plasma, thus converting the prophenoloxidase system to its active form. The

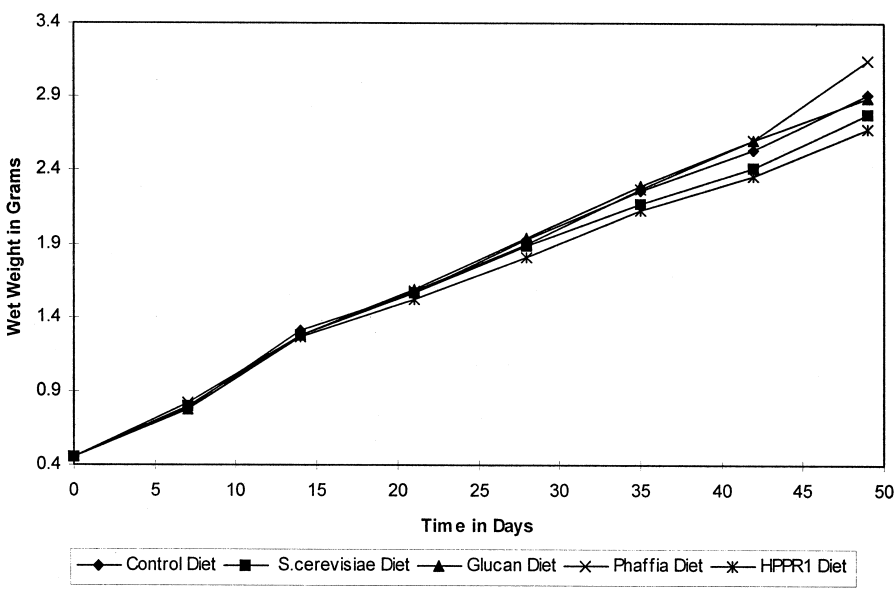

Fig. 1. Mean weight of *P. vannamei* juveniles throughout the 7-week feeding trial.

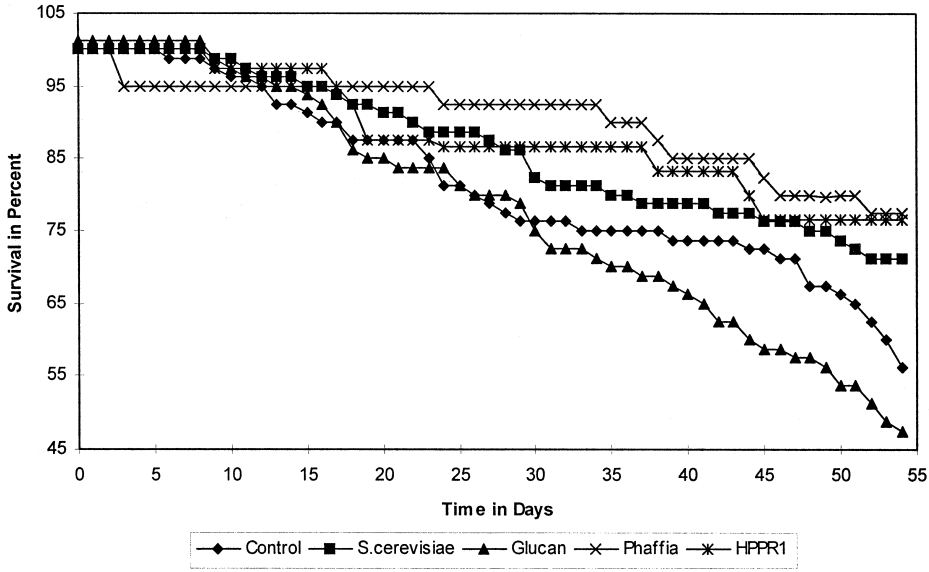

Fig. 2. Survival of *P. vannamei* throughout the 7-week feeding trial.

glucan-binding protein can also act as an opsonin, stimulating phagocytic uptake of yeast particles by isolated blood cells (Cerenius et al., 1994). β-1,3 glucans have been used successfully as immunostimulants for enhancing the nonspecific defence systems

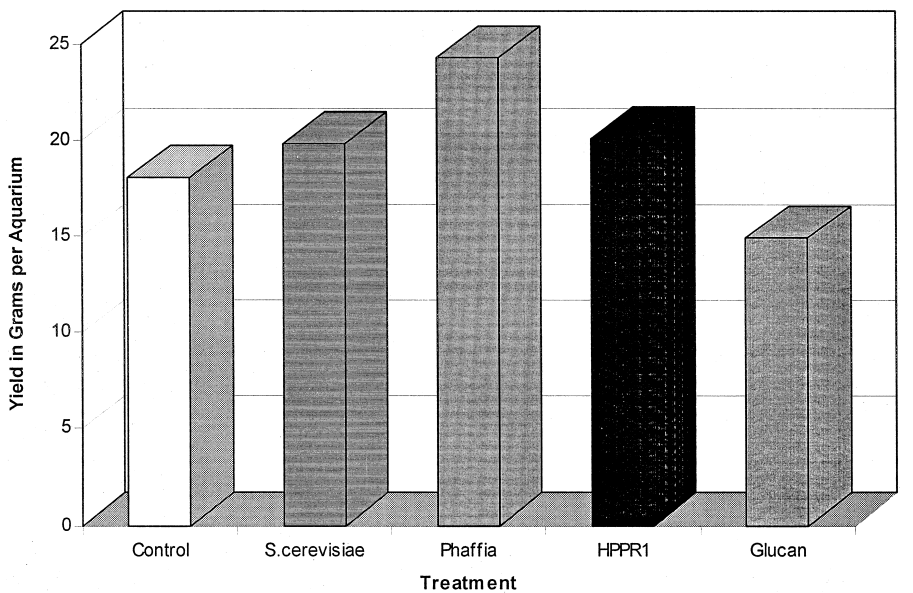

Fig. 3. Mean final biomass of *P. vannamei* after the 7-week feeding trial.

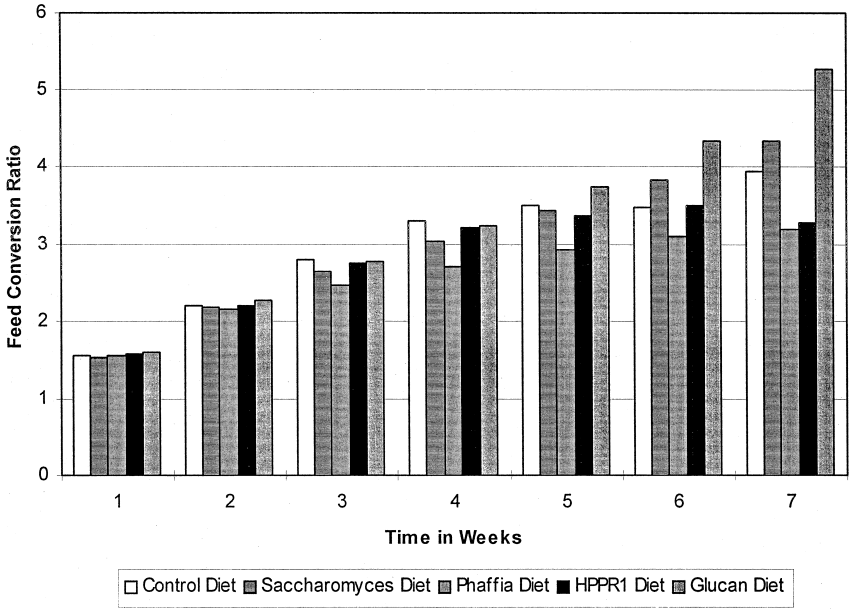

Fig. 4. Feed conversion ratio of *P. vannamei* fed on five different diets.

of a wide range of animals such as swine and including shrimp. Doses as low as 10^{-7} g/crab have been found to effectively diminish the cell count in *Carcinus maenas* (Smith et al., 1984). Sung et al. (1994) studied the effects of β-glucan extracted from *S. cerevisiae* on *P. monodon* postlarvae by immersion and concluded that concentrations of 0.5 and 1 mg/ml, gave short-term protection lasting 18 days when challenged with *V. vulnificus*.

Robertsen et al. (1990) showed that, although the use of β-glucan from *S. cerevisiae* cell walls enhanced resistance of *Salmo salar* against the causative agent of enteric redmouth disease and vibriosis, the method of β-glucan preparation was of paramount importance in achieving this effect and that the dissimilar performance of the different glucans may be due to differing glycosidic linkages. Matsuo and Miyazono (1993) demonstrated that at 56 days, the feeding of peptidoglycan, a type of β-glucan, to rainbow trout, resulted in lower levels of protection than at 28 days. It appears that compounds like β-glucan, designed as immunostimmulants, do not follow a linear dose/response relationship (Bliznakov and Adler, 1972, cited in Raa, 1996) but that a distinct maximum can often be observed at an intermediate concentration with higher concentrations often giving an absence of the effect or even toxicity (Floch et al., 1987, cited in Raa, 1996).

It is possible that the negative effect on survival of *P. vannamei*, found for β-glucan in this study, could be due to the dose administered, especially when taking into account that the control diet gave relatively better survival, though not at a significant level. These findings suggest that there is need for caution when administering β-glucan long-term and/or in high doses.

The *S. cerevisiae, P. rhodozyma* and experimental yeast diets on the other hand gave good performance with survival between 71% and 76% when compared to the control and more so when compared to the glucan treatment. Dehasque et al. (1995a) report that Atlantic salmon fry, fed a diet containing baker's yeast at 2%, displayed better survival than control animals when challenged with *V. anguillarum*, while juvenile European seabass showed similar results when fed a diet containing experimental treated yeast at 1% (Dehasque et al., 1995b). Dehasque et al. (1995a) further found that brewer's yeast, chemically treated to enhance its digestibility and to increase access to the glucans on the surface of the yeast, gave better survival than fresh yeast. It may therefore be possible to further enhance the performance of the yeasts tested through chemical treatment.

3.2. Phenoloxidase activity

The results for phenoloxidase activity, found for *P. vannamei*, fed the five different treatments, are shown in Fig. 5. Great variation was found for phenoloxidase activity of the animals on all diets except the animals fed the *Phaffia* diet which had the lowest and most uniform values overall, with significantly lower values ($P = 0.003$) than the control diet, the *S. cerevisiae* diet and the experimental yeast diet but not the β-glucan diet. Values for the β-glucan diet though are generally also higher than for the *Phaffia* diet though not at a statistically significant level, mainly due to the great variability of the individual readings.

3.3. Bacterial clearance

Fig. 6 shows the results of bacterial clearance from the hemolymph of the shrimp after a challenge with a pathogenic *V. harveyi* strain BP05. Results are variable and

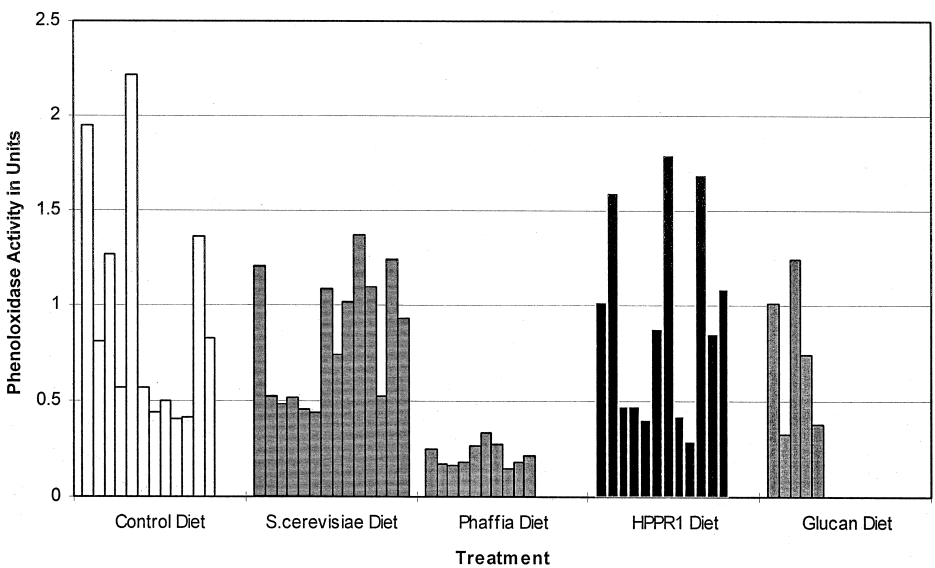

Fig. 5. Phenoloxidase activity of whole hemolymph of *P. vannamei*.

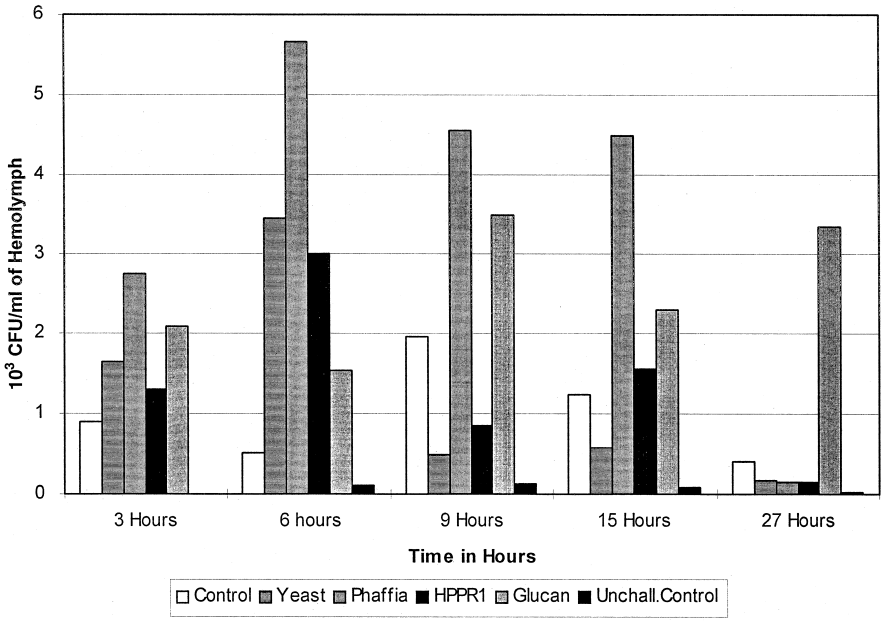

Fig. 6. Bacterial clearance of *V. harveyi* from the hemolymph of *P. vannamei*.

differ between individual animals of the same treatment but there is a downward trend to reduced numbers over time for all treatments, down to the base level of 0 to 10^2 CFU/ml hemolymph, detected for the nonchallenged control shrimp. Fifteen hours post-immersion, no significant difference ($P = 0.346$) was detected for any of the diets, when compared to the animals on the control diet or the unchallenged control animals. Statistical analysis of the data was not possible 27 h post-immersion due to several mortalities of the animals on the β-glucan treatment, leaving only two animals from this treatment for sampling. It is interesting to note though that the animals on the *Phaffia* diet displayed higher numbers of *V. harveyi* in the hemolymph throughout, until 27 h post-immersion where the numbers were back to the base level detected for nonchallenged shrimp, while the animals on the glucan diet did not seem to follow the general trend to reduced numbers over time. It thus appears that the animals of the β-glucan treatment were less effective in clearing *V. harveyi* from the hemolymph, as can also be seen in that mortalities were only recorded for animals of this treatment.

4. Conclusions

It can be concluded that *P. rhodozyma*, included at 1% in the diet, had a positive effect on weight, final biomass and survival of juvenile *P. vannamei*. Phenoloxidase activity, which was expected to be highest for these animals, was found to give the lowest activity which was also the most uniform. The animals tested for phenoloxidase activity, were not challenged with a pathogen prior to sampling and treatment was last

administered 20 h before taking hemolymph samples. This could indicate that with *Phaffia*, activation of the immune system is a quick response which could already have been back to a base level at the time of sampling (Raa, 1996), thus positively strengthening shrimp against potential pathogens. The overall positive effect of *P. rhodozyma* might be attributed to the presence of carotenoid pigment. *P. rhodozyma* is a yeast that can produce > 3000 μg total carotenoid in cultured strains (Johnson and An, 1991). In recent years, carotenoid pigments are receiving increased attention in shrimp nutrition and evidence is becoming stronger supporting the vital role of carotenoid pigments in crustaceans and indicating that they can no longer be considered only as color-giving pigments (Menasveta et al., 1993) playing an important role in the physiology and overall health of animals (Torrissen, 1990). In fact, it has been proposed to classify carotenoids as one of the fat-soluble vitamins. Glucan on the other hand, gave reduced survival and the animals on this diet were observed to be much more prone to stress (mortalities when handling, etc.) than all other animals. *S. cerevisiae* gave increased survival and there does appear to be an immunostimulatory effect, but this is not as pronounced as with the shrimp of the *Phaffia* treatment. The experimental yeast also gave variable results. Survival was increased but growth and biomass were reduced though not significantly.

The increased performance of the yeasts tested, especially *P. rhodozyma*, gives scope for the potential use as a probiont/immunostimulant but studies on dosage, but also different types of treated yeast are necessary, while for the use of β-glucan, more studies are needed to understand the effects and mode of action of these potential immunostimulants.

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