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Influence of different yeast cell-wall mutants on performance and protection against pathogenic bacteria (*Vibrio campbellii*) in gnotobiotically-grown *Artemia*

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Abstract

A selection of isogenic yeast strains (with deletion for genes involved in cell-wall synthesis) was used to evaluate their nutritional and immunostimulatory characteristics for gnotobiotically-grown *Artemia*. In the first set of experiments the nutritional value of isogenic yeast strains (effected in mannoproteins, glucan, chitin and cell-wall bound protein synthesis) for gnotobiotically-grown *Artemia* was studied. Yeast cell-wall mutants were always better feed for *Artemia* than the isogenic wild type mainly because they supported a higher survival but not a stronger individual growth. The difference in *Artemia* performance between WT and mutants feeding was reduced when stationary-phase grown cells were used. These results suggest that any mutation affecting the yeast cell-wall make-up is sufficient to improve the digestibility in *Artemia*. The second set of experiments, investigates the use of a small amount of yeast cells in gnotobiotic *Artemia* to overcome pathogenicity of *Vibrio campbellii* (VC). Among all yeast cell strains used in this study, only mnn9 yeast (less cell-wall bound mannoproteins and more glucan and chitin) seems to completely protect *Artemia* against the pathogen. Incomplete protection against the pathogen was obtained by the gas1 and chs3 mutants, which are lacking the gene for a particular cell-wall protein and chitin synthesis, respectively, resulting in more glucan. The result with the chs3 mutant is of particular interest, as its nutritional value for *Artemia* is comparable to the wild type. Hence, only with the chs3 strain, in contrast to the gas1 or mnn9 strains, the temporary protection to VC is not concomitant with a better growth performance under non-challenged conditions, suggesting non-interference of general nutritional effects.

Keywords: *Artemia*; Gnotobiotic culture; *Saccharomyces cerevisiae*; Isogenic yeast mutants; Immune ability; *Vibrio campbellii*

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1. Introduction

Immunomodulation of larval fish has been proposed as a potential method for improving larval survival by increasing the innate responses of the developing animals until their adaptive immune response is sufficiently developed to increase an effective response to the pathogen [1].

Invertebrates are not equipped with cells that are analogous to antibody producing lymphocytes in vertebrates. 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 any specific diseases .Yet, a recent study in the copepod *Macrocyclops albidus* showed that the defence system of this invertebrate species reacted more efficiently after a previous encounter with an antigenically similar parasite, implying that a specific memory may exist [3]. Furthermore, exposure of shrimp to inactivated *Vibrio* spp. has been reported to provide some protection [4–6]. 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 [7]. Such substances may reduce the risk of disease outbreaks if administered prior to a situation known to result in stress and impaired general performance (e.g. handling stress, change of temperature or other environmental parameters, weaning from live to artificial feeds) or prior to an expected increase in exposure to pathogenic micro-organisms and parasites (e.g. spring and autumn blooms in marine environment, transfer to engrowing systems).

Several immunostimulants have been used in vertebrate and invertebrate culture, to induce protection against a wide range of diseases: i.e. β-glucans [8–11], chitin [12–14], mannoproteins [15], lipopolysaccharides [16], peptidoglycans [5,17] and dead bacteria [4,18,19].

Marques et al. [20,21] have recently developed and validated the usefulness of an *Artemia* gnotobiotic test system allowing to study the effect of food composition on survival and growth in the presence or absence of a pathogen. Baker's yeast *Saccharomyces cerevisiae*, which has been found to be a good immune enhancer in some aquatic organism, is an excellent source of β-glucans and chitin. These compounds together with mannoproteins constitute the major compounds of the yeast cell wall [22]. The present study aims to identify the critical cell-wall components that induce pathogen-protection in *Artemia*. The effect of isogenic yeast deletion mutants (eight strains), carrying a null mutation in a gene involved in cell-wall synthesis, was evaluated in a gnotobiotic *Artemia* test system. Firstly, *Artemia* performance was examined with the null-mutant yeast cells, harvested in exponential and/or stationary growth phase. In a second stage, these feed sources were tested in combination with a *Vibrio campbellii* challenge.

2. Methodology

2.1. Axenic culture of yeast

To verify the digestibility of live baker's yeast (*S. cerevisiae*) by *Artemia*, seven different null-mutants of yeast (isogenic deletion strains derived from baker's yeast strain BY4741) and the wild type strain (WT) (genotype described in Table 1) were fed to *Artemia*. All strains were provided by EUROSCARF (University of Frankfurt, Germany).

Yeast cultures were performed according to procedures previously described by Marques et al. [20], using minimal Yeast Nitrogen Base culture medium (YNB).

Yeasts were harvested by centrifugation (±800 × *g* for 10 min), either in the exponential growth phase (after 20 h; "exp.yeast") or in the stationary growth phase (after 3 days; "stat.yeast"). Yeast cell concentrations were determined with a Bürker haemocytometer. Yeast suspensions were stored at 4 °C until the end of each experiment (maximum storage of one week).

2.2. Bacterial strains and growth conditions

Two bacterial strains were selected, i.e. *Aeromonas hydrophila* strain LVS3 [23–25] for its positive effect on *Artemia* performance when fed sub-optimally and *Vibrio campbellii* strain LMG21363 (VC) for its pathogenic effect towards *Artemia* and shrimp [25–27]. The two bacterial strains were cultured and harvested according to procedures previously described by Marques et al. [25]. Pure cultures of the two bacterial strains were obtained from the Laboratory of Microbial Ecology and Technology, Gent University, and from the Laboratory of Microbiology,

Table 1
Genotype of all yeast strains used as feed for *Artemia* and description of each gene mutation in the development of cell-wall components

Strains	Genotype	Phenotype (cell-wall changes)	Reference
WT	BY4741; *Mat* a; *his* 3Δ1; *leu* 2Δ0; *met* 15Δ0; *ura* 3Δ0	Control yeast	Dallies et al. [43]; Klis et al. [35]; Magnelli et al. [22]; Marques et al. [20,21]
mnn9	BY4741; *Mat* a; *his* 3Δ1; *leu* 2Δ0; *met* 15Δ0; *ura* 3Δ0; YPL050c::kanMX4	Less mannan, higher chitin, higher β-glucans	Klis et al. [35]; Magnelli et al. [22]; Marques et al. [20,21]
mnn6	BY4741; *Mat* a; *his* 3Δ1; *leu* 2Δ0; *met* 15Δ0; *ura* 3Δ0; YPL053c::kanMX4	Less phosphomannan	Karson and Ballou [44]; Wang and Nakayama [45]; Jigami and Odani [34]
fks1	BY4741; *Mat* a; *his* 3Δ1; *leu* 2Δ0; *met* 15Δ0; *ura* 3Δ0; YLR342w::kanMX4	Less β-1,3 glucans, higher chitin	Dallies et al. [43]; Magnelli et al. [22]; Martin-Yken et al. [30]; Pagé et al. [46]; Aguilar-Uscanga and Francois [29]
knr4	BY4741; *Mat* a; *his* 3Δ1; *leu* 2Δ0; *met* 15Δ0; *ura* 3Δ0; YGR229c::kanMX4	Less β-1,3 glucans, higher chitin	Dallies et al. [43]; Magnelli et al. [22]; Martin-Yken et al. [30]; Pagé et al. [46]; Aguilar-Uscanga and Francois [29]
kre6	BY4741; *Mat* a; *his* 3Δ1; *leu* 2Δ0; *met* 15Δ0; *ura* 3Δ0; YPR159w::kanMX4	Less β-1,6 glucans, higher chitin	Magnelli et al. [22]; Aguilar-Uscanga and Francois [29]; Martin-Yken et al. [30]; Pagé et al. [46]
chs3	BY4741; *Mat* a; *his* 3Δ1; *leu* 2Δ0; *met* 15Δ0; *ura* 3Δ0; YBR023c::kanMX4	Less chitin	Valdivieso et al. [47]; Cabib et al. [31]; Klis et al. [35]; Magnelli et al. [22];
gas1	BY4741; *Mat* a; *his* 3Δ1; *leu* 2Δ0; *met* 15Δ0; *ura* 3Δ0; YMR307w::kanMX4	Less integration of yeast cell adhesion proteins into the cell wall less β-1,3 glucans, higher chitin	De Nobel et al. [38]; Popolo et al. [48]; Lipke and Ovalle [33]; Magnelli et al. [22]

Gent 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^{-1}, BD Biosciences) and agar bacteriological grade (20 g l^{-1}, ICN). For each bacterial strain a single colony was selected from the plate and incubated overnight at 28 °C in 50 ml Difco™ marine broth 2216 on a shaker (150 rpm). Stationary-grown bacteria were harvested by centrifugation (15 min; ±2200 × *g*); the supernatant were discarded and the pellet resuspended in 20 ml filtered autoclaved sea water (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^{-1}, according to McFarland standard (Biomerieux, Marcy l'Etoile, France).

Bacteria were resuspended in filtered autoclaved sea water (FASW) and their densities were determined by spectrophotometry (OD_{550}), assuming that an optical density of 1.000 corresponds to 1.2×10^9 cells ml^{-1}, according to McFarland standard (Biomerieux, Marcy l'Etoile, France).

At day 3, challenge tests were performed with live VC. For that purpose, in a laminar flow hood, the pathogen was provided to each replicate at a density of 5×10^6 cells ml^{-1}. Dead LVS3 was provided to *Artemia* using aliquots of autoclaved concentrated bacteria (autoclaving at 120 °C for 20 min). After autoclaving, bacteria were plated to check if they were effectively killed by this method. For this purpose, 100 μl of the culture medium were transferred to marine agar (MA; $n = 3$), containing Difco™ marine broth 2216 (BD Biosciences, 3.74% w/v) and agar bacteriological grade (ICN, 2% w/v). Absence of bacterial growth was monitored after incubating plates for 5 days at 28 °C. Autoclaving treatment was 100% effective, since no bacterial growth was observed on the MA after 5 days of incubation. Dead and live bacterial suspensions were stored at 4°C until the end of each experiment.

2.3. Yeast and bacterial ash-free content

To determine the yeast and bacterial ash-free dry weight (AFDW), 50 ml of each culture sample were filtered on pre-dried filters (pore size 0.45 μm, two replicate per culture). Filters were subsequently dried at 60 °C for 24 h and weighed. Afterwards they were combusted at 600 °C for 6 h to determine the ash content. The AFDW was calculated as the difference between dry weight and ash weight. The DW and AFDW of control (filter only, $n = 2$) were subtracted from all samples. The AFDW of the yeast strains and the bacteria is presented in Table 2.

Table 2
Average ash-free dry weight (AFDW) of seven different null-mutants of yeast (isogenic strains derived from BY 4741) and the wild type strain (WT) harvested in the exponential and stationary growth phase, together with AFDW of dead LVS3 and live VC bacteria expressed in mg/10^9 cells

Strains	AFDW (mg/10^9 cells)		AFDW (mg/Falcon tube)		p-value
	Exponential phase	Stationary phase	Exponential phase	Stationary phase	Exponential vs stationary phase AFDW (mg/Falcon tube)
WT	15.24 ± 0.18^{f}	13.69 ± 0.07^{d}	1.60 ± 0.02^{e}	1.44 ± 0.01^{de}	0.014
mnn9	54.67 ± 1.66^{a}	36.40 ± 7.23^{a}	5.74 ± 0.17^{a}	3.82 ± 0.75^{a}	0.161
mnn6	17.09 ± 0.37^{e}	11.83 ± 0.10^{de}	1.79 ± 0.04^{d}	1.24 ± 0.01^{e}	0.013
fks1	18.90 ± 1.41^{d}	17.73 ± 0.28^{c}	1.98 ± 0.13^{d}	1.86 ± 0.0^{cd}	0.644
knr4	14.77 ± 0.26^{f}	13.17 ± 0.13^{d}	1.55 ± 0.03^{e}	1.38 ± 0.10^{de}	0.037
kre6	34.54 ± 1.41^{b}	24.52 ± 1.25^{b}	3.63 ± 0.28^{b}	2.57 ± 0.13^{b}	0.076
chs3	16.4 ± 0.12^{e}	11.0 ± 0.40^{e}	1.72 ± 0.01^{d}	1.15 ± 0.04^{e}	0.020
gas1	29.09 ± 0.86^{c}	20.30 ± 2.60^{bc}	13.05 ± 0.09^{c}	2.13 ± 0.28^{bc}	0.1
Live LVS3	–	0.2186 ± 0.02^{f}	–	0.023 ± 0.01^{f}	–
Dead LVS3	–	0.2725 ± 0.02^{f}	–	0.029 ± 0.01^{f}	–
Live VC	–	0.1134 ± 0.01^{f}	–	0.034 ± 0.01^{f}	–

Values of AFDW are presented with the respective standard deviation (mean ± SD). Values in the same column showing the same superscript letter are not significantly different ($p_{\text{Tukey}} > 0.05$). p-values obtained for direct comparison of AFDW (mg/Falcon tube) of different yeast cell strains, harvested in exponential and stationary growth phase were included. Significant differences were obtained when $p_{\text{Tukey}} < 0.05$.

2.4. *Artemia gnotobiotic culture*

Experiments were performed with *Artemia franciscana* cysts, originating from Great Salt Lake, Utah, USA (EG ® type, INVE Aquaculture, Belgium). Bacteria-free cysts and nauplii were obtained using the procedure described by Marques et al. [20]. After hatching, 20 nauplii (Instar II) were picked and transferred to Falcon tubes containing 30 ml of FASW together with the amount of feed scheduled for day 1. Feeding rates were intended to provide *ad libitum* ratios but avoiding excessive feeding in order not to affect the water quality in the test tubes, except in Experiments 4 and 5 (treatments 19 and 20) where nauplii were overfed (5.74 mg AFDW/FT) (Table 5, feeding regime: (d) in order to verify the effect of overfeeding. Each treatment consisted of four Falcon tubes (replicates). Falcon tubes were placed on a rotating rod at 4 cycles per min, exposed to constant incandescent light (± 41 μEm^{-2}) at 28 °C. Tubes were being transferred to the laminar flow just once per day for feeding.

2.5. *Method used to verify axenity*

Axenity of feed, decapsulated cysts and *Artemia* cultures were checked at the end of each experiment using a combination of plating (MA) and live counting (using tetrazolium salt MTT staining following the procedure described by Marques et al. [20,21]. 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.

2.6. *Experimental design*

In Experiment 1, all live and axenic yeast strains (WT and seven null mutants) were harvested in the exponential growth phase and used as feed for the *Artemia*.

In Experiment 2, stationary-grown live and axenic yeast strains (the same strains as used in Exp. 1) were used as feed for nauplii. In both experiments, a modified feeding schedule was adopted from Coutteau et al. [28] and Marques et al. [20]. The feeding schedule resulted in an equal amount of yeast-cell particles per treatment being offered to *Artemia*. Both experiments were performed twice (A and B), to verify the reproducibility of the results.

In Experiment 3, an equal amount of feed was provided to *Artemia* (Table 5). As the AFDW per cell of the yeast mutants is different (see Table 2), this resulted in different amount of yeast cells being offered. Each feed was tested in four replicates.

In Experiments 4 and 5, all treatments were fed with an equal amount of yeast (in terms of AFDW). Yeast strains (in exponential and/or stationary growth phase) were provided daily in small but equal amounts, in combination with dead LVS3 (as a major part of the feed) to *Artemia* (Table 5 – feeding regime for Exps. 4 and 5). As a control, *Artemia* was fed only dead LVS3 (Table 5 – feeding regime: (c). Challenge tests were performed with live VC at a density of 5×10^6 cells ml^{-1} added at day 3.

2.7. Survival and growth of Artemia

Survival and growth of *Artemia* nauplii were determined according to procedures described by Marques et al. [20,21]. At the end of Experiments 1, 2 and 3 (day 6 after hatching) the number of swimming larvae was determined and survival percentage was calculated. Living larvae were fixed with Lugol's solution to measure their individual length (growth calculation), using a dissecting microscope equipped with a drawing mirror, a digital plan measure and the software Artemia 1.0® (Marnix Van Domme). In order to integrate the results of survival and growth, the criterion "total length" was introduced, i.e. total millimeters of *Artemia* per Falcon tube or mm/FT = number of survivors × mean individual length.

In Experiments 4 and 5 the survival percentage for each treatment was determined daily. 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 preserve the axenity.

Values of larval survival (percentage) were arcsin transformed, while values of individual length and total length were logarithmic or square root transformed to satisfy normal distribution and homocedasticity requirements. Differences on survival, individual length and total length of *Artemia* fed with different feeds, were studied with analysis of variances (ANOVA) and multiple comparisons of Tukey's range, tested at 0.05 level of probability, using the software Spss 11.5 for Windows.

3. Results

3.1. Artemia performance fed live yeast cells

Artemia nauplii were fed with seven different isogenic mutant strains of baker's yeast (*Saccharomyces cerevisiae*) (Table 1) and compared with nauplii fed wild type yeast under gnotobiotic condition. In all cases equal amounts of yeast cells were offered. The results presented in Tables 3 and 4 (results obtained in Exps. 1 and 2) show that independently of the growth stage, the yeast genetic background has a big influence on *Artemia* performance. Compared with WT yeast, total biomass production of nauplii was significantly improved when the exp-grown isogenic yeast mutant strains were used as feed, due to both significant higher survival and/or individual length (Table 3). Among them, the mnn9 yeast strain supported the best nauplii performance.

Table 3
Experiment 1: average survival (%), individual length (mm) and total length (mm per Falcon tube-FT) of *Artemia* nauplii fed with live yeast cells (harvested in exponential growth phase) after 5 days: effect of growth stage and genetic background

Strains	A			B		
	Survival (%)	Individual length (mm)	Total length (mm/FT)	Survival (%)	Individual length (mm)	Total length (mm/FT)
WT	32 ± 6^{c}	1.3 ± 0.1^{f}	8.6 ± 1.7^{d}	29 ± 7^{c}	2.2 ± 0.1^{cd}	12.5 ± 3.3^{d}
mnn9	87 ± 9^{a}	4.0 ± 0.4^{a}	70.7 ± 7.7^{a}	87 ± 6^{a}	3.9 ± 0.2^{a}	68.7 ± 5.0^{a}
mnn6	64 ± 7^{bc}	1.8 ± 0.1^{de}	23.5 ± 2.6^{c}	52 ± 6^{bc}	1.8 ± 0.2^{d}	19.0 ± 2.3^{cd}
fks1	55 ± 10^{b}	2.1 ± 0.1^{c}	23.6 ± 4.6^{c}	50 ± 12^{bc}	2.2 ± 0.3^{cd}	20.0 ± 9.0^{cd}
gas1	61 ± 5^{b}	3.2 ± 0.2^{b}	39.0 ± 3.0^{b}	64 ± 5^{b}	3.3 ± 0.1^{b}	42.0 ± 3.0^{b}
knr4	62 ± 6^{b}	2.0 ± 0.2^{cd}	25.0 ± 3.4^{c}	50 ± 12^{bc}	2.3 ± 0.1^{c}	23.0 ± 5.6^{cd}
kre6	45 ± 4^{bc}	2.0 ± 0.1^{cd}	18 ± 1.6^{c}	58 ± 13^{b}	2.5 ± 0.1^{c}	29.0 ± 6.0^{c}
chs3	55 ± 5^{b}	1.7 ± 0.2^{e}	18.5 ± 2.0^{c}	42 ± 5^{bc}	2.0 ± 0.2^{cd}	17.0 ± 2.0^{cd}

Means were put together with the standard deviation (mean ± SD). Each experiment was repeated twice A and B. Each feed was tested in four replicates. Values in the same column showing the same superscript letters are not significantly different ($p_{Tukey} > 0.05$).

Table 4
Experiment 2: average survival (%), individual length (mm) and total length (mm per Falcon tube-FT) of *Artemia* nauplii fed with live yeast cells (harvested in the stationary growth phase) after 5 days: effect of growth stage and genetic background

Strains	A			B		
	Survival (%)	Individual length (mm)	Total length (mm/FT)	Survival (%)	Individual length (mm)	Total length (mm/FT)
WT	15 ± 7^{d}	1.75 ± 0.2^{bcd}	5.2 ± 2.5^{c}	25 ± 4^{c}	1.7 ± 0.2^{bc}	8.5 ± 1.4^{c}
mnn9	68 ± 5^{a}	3.3 ± 0.2^{a}	45.6 ± 3.2^{a}	71 ± 4^{a}	2.8 ± 0.1^{a}	40.0 ± 2.7^{a}
mnn6	40 ± 4^{bc}	1.8 ± 0.1^{bc}	14.4 ± 1.5^{b}	38 ± 8^{bc}	1.3 ± 0.1^{d}	9.8 ± 1.9^{bc}
fks1	18 ± 3^{d}	1.7 ± 0.2^{bcd}	6.6 ± 0.9^{c}	28 ± 13^{bc}	1.6 ± 0.2^{bc}	9.4 ± 1.9^{bc}
gas1	35 ± 4^{bc}	2.0 ± 0.3^{b}	14.3 ± 1.7^{b}	40 ± 7^{b}	1.8 ± 0.1^{b}	14.9 ± 2.6^{b}
knr4	48 ± 5^{b}	1.3 ± 0.2^{d}	13.2 ± 1.3^{b}	35 ± 4^{bc}	1.5 ± 0.1^{cd}	10.4 ± 1.2^{bc}
kre6	40 ± 13^{bc}	1.6 ± 0.1^{cd}	12.6 ± 4.0^{b}	32 ± 15^{b}	1.6 ± 0.2^{bcd}	12.2 ± 2^{bc}
chs3	29 ± 8^{cd}	1.7 ± 0.2^{bcd}	9.7 ± 2.9^{bc}	25 ± 6^{bc}	1.9 ± 0.1^{b}	9.3 ± 2.2^{bc}

Means were put together with the standard deviation (mean ± SD). Each experiment was repeated twice A and B. Each feed was tested in four replicates. Values in the same column showing the same superscript letters are not significantly different ($p_{Tukey} > 0.05$).

Also the use of the mnn6 mutant resulted in a significantly improved total biomass production of *Artemia*. In this treatment a higher biomass production was obtained due to a considerable increase in survival. Using knr4 and fks1 (less β-1,3 glucans) and kre6 (less β-1,6 glucans) as feed resulted in less *Artemia* biomass production compared to the mnn9 yeast strain but significantly more *Artemia* biomass production compared to the WT yeast. The chitin-defective yeast strain (chs3) supported a small increase in *Artemia* biomass production compared to the WT-treatment, mainly due to better nauplii survival. Finally, using the gas1 mutant as food (this strain is lacking an important cell-wall protein involved in cross-linking the major cell-wall components) resulted in better nauplii performance compared to WT yeast.

Higher total biomass (except for fks1 fed *Artemia*) production (compared to WT) in *Artemia* fed mutant stat-grown yeast cells were mainly due to higher nauplii survival rather than stronger individual growth. Only with stat-grown mnn9 cells higher survival and stronger individual growth contributed to more biomass production. When exp-yeast cells were fed to the nauplii, significant higher total *Artemia* biomass production (mostly due to higher nauplii survival) values were observed in all cases compared with stat-yeast cells possibly because the AFDW of yeast cells in the exponential growth phase was higher than in the stationary growth phase (Table 2) (although not significantly different in the mnn9, gas1 and fks1 yeast strains).

In the experiments described above equal amounts of yeast cell particles (Table 5 – feeding regime for Exp. 3) were supplied as food, resulting, however, in different amounts of AFDW of food offered to *Artemia* (see Table 2). This could have been the reason for the considerable higher *Artemia* biomass production e.g. with mnn9 yeast. Hence, the feeding experiment was repeated, this time offering equal amounts of food expressed as AFDW. In all cases, feeding mutant-yeast cells resulted in better nauplii survival than feeding WT yeast. Also in this experiment, the mnn9-fed *Artemia* presented the highest biomass production (Table 6). *Artemia* biomass production was equal after feeding WT,

Table 5
Feeding regimes in the 3 experiments (Exp.) performed. Daily and average total ash-free dry weight (AFDW), expressed in μg/FT of yeast cells and dead bacteria (LVS3) supplied to *Artemia* in Experiments 3, 4 and 5

	Feeding regime										Total AFDW (μg/FT) offered
	Day1		Day2		Day3		Day4		Day5		
	Y	DB	Y	DB	Y	DB	Y	DB	Y	DB	
Exp. 3	124	0	248	0	248	0	324	0	496	0	1440
Exps. 4 and 5											
(a)	25	221	50	442	50	442	62	592	100	886	2870
(b)	50	197	100	394	100	394	124	525	200	786	2870
(c)	0	246	0	492	0	492	0	656	0	984	2870
(d)	0	492	0	984	0	984	0	1312	0	1968	5740

Challenge tests were performed with live *Vibrio campbellii* (VC) at a density of 5×10^{6} cells ml^{-1} added at day 3 in Experiments 4 and 5.
(a) Dead LVS3 + yeast 5%; (b) Dead LVS3 + yeast 10%; (c) The treatment dead LVS3 X; and (d) The treatment: dead LVS3 2X.
X = the total amount of feed offered (2870 μg AFDW/FT); Y = yeast (wild type and isogenic yeast mutants added at 5% or 10%); DB = dead bacterium LVS3.

Table 6
Experiment 3: average survival (%), individual length (mm) and total length (mm/FT) of *Artemia* nauplii fed with live yeast cells (harvested in the stationary growth phase) for 5 days: effect of genetic background

Strains	Survival (%)	Individual length (mm)	Total length (mm/FT)
WT	18 ± 6^{c}	1.74 ± 0.1^{e}	6.1 ± 2.2^{d}
mnn9	63 ± 6^{a}	2.44 ± 0.1^{a}	30.6 ± 3.2^{a}
mnn6	35 ± 9^{b}	1.8 ± 0.1^{e}	12.6 ± 3.3^{bc}
fks1	28 ± 7^{bc}	1.67 ± 0.1^{e}	9.2 ± 2.2^{cd}
gas1	38 ± 6^{b}	2.11 ± 0.1^{b}	15.8 ± 2.7^{b}
knr4	43 ± 6^{b}	1.53 ± 0.1^{d}	13.0 ± 2.0^{bc}
kre6	33 ± 7^{b}	1.33 ± 0.1^{e}	8.6 ± 1.7^{cd}
chs3	33 ± 6^{b}	1.81 ± 0.1^{c}	11.8 ± 2.3^{bcd}

All treatments were fed with an equal amount of feed corresponding to an AFDW (1.44 ± 0.01 mg/FT) (see Table 5 – Exp. 3) (for instance fed with WT-YNB yeast cells). Means were put together with the standard deviation (mean ± SD). Each feed was tested in four replicates. Values in the same column showing the same superscript letters are not significantly different ($p_{Tukey} > 0.05$).

fks1, kre6 and chs3 cells although the mutants (kre6 and knr4) display, respectively, a higher or equal AFDW as compared with WT yeast.

3.2. Protection effect by bacteria (LVS3) and yeast

The effect of feeding two different amounts of dead bacteria (autoclaved LVS3) to the nauplii (either challenged or not with a live pathogen) is presented in Table 7 (Exp. 4). Unchallenged, there is no effect on survival in the two feeding regime. Yet, nauplii fed with dead LVS3 (5.74 mg AFDW/FT) (Table 5, feeding regime: (d) yielded a significantly higher survival in the challenge test as compared to the nauplii fed with only half of this amount (Table 7, lines 20 and 22). The results of supplying a low amount of WT or isogenic yeast mutants to the nauplii fed dead LVS3 are presented in Tables 7 and 8. Most of the non-challenged nauplii fed solely with dead LVS3 or both dead LVS3 and yeast cells survived until day 6 (68 % or higher). In most treatments, challenged nauplii fed only with dead LVS3 or both dead LVS3 and yeast cells (WT, mnn6, fks1, knr4, kre6) died before day 5. Yet, exceptions occurred when the nauplii were fed both dead LVS3 and mnn9, gas1 and chs3 (only exp-yeast) yeast strains. Only mmn9 yeast cells can protect nauplii against the live pathogen until day 6 (Tables 7 and 8, line 4). However, the addition of 5% of the total AFDW in the form of mnn9 yeast cells was not sufficient to keep the nauplii alive until day 6 (Tables 7 and 8, line 6). In all yeast strains, the exp-yeasts provided a better protection against the pathogen than stat-yeasts, but in most cases, this protection lasted only for a short period (one day after challenge). The mnn9 cells supported a high protection until the end of the experiments against the VC pathogen when offered as exp-grown or stat-grown cells (Tables 7 and 8, line 4).

4. Discussion

Marques et al. [20,21] have shown that yeast digestion by *Artemia* can be significantly improved by manipulating the genetic characteristics of the yeast, the growth stage and the medium used. This study confirms that the genetic background of the yeast strain used, has a strong influence on the *Artemia* performance (Exps. 1, 2 and 3).

In this study, a yeast strain containing low concentrations of mannoproteins in the cell wall, such as the mnn9 mutant, always supported a high *Artemia* biomass production (i.e. best nauplii growth as well as highest survival rate). According to Coutteau et al. [28], β-glucanase activity is detected in the digestive tract of *Artemia* but no mannase activity, making the external mannoprotein layer of the yeast cell wall probably the major barrier for the digestion of the yeast cell by the *Artemia* nauplii. Therefore, it is likely that the digestive enzymes of *Artemia* (such as β-glucanase) can easily enter and provide suitable digestion of yeast cells with reduced mannoprotein content.

An improved *Artemia* performance was also obtained with yeast mutants with reduced β-glucans (fks1, knr4, kre6 and gas1) and chitin (chs3) levels as compared to the nauplii fed WT-yeast cells (especially with exp-grown yeast cells as food). According to Aguilar-Uscanga and Francois [29], β-1,3 glucans and especially β-1,6 glucans provide anchorage to most cell-wall mannoproteins and are also covalently linked with chitin, contributing to the modular structure of the cell wall. β-1,3 glucans also contribute to the rigidity and integrity of the cell wall, and determine the cell

Table 7
Experiment 4: mean daily survival (%) of *Artemia* fed daily with dead LVS3 and yeast cell strains harvested in the exponential growth phase

TN		A – survival (%)					B – survival (%)				
		Day 2	Day 3	Day 4	Day 5	Day 6	Day 2	Day 3	Day 4	Day 5	Day 6
1	Dead LVS3 + WT 10%	93 ± 3^a	86 ± 3^a	81 ± 5^a	74 ± 4^a	68 ± 3^a	91 ± 3^a	88 ± 3^a	83 ± 3^a	73 ± 3^a	66 ± 3^a
2	Dead LVS3 + WT 10% + VC D3	91 ± 3^a	88 ± 3^a	56 ± 5^b	0^b	0^b	88 ± 3^a	86 ± 3^a	55 ± 4^b	0^b	0^b
3	Dead LVS3 + mnn9 10%	98 ± 3^a	94 ± 3^a	90 ± 4^a	86 ± 3^a	83 ± 3^a	96 ± 3^a	93 ± 3^a	91 ± 3^a	84 ± 3^a	80 ± 4
4	Dead LVS3 + mnn9 10% + VC D3	96 ± 3^a	93 ± 3^a	86 ± 3^a	80 ± 4^a	76 ± 3^a	98 ± 3^a	93 ± 3^a	88 ± 3^a	81 ± 5^a	76 ± 3^a
5	Dead LVS3 + mnn9 5%	98 ± 3^a	93 ± 3^a	86 ± 3^a	80 ± 4^a	75 ± 4^a	98 ± 3^a	93 ± 3^a	88 ± 3^a	84 ± 3^a	76 ± 3^a
6	Dead LVS3 + mnn9 5% + VC D3	99 ± 3^a	89 ± 3^a	74 ± 3^a	31 ± 5^b	0^b	98 ± 3^a	91 ± 3^a	81 ± 3^a	35 ± 4^b	0^b
7	Dead LVS3 + mnn6 10%	93 ± 3^a	89 ± 3^a	83 ± 5^a	79 ± 3^a	73 ± 3^a	91 ± 3^a	89 ± 3^a	81 ± 3^a	78 ± 3	70 ± 4^a
8	Dead LVS3 + mnn6 10% + VC D3	91 ± 3^a	88 ± 3^a	66 ± 5^b	0^b	0^b	96 ± 3^a	86 ± 3^a	70 ± 4^b	0^b	0^b
9	Dead LVS3 + fks1 10%	93 ± 3^a	86 ± 3^a	83 ± 3^a	79 ± 3^a	70 ± 4^a	93 ± 3^a	86 ± 3^a	81 ± 3^a	78 ± 3^a	73 ± 3^a
10	Dead LVS3 + fks1 10% + VC D3	93 ± 3^a	88 ± 3^a	64 ± 5^b	0^b	0^b	91 ± 3^a	88 ± 3^a	66 ± 5^b	0^b	0^b
11	Dead LVS3 + knr4 10%	94 ± 3^a	88 ± 3^a	79 ± 3^a	78 ± 4^a	73 ± 3^a	93 ± 3^a	86 ± 3^a	81 ± 3^a	75 ± 4^a	69 ± 3^a
12	Dead LVS3 + knr4 10% + VC D3	93 ± 3^a	86 ± 3^a	63 ± 3^b	0^b	0^b	93 ± 3^a	88 ± 3^a	66 ± 3^b	0^b	0^b
13	Dead LVS3 + kre6 10%	94 ± 3^a	88 ± 3^a	80 ± 4^a	75 ± 4^a	71 ± 3^a	93 ± 3^a	88 ± 3^a	83 ± 3^a	78 ± 3^a	70 ± 4^a
14	Dead LVS3 + kre6 10% + VC D3	94 ± 3^a	88 ± 3^a	60 ± 4^b	0^b	0^b	93 ± 3^a	86 ± 3^a	65 ± 4^b	0^b	0^b
15	Dead LVS3 + gas1 10%	93 ± 3^a	88 ± 3^a	84 ± 3^a	81 ± 3^a	73 ± 3^a	94 ± 3^a	89 ± 3^a	86 ± 3^a	78 ± 3^a	71 ± 5^a
16	Dead LVS3 + gas1 10% + VC D3	94 ± 3^a	86 ± 3^a	50 ± 4^b	36 ± 4^b	0^b	93 ± 3^a	88 ± 3^a	53 ± 6^b	33 ± 6^b	0^b
17	Dead LVS3 + chs3 10%	94 ± 3^a	86 ± 3^a	79 ± 5^a	79 ± 5^a	75 ± 3^a	96 ± 3^a	88 ± 3^a	81 ± 5^a	75 ± 4^a	74 ± 3^a
18	Dead LVS3 + chs3 10% + VC D3	94 ± 3^a	88 ± 3^a	75 ± 6^a	34 ± 5^b	0^b	98 ± 3^a	86 ± 3^a	61 ± 5^a	28 ± 3^b	0^b
19	Dead LVS3(2X)	98 ± 3^a	93 ± 3^a	84 ± 3^a	78 ± 3^a	73 ± 3^a	98 ± 3	89 ± 3^a	85 ± 4^a	78 ± 3^a	74 ± 3^a
20	Dead LVS3(2X) + VC D3	98 ± 3^a	86 ± 3^a	77 ± 3^a	53 ± 3^b	44 ± 3^b	99 ± 3^a	86 ± 3^a	78 ± 3^a	65 ± 4^b	51 ± 3^b
21	Dead LVS3 (X)	98 ± 3^a	90 ± 3^a	83 ± 3^a	76 ± 3^a	69 ± 5^a	96 ± 3^a	91 ± 3^a	83 ± 3^a	75 ± 46	8 ± 3
22	Dead LVS3 (X) + VC D3	96 ± 3^a	89 ± 3^a	56 ± 5^b	0^b	0^b	98 ± 3^a	93 ± 3^a	60 ± 4^b	0^b	0^b

The yeast cells constituted either 574 ± 0.2 µg/FT or 287 ± 0.2 µg/FT of the total AFDW supplied. The challenged test was performed with *Vibrio campbellii* (VC) added at day 3. Each experiment was repeated twice: A and B. Each feed was tested in four replicates. The total amount of feed offered is equal to 2870 µg AFDW/FT. Means were put together with the standard deviation (mean ± SD). Survival in the challenge test was compared directly to the survival of non-challenged *Artemia*. Values showing the same superscript letter are not significantly different ($p > 0.05$).

shape [30]. As a consequence, a lack of β-glucans in the yeast cell wall might result in less covalent linkage between the three cell-wall compounds, resulting in a more permeable and digestible cell wall in comparison to the WT strain. Although in a WT-yeast strain chitin concentration makes up only 1–2% of the cell-wall dry mass [22], it plays a key role in yeast cell growth and division and is attached covalently to β-1,3 glucans, β-1,6 glucans and mannoproteins [31]. Therefore, the better results obtained with nauplii fed chitin-defective yeast could also be due to an enhanced digestibility of chs3 cells by *Artemia*, caused by reduced linkage between the three cell-wall components.

In the gas1 yeast mutant, the production of the glycosylphosphatidylinositol (GPI) anchored protein is inhibited resulting in a non-proper fiber assembly of the cell wall (defective architecture) as well as in reduced β-glucans [32,33]. This apparently results in an eventual digestibility of gas1 cells by *Artemia*. Compared to WT-fed nauplii, yeast strains with reduced phosphomannan levels in the cell wall (mnn6) always gave higher *Artemia* performance mainly due to a higher nauplii survival. This fact could be due to interference of phosphomannans in phosphodiester cross-linking of mannoproteins to β-glucans [34]. In the experiments shown in Tables 3 and 4, equal amounts of yeast cell particles (feeding schedule of Exps. 1 and 2) are offered. This results in different amounts of AFDW being supplied (Table 2). We therefore, in a further experiment, supplied exactly equal amount of AFDW (see Table 5 – feeding regime for Exp. 3) of the different yeast cells to *Artemia* (Table 6). Consequently, in these experiments, the feed

Table 8
Experiment 5: mean daily survival (%) of *Artemia* fed daily with dead LVS3 and yeast cell strains harvested in the stationary growth phase

TN		A – survival (%)					B – survival (%)				
		Day 2	Day 3	Day 4	Day 5	Day 6	Day 2	Day 3	Day 4	Day 5	Day 6
1	Dead LVS3 + WT 10%	93 ± 3^a	88 ± 3^a	79 ± 5^a	75 ± 3^a	65 ± 4^a	91 ± 3^a	88 ± 3^a	76 ± 3^a	73 ± 3^a	6 ± 5^a
2	Dead LVS3 + WT 10% + VC D3	93 ± 3^a	89 ± 3^a	46 ± 5^b	0^b	0^b	94 ± 3^a	89 ± 3^a	45 ± 6^b	0^b	0^b
3	Dead LVS3 + mnn9 10%	99 ± 3^a	96 ± 3^a	91 ± 3^a	86 ± 3^a	83 ± 3^a	98 ± 3^a	94 ± 3^a	89 ± 5^a	86 ± 3^a	80 ± 4^a
4	Dead LVS3 + mnn9 10% + VC D3	98 ± 3^a	95 ± 3^a	86 ± 3^a	74 ± 3^a	69 ± 3^a	98 ± 3^a	93 ± 3^a	86 ± 3^a	73 ± 3^a	69 ± 3^a
5	Dead LVS3 + mnn9 5%	94 ± 3^a	89 ± 3^a	81 ± 5^a	75 ± 4^a	73 ± 3^a	98 ± 3^a	91 ± 3^a	80 ± 6^a	75 ± 4^a	71 ± 3^a
6	Dead LVS3 + mnn9 5% + VC D3	91 ± 3^a	88 ± 3^a	61 ± 5^a	23 ± 3^b	0^b	96 ± 3^a	90 ± 4^a	64 ± 3^b	25 ± 4^b	0^b
7	Dead LVS3 + mnn6 10%	99 ± 3^a	91 ± 3^a	84 ± 3^a	78 ± 3^a	70 ± 4^a	99 ± 3^a	93 ± 3^a	83 ± 3^a	76 ± 5^a	69 ± 5^a
8	Dead LVS3 + mnn6 10% + VC D3	98 ± 3^a	90 ± 4^a	43 ± 3^b	0^b	0^b	99 ± 3^a	91 ± 3^a	45 ± 4^b	0^b	0^b
9	Dead LVS3 + fks1 10%	98 ± 3^a	91 ± 3^a	81 ± 3^a	76 ± 3^a	68 ± 3^a	98 ± 3^a	91 ± 3^a	84 ± 3^a	75 ± 4^a	69 ± 3^a
10	Dead LVS3 + fks1 10% + VC D3	96 ± 3^a	90 ± 4^a	41 ± 5^a	0^b	0^b	96 ± 3^a	90 ± 4^a	39 ± 5^b	0^b	0^b
11	Dead LVS3 + knr4 10%	95 ± 3^a	89 ± 3^a	85 ± 4^a	78 ± 3^a	71 ± 5^a	96 ± 3^a	91 ± 3^a	84 ± 3^a	76 ± 5^a	74 ± 3^a
12	Dead LVS3 + knr4 10% + VC D3	99 ± 3^a	88 ± 3^a	46 ± 5^b	0^b	0^b	98 ± 3^a	93 ± 3^a	50 ± 4^b	0^b	0^b
13	Dead LVS3 + kre6 10%	98 ± 3^a	84 ± 3^a	79 ± 3^a	78 ± 3^a	69 ± 5^a	96 ± 3^a	88 ± 3^a	78 ± 3^a	79 ± 3^a	71 ± 5^a
14	Dead LVS3 + kre6 10% + VC D3	96 ± 3^a	86 ± 3^a	41 ± 5^b	0^b	0^b	98 ± 3^a	86 ± 3^a	38 ± 3^b	0^b	0^b
15	Dead LVS3 + gas1 10%	91 ± 3^a	88 ± 3^a	84 ± 3	73 ± 3^a	69 ± 3^a	93 ± 3^a	89 ± 3^a	83 ± 3^a	74 ± 5^a	71 ± 3^a
16	Dead LVS3 + gas1 10% + VC D3	90 ± 3^a	89 ± 3^a	43 ± 3^b	25 ± 4^b	0^b	93 ± 3^a	86 ± 3^a	46 ± 3^b	28 ± 3^b	0^b
17	Dead LVS3 + chs3 10%	98 ± 3^a	88 ± 3^a	83 ± 3^a	75 ± 4^a	68 ± 3^a	96 ± 3^a	89 ± 5^a	84 ± 3^a	78 ± 3^a	70 ± 4^a
18	Dead LVS3 + chs3 10% + VC D3	98 ± 3^a	89 ± 3^a	68 ± 3^b	0^b	0^b	98 ± 3^a	88 ± 3^a	66 ± 5^b	0^b	0^b

The yeast cells constituted either 5% or 10% of the total AFDW supplied. The challenged test was performed with *Vibrio campbellii* (VC) added at day 3. Each experiment was repeated twice: A and B. Each feed was tested in four replicates. Means were put together with the standard deviation (mean ± SD). Survival in the challenge test was compared directly to the survival of non-challenged *Artemia*. Values showing the same superscript letter are not significantly different ($p > 0.05$).

particle concentration, just after the feeding, was different with the various mutants. Also in this case mnn9-fed *Artemia* outperformed WT-fed *Artemia* (both in survival and individual growth). With the other mutants, *Artemia* biomass production improved mainly through higher survival. With two mutants, namely fks1 and chs3, *Artemia* biomass production was equal as in the experiment where WT-cells were offered.

In the present study the *Artemia* nauplii displayed a higher performance when fed with an exp-grown cells compared with *Artemia* nauplii fed with stat-grown yeast strains. According to Klis et al. [35], yeast cells entering the stationary phase of growth will form different cell walls, i.e. thicker, more resistant to enzymatic breakdown and less permeable to macromolecules. The level of mannosyl phosphorylation of cell-wall proteins increases in the late-exponential and stationary phase of growth [36]. In addition more extensive cross-linking (through disulfide bridges) between the polysaccharide components of the cell wall (mannoproteins, glucans and chitin) is taking place in the stationary phase [31,37,38]. In conclusion, it seems that the density of covalent linkage between the three cell-wall compounds of the yeast cell plays an important role in their digestibility by *Artemia*. In addition to that, high amounts of cell-wall chitin and glucans in combination with low amounts of mannoproteins favour *Artemia* biomass production under gnotobiotic condition [20,21].

According to Raa [2] improvements in the health status of aquatic organisms can be achieved by balancing the diet with regards to nutritional factors. This phenomenon is identified as nutritional immunology, since some nutritional factors are so closely linked with biochemical processes of the immune system that significant health benefits can be obtained by adjusting the concentration of such factors. Inadequate food or imbalances in the nutrient composition of the diet will affect growth and general performance of an animal, most likely, also the biochemical process of the immune system [2]. In this study nauplii fed with dead LVS3 (5.74 mg AFDW/FT) (Table 5 – feeding

regime: (d), presented significantly higher survival after challenge in comparison to nauplii fed solely with half of this amount (Table 7). This experiment clearly illustrated that the outcome of the challenge with *V. campbellii* under gnotobiotic condition is very much dependent on the overall condition of the nauplii. These results are also consistent with the perception that *Vibrio* spp. are opportunistic pathogens. Therefore in all challenge experiments, in which the effect of yeast mutants were tested in small quantities, the total AFDW supplied was kept constant. The mnn9-fed *Artemia* could resist detrimental effect of pathogenic VC until the end of the experiments as previously reported by Marques et al. [25] (Tables 7 and 8). Nevertheless, the addition of 5% of mnn9 yeast was not able to protect nauplii against VC until day 6. The mnn9 yeast has a null mutation resulting in phenotypically increased amounts of cell-wall bound chitin and glucans in combination with reduced amount of mannose linked to mannoproteins, and probably a reduced density of covalent linkages between these three yeast cell-wall constituents and/or nature of the covalent bonds, in comparison to the WT strain [22,29]. The protection provided by the *mnn9* yeast could be the effect of general improvements in *Artemia* health condition due to extra (or better quality) nutrients available in this yeast or due to a stimulation of a non-specific immune response by some compounds, such as β-glucans or chitin that are present in the yeast cell wall. Vismara et al. [39] considerably increased *Artemia* resistance to stress conditions, such as poor growth medium quality and daily handling, by administering daily nauplii with a mutant of *Euglena gracilis* presenting high amount of β-glucans (and thus enabling its response against disease). In contrast to mnn9 yeast, which has both strong nutritional and/or immunologic characteristics, gas1 cells have good nutritional effects, and protect nauplii temporarily against the pathogen in the challenge test. Furthermore, weak or no nutritional and protection effects were observed with fks1, knr4, mnn6 and kre6 yeast cells (Tables 9 and 10). Yet, interestingly, temporary protection against VC was obtained by adding chs3 in the diet, while this mutant has hardly any effect on individual growth (Table 7). Using the described set of yeast mutants, a full or partial protection against VC can be associated with increased glucan and chitin in the cell wall (e.g. mnn9 but also gas1) and reduced chitin and increased glucan in the cell wall (chs3). This seems to suggest that chitin as such is not involved in the protection against VC. Rather the results indicate that glucan as such is the potential active compound.

β-glucans have been identified as specific immunostimulants activating the aquatic organisms immune system and protecting them from adverse conditions [7]. For example, yeast *Saccharomyces cerevisiae* has been found to be

Table 9
Summary table of results obtained in the challenged and non-challenged experiments

Yeast strains	Phenotype	Non-challenged experiments (Exps. 1–3)			Challenged experiments (Exps. 4 and 5)		
		Survival (D6) (%)	II. (mm)	TBP (mm/FT)	Survival (D4) (%)	Survival (D5) (%)	Survival (D6) (%)
WT	Control yeast	C	C	C	+	–	–
mnn9	Less mannan, higher chitin, higher β-glucans	A	A	A	+	+	+
mnn6	Less phosphomannan	B	C	C	+	–	
fks1	Less β-1,3 glucans, higher chitin	B	C	C	+	–	–
knr4	Less β-1,3 glucans, higher chitin	B	C	C	+	–	–
kre6	Less β-1,6 glucans, higher chitin	B	C	C	+	–	–
chs3	Less chitin	B	C	C	+	⊕	–
gas1	Less integration of yeast cell adhesion. proteins into the cell wall, less β-1,3 glucans, higher chitin	B	B	B	+	⊕	–

A and B mean respectively statistically different ($p < 0.05$) strong and moderate positive effect of yeast feed on *Artemia* performance in comparison to the wild type yeast strain. C means no statistical different effect of feed on *Artemia* performance in comparison to the wild type yeast strain. "+" means protection (no significant difference in survival rate between challenged and non-challenged treatments) provided by small amounts of yeast feed (5 or 10%) on *Artemia* performance when fed with dead LVS3 and challenged with *Vibrio campbellii* . "–" means no protection (significant difference in survival rate between challenged and non-challenged treatments). ⊕ means that the feed was only protecting partially against the pathogen. D4, D5 and D6 correspond, respectively, to day 4, 5 and 6.

Table 10
Experimental design of the 5 experiments (Exp.) performed

		Day 1 (start)		Day 2		Day 3		Day 4		Day 5	Day 6 (harvest)
Exps. 1–3	(a)	Y	→	Y	→	Y	→	Y	→	Y	→
Exps. 4 and 5	(b)	DB + Y	→	DB + Y	→	DB + Y	→	DB + Y	→	DB + Y	→
	(c)	DB + Y	→	DB + Y	→	DB + Y + P	→	DB + Y	→	DB + Y	→
	(d)	DB (X)	→	DB (X)	→	DB (X)	→	DB (X)	→	DB (X)	→
	(e)	DB (X)	→	DB (X)	→	DB (X) + P	→	DB (X)	→	DB (X)	→
	(f)	DB (2X)	→	DB (2X)	→	DB (2X)	→	DB (2X)	→	DB (2X)	→
	(g)	DB (2X)	→	DB (2X)	→	DB (2X) + P	→	DB (2X)	→	DB (2X)	→

(a–g) correspond to the treatments performed; Y = yeast strains (wild type or isogenic yeast mutants). Yeast strains were added either at an equal amount of yeast cell particles (Exps. 1 and 2) or an equal amount of feed (Exp. 3), or 5 or 10% (Exps. 4 and 5); DB = dead bacterium LVS3; X = the total amount of dead LVS3 offered over the full experimental period (2870 μg AFDW/FT); P = pathogen (*Vibrio campbellii*). See Table 1.

a good enhancer of the trout immune system [40]. Patra and Mohamed [41] showed that *Artemia* supplemented with *Saccharomyces boulardi* were protected against *Vibrioi harveyi*. Immunostimulant properties of wild type yeast (WT) and fks1 mutant strain (resulting in fivefold higher cell-wall bound chitin) were administrated to the diet to gilthead sea bream (*Sparus aurata* L.) for six weeks under non-axenic conditions [42]. The results showed that chitin-enrichment in the fks1 strain may be responsible for increasing the innate immune responses resulting in beneficial effects on fish performance. The latter findings are not supported by our results.

In conclusion, the mnn9 yeast strain, even in small quantities, can protect *Artemia* nauplii against pathogenic bacteria, suggesting that this yeast strain is stimulating the innate immune response. It seems probable that mnn9 cells protect nauplii either through their higher concentration of β-glucans in the cell wall and/or the higher availability of β-glucans to nauplii. However, an overall nutritional stimulation by mnn9 with positive effect on the immunological status cannot be excluded. Using chs3 strain (in comparison to WT) as feed has very little extra effect on the growth and survival. Yet, this feed can temporarily protect *Artemia* against VC.

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