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Immunostimulatory nature of β-glucans and baker's yeast in gnotobiotic *Artemia* challenge tests

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Abstract

The use of new preventive approaches such as immunostimulants to reduce stress and mortalities, to maintain good health of cultured organisms and to stimulate the non-specific defence mechanism, is becoming increasingly important in aquaculture. Yet detailed analysis reveals that in most experiments the validity of some conclusions with respect to the benefit of immunostimulation is still doubtful, especially in invertebrates. The use of standardized trials under controlled rearing conditions, complemented with fundamental research on defence mechanisms can provide unequivocal evidence for the beneficial effects of immunostimulants in reducing invertebrate susceptibility to diseases or infections. This study investigated the use of small amounts of baker's yeast *Saccharomyces cerevisiae* and glucan particles (obtained from baker's yeast) in gnotobiotic *Artemia* to overcome the pathogenicity of two organisms: *Vibrio campbellii* and *V proteolyticus*. *Artemia* supplemented with small quantities of a yeast strain presenting higher concentrations of β-glucans or with glucan particles seemed to completely resist the detrimental effects of both pathogens. The higher amount and/or availability of β-glucans in that yeast might play an essential role in such protection, as most probably glucans stimulate the immune response of the nauplii.

Keywords: *Artemia*; Gnotobiotic culture; *Saccharomyces cerevisiae*; β-glucans; Immune ability; *Vibrio campbellii*; *Vibrio proteolyticus*

1. Introduction

Diseases are still a major constraint to sustainable aquaculture production, especially for the farming of invertebrates [1]. In intensive systems, fish and shellfish species are often exposed to stressful conditions, eventually becoming more susceptible to microbial infections, especially in their larval stages [2]. In order to protect larvae, it is common practice to disinfect the water before use and to apply chemotherapy (e.g. antibiotics) [3]. Yet such practices are undesirable since they promote the selection and dissemination of antibiotic-resistant bacteria in both the target-organisms, as well as in the environment [2]. Nowadays, the use of preventive approaches, essential for further development of more sustainable aquaculture practices, are becoming increasingly important, e.g. vaccines, immunostimulants and probiotics.

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Contrary to the vertebrates, which have both an innate and a specific immune system, the defence mechanism in invertebrates depends mainly on a non-specific or innate immune response to fight infectious diseases [4]. The invertebrate innate immune system presumably recognizes a wide diversity of pathogens, represented by common molecular patterns (e.g. lipopolysaccharides or peptidoglycans from bacteria, or β-glucans from bacteria and fungi) rather than structures specific for particular microbes [5]. In cases where disease outbreaks are cyclic and can be predicted, immunostimulants may be used to elevate the non-specific defence mechanisms, to reduce stress and mortalities, and to maintain the health of cultured organisms [6]. Although the exact mechanism is still not yet completely understood, several immunostimulants are being applied in vertebrate and invertebrate cultures, to induce and build up protection against a wide range of diseases: e.g. β-glucans [7–10], chitin [11], mannoproteins [12], lipopolysaccharides [13], peptidoglycans [14,15] and dead bacteria [16–18]. Nevertheless, rigorous analysis of the results obtained in most experiments reveals that the validity of some conclusions with respect to the benefit of immunostimulation is limited, due mainly to poor experimental design, to the absence of any statistical analysis and to the poor reproducibility of results [2]. Therefore, these authors argued that there is an urgent need to provide unequivocal evidence of the beneficial effects of immunostimulants in reducing invertebrate susceptibility to disease or infection using standardized trials under controlled rearing conditions, complemented with fundamental research on defence mechanisms. These trials could be performed with gnotobiotically-cultured invertebrates (animals cultured in a totally controlled microbial environment).

The brine shrimp *Artemia* has been recently shown to be instrumental in the development of a gnotobiotic test system for studying the effect of food composition on survival and growth in the presence or absence of a challenge test [19–21]. *Artemia* is a crucial part of the live food chain in the farming of fish and shellfish larvae [22] and is also widely used in hatcheries to deliver immunostimulants to larvae (using the bioencapsulation technique, whereby specific compounds are incorporated in live prey organisms) [23]. Since *Artemia* can take up a wide variety of feed particles as it is a non-selective particle filter feeder able to ingest a maximum particle size of 25–30 μm for nauplii and 50 μm for adults [24,25], axenically grown baker's yeast can be offered as feed. The Baker's yeast *Saccharomyces cerevisiae* is an excellent source of β-glucans and chitin, which are mainly present in the yeast cell wall as major compounds together with mannoproteins [26].

The present study investigates the use of Baker's yeast and glucan particles in gnotobiotic *Artemia* to overcome the virulence of two pathogenic bacterial strains. For that purpose, commercially available particles of glucans obtained from *S. cerevisiae* and intact cells of two strains of live Baker's yeast were offered as feed to gnotobiotic *Artemia*.

2. Materials and methods

2.1. Axenic cultures of yeast

Two different strains of Baker's yeast *Saccharomyces cerevisiae* were provided to *Artemia*: the wild type strain (WT) (BY4741, genotype *Mat a*; *his3Δ1*; *leu2Δ0*; *met15Δ0*; *ura3Δ0*) and its mnn9 isogenic mutant (BY4741; genotype *Mat a*; *his3Δ1*; *leu2Δ0*; *met15Δ0*; *ura3Δ0*; YPL050c::kanMX4), which has a null mutation resulting in a lower concentration of mannose linked to mannoproteins, and higher concentrations of chitin and glucans in the cell wall [19,26]. Both strains were provided by the European *Saccharomyces cerevisiae* Archive for Functional Analysis, University of Frankfurt, Germany. The procedures used in the present study to culture both yeasts were identical to the methods described by Marques et al. [19]. A complete Yeast Extract Peptone Dextrose medium was used to culture the WT yeast, while a minimal Yeast Nitrogen Based medium was used to culture the mnn9 yeast. The WT strain was harvested by centrifugation ($\pm 800 \times g$ for 10 min) in the stationary growth phase [using a spectrophotometer to measure the optical density (OD) of the yeast culture at a wavelength of 600 nm after 3 days of culture; OD_{600}: ±10.600 for WT], while mnn9 yeast was harvested in the exponential growth phase (after 20 h of culture; OD_{600}: ±0.700). All handlings were performed in a laminar flow hood to maintain sterility. Yeasts were resuspended in filtered and autoclaved seawater (FASW, 0.2 μm) and their densities were determined by measuring twice the cell concentration, using a Bürker haemocytometer. Suspensions were stored at 4 °C and provided to *Artemia* until the end of each experiment. The following 2 feeds were chosen because of the protection they provide against pathogenic bacteria: no protection (WT yeast) and protection (mnn9 yeast) [21].

2.2. Bacterial strains and growth conditions

Three bacterial strains were examined: strain LVS 3 (*Aeromonas hydrophila*), *Vibrio campbellii* strain LMG21363 (VC) and *Vibrio proteolyticus* strain CW8T2 (VP). LVS 3 was selected because of its positive effect on growth and survival in sub-optimally fed *Artemia* [21,27,28], while the other two bacterial strains were previously characterized as pathogenic bacteria for *Artemia* [21,27–30]. Pure cultures of the 3 bacterial strains were obtained from the Laboratory of Microbial Ecology and Technology, Ghent University, and from the Laboratory of Microbiology, Ghent University. The bacterial strains were stored at −80 °C and grown overnight at 28 °C on marine agar, containing Difco™ marine broth 2216 (37.4 g/l, BD Biosciences) and agar bacteriological grade (20 g/l, ICN). For each bacterial strain a single colony was selected from the plate and incubated overnight at 28 °C in Difco™ marine broth 2216 on a shaker (150 rpm). Bacteria were harvested by centrifugation (15 min; ±2200 × *g*), the supernatant was discarded and the pellet resuspended in 20 ml FASW. Bacterial densities were determined by spectrophotometry (OD_{550}), assuming that an optical density of 1.000 corresponds to 1.2×10^9 cells/ml, according to the McFarland standard (BioMerieux, Marcy L'Etoile, France). Dead LVS 3 was fed to *Artemia* using aliquots of autoclaved bacteria (autoclaving at 120 °C for 20 min). In order to check if the bacteria were effectively killed by autoclaving, LVS 3 was plated after being exposed to this method. For this purpose, 100 µl of the culture medium were transferred to marine agar ($n = 3$), containing Difco™ marine broth 2216 (BD Biosciences, 3.74% w/v) and bacteriological grade agar (ICN, 2% w/v). Absence of bacterial growth was monitored after incubating the plates for 5 days at 28 °C. The autoclaving treatment was 100% effective, since no bacterial growth was observed on the marine agar after 5 days of incubation. Dead and live bacterial suspensions were stored at 4 °C until the end of each experiment.

2.3. Artemia gnotobiotic culture

Experiments were performed with *Artemia franciscana* (Kellog) cysts, originating from Great Salt Lake, Utah, USA (EG® Type, INVE Aquaculture NV, Belgium). Bacteria-free cysts and nauplii were obtained *via* decapsulation according to the procedure described by Sorgeloos et al. [25]. During decapsulation 0.22-µm filtered aeration was provided. All manipulations were carried out under a laminar flow hood and all necessary tools were previously autoclaved at 120 °C for 20 min. Decapsulated cysts were washed carefully with FASW over a 50 µm pore size sterile net and transferred to a sterile 50 ml screw cap Falcon tube (TRP®, γ-irradiated) containing 30 ml of FASW. The tube was capped and placed on a rotator at 4 cycles per min, exposed to constant incandescent light (±41 μEm^{-2}) at 28 °C for 18–20 h. Twenty axenic nauplii (Instar II) were picked and transferred to sterile 50 ml tubes containing 30 ml of FASW, together with the amount of feed scheduled for day 1 (see further for feeding schedule). Each treatment was set up in 4 replicates. Falcon tubes were placed on a rotator at 4 cycles per min, exposed to constant incandescent light (± 41 μEm^{-2}) at 28 °C, being transferred to the laminar flow just once per day for feed addition. The daily feeding schedule was adapted from Coutteau et al. [31] and Marques et al. [19], and was intended to provide *ad libitum* ratios, avoiding excessive feeding, in order not to affect the water quality in the test tubes. The total amount of dead LVS 3 provided to *Artemia* was approximately 3.7×10^9 cells/tube (added daily using the same fraction as of yeast − 9:18:18:24:36), corresponding to an ash free dry weight (AFDW) (1.06 ± 0.06 mg/tube) normally provided to nauplii fed with WT yeast cells [19–21]. The total AFDW of yeast and bacteria added to *Artemia* in experiments 1–3 is presented in Table 1. The AFDW of yeast and bacteria were calculated according to the procedures described by Marques et al. [21].

Table 1

Average total ash free dry weight (AFDW), expressed in mg/Falcon tube (mg/FT), of yeast (WT and mnn9), dead and live bacteria (LVS 3, VC and VP), and glucans supplied to *Artemia* in experiments 1–3 ($n = 2$)

Experiment 1–2	AFDW (µg/FT)	Experiment 3	AFDW (µg/FT)
dead LVS 3	1057.95 ± 59.63	WT	1057.95 ± 84.76
WT 10%	105.79 ± 8.48	dead LVS 3	1057.95 ± 59.63
WT 1%	10.58 ± 0.85	live VC D3	37.98 ± 0.14
mnn9 10%	260.18 ± 7.90	live VP D3	36.02 ± 0.54
mnn9 1%	26.02 ± 0.79	–	–
live VC D3	37.98 ± 0.14	–	–
live VP D3	36.02 ± 0.54	–	–

D3 means that products were added on day 3. Values of AFDW are presented with the respective standard deviation (mean ± SD).

2.4. Methods used to verify axenity

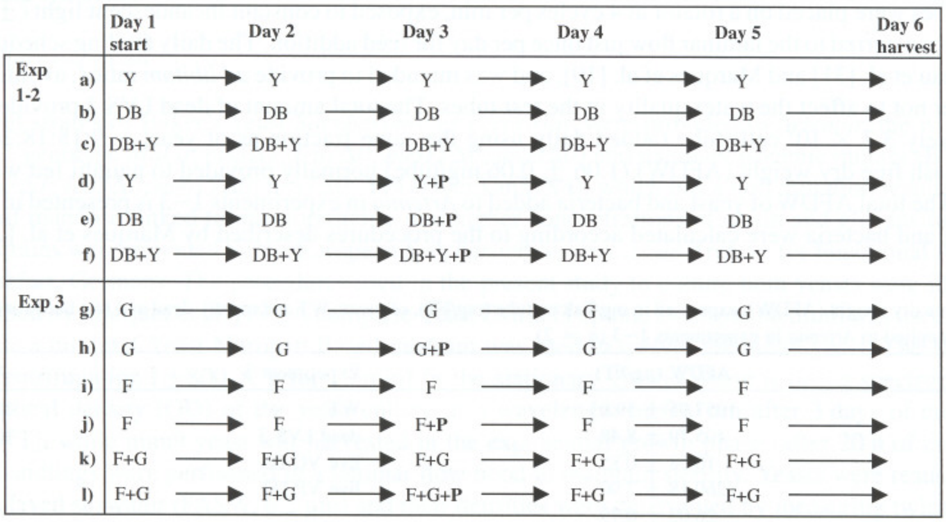
Axenity of feed, decapsulated cysts and *Artemia* cultures was checked at the end of each experiment using a combination of plating (marine agar) and live counting (using tetrazolium salt MTT live staining), following the procedures described by Marques et al. [19–21]. Absence of bacteria was monitored by transferring 100 μl of culture medium to Petri plates with marine agar 2216 ($n = 2$). Plates were incubated for 5 days at 28 °C. As for live counting, each sample was stained with tetrazolium salt MTT (-3-(4,5-dimethylthazol-2-yl)-2,5-diphenyl tetrazolium bromide) (Sigma, 0.5% w/v) in a sterile recipient (1 part of MTT to 9 parts of sample) and incubated at 30 °C for 30 min. Under a light microscope (1000× magnification), live bacterial detection was performed. The MTT blue stains all viable/living cells remaining in a culture [32], making the detection of bacterial contaminations in a culture medium easier. Contaminated culture tubes were not considered for further analysis and the treatment was repeated.

2.5. Glucan particles

Pure insoluble glucan particles obtained from the Baker's yeast *Saccharomyces cerevisiae* (Sigma, 20 mg) were aseptically transferred to sterile 50 ml Falcon tubes and homogeneously suspended in FASW. Contaminations were checked by plating the suspension on marine agar (100 μl, $n = 2$). Absence of bacterial growth was monitored after incubating the plates for 5 days at 28 °C. No bacterial growth was detected on marine agar after the incubation period. An optical laser particle size analyser (Mastersizer MSX-17, Malvern Instruments Ltd., Malvern, Works, UK; resolution 0.05–900 μm) was used to determine the diameter of the glucan particles present in the suspension, using the software Malvern Mastersizer S version 2.19® (Malvern Instruments Ltd., Malvern, Works, UK). Once the volume percentage of particles sizing less than 50 μm (maximum particle size that can be ingested by *Artemia*) was calculated, the total amount of glucans needed per tube was established, according to previous results obtained by Marques et al. [19,21]. These authors determined the amount of glucans present in the cell wall of the WT yeast cultured in the complete yeast extract peptone dextrose medium and harvested in the stationary growth phase (±128.33 μg/tube) and noticed the lack of *Artemia* protection against pathogenic bacteria when fed with this yeast. The suspension of glucans was stored at 4 °C until the end of the experiment being provided daily in equal portions per day to *Artemia* (26 μg/tube/day).

2.6. Experimental design

This study comprises 3 experiments and their experimental design is schematized in Fig. 1. In Experiments 1 and 2, respectively WT or mnn9 yeast were daily provided to *Artemia* in small amounts. As a result, *Artemia* were fed either



		Day 1 start	Day 2	Day 3	Day 4	Day 5	Day 6 harvest
Exp 1-2	a)	Y →	Y →	Y →	Y →	Y →	
	b)	DB →	DB →	DB →	DB →	DB →	
	c)	DB+Y →	DB+Y →	DB+Y →	DB+Y →	DB+Y →	
	d)	Y →	Y →	Y+P →	Y →	Y →	
	e)	DB →	DB →	DB+P →	DB →	DB →	
	f)	DB+Y →	DB+Y →	DB+Y+P →	DB+Y →	DB+Y →	
Exp 3	g)	G →	G →	G →	G →	G →	
	h)	G →	G →	G+P →	G →	G →	
	i)	F →	F →	F →	F →	F →	
	j)	F →	F →	F+P →	F →	F →	
	k)	F+G →	F+G →	F+G →	F+G →	F+G →	
	l)	F+G →	F+G →	F+G+P →	F+G →	F+G →	

Fig. 1. Experimental design of the 3 experiments (Exp) performed. Legend: a)–l) correspond to the treatments performed; Y – yeast (wild type or mnn9 yeast added at 1% or 10%); DB – dead bacterium LVS 3; P – pathogen (*Vibrio campbellii* or *V. proteolyticus*); G – glucan particles; F – feed provided (WT yeast, dead LVS 3 or a mixture of WT yeast and dead LVS 3).

with dead LVS 3 or with a combination of dead LVS 3 and yeast cells (1 or 10% of the totally offered AFDW, divided in equal parts per day, to avoid the possibility that those yeast cells could be used as major feed sources by *Artemia*). As a control, *Artemia* was only fed with yeast (1 or 10% of the normally offered total AFDW). At day 3, challenge tests were performed with VC or VP. For that purpose, in a laminar flow hood, the pathogen was provided to each replicate at a density of approximately 5×10^6 cells/ml. These experiments were repeated to verify the reproducibility of the results.

In experiment 3, glucan particles were daily added to *Artemia* using the poor-performing WT yeast, dead LVS 3 or a combination of WT and dead LVS 3 (WT + LVS3) as major feed sources. In these treatments, *Artemia* were fed either with the major feed sources or with a combination of the major feed sources with glucans particles (provided in equal parts per day, to avoid the possibility that those glucans could be used as a major feed source by *Artemia*). Challenge tests were performed with VC or VP added on day 3. As a control *Artemia* was only fed with glucan particles (provided in equal parts per day) and challenged (or not) with the pathogens. This experiment was performed twice to verify the reproducibility of the results.

In each experiment, *Artemia* performance in the control treatments was compared to results previously obtained by Marques et al. [19–21], in order to evaluate the reproducibility. If significant differences were detected, data were rejected and all the treatments linked with that specific feed combination were repeated. Yeasts or glucans were considered to protect the *Artemia* nauplii against a specific pathogen when their use significantly improved *Artemia* performance in comparison to challenged nauplii not supplied with the respective compound.

2.7. Survival and growth of Artemia

The survival percentage was determined daily for each treatment. For this purpose, the number of live *Artemia* was registered before feeding or adding any bacteria by exposing each transparent Falcon tube to an incandescent light without opening the tube to maintain the gnotobiotic environment. At the end of each experiment (day 6 after hatching), live *Artemia* were fixed with Lugol's solution to measure their individual length (IL), using a dissecting microscope equipped with a drawing mirror, a digital plan measure and the software Artemia 1.0® (courtesy Marnix Van Damme). As a criterion that combines both the effects of survival and IL, the total biomass production (TBP; expressed in mm per Falcon tube) was determined according to the following equation: TBP = number of survivors × mean IL.

2.8. Statistics

The percentages of larval survival were arcsine transformed, while values of IL and TBP were logarithmic or square root transformed, whenever necessary, to satisfy normal distribution and homocedasticity requirements. Differences in survival, IL and TBP of *Artemia* cultured in different conditions were investigated with analysis of variance (ANOVA) and Tukey's multiple comparison range. All statistical analyses were tested at 0.05 level of probability.

3. Results

3.1. Small amounts of yeast

In a first challenge test with VC or VP, the effects of supplying low amounts of WT or mnn9 yeasts to nauplii fed with dead LVS 3 as the major feed source were studied. Results presented in Table 2 (Exp 1) indicate that non-challenged nauplii fed solely with dead LVS 3 (treatments b) or with a mixture of dead LVS 3 and WT yeast (1% or 10% – treatments c) survived until day 6 with low TBP. Yet, the addition of 1% and 10% (treatments c) of mnn9 to non-challenged nauplii fed with dead LVS 3 (Table 3 – Exp 2) significantly improved TBP in comparison to nauplii fed solely with dead LVS 3 (treatments b), due to both improved survival and IL.

Even providing only 10% of the normal amount of AFDW (of mnn9 yeast) in the absence of bacteria (Table 3 – treatment a) was still enough to keep 65% of the nauplii alive until day 6 with a relatively high TBP, while nauplii only supplied with 1% of the normal amount of AFDW of this yeast died on day 5 (treatment a). Yet, in terms of AFDW, the amounts of mnn9 yeast provided to *Artemia* were relatively low (Table 1).

Table 2
Experiment 1 – Mean daily survival (%), individual length (IL) (mm) and total biomass production (TBP) (mm per Falcon tube – FT) of *Artemia* fed daily with various feed combinations

T	Experiment 1	Survival (%)					IL (mm)	TBP (mm/FT)
		Day 2	Day 3	Day 4	Day 5	Day 6		
a)	WT 10%	26 ± 12^{d}	20 ± 8^{c}	8 ± 5^{c}	0^{c}	0^{c}	–	0.00^{c}
b)	dead LVS3	66 ± 9^{ab}	55 ± 17^{ab}	33 ± 17^{ab}	14 ± 6^{b}	8 ± 3^{b}	1.36 ± 0.18^{a}	2.04 ± 0.79^{b}
c)	dead LVS3 + WT 10%	70 ± 30^{abc}	50 ± 14^{ab}	14 ± 18^{bcd}	6 ± 13^{bc}	6 ± 13^{abc}	1.31 ± 0.18^{a}	1.64 ± 3.28^{bc}
d)	WT 10% + VC D3	34 ± 13^{cd}	24 ± 6^{c}	0^{d}	0^{c}	0^{c}	–	0.00^{c}
e)	dead LVS3 + VC D3	70 ± 15^{ab}	64 ± 17^{a}	3 ± 5^{cd}	0^{c}	0^{c}	–	0.00^{c}
f)	dead LVS3 + WT 10% + VC D3	64 ± 9^{ab}	58 ± 12^{a}	9 ± 8^{bc}	0^{c}	0^{c}	–	0.00^{c}
d)	WT 10% + VP D3	29 ± 18^{cd}	23 ± 17^{bc}	3 ± 5^{cd}	1 ± 3^{c}	1 ± 3^{c}	1.50^{a}	0.38 ± 0.75^{c}
e)	dead LVS3 + VP D3	79 ± 11^{a}	61 ± 8^{a}	16 ± 10^{bc}	0^{c}	0^{c}	–	0.00^{c}
f)	dead LVS3 + WT 10% + VP D3	59 ± 6^{b}	56 ± 8^{a}	54 ± 8^{a}	45 ± 13^{a}	36 ± 18^{a}	1.68 ± 0.33^{a}	12.18 ± 6.04^{a}
a)	WT 1%	34 ± 25^{bc}	26 ± 17^{bcd}	0^{d}	0^{c}	0^{c}	–	0.00^{c}
b)	dead LVS3	66 ± 9^{ab}	55 ± 17^{ab}	33 ± 17^{ab}	14 ± 6^{a}	8 ± 3^{a}	1.36 ± 0.18^{b}	2.04 ± 0.79^{a}
c)	dead LVS3 + WT 1%	73 ± 9^{a}	58 ± 15^{a}	45 ± 14^{a}	11 ± 8^{ab}	8 ± 5^{ab}	1.20 ± 0.13^{b}	1.80 ± 1.20^{ab}
d)	WT 1% + VC D3	25 ± 12^{c}	21 ± 14^{cd}	0^{d}	0^{c}	0^{c}	–	0.00^{c}
e)	dead LVS3 + VC D3	70 ± 15^{ab}	64 ± 17^{a}	3 ± 5^{cd}	0^{c}	0^{c}	–	0.00^{c}
f)	dead LVS3 + WT 1% + VC D3	59 ± 21^{ab}	50 ± 26^{abc}	6 ± 3^{c}	1 ± 3^{bc}	1 ± 3^{bc}	1.56^{a}	0.39 ± 0.78^{bc}
d)	WT 1% + VP D3	21 ± 6^{c}	16 ± 5^{d}	0^{d}	0^{c}	0^{c}	–	0.00^{c}
e)	dead LVS3 + VP D3	79 ± 11^{a}	61 ± 8^{a}	16 ± 10^{bc}	0^{c}	0^{c}	–	0.00^{c}
f)	dead LVS3 + WT 1% + VP D3	59 ± 17^{ab}	50 ± 14^{ab}	39 ± 12^{a}	14 ± 14^{abc}	6 ± 9^{abc}	1.73 ± 0.35^{a}	2.16 ± 3.27^{abc}

The WT yeast cells constituted either 1% or 10% of the total AFDW supplied. As a control, nauplii were not supplied with dead LVS3 but only with these reduced amounts of yeast cells. The challenge test was performed with *Vibrio campbellii* (VC) or *Vibrio proteolyticus* (VP) added on day 3 (D3). The first column in the table refers to the type of treatment (see Fig. 1). Values are presented with the respective standard deviation (mean ± SD). Values in the same column showing the same superscript letter are not significantly different ($n = 4$; $p_{\mathrm{Tukey}} > 0.05$).

Table 3
Experiment 2 – Mean daily survival (%), individual length (IL) (mm) and total biomass production (TBP) (mm per Falcon tube – FT) of *Artemia* fed daily with dead LVS 3 and mnn9

T	Experiment 2	Survival (%)					IL (mm)	TBP (mm/FT)
		Day 2	Day 3	Day 4	Day 5	Day 6		
a)	mnn9 10%	81 ± 11^{abc}	78 ± 10^{abc}	68 ± 13^{bc}	65 ± 14^{bc}	65 ± 14^{abc}	2.10 ± 0.35^{a}	27.30 ± 5.94^{bc}
b)	dead LVS3	66 ± 9^{c}	55 ± 17^{c}	33 ± 17^{de}	14 ± 6^{d}	8 ± 3^{d}	1.36 ± 0.18^{b}	2.04 ± 0.79^{d}
c)	dead LVS3 + mnn9 10%	89 ± 3^{a}	88 ± 3^{a}	86 ± 5^{ab}	84 ± 6^{ab}	80 ± 7^{a}	2.65 ± 0.43^{a}	42.40 ± 3.75^{a}
d)	mnn9 10% + VC D3	73 ± 9^{bc}	73 ± 9^{bc}	69 ± 6^{c}	59 ± 8^{c}	50 ± 7^{c}	2.29 ± 0.37^{a}	22.90 ± 3.24^{c}
e)	dead LVS3 + VC D3	70 ± 15^{bc}	64 ± 17^{bc}	3 ± 5^{f}	0^{e}	0^{e}	–	0.00^{e}
f)	dead LVS3 + mnn9 10% + VC D3	89 ± 8^{ab}	85 ± 8^{ab}	80 ± 4^{abc}	74 ± 5^{b}	71 ± 9^{ab}	2.62 ± 0.45^{a}	37.34 ± 4.47^{ab}
d)	mnn9 10% + VP D3	66 ± 13^{c}	61 ± 18^{bc}	59 ± 22^{cd}	58 ± 21^{bc}	50 ± 21^{bc}	2.37 ± 0.49^{a}	23.70 ± 9.87^{bc}
e)	dead LVS3 + VP D3	79 ± 11^{abc}	61 ± 8^{c}	16 ± 10^{ef}	0^{e}	0^{e}	–	0.00^{e}
f)	dead LVS3 + mnn9 10% + VP D3	91 ± 5^{a}	91 ± 5^{a}	90 ± 4^{a}	89 ± 5^{a}	86 ± 8^{a}	2.52 ± 0.41^{a}	43.47 ± 3.78^{a}
a)	mnn9 1%	48 ± 19^{bcd}	28 ± 17^{bcd}	1 ± 3^{e}	0^{c}	0^{d}	–	0.00^{d}
b)	dead LVS3	66 ± 9^{ab}	55 ± 17^{abc}	33 ± 17^{bc}	14 ± 6^{b}	8 ± 3^{c}	1.36 ± 0.18^{b}	2.04 ± 0.79^{c}
c)	dead LVS3 + mnn9 1%	83 ± 16^{a}	68 ± 12^{a}	65 ± 9^{a}	64 ± 9^{a}	64 ± 9^{a}	1.73 ± 0.34^{ab}	22.06 ± 2.95^{a}
d)	mnn9 1% + VC D3	33 ± 9^{d}	18 ± 12^{d}	0^{e}	0^{c}	0^{d}	–	0.00^{d}
e)	dead LVS3 + VC D3	70 ± 15^{ab}	64 ± 17^{a}	3 ± 5^{de}	0^{c}	0^{d}	–	0.00^{d}
f)	dead LVS3 + mnn9 1% + VC D3	63 ± 20^{abc}	51 ± 17^{abc}	19 ± 8^{c}	0^{c}	0^{d}	–	0.00^{d}
d)	mnn9 1% + VP D3	38 ± 13^{cd}	23 ± 16^{cd}	0^{e}	0^{c}	0^{d}	–	0.00^{d}
e)	dead LVS3 + VP D3	79 ± 11^{a}	61 ± 8^{a}	16 ± 10^{cd}	0^{c}	0^{d}	–	0.00^{d}
f)	dead LVS3 + mnn9 1% + VP D3	59 ± 17^{abc}	56 ± 15^{ab}	55 ± 14^{ab}	43 ± 13^{a}	30 ± 14^{b}	1.86 ± 0.33^{a}	11.16 ± 5.26^{b}

The mnn9 yeast cells constituted either 1% or 10% of the total AFDW supplied. The challenge test was performed with *Vibrio campbellii* (VC) and *Vibrio proteolyticus* (VP) added on day 3 (D3). The first column in the table refers to the type of treatment (see Fig. 1). Values are presented with the respective standard deviation (mean ± SD). Values in the same column showing the same superscript letter are not significantly different ($n = 4$; $p_{\mathrm{Tukey}} > 0.05$).

All nauplii challenged with both pathogens and not supplied with WT or mnn9 yeast died before day 5 (Tables 2 and 3 – treatments e). The addition of 1 or 10% of WT yeast did not significantly improve *Artemia* performance when challenged with VC (Table 2 – treatments f). When challenged with VP, nauplii supplemented with 1% of WT yeast (treatment f) were only able to significantly improve survival on day 4. Yet, significantly higher TBP occurred when 10% of WT yeast was provided to nauplii challenged with VP (treatment f), in comparison to challenged nauplii not supplied with this yeast (treatment e), due to higher survival. The addition of 1% of mnn9 yeast was not able to significantly improve *Artemia* performance when challenged with VC (Table 3 – treatment f) in comparison to challenged nauplii not supplied with the mnn9 yeast (treatment e), except for survival on day 4. Yet, challenged nauplii supplied with 10% (VC and VP – treatments f) and 1% (VP – treatment f) of mnn9 yeast, significantly improved TBP in comparison to nauplii solely fed with LVS 3 (treatments e), mainly due to higher survival.

3.2. Particles of glucan

The particle diameter distribution of commercial glucans is presented in Fig. 2. Only 12.11% of the particles had a diameter lower than 50 μm, which is considered to be the upper limit for possible uptake by *Artemia.* In order to provide enough amounts of glucans (130 μg/tube or 26 μg/tube/day) that could be effectively ingested by *Artemia*, it was necessary to provide a higher amount of glucans (±1 mg/tube or ±200 μg/tube/day).

The effect of glucans on the protection of nauplii fed with WT yeast, dead LVS 3 or a combination of dead LVS 3 and WT yeast (called mixture) was tested in a challenge test with VC and VP. Results presented in Table 4 (Exp 3) indicate that non-challenged nauplii supplied solely with glucans (treatment g) were only able to survive until day 4, suggesting that glucans are a poor feed source. *Artemia* fed solely with WT yeast or dead LVS 3 (treatments i) could survive until day 6, although with low TBP, while nauplii fed with the mixture (treatment i) presented a relatively high TBP. This improvement was probably related to the higher AFDW provided (Table 1). The addition of glucans to non-challenged nauplii fed WT yeast or dead LVS 3 (treatments k) allowed them to significantly improve TBP, mostly due to higher survival. No major improvements were observed in nauplii TBP when fed with the mixture and glucans (treatment k).

When challenged with VC or VP, *Artemia* fed with dead LVS 3 (treatments j) died before day 5 (Table 4), while the addition of glucans was able to significantly improve *Artemia* TBP challenged with both pathogens (treatments l), mostly due to improvements in survival. Similarly, *Artemia* fed with WT yeast and challenged with VC died before day 5 (treatment j), while 4% of the nauplii fed with this yeast and challenged with VP were able to survive until the end of the experiment (treatment j), although with a low TBP. The addition of glucans to *Artemia* fed with this yeast significantly improved their performance when challenged with VC or VP (treatments l), mostly due to improvements in survival. Nauplii challenged with both pathogens and fed with the mixture (treatment j) presented significantly lower TBP in comparison to non-challenged nauplii (treatment i), due both to lower survival and IL. Yet, the addition of glucans to nauplii fed with the mixture and challenged with both pathogens (treatment l) significantly improved TBP, in comparison to challenged nauplii not supplied with glucans (treatment j), mostly due to higher survival.

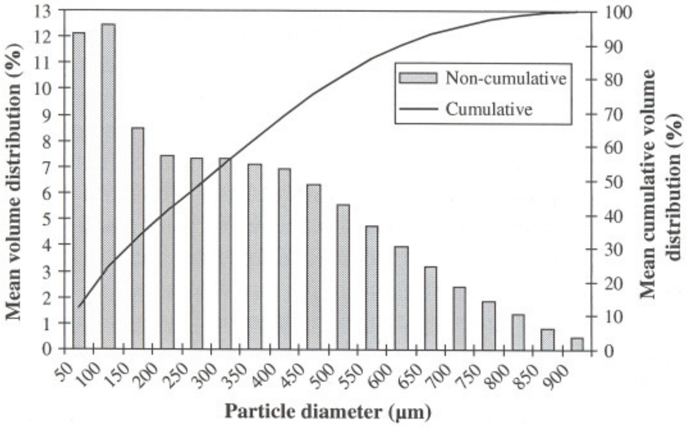


Fig. 2. Non-cumulative mean volume distribution (%– grey bars) and cumulative mean volume distribution (%– black line) of the diameter of glucan particles (μm) used in experiment 4.

Table 4

Experiment 3 – Mean daily survival (%), individual length (IL) (mm) and total biomass production (TBP) (mm per Falcon tube – FT) of *Artemia* fed daily with WT yeast, dead LVS 3 or a combination of WT and dead LVS 3 (WT + dead LVS3)

T	Experiment 3	Survival (%)					IL (mm)	TBP (mm/FT)
		Day 2	Day 3	Day 4	Day 5	Day 6		
g)	Glucans	86 ± 9^{a}	51 ± 6^{a}	3 ± 5^{a}	0^{a}	0^{a}	–	0^{a}
h)	Glucans + VC D3	84 ± 5^{a}	46 ± 8^{a}	0^{a}	0^{a}	0^{a}	–	0^{a}
h)	Glucans + VP D3	91 ± 7^{a}	54 ± 5^{a}	0^{a}	0^{a}	0^{a}	–	0^{a}
i)	WT	49 ± 14^{b}	35 ± 19^{b}	26 ± 19^{c}	13 ± 10^{c}	4 ± 5^{bc}	1.19 ± 0.15^{a}	0.89 ± 1.14^{bc}
j)	WT + VC D3	48 ± 18^{b}	36 ± 13^{b}	4 ± 5^{d}	0^{d}	0^{c}	–	0^{c}
j)	WT + VP D3	41 ± 14^{b}	35 ± 7^{b}	16 ± 5^{c}	8 ± 6^{c}	4 ± 5^{bc}	1.32 ± 0.09^{a}	0.99 ± 1.26^{bc}
k)	WT + Glucans	93 ± 10^{a}	83 ± 9^{a}	80 ± 4^{a}	75 ± 4^{a}	43 ± 12^{a}	1.36 ± 0.20^{a}	11.56 ± 3.24^{a}
l)	WT + Glucans + VC D3	83 ± 6^{a}	76 ± 11^{a}	64 ± 8^{b}	50 ± 11^{b}	29 ± 13^{a}	1.51 ± 0.25^{a}	8.68 ± 3.97^{a}
l)	WT + Glucans + VP D3	88 ± 3^{a}	88 ± 3^{a}	73 ± 10^{ab}	39 ± 9^{b}	19 ± 13^{ab}	1.37 ± 0.22^{a}	5.14 ± 3.60^{ab}
i)	dead LVS3	81 ± 11^{a}	64 ± 11^{cd}	48 ± 21^{b}	24 ± 12^{bc}	20 ± 14^{bc}	1.44 ± 0.23^{a}	5.76 ± 4.07^{bc}
j)	dead LVS3 + VC D3	86 ± 6^{a}	65 ± 13^{bcd}	3 ± 3^{c}	0^{d}	0^{d}	–	0^{d}
j)	dead LVS3 + VP D3	83 ± 12^{a}	64 ± 5^{d}	5 ± 4^{c}	0^{d}	0^{d}	–	0^{d}
k)	dead LVS3 + Glucans	96 ± 5^{a}	90 ± 11^{a}	88 ± 9^{a}	79 ± 15^{a}	48 ± 10^{a}	1.44 ± 0.27^{a}	13.68 ± 3.00^{a}
l)	dead LVS3 + Glucans + VC D3	91 ± 8^{a}	85 ± 7^{ab}	53 ± 23^{b}	11 ± 3^{c}	5 ± 4^{c}	1.57 ± 0.26^{a}	1.57 ± 1.28^{c}
l)	dead LVS3 + Glucans + VP D3	89 ± 6^{a}	79 ± 8^{abc}	64 ± 15^{ab}	41 ± 17^{b}	28 ± 9^{b}	1.57 ± 0.25^{a}	8.64 ± 2.72^{ab}
i)	WT + dead LVS3	90 ± 7^{ab}	79 ± 8^{b}	76 ± 10^{a}	76 ± 10^{ab}	75 ± 9^{ab}	1.75 ± 0.32^{a}	26.25 ± 3.20^{ab}
j)	WT + dead LVS3 + VC D3	88 ± 3^{b}	83 ± 6^{ab}	41 ± 19^{c}	25 ± 16^{c}	15 ± 9^{c}	1.55 ± 0.21^{a}	4.65 ± 2.83^{d}
j)	WT + dead LVS3 + VP D3	96 ± 5^{ab}	95 ± 4^{a}	69 ± 9^{bc}	61 ± 16^{b}	51 ± 19^{b}	1.59 ± 0.23^{a}	16.30 ± 6.00^{c}
k)	WT + dead LVS3 + Glucans	100 ± 0^{a}	93 ± 6^{ab}	91 ± 5^{a}	85 ± 7^{a}	76 ± 3^{a}	1.90 ± 0.36^{a}	28.98 ± 0.95^{a}
l)	WT + dead LVS3 + Glucans + VC D3	93 ± 3^{ab}	90 ± 4^{ab}	88 ± 6^{a}	81 ± 9^{ab}	70 ± 11^{ab}	1.58 ± 0.28^{a}	22.12 ± 3.41^{b}
l)	WT + dead LVS3 + Glucans + VP D3	96 ± 5^{ab}	91 ± 9^{ab}	84 ± 6^{ab}	84 ± 6^{a}	78 ± 18^{ab}	1.88 ± 0.35^{a}	29.14 ± 6.95^{ab}

Particles of glucans were supplied daily (glucans). The challenge test was performed with *Vibrio campbellii* (VC) or *Vibrio proteolyticus* (VP) added on day 3 (D3). The first column in the table refers to the type of treatment (see Fig. 1). Values are presented with the respective standard deviation (mean ± SD). Values in the same column showing the same superscript letter are not significantly different ($n = 4$; $p_{\mathrm{Tukey}} > 0.05$).

4. Discussion

Yeast cells are surrounded by a cell wall composed mostly of β1,3- and β1,6-glucans, mannoproteins and chitin [26]. The mannoproteins are mainly present in the outer cell wall layer. In WT yeast cultured in YEPD medium and harvested in the stationary growth phase 48% of the cell wall is mannoproteins [19]. Since *Artemia* do not have digestive enzymes in their gut able to process these mannoproteins [31], the yeast content is probably less accessible to other digestive enzymes, and thus the WT yeast cannot be properly digested. Consequently, animals fed with WT yeast present poor growth and survival, as demonstrated by Marques et al. [19–21]. The mnn9 mutation changes considerably the yeast cell wall composition, resulting in increased proportional presence of cell wall bound chitin and glucans in combination with reduced mannoproteins [19,26,33]. The distribution of the cell wall constituents also change (e.g. chitin is more evenly distributed through the cell wall in mnn9), causing an effect on the way the cell wall compounds are covalently linked to each other [34]. This change increases cell wall permeability and decreased integrity in the mnn9 yeast [35], as well as an improved yeast digestibility to *Artemia*, thus allowing nauplii to perform better in comparison to WT yeast [19,20]. Interestingly, *Artemia* fed with the mnn9 yeast was also always protected against different pathogenic bacteria, while nauplii fed with WT yeast could not resist the detrimental effects of the same pathogens [21]. Although the authors were not able to find the exact mechanism involved in such protection, β-glucans and chitin (present in higher concentrations in the mnn9 yeast cell wall) were hypothesized to play an important role in the protection of *Artemia* against pathogenic bacteria. This hypothesis is corroborated by Vismara et al. [36], who obtained a significant increase in *Artemia* resistance against stress conditions, by daily administering nauplii with a *Euglena gracilis* mutant rich in β-glucans. Other hypotheses can be formulated to explain the protection provided by the mnn9 yeast: it might be the result of an improved condition of *Artemia* fed with a more nutritious diet; or mnn9 yeast might stimulate/activate the digestive physiology (enzyme secretion) in the same way as previously described for European sea bass *Dicentrarchus labrax* supplied with microalgae [37] and Baker's yeast [38].

To clarify the contribution of mnn9 yeast in the protection of *Artemia* against VC and VP, it was necessary to differentiate the nutritional features of mnn9 from its immune abilities. For that purpose, small amounts of mnn9 and WT yeast were daily supplemented to nauplii fed with the poor-performing dead LVS 3, which could not protect *Artemia* against both pathogens. As expected, *Artemia* supplied with the non-protective WT yeast (1% or 10%) were not able to withstand the challenge with VC (Tables 2 and 5; treatment f). Yet, total protection was achieved when 10% or even 1% of WT yeast was provided to *Artemia* challenged with VP. *Artemia* supplied with 10% of mnn9 yeast were completely protected against VC and VP (treatment f), while nauplii provided with 1% of this yeast presented total protection against VP, but not against VC (although some residual effects were observed on survival at day 4) (Tables 3 and 5). Additionally, in most treatments where protection was achieved with the mnn9 yeast, general improvements in nauplii IL were observed. The addition of small amounts of yeast to *Artemia* suggests that the protection against both pathogens was not only due to better animal condition, but eventually due to an enhancement of their immune response. Yet, results obtained with VC were inconclusive as nauplii did survive when supplied solely with 10% of mnn9 (treatment a). Improvements in the health status of aquatic organisms can be achieved by balancing their diets with regard to nutritional factors, a phenomenon called nutritional immunology, since some nutritional factors are so intimately linked with biochemical processes of the immune system that significant health benefits can be obtained by adjusting the concentration of such factors [6].

Interestingly, unchallenged nauplii offered a mixture of mnn9 yeast (only 1% of the total AFDW provided) and a dead bacterial strain presented significant improvements (Table 3; treatment c), especially in the survival rate, in comparison to unchallenged nauplii supplied solely with the dead bacterium (treatment b). This result seems to suggest that the mnn9 yeast presents unknown compounds that enable *Artemia* to better digest dead bacteria, e.g., by stimulating/activating the naupliar digestive physiology (enzyme secretion), in the same way as previously described for European sea bass supplied with baker's yeast [38]. Since a similar improvement in *Artemia* survival was observed in Experiment 3, when glucan particles obtained from the baker's yeast *S. cerevisiae* were provided to unchallenged nauplii, most likely glucans present in the mnn9 yeast were responsible for such enhancement.

In the final set of experiments (Table 4), glucan particles were fed to *Artemia*, together with non-protective feeds, to evaluate the role of such particles in the protection against VC and VP. In the absence of a challenge, pure glucan particles were not able to boost the IL of *Artemia* nauplii in a significant way (although it had a clear effect on survival) (treatments k), suggesting that β-glucan is not a major nutrient source. Nonetheless, almost all challenged nauplii were completely protected against both pathogens when supplied daily with small amounts of glucans (26 μg/tube) (Tables 4 and 5), except when nauplii were fed solely with dead LVS 3 (glucans could avoid detrimental effects of VP, but only partly the effect of VC). Although not confirmed for *Artemia*, β-glucans, lipopolysaccharides and dead bacterial cells have specific recognition proteins in shrimp haemocytes [39], which play important roles in the crustacean immune system [2,40], improving their innate immune response against diseases.

Table 5
Summary table of results obtained in the challenge experiments: "+" means protection (=significantly higher in comparison to challenged nauplii supplied solely with feeds) and "=" means no protection (=no significant differences) provided by small amounts of WT or mnn9 yeast and by glucan particles on *Artemia* performance, when fed with different feeds and challenged with *V. campbellii* and *V. proteolyticus*

	Vibrio campbellii				*Vibrio proteolyticus*			
	Survival D4 (%)	Survival D6 (%)	IL (mm)	TBP (mm/FT)	Survival D4 (%)	Survival D6 (%)	IL (mm)	TBP (mm/FT)
Experiments 1 and 2								
Dead LVS 3								
10% WT	=	=	nd	=	⊕	⊕	nd	⊕
1% WT	=	=	nd	=	⊕	=	nd	=
10% mnn9	⊕	⊕	nd	⊕	⊕	⊕	nd	⊕
1% mnn9	⊕	=	nd	=	⊕	⊕	nd	⊕
Experiment 3								
WT + Glucans	⊕	⊕	nd	⊕	⊕	=	=	=
LVS 3 + Glucans	⊕	⊕	nd	⊕	⊕	⊕	nd	⊕
WT + LVS 3 + Glucans	⊕	⊕	=	⊕	=	=	=	=

A circle around the "+" means that the feed was not protecting or only protecting partly nauplii against the specific pathogen; "nd" means not determined due to no survivals. D1, D4 and D6 means that the compound was added respectively on days 1, 4 and 6.

In conclusion, the mnn9 yeast (but not the WT yeast strain), even when supplied in small quantities, protected *Artemia* nauplii against pathogenic bacteria, suggesting that this yeast strain is able to stimulate the innate immune response. Similarly, the addition of pure glucans to *Artemia* fed with poor performing or better quality feeds totally protected this organism against VC and VP, most probably by direct stimulation of the naupliar immune system. The mnn9 yeast cells seem to fulfil a double function, as they are both boosting IL of *Artemia* and protecting them in a challenge test. In view of the fact that the mnn9 yeast cell contains more β-glucans than the WT yeast cell [19,26,33], it is tempting to speculate that mnn9 cells, at least in part, protect nauplii through either their higher concentration of β-glucans in the cell wall and/or the higher bio-availability of β-glucans to the *Artemia* nauplii. The mnn9 null mutation causes a considerable change in the way the major yeast cell wall components are covalently linked to each other [34].

The present gnotobiotic *Artemia* challenge test can be used as a unique system for differentiating between nutritional effects and the real immune stimulatory abilities of a specific compound, especially if combined with other tools, such as the measurement of immune parameters and gene expression analysis. It will be interesting to investigate whether the mnn9 mutant yeast cell can be applied in open cultures with other aquaculture target species and against other pathogens. In addition, gene expression studies in these gnotobiotically-grown *Artemia* should provide further documentation on how yeast cells and/or specific compounds have a direct effect on the innate immune system.

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References

[1] Bachère E. Anti-infectious immune effectors in marine invertebrates: potential tools for disease control in larviculture. Aquaculture 2003;227:427–38.

[2] Smith V, Brown J, Hauton C. Immunostimulation in crustaceans: does it really protect against infection? Fish Shellfish Immunol 2003;15: 71–90.

[3] Vadstein O. The use of immunostimulation in marine larviculture: possibilities and challenges. Aquaculture 1997;155:401–17.

[4] Kurtz J, Franz K. Evidence for memory in invertebrate immunity. Nature 2003;425:37–8.

[5] Söderhäll K, Cerenius L, Johansson M. The prophenoloxidase activating system in invertebrates. In: Söderhäll K, et al., editors. New Directions in Invertebrate Immunology. Fair Haven, New Jersey, USA: SOS Publications; 1996.

[6] Raa J. The use of immune-stimulants in fish and shellfish feeds. In: Cruz-Suárez L, et al., editors. Avances en Nutrición Acuícola V. Memorias del V Simposium Internacional de Nutrición Acuícola November 19–22, 2000. Mérida, Yucatán, Mexico; 2000.

[7] Sung H, Yang Y, Song Y. Enhancement of microbicidal activity in the tiger shrimp, *Penaeus monodon*, *via* immunostimulation. J Crust Biol 1996;16:278–84.

[8] Sritunyalucksana K, Sithisarn P, Withayachumnarnkul B, Flegel T. Activation of prophenoloxidase, agglutinin and antibacterial activity in haemolymph of the black tiger prawn, *Penaeus monodon*, by immunostimulants. Fish Shellfish Immunol 1999;9:21–30.

[9] Burgents J, Burnett K, Burnett L. Disease resistance of Pacific white shrimp, *Litopenaeus vannamei*, following the dietary administration of a yeast culture food supplement. Aquaculture 2004;231:1–8.

[10] Misra C, Das B, Pradhan J, Pattnaik P, Sethi S, Mukherjee S. Changes in lysosomal enzyme activity and protection against *Vibrio* infection in *Macrobrachium rosenbergii* (De Man) post larvae after bath immunostimulation with β-glucan. Fish Shellfish Immunol 2004;17:389–95.

[11] Anderson D, Siwicki A. Duration of protection against *Aeromonas salmonicida* in brook trout immunostimulated with glucan or chitosan by injection or immersion. Prog Fish Culturist 1994;56:258–61.

[12] Tizard I, Carpenter R, McAnalley B, Kemp M. The biological activities of mannans and related complex carbohydrates. Mol Biother 1989;1:290–6.

[13] Takahashi Y, Kondo M, Itami T, Honda T, Inagawa H, Nishizawa T, et al. Enhancement of disease resistance against penaeid acute viraemia and induction of virus-inactivating in haemolymph of kuruma shrimp, *Penaeus japonicus*, by administration of *Pantoea agglomerans* lipopolysaccharide (LPS). Fish Shellfish Immunol 2000;10:555–8.

[14] Boonyaratpalin S, Boonyaratpalin M, Supamattaya K, Toride Y. Effects of peptidoglycan (PG) on growth, survival, immune response, and tolerance to stress in black tiger shrimp, *Penaeus monodon*. In: Shariff M, et al., editors. Diseases in Asian Aquaculture II. Fish health section. Manila, Philippines: Asian Fisheries Society; 1995.

[15] Itami T, Asano M, Tokushige K, Kubono K, Nakagawa A, Takeno N, et al. Enhancement of disease resistance of kuruma shrimp, *Penaeus japonicus*, after oral administration of peptidoglycan derived from *Bifidobacterium thermophilium*. Aquaculture 1998;164:277–88.

[16] Keith I, Paterson W, Aidrie D, Boston L. Defense mechanisms of the American lobster (*Homarus americanus*): vaccination provided protection against gaffkemia infections in laboratory and field trials. Fish Shellfish Immunol 1992;2:109–19.

[17] Alabi A, Jones D, Latchford J. The efficacy of immersion as opposed to oral vaccination of *Penaeus indicus* larvae against *Vibrio harveyi*. Aquaculture 1999;178:1–11.

[18] Vici V, Singh B, Bhat S. Application of bacterins and yeast *Acremonium dyosporii* to protect the larvae of *Macrobrachium rosenbergii* from vibriosis. Fish Shellfish Immunol 2000;10:559–63.

[19] Marques A, François J, Dhont J, Bossier P, Sorgeloos P. Influence of yeast quality on performance of gnotobiotically-grown *Artemia*. J Exp Mar Biol Ecol 2004;310:247–64.

[20] Marques A, Dhont J, Sorgeloos P, Bossier P. Evaluation of different yeast cell wall mutants and microalgae strains as feed for gnotobiotically-grown brine shrimp *Artemia franciscana*. J Exp Mar Biol Ecol 2004;312:115–36.

[21] Marques A, Dinh T, Ioakeimidis C, Huys G, Swings J, Verstraete W, et al. Effects of bacteria on *Artemia* cultured in different gnotobiotic environments. Appl Environ Microbiol 2005;71:4307–17.

[22] Triantaphyllidis G, Abatzopoulos T, Sorgeloos P. Review of the biogeography of the genus *Artemia* (Crustacea, Anostraca). J Biogeogr 1998;25:213–26.

[23] Patra S, Mohamed K. Enrichment of *Artemia* nauplii with the probiotics yeast *Saccharomyces boulardii* and its resistance against a pathogenic *Vibrio*. Aquacult Int 2003;11:505–14.

[24] Dobbeleir J, Adam N, Bossuyt E, Bruggeman E, Sorgeloos P. New aspects of the use of inert diets for high density culturing of brine shrimp. In: Persoone G, et al., editors. The Brine Shrimp Artemia – ecology, culturing and use in aquaculture, vol. 3. Wetteren, Belgium: Universa Press; 1980.

[25] Sorgeloos P, Lavens P, Léger P, Tackaert W, Versichele D. Manual for the culture and use of brine shrimp *Artemia* in Aquaculture. Belgium: Artemia Reference Center, Faculty of Agriculture, State University of Ghent; 1986.

[26] Magnelli P, Cipollo J, Abeijon C. A refined method for the determination of *Saccharomyces cerevisiae* cell wall composition and β-1,6-glucan fine structure. Anal Biochem 2002;301:136–50.

[27] Verschuere L, Rombaut G, Huys G, Dhont J, Sorgeloos P, Verstraete W. Microbial control of the culture of *Artemia* juveniles through preemptive colonization by selected bacterial strains. Appl Environ Microbiol 1999;65:2527–33.

[28] Verschuere L, Heang H, Criel G, Sorgeloos P, Verstraete W. Selected bacterial strains protect *Artemia* spp. from pathogenic effects of *Vibrio proteolyticus* CW8T2. Appl Environ Microbiol 2000;66:1139–46.

[29] Soto-Rodriguez S, Roque A, Lizarraga-Partida M, Guerra-Flores A, Gomez-Gil B. Virulence of luminous vibrios to *Artemia franciscana* nauplii. Dis Aquat Organ 2003;53:231–40.

[30] Gomez-Gil B, Soto-Rodríguez S, García-Gasca A, Roque A, Vazquez-Juarez R, Thompson F, et al. Molecular identification of *Vibrio harveyi*-related isolates associated with diseased aquatic organisms. Microbiology 2004;150:1769–77.

[31] Coutteau P, Lavens P, Sorgeloos P. Baker's yeast as a potential substitute for live algae in aquaculture diets: *Artemia* as a case study. J World Aquacult Soc 1990;21:1–8.

[32] Sladowski D, Steer S, Clothier R, Balls M. An improved MTT assay. J Immunol Methods 1993;157:203–7.

[33] Aguilar-Uscanga B, François J. A study of the yeast cell wall composition and structure in response to growth conditions and mode of cultivation. Lett Appl Microbiol 2003;37:268–74.

[34] Klis K, Mol P, Hellingwerf K, Brul S. Dynamics of cell wall structure in *Saccharomyces cerevisiae*. FEMS Microbiol Rev 2002;26:239–56.

[35] De Nobel J, Klis F, Priem J, Munnik T, Van den Ende H. The glucanase-soluble mannoproteins limit cell wall porosity in *Saccharomyces cerevisiae*. Yeast 1990;6:491–9.

[36] Vismara R, Vestri S, Frassanito A, Barsanti L, Gualtieri P. Stress resistance induced by paramylon treatment in *Artemia* sp. J Appl Phycol 2004;16:61–7.

[37] Cahu C, Zambonino J, Péres A, Quazuguel P, Le Gall M. Algal addition in sea bass (*Dicentrarchus labrax*) larvae rearing: effect on digestive enzymes. Aquaculture 1998;161:479–89.

[38] Tovar D, Zambonino J, Cahu C, Gatesoupe F, Vázquez-Juárez R, Lésel R. Effect of live yeast incorporation in compound diet on digestive enzyme activity in sea bass (*Dicentrarchus labrax*) larvae. Aquaculture 2002;204:113–23.

[39] Cheng W, Liu C, Tsai C, Chen J. Molecular cloning and characterization of a pattern recognition molecule, lipopolysaccharide and β-1,3-glucan binding protein (LGBP) from the white shrimp *Litopenaeus vannamei*. Fish Shellfish Immunol 2005;18:297–310.

[40] Brown G, Gordon S. Immune recognition of fungal β-glucans. Micro Review. Cell Microbiol 2005;7:471–9.