Enhanced disease resistance in *Artemia* by application of commercial β-glucans sources and chitin in a gnotobiotic *Artemia* challenge test

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Abstract

The anti-infectious potential of a selection of putative immunostimulants including six commercial β-glucans (all extracted from baker’s yeast *Saccharomyces cerevisiae* except for Laminarin) and chitin particles were verified in *Artemia nauplii* by challenging them under gnotobiotic conditions with the pathogen *Vibrio campbellii*. Under the described experimental conditions, no differential macroscopic nutritional effect (e.g. growth) was observed among the products. Significant increased survival was observed with β-glucan (Sigma) and Zymosan and to a lesser extent with MacroGard in challenged nauplii. A poor correlation was found between survival values of the challenged *Artemia* and the product compositions (such as chitin, mannose and β-glucan content) indicating that the quality of β-glucans (e.g. the ratio of β-1,3 and β-1,6 glucan, the molecular weight, the dimensional structure, type and frequency of branches), eventually in combination with other unidentified compounds, is more important than the amount of product offered. This small-scale testing under gnotobiotic conditions using freshly hatched *Artemia nauplii* allows for a rapid and simultaneous screening of anti-infectious and/or putative immunostimulatory polymers, and should be combined with studies on cellular and humoral immune responses in order to gain more quantitative insight into their functional properties. © 2007 Elsevier Ltd. All rights reserved.

Keywords: *Artemia*; Gnotobiotic culture; Disease resistance; β-Glucan; *Vibrio campbellii*

1. Introduction

Diseases are still a major constraint to sustainable aquaculture production, especially for the farming of invertebrates [1]. According to Raa [2], invertebrates are apparently entirely dependent on non-specific immune mechanisms to cope with infections, as they lack the specific immunological “memory” that is found in fish and warm-blooded
animals. As a result, it does not seem to make sense to vaccinate them against specific diseases. Nowadays, the use of preventive and environment-friendly approaches such as probiotics, immunostimulants, antibacterial peptides and quorum-sensing systems are becoming increasingly important in aquaculture [1,3–5]. However, the application of such technologies must be based on a thorough understanding of the mechanisms involved and the putative consequences. An essential part of that understanding can be provided by studies of host—microbial interactions. A key experimental approach to study these interactions is to first define the functioning of the host in the absence of bacteria and then to evaluate the effect of adding a single or defined population of microbes, or certain compounds, i.e. under axenic or gnotobiotic conditions [6–8]. Marques et al. [7,8] have recently developed and validated the usefulness of an Artemia gnotobiotic test system allowing studying the nutritional effect of food composition as well as the host—microbial interaction to be studied.

The use of specific biological compounds (immunostimulants) that enhance immune responses of target organisms, rendering animals more resistant to diseases, may be an excellent preventive tool against pathogens [9]. Several immunostimulants have been used in vertebrate and invertebrate culture, to induce protection against a wide range of diseases: i.e. β-glucans [10–13], chitin [14–16], mannoproteins [17], lipopolysaccharides [18], peptidoglycans [18,19] and dead bacteria [18,20,21]. Nevertheless, rigorous analysis of the results obtained in most experiments revealed that the validity of some conclusions with respect to the benefit of immunostimulation is limited, due to mainly poor experimental design, to the absence of any statistical analysis and to poor reproducibility of the results [22]. Therefore, Smith et al. [22] argued that there is an urgent need to provide unequivocal evidence of the beneficial effects of immunostimulants 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 present study aims to verify the putative anti-infectious effect of some commercial β-glucans and chitin particles in a gnotobiotic Artemia challenge test system using the opportunistic pathogenic bacterium Vibrio campbellii.

2. Methodology

2.1. Bacterial strains and growth conditions

Two bacterial strains were used, i.e. Aeromonas hydrophila strain LVS3 for its positive effect on Artemia performance when fed at sub-optimal concentration [23–25] and Vibrio campbellii (VC) strain LMG21363 for its pathogenic effect towards Artemia and shrimp [23–26]. The two bacterial strains were cultured and harvested according to procedures described by Marques et al. [25]. Bacteria were resuspended in filtered and autoclaved seawater (FASW, 0.2 μm) and their densities determined by spectrophotometry (OD550), assuming that an optical density of 1.000 corresponds to 1.2 × 10^8 cells/ml, according to the McFarland standard (Biomerieux, Marcy l’Etoile, France). Dead LVS3 was fed to Artemia using aliquots of autoclaved bacteria (autoclaving at 120 °C for 20 min). At day 3, challenge tests were performed with live VC according to a procedure described by Soltanian et al. [27].

The composition of the commercial β-glucans was determined using high performance anionic exchange chromatography (HPAEC) (Dionex Bio-LC50 system, Sunnyvale, USA), according to the methodologies described by Dallies et al. [28].

2.2. Artemia gnotobiotic culture

Experiments were performed with Artemia franciscana cysts, originating from Great Salt Lake, Utah, USA (EG® type, INVE Aquaculture SA, Dendermonde, Belgium). All manipulations were carried out under a laminar flow hood and all necessary tools were previously autoclaved at 120 °C for 20 min. Bacteria-free cysts and nauplii were obtained using the decapsulation procedure described by Sorgeloos et al. [29] and Marques et al. [7]. After hatching, 20 axenic nauplii (Instar II) were transferred to Falcon tubes containing 30 ml of FASW together with the amount of feed scheduled for day 1. The daily feeding schedule was adopted from Soltanian et al. [27] and is intended to provide ad libitum ratios but avoiding excessive feeding in order not to affect the water quality in the test tubes. In all experiments the total amount of dead LVS3 provided to Artemia was approximately 10.5 × 10^9 cells/FT (distributed in five daily feeding portions; daily fraction (in %) 9:17:17:23:34). Each treatment consisted of four Falcon tubes (replicates), placed
on a rotating rod at four cycles per minute and exposed to constant incandescent light (±41 µE m⁻²) at 28 °C. Tubes were being transferred to the laminar flow just once per day for feeding.

2.3. Method 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 after staining with tetrazolium salt MTT (-3-(4,5–dimethylthazol-2-yl)-2,5-diphenyl tetrazolium bromide) (Sigma, 0.5% w/v) following the procedure described by Marques et al. [30]. In challenge treatments, the axenity of Artemia culture was always checked before challenge using the same methods. Contaminated culture tubes were not considered for further analysis and the treatment was repeated.

2.4. Particles of glucans and chitin

Insoluble particles of chitin (Sigma, from crab shell, 1 g) and five commercial β-glucans, namely, Biorigin (Beta-mune, Brazil), MacroGard (MacroGard®, Biotec—Mackzymal, Norway), Immunowall (Brazil), Zymosan (Sigma, 1 g) and the β-glucan (Sigma, 100 mg) (all obtained from baker’s yeast Saccharomyces cerevisiae) in addition to a soluble β-glucan (Laminarin, Sigma, 500 mg) extracted from Laminaria digitata, were tested in the Artemia gnotobiotic challenge test system to verify their potential to protect Artemia nauplii against the pathogenic VC. Non-sterile compounds (Biorigin, Immunowall and MacroGard) were suspended in absolute ethanol (100%) in sterile 50 ml Falcon tubes with loose caps and put at 28 °C to dry. Particles were aseptically transferred to sterile 50 ml Falcon tubes and homogeneously suspended in FASW. Contamination was checked by plating the suspension on MA (100 µl, n = 2). Absence of bacterial growth was monitored after incubating plates for 5 days at 28 °C. No bacterial growth was detected on marine agar after 5 days of incubation. An optical laser particle size analyser (Mastersizer MSX-17, Malvern Instruments Ltd., Malvern, Works, UK; resolution 0.05–900 µm; software Malvern Mastersizer S version 2.19®) was used to determine the diameter of the particles present in the suspension. Each product had its specific particle distribution curve. The volume percentage of particles, sizing less than 50 µm (maximum particle size that can be ingested by Artemia) was calculated (Table 2, second column). In a classical 6-day experiment [7] 1.06 mg AFDW (ash free dry weight) of yeast cells are offered to Artemia per tube. According to Marques et al. [7], this corresponds on average, depending on the yeast strains, to 213 µg of cell wall material. Here it was decided, on an arbitrary basis, to add 128 µg of ingestible product (being 60%). In order to provide equal amounts of ingestible particles it was necessary to adjust the feeding regime for each product (see legend Table 2). Experiments in which three times as many particles of the respective products were offered, were also performed. The suspension of particles was stored at 4 °C until the end of the experiment. It was verified whether the particles in the different commercial preparations could display a differential adhesion of Vibrio cells, potentially influencing the number of pathogenic bacteria (VC) ingested by Artemia in the challenge test. However, no bacterial adherence to particles was observed when checked under the microscope (1000×) over a period of 48 h of contact between them (results not shown).

2.5. Experimental design

This study comprises two experiments and their experimental design is schematised in Fig. 1. In experiments 1 and 2, Artemia nauplii were fed daily with poor-performing and non-protective dead LVS3 [27] (a total 10.5 × 10⁹ cells/Falcon tube (FT) for 6 days) as a major feed source in combination with small amount of glucans and chitin particles (approximately 2% in dry weight of the total amount of feed offered). Glucan and chitin additions were divided in equal parts per day, to avoid the possibility that those particles could be used as major feed source by Artemia) (Fig. 1). At day 3, challenge tests were performed with live VC. For that purpose, in a laminar flow hood, the pathogen was added to each replicate at a density of 5 × 10⁶ cells/ml. As a control Artemia was only fed with the dead LVS3 and challenged (or not) with the pathogen. These experiments were repeated to verify the reproducibility of the results.

2.6. 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 bacteria by counting with the naked eye while 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 \emph{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/C210 (courtesy of Marnix Van Damme). As a criterion that combines both the effects of survival and IL, the total biomass production (TBP) was determined according to the following equation: 

\[
\text{TBP (millimetres per Falcon tube)} = \frac{\text{number of survivors}}{\text{mean IL.}}
\]

The relative percentage survival (RPS) value was determined in \emph{Artemia} fed with every product or not (control) according to the following equation: 

\[
\text{RPS (\%)} = \frac{\text{(percentage of surviving challenged Artemia)}}{\text{(percentage of surviving unchallenged Artemia)}} \times 100.
\]

Regression analysis was carried out between \emph{Artemia} survival (arcsine transformed values) and polymer concentration (chitin, mannose and \(\beta\)-glucans) in each product in order to test for possible correlation between these parameters.

### 2.7. Statistics

Values of larval survival (percentage) were arcsin transformed, while values of IL and TBP were logarithmic or square root transformed to satisfy normal distribution and homoscedasticity requirements. Differences in survival, RPS, IL and TBP of \emph{Artemia} cultured in different conditions were investigated with analysis of variances (ANOVA) and Tukey’s multiple comparison range. All statistical analyses were tested at the 0.05 level of probability, using the software SPSS 11.5 for Windows.

### 3. Results

The polymer composition of the commercial glucans used in this study is shown in Table 1. The composition analysis of the products indicates that the percentage of each polymers (chitin, mannose and \(\beta\)-glucans) varies between the products (see Table 1). The percentage of ingestible (lower than 50 \(\mu\)) particles and total amount of \(\beta\)-glucans offered to \emph{Artemia} is presented in Table 2 (the second and fourth column respectively). Except for Immunowall, all components contain a high proportion of \(\beta\)-glucan in their sugar fraction. The purity of the compounds (e.g. percent of sugar) is, however, very variable. The effect of \(\beta\)-glucans and chitin particles on the survival of nauplii fed with dead LVS3 was tested in a challenge test with VC. The results are presented in Tables 3 and 4 (experiments 1 and 2). No significant difference was observed in \emph{Artemia} survival until day 3 (before the challenge test, data not shown). The results indicate...
that some of the challenged nauplii fed only with dead LVS3 (control treatment) could survive until day 6 although in general the performance was low (low survival, low RPS and low TBP). The addition of some products (Zymosan, β-glucan (Sigma) and MacroGard) was able to significantly improve the TBP in challenged nauplii (in comparison to challenged nauplii which did not receive these compounds), mainly due to higher Artemia survival values (Tables 3 and 4; treatments 2, 8 and 14 vs 18). On the other hand, the TBP of Artemia was always significantly reduced by challenging the nauplii, irrespective of the treatment. Not a single compound had a significant effect on Artemia growth (IL) under the described experimental conditions, as illustrated by identical average individual length values over all treatments in the absence of challenge. Moreover, application of higher concentrations of these products (up to three times) did not change the obtained challenge data (data not shown).

4. Discussion

β-Glucans have been successfully used to enhance resistance of crustaceans against bacterial and viral infections [31–37]. The present study confirms the results of Marques et al. [30]. That study showed that a daily addition of small amounts of β-glucan (Sigma) to Artemia, fed with a poor performing feed, enhanced resistance of this organism against the pathogenic VC, while Artemia solely fed with dead LVS3 cells could not resist this pathogen. In this study, the addition of β-glucan (Sigma) was able to significantly improve Artemia RPS and TBP when challenged with VC (mostly due to an improvement in survival) compared to the challenged nauplii which did not receive that glucan (Tables 3 and 4; line 8 vs 18). The administration of different forms of β-glucan in the diet of different shrimp species has been shown to result in an enhancement of protection against various pathogens [32–40]. This increased resistance has been attributed to the stimulation of the phagocytic activity of haemocytes [32]. Nevertheless, in some cases no beneficial effects were observed using β-glucans. It was reported that the survival of juvenile Litopenaeus vannamei fed a glucan-supplemented diet was reduced with respect to control animals over a 7-week period [41].

Bath administration of glucan has been proven to be a suitable procedure to enhance the immune response and disease resistance in shrimp [42–45]. However, such enhancement varies with dose and type of glucan, feeding

<table>
<thead>
<tr>
<th>Product</th>
<th>Sugar (% dry weight)</th>
<th>Chitin (%)</th>
<th>Mannose (%)</th>
<th>β-Glucan (%)</th>
<th>Glucan (% dry weight)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Zymosan</td>
<td>71.5</td>
<td>1.7</td>
<td>14.0</td>
<td>84.3</td>
<td>60.2</td>
</tr>
<tr>
<td>β-Glucan (Sigma)</td>
<td>54.8</td>
<td>2.9</td>
<td>5.5</td>
<td>91.6</td>
<td>50.2</td>
</tr>
<tr>
<td>Laminarin</td>
<td>91.4</td>
<td>0</td>
<td>0.8</td>
<td>91.2</td>
<td>83.3</td>
</tr>
<tr>
<td>Biorigin</td>
<td>72.7</td>
<td>1.4</td>
<td>2.6</td>
<td>96.0</td>
<td>69.8</td>
</tr>
<tr>
<td>MacroGard</td>
<td>50.0</td>
<td>2.0</td>
<td>7.0</td>
<td>91.0</td>
<td>45.5</td>
</tr>
<tr>
<td>Immunowall</td>
<td>37.0</td>
<td>1.6</td>
<td>43.4</td>
<td>55.0</td>
<td>20.3</td>
</tr>
</tbody>
</table>

Table 1
Differential polymer distribution (chitin, mannose and β-glucan) of six commercial glucans tested in the gnotobiotic Artemia challenge test

<table>
<thead>
<tr>
<th>Product</th>
<th>Percentage of ingestible particles (size less than 50 μm)</th>
<th>Particles offered (μg/tube per day)</th>
<th>Total dry weight (DW) of ingestible glucan offered (μg/tube per day)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Zymosan</td>
<td>100</td>
<td>26</td>
<td>15.7</td>
</tr>
<tr>
<td>β-Glucan (Sigma)</td>
<td>12.11</td>
<td>214</td>
<td>13.1</td>
</tr>
<tr>
<td>Biorigin</td>
<td>18</td>
<td>145</td>
<td>18.2</td>
</tr>
<tr>
<td>MacroGard</td>
<td>74</td>
<td>35</td>
<td>11.8</td>
</tr>
<tr>
<td>Immunowall</td>
<td>100</td>
<td>26</td>
<td>5.3</td>
</tr>
<tr>
<td>Chitin</td>
<td>60</td>
<td>21</td>
<td>—</td>
</tr>
<tr>
<td>Laminarin</td>
<td>soluble</td>
<td>26</td>
<td>21.7</td>
</tr>
</tbody>
</table>

Only the particles less than 50 μm are considered to be ingestible by Artemia nauplii. The amount of particles offered to Artemia was calculated as 128 μg/5 feedings/% ingestible particles/100. The total amount of ingestible glucan offered was calculated as the amount of particles offered × % sugar content/100 × % glucan content/100. (For sugar content and glucan content, see Table 1, columns 2 and 5 respectively).
Table 3
Experiment 1: mean daily survival (%), relative percentage survival (%) (RPS), individual length (IL) (mm) and total biomass production (TBP) (mm per Falcon tube (FT)) of *Artemia* fed daily with dead LVS3 alone or in combination with different types of glucan and chitin particles after 5 days

<table>
<thead>
<tr>
<th>Treatment number</th>
<th>Survival (%)</th>
<th>Day 4</th>
<th>Day 5</th>
<th>Day 6</th>
<th>RPS (%)</th>
<th>IL (mm)</th>
<th>TBP (mm/FT)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Dead LVS3 + Zymosan</td>
<td>83 ± 3abc</td>
<td>79 ± 5abc</td>
<td>75 ± 4abc</td>
<td>82 ± 5a</td>
<td>1.6 ± 0.1a</td>
<td>23.5 ± 1.5ab</td>
<td></td>
</tr>
<tr>
<td>2. Dead LVS3 + Zymosan + VC (D3)</td>
<td>78 ± 3abc</td>
<td>70 ± 4bcd</td>
<td>61 ± 2b</td>
<td>1.5 ± 0.1a</td>
<td>19.4 ± 0.8b</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3. Dead LVS3 + Laminarin</td>
<td>84 ± 5abc</td>
<td>81 ± 3b</td>
<td>79 ± 3ab</td>
<td>22 ± 7cd</td>
<td>1.5 ± 0.1a</td>
<td>24.0 ± 0.8c</td>
<td></td>
</tr>
<tr>
<td>4. Dead LVS3 + Laminarin + VC (D3)</td>
<td>64 ± 5a</td>
<td>81 ± 8a</td>
<td>58 ± 4f</td>
<td>1.4 ± 0.1a</td>
<td>3.5 ± 1.4c</td>
<td></td>
<td></td>
</tr>
<tr>
<td>5. Dead LVS3 + chitin</td>
<td>89 ± 3d</td>
<td>86 ± 3b</td>
<td>83 ± 3b</td>
<td>10 ± 5d</td>
<td>1.5 ± 0.1a</td>
<td>20.3 ± 1.5bcd</td>
<td></td>
</tr>
<tr>
<td>6. Dead LVS3 + chitin + VC (D3)</td>
<td>63 ± 3e</td>
<td>80 ± 7e</td>
<td>65 ± 7f</td>
<td>1.4 ± 0.1a</td>
<td>2.5 ± 1.4cd</td>
<td></td>
<td></td>
</tr>
<tr>
<td>7. Dead LVS3 + glucan</td>
<td>86 ± 3b</td>
<td>79 ± 5abc</td>
<td>78 ± 3bc</td>
<td>84 ± 9c</td>
<td>1.6 ± 0.1a</td>
<td>23.1 ± 0.8ab</td>
<td></td>
</tr>
<tr>
<td>8. Dead LVS3 + glucan + VC (D3)</td>
<td>79 ± 3bde</td>
<td>69 ± 5de</td>
<td>65 ± 7bc</td>
<td>1.4 ± 0.1a</td>
<td>18.7 ± 0.8c</td>
<td></td>
<td></td>
</tr>
<tr>
<td>9. Dead LVS3 + glucan + chitin</td>
<td>84 ± 3bcd</td>
<td>80 ± 4bc</td>
<td>75 ± 4abc</td>
<td>88 ± 3a</td>
<td>1.6 ± 0.1a</td>
<td>23.6 ± 1.3b</td>
<td></td>
</tr>
<tr>
<td>10. Dead LVS3 + glucan + chitin + VC (D3)</td>
<td>80 ± 4bcd</td>
<td>70 ± 4bcd</td>
<td>66 ± 5b</td>
<td>1.5 ± 0.1a</td>
<td>20.3 ± 1.5bc</td>
<td></td>
<td></td>
</tr>
<tr>
<td>11. Dead LVS3 + Biorigin</td>
<td>84 ± 3abc</td>
<td>78 ± 3abc</td>
<td>70 ± 4c</td>
<td>23 ± 9cd</td>
<td>1.5 ± 0.1a</td>
<td>21.4 ± 1.2abcd</td>
<td></td>
</tr>
<tr>
<td>12. Dead LVS3 + Biorigin + VC (D3)</td>
<td>70 ± 4ef</td>
<td>36 ± 6ef</td>
<td>16 ± 6ef</td>
<td>1.4 ± 0.1a</td>
<td>4.6 ± 1.8bc</td>
<td></td>
<td></td>
</tr>
<tr>
<td>13. Dead LVS3 + MacroGard</td>
<td>83 ± 3abc</td>
<td>78 ± 3abc</td>
<td>74 ± 3abc</td>
<td>63 ± 3b</td>
<td>1.6 ± 0.1a</td>
<td>23.1 ± 0.8ab</td>
<td></td>
</tr>
<tr>
<td>14. Dead LVS3 + MacroGard + VC (D3)</td>
<td>71 ± 4de</td>
<td>56 ± 3de</td>
<td>46 ± 3d</td>
<td>1.4 ± 0.1a</td>
<td>13.3 ± 0.7c</td>
<td></td>
<td></td>
</tr>
<tr>
<td>15. Dead LVS3 + Immunowall</td>
<td>83 ± 3abc</td>
<td>76 ± 3abc</td>
<td>70 ± 4c</td>
<td>36 ± 12c</td>
<td>1.5 ± 0.1a</td>
<td>21.2 ± 1.4abc</td>
<td></td>
</tr>
<tr>
<td>16. Dead LVS3 + Immunowall + VC (D3)</td>
<td>65 ± 4f</td>
<td>41 ± 6e</td>
<td>25 ± 7e</td>
<td>1.4 ± 0.1a</td>
<td>7.0 ± 2.0f</td>
<td></td>
<td></td>
</tr>
<tr>
<td>17. Dead LVS3</td>
<td>83 ± 3abc</td>
<td>78 ± 3abc</td>
<td>70 ± 6b</td>
<td>22 ± 7cd</td>
<td>1.4 ± 0.1a</td>
<td>19.4 ± 1.6d</td>
<td></td>
</tr>
<tr>
<td>18. Dead LVS3 + VC (D3)</td>
<td>68 ± 3e</td>
<td>28 ± 6ef</td>
<td>15 ± 4ef</td>
<td>1.4 ± 0.1a</td>
<td>4.1 ± 1.1e</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

The challenge test was performed with *Vibrio campbelli* (VC) added on day 3 (D3). The first column in the table refers to the type of treatments (see Fig. 1). Each feed was tested in four replicates. Values are presented with the respective standard deviation (mean ± SD). Values in the same column showing the same superscript letter are not significantly different (*p* < 0.05).

Table 4
Experiment 2: mean daily survival (%), relative percentage survival (%) (RPS), individual length (IL) (mm) and total biomass production (TBP) (mm per Falcon tube (FT)) of *Artemia* fed daily with dead LVS3 alone or in combination with different types of glucan and chitin particles after 5 days

<table>
<thead>
<tr>
<th>Treatment number</th>
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<th>Day 5</th>
<th>Day 6</th>
<th>RPS (%)</th>
<th>IL (mm)</th>
<th>TBP (mm/FT)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Dead LVS3 + Zymosan</td>
<td>85 ± 4abc</td>
<td>79 ± 3abc</td>
<td>74 ± 4abc</td>
<td>86 ± 5a</td>
<td>1.5 ± 0.1ab</td>
<td>22.1 ± 1.2a</td>
<td></td>
</tr>
<tr>
<td>2. Dead LVS3+Zymosan + VC (D3)</td>
<td>79 ± 5abcd</td>
<td>69 ± 8c</td>
<td>64 ± 2cd</td>
<td>1.4 ± 0.1ab</td>
<td>17.6 ± 2.5bc</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3. dead LVS3 + Laminarin</td>
<td>83 ± 3abc</td>
<td>81 ± 3a</td>
<td>79 ± 3a</td>
<td>16 ± 5cd</td>
<td>1.5 ± 0.1ab</td>
<td>23.2 ± 0.7a</td>
<td></td>
</tr>
<tr>
<td>4. Dead LVS3 + Laminarin + VC (D3)</td>
<td>61 ± 3c</td>
<td>29 ± 3ef</td>
<td>13 ± 5f</td>
<td>1.3 ± 0.1abc</td>
<td>4.3 ± 1.3ce</td>
<td></td>
<td></td>
</tr>
<tr>
<td>5. Dead LVS3 + chitin</td>
<td>79 ± 4bcd</td>
<td>75 ± 4ab</td>
<td>73 ± 3abc</td>
<td>19 ± 10cd</td>
<td>1.5 ± 0.1ab</td>
<td>22.0 ± 9a</td>
<td></td>
</tr>
<tr>
<td>6. Dead LVS3 + chitin + VC (D3)</td>
<td>66 ± 5fg</td>
<td>21 ± 8f</td>
<td>16 ± 8f</td>
<td>1.4 ± 0.1ab</td>
<td>3.8 ± 2.1c</td>
<td></td>
<td></td>
</tr>
<tr>
<td>7. Dead LVS3 + glucan</td>
<td>83 ± 4abc</td>
<td>80 ± 4ab</td>
<td>74 ± 3abc</td>
<td>84 ± 10a</td>
<td>1.5 ± 0.1a</td>
<td>21.9 ± 0.8a</td>
<td></td>
</tr>
<tr>
<td>8. Dead LVS3 + glucan + VC (D3)</td>
<td>76 ± 3cde</td>
<td>71 ± 3abc</td>
<td>65 ± 7cd</td>
<td>1.4 ± 0.1b</td>
<td>16.6 ± 2.3c</td>
<td></td>
<td></td>
</tr>
<tr>
<td>9. Dead LVS3 + glucan + chitin</td>
<td>80 ± 4bcd</td>
<td>80 ± 4ab</td>
<td>74 ± 6</td>
<td>92 ± 6a</td>
<td>1.5 ± 0.1b</td>
<td>22.7 ± 1.2a</td>
<td></td>
</tr>
<tr>
<td>10. Dead LVS3 + glucan + chitin + VC (D3)</td>
<td>75 ± 4abc</td>
<td>69 ± 5bc</td>
<td>67 ± 3bcd</td>
<td>1.3 ± 0.1ab</td>
<td>16.8 ± 1.7bc</td>
<td></td>
<td></td>
</tr>
<tr>
<td>11. Dead LVS3 + Biorigin</td>
<td>85 ± 4ab</td>
<td>78 ± 3ab</td>
<td>71 ± 3abc</td>
<td>28 ± 9cd</td>
<td>1.5 ± 0.1a</td>
<td>21.6 ± 0.8a</td>
<td></td>
</tr>
<tr>
<td>12. Dead LVS3 + Biorigin + VC (D3)</td>
<td>73 ± 3def</td>
<td>40 ± 7de</td>
<td>20 ± 7ef</td>
<td>1.4 ± 0.1ab</td>
<td>5.5 ± 2.6c</td>
<td></td>
<td></td>
</tr>
<tr>
<td>13. Dead LVS3 + MacroGard</td>
<td>83 ± 3abc</td>
<td>74 ± 3ab</td>
<td>71 ± 3abc</td>
<td>68 ± 8b</td>
<td>1.5 ± 0.1b</td>
<td>21.7 ± 0.8a</td>
<td></td>
</tr>
<tr>
<td>14. Dead LVS3 + MacroGard + VC (D3)</td>
<td>73 ± 3def</td>
<td>61 ± 3f</td>
<td>49 ± 5d</td>
<td>1.5 ± 0.1ab</td>
<td>14.2 ± 1.4c</td>
<td></td>
<td></td>
</tr>
<tr>
<td>15. Dead LVS3 + Immunowall</td>
<td>81 ± 5abcd</td>
<td>71 ± 5abc</td>
<td>69 ± 4abc</td>
<td>41 ± 6a</td>
<td>1.5 ± 0.1b</td>
<td>20.4 ± 0.7ab</td>
<td></td>
</tr>
<tr>
<td>16. Dead LVS3 + Immunowall + VC (D3)</td>
<td>71 ± 5deg</td>
<td>43 ± 6d</td>
<td>26 ± 5e</td>
<td>1.3 ± 0.1ab</td>
<td>7.6 ± 1.3d</td>
<td></td>
<td></td>
</tr>
<tr>
<td>17. Dead LVS3</td>
<td>86 ± 3a</td>
<td>79 ± 3ab</td>
<td>74 ± 3ab</td>
<td>24 ± 4cd</td>
<td>1.4 ± 0.1ab</td>
<td>20.9 ± 1.1a</td>
<td></td>
</tr>
<tr>
<td>18. Dead LVS3 + VC (D3)</td>
<td>63 ± 3e</td>
<td>28 ± 3f</td>
<td>18 ± 3ef</td>
<td>1.3 ± 0.1b</td>
<td>4.5 ± 0.7ce</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

The challenge test was performed with *Vibrio campbellii* (VC) added on day 3 (D3). The first column in the table refers to the type of treatments (see Fig. 1). Each feed was tested in four replicates. Values are presented with the respective standard deviation (mean ± SD). Values in the same column showing the same superscript letter are not significantly different (*p* < 0.05).
regime, test animal [46] and developmental stage of the target organism. Furthermore, the biological effects of immunostimulants are highly dependent on the specificity of the receptors on the target cells recognising them as potential high-risk molecules and triggering defence pathways [47]. Some authors mentioned that a high level of β-1,3 glucan directly induced the respiratory burst, which after a period can exhaust the immune cells resulting in immunosuppression or feedback regulation [45,46,48,49]. Therefore, in the present study only small amounts of putative immunoenhancers were applied to Artemia likely not to over-stimulate the immune system. However, when the concentration of the applied β-glucans was increased (up to 3-fold) no changes were observed in the results.

Zymosan is one of the commercial β-glucans used in this study. It was described as a crude yeast cell-wall preparation of S. cerevisiae containing a relatively crude mixture of proteins, lipids and polysaccharides that was able to stimulate non-specific immunity [50,51]. We obtained a significant increased resistance against VC (high RPS value) when Artemia nauplii were supplemented with small amounts of Zymosan (Tables 3 and 4; lines 2 vs 18). Actually, the results are very similar to the results obtained by using β-glucan (Sigma) in this study. Although Zymosan is not a very pure β-glucan (only 84% of the sugars are made up of β-glucan; see Table 1), apparently the level and the type of β-glucan it contains is appropriate to upregulate defence responses of Artemia, as does β-glucan (Sigma). In crayfish, Zymosan can activate the prophenoloxidase (pro PO) system, which is considered to be an important component in the innate defence of arthropods [52]. Sung et al. [43] showed that Zymosan treatment in shrimp via immersion significantly increased anti-E. coli activity of plasma, as well as superoxide anion (O$_2^-$) and PO activity of shrimp haemocytes. These enhanced microbicidal reactions increased the clearance ability of haemolymph against the invasive pathogen Vibrion vulnificus [42].

MacroGard, a cell-wall extract from Saccharomyces cerevisiae, is another type of β-glucan tested in the gnotobiotic challenge test system. Administration of MacroGard by immersion has been shown to cause a transient increase in phenoloxidase enzyme activity and superoxide production (O$_2^-$) in P. monodon [42]. Similarly, immersion of shrimp post-larvae in a suspension of MacroGard enhanced growth performance, immune response, and disease resistance in black tiger shrimp [53]. In the present study, MacroGard improved Artemia survival, RPS and TBP, providing some level of pathogen resistance when challenged with VC (Tables 3 and 4; line 14 vs 18), although the results, for unknown reasons, were not as pronounced as with β-glucan (Sigma) or Zymosan.

Laminarin (a water-soluble beta-1,3-glucan derived from the brown algae Laminaria digitata), was the other type of commercial β-glucan tested in the present study. Although some investigations documented positive immunostimulatory effects of Laminarin, no beneficial effect was observed in this study (low RPS and low TBP in challenged nauplii) (Tables 3 and 4; lines 4 vs 18). The effects of Laminarin on the haemocytes of the freshwater crayfish, Astacus astacus, and the shore crab, Carcinus maenas, were studied in vitro and in vivo to determine the role of the PO activation system, in the cellular defence reactions of crustaceans [54]. In vitro, phagocytosis of the bacterium Moraxella sp. was significantly raised by addition of Laminarin. In vivo, injection of Laminaran (0.2 mg/ml haemolymph) into the haemocoele of A. astacus or C. maenas caused a rapid, marked reduction in the number of circulating haemocytes, indicating that a cellular defence reaction was initiated [54]. So, although Laminarin seems to have a proven in vitro and in vivo effect (the latter after injection into the host), there was no effect in this study. This might be due to the solubility of this compound, resulting in non-ingestion by Artemia, preventing exposure of this compound to cells responsible for the enhanced pathogen resistance. Sritunyalucksana et al. [55] assessed the effect of Laminarin in vitro by measuring PO, agglutinin and antibacterial activities in black tiger shrimp. Interestingly, their results showed a reduction in PO and antibacterial activities following Laminarin treatment. Furthermore, Muñoz et al. [56] reported no significant effect of using Laminarin on haemolymph PO activity in three clam species. In freshwater crayfish Astacus astacus, injections with Laminarin resulted in increased levels of prophenoloxidase mRNA in the haemocytes, whereas the levels of several other transcripts such as actin or the blood cell adhesion protein peroxinectin remained unchanged [57]. In standard infection experiments conducted with the fungal parasite Aphanomyces astaci, the accumulated mortality reached 50% within 4 days in the infected control crayfish, whereas the same mortality was reached after 9 days in the treated animals. In summary, the literature data and the results reported here suggest that the immunostimulatory effect of Laminarin is dependent on the experimental set-up and further experiments are needed to clarify under which conditions Laminarin can be beneficial to the host.

In the present study the application of two commercial β-glucans, namely Biorigin and Immunowall, had no favourable effect on Artemia nauplii challenged with pathogenic VC (Tables 3 and 4; lines 12 and 16 vs 18). Although the level of β-glucan in Biorigin is even higher than in MacroGard (see Table 1), for unknown reasons it could not contribute to pathogen resistance.
Immunomodulatory effects of chitin (chitin is a β-1,4-linked polymer of N-acetyl-D-glucosamine and a common constituent of insect, exoskeleton, crustacean shells and fungal cell walls [58]) and chitosan have been reported by many workers [59,60]. The present study showed no significant effect of chitin in the *Artemia* challenge test (Tables 3 and 4; lines 6 vs 18). Furthermore, the addition of chitin to the β-glucan treated animals (Sigma), could not further improve the level of pathogen resistance obtained by β-glucan (Sigma) itself. White shrimp, *Litopenaeus vannamei* injected with chitin at 6 μg/g, showed increased phagocytic activity and resistance against *Vibrio alginolyticus* infection [16]. It is known that many external (environmental) and internal factors may influence the effects of a particular immunostimulant on the fish immune system [61]. One such factor is the way by which the immunostimulant is administered. Different administration protocols (e.g. immersion, injection, oral) have produced different results, even with the same substance [62]. Therefore, it is suggested that ingestion of chitin is unlikely to be an appropriate way to induce the immune system in gnotobiotic *Artemia*.

In the present study, VC never had a significant negative impact on individual growth. However in combination with the survival values, total biomass production was always significantly lower compared to the respective controls (irrespective of the compound tested), indicating that VC still had an adverse effect on *Artemia*, even in the presence of a protective agent like glucan (Sigma) and Zymosan (Tables 3 and 4, treatments 1 vs 2, and 7 vs 8). We can only speculate on the mechanism involved. VC could possibly influence food conversion rate (FCR) for instance through a reduced resorption of nutrients or through an increased transit of the feed in the gastro-intestinal tract. Alternatively VC could affect the food uptake rate (FUR) resulting in poorer performance. A more targeted study would be needed to verify in what way total biomass production is affected even in the absence of a significant reduced survival.

The values in Table 1 indicate that the sugar concentration is different in the various compounds applied in this study. Also, the level of β-glucan within the sugar fraction varies between these products. Therefore, the total amount of β-glucans (dry weight) offered to *Artemia* was calculated (see Table 2). In fact, different amounts of β-glucans from these compounds were supplied to *Artemia*. Therefore, one could expect that the differences observed in this study could actually be due to different amounts of β-glucans (from different products) being supplied to *Artemia*. For instance, if Immunowall could not protect *Artemia* in the challenge test, it could be argued that this was due to the low β-glucan concentration in this product (offered to *Artemia*) in comparison to Zymosan or β-glucans (Sigma). However, when higher amounts of the products (up to 3-fold) were provided to *Artemia*, the results did not change. In addition, compared with Zymosan or β-glucan (Sigma), the higher amount of β-glucan present in Biorigin could not provide protection to challenged nauplii. Also, no correlation could be found between the chemical composition of the tested compounds and their protective effect in the challenged group. Therefore, it can be postulated that the induced protection against VC is not only dependent on the amount of β-glucan offered, but rather the quality (molecular weight, three dimensional structure, type and frequency of branches [44]) of the products must also be important. Interestingly, it was shown that the adsorption affinity of β-glucans to mycotoxins (e.g. Zearalenone (ZEN) produced by numerous *Fusarium* species) is highly dependent on the three-dimensional structure of these products [63].

5. Conclusion

This is the first time that these different commercial glucans and chitin particles have been tested simultaneously in a standardized *Artemia* challenge test. Because of the gnotobiotic conditions, this system can be used as a unique tool for testing anti-infectious properties of a specific compound with limited nutritional interference. Therefore, this challenge test, complemented with other tools such as measurement of immune parameters and gene expression analysis, can provide further documentation on the exact impact of putative immunostimulants on immunity and disease resistance in *Artemia*, probably offering further insight into the innate immune response in crustaceae.

Acknowledgments

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References


