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Effects of Bacteria on *Artemia franciscana* Cultured in Different Gnotobiotic Environments

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The use of probiotics is receiving considerable attention as an alternative approach to control microbiota in aquaculture farms, especially in hatching facilities. However, application with consistent results is hampered by insufficient information on their modes of action. To investigate whether dead bacteria (allowing investigation of their nutritional effect) or live bacteria (allowing evaluation of their probiotic effect) have any beneficial effect towards *Artemia franciscana* and, subsequently, if live bacteria have probiotic effects beyond the effects observed with dead bacteria, a model system was employed using gnotobiotic *Artemia* as a test organism. Nauplii were cultured in the presence of 10 bacterial strains combined with four different major axenic live feeds (two strains of *Saccharomyces cerevisiae* and two strains of *Dunaliella tertiolecta*) differing in their nutritional values. In combination with poor- and medium-quality live feeds, dead bacteria exerted a strong effect on *Artemia* survival but a rather weak or no effect on individual length and constituted a maximum of only 5.9% of the total ash-free dry weight supplied. These effects were reduced or even disappeared when medium- to good-quality major feed sources were used, possibly due to improvements in the health status of *Artemia*. Some probiotic bacteria, such as GR 8 (*Cytophaga* spp.), improved (not always significantly) the performance of nauplii beyond the effect observed with dead bacteria, independently of the feed supplied. The present approach can be an excellent system to study the exact mode of action of bacteria, especially if combined with challenge tests or other types of analysis (e.g., transcriptome and proteonomic analysis).

Mass production of juvenile stages of aquatic organisms and their live preys for aquaculture obliges the use of intensive culture systems, which often leads to high mortalities caused mostly by pathogenic or opportunistic bacteria (5). The addition of high amounts of feed to these systems constitutes an excellent medium for the proliferation of heterotrophic bacteria, including opportunistic pathogens (49).

Until recently, one of the most frequent procedures used to avoid the establishment of undesirable bacteria in a target organism was the administration of antibiotics in the water (7). However, this practice promotes the selection and dissemination of antibiotic-resistant bacteria in the target organism and throughout the environment (19). For this reason, there is an urgent need to control the microbiota in hatching facilities by using alternative approaches.

Among other options (such as immunostimulation), the use of probiotics is receiving considerable attention (3, 15, 18, 22, 34, 52, 53). The application of a single probiotic strain or a combination of strains either in the rearing water or in live feed aims at a permanent or transient beneficial colonization of the host. However, none of those studies have provided sufficient evidence on the mode of action of the so-called "probionts" to allow for their application with consistent beneficial effects to the larvae. These effects could be partly, or entirely, due to a general improvement of the larval condition, for instance, as a consequence of the addition of essential nutrients by probionts. Moreover, although antimicrobial effects have already been shown in vitro (41), a clear demonstration of the causal link between beneficial effects of the probiont and in vivo suppression of a pathogen is rarely provided. According to Verschuere et al. (52), the two putative features of a probiont (i.e., nutritional and disease control) should be examined separately, although it is conceivable that a combination of a nutritional effect and disease control yields the best probiotic effect.

In order to study the effects of microorganisms more accurately, a model system was employed using the brine shrimp, *Artemia franciscana*, as a test organism. *Artemia* is one of the most important live feeds for commercial production of fish and shellfish larvae (45). This organism can be fed on a wide variety of feeds since it is a continuous nonselective and particle filter feeder (45). The most commonly used feed sources include live microalgae, such as *Dunaliella tertiolecta* (11, 31), and baker's yeast (11), since they provide the basic nutrients for development of nauplii. *Artemia* is an excellent model organism to study the modes of action of probiotic and pathogenic bacteria, as it can easily be cultured under gnotobiotic conditions (29, 30) and can be used as a vector for transferring probiotics to larvae of target species.

The aim of the present study was not to select the probiotic strains to improve the mass rearing of the shrimp but to inves-

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TABLE 1. Identification of all bacterial strains tested and their average AFDW[a]

Strain	Identification		AFDW (μg/FT)				Ratio of amt of bacteria/amt of feed added			
	Gram	Identity	Live	Norvanol treated	Autoclaved	γ-Irradiated	WT	mnn9	DT CCAP 19/6B	DT CCAP 19/27
LVS2	+	*Bacillus* sp.	45.87 ± 3.87^{A}		33.92 ± 0.87^{B}	34.05 ± 1.63^{B}	0.043	0.016	0.017	0.020
LVS3	−	*Aeromonas hydrophila*	35.17 ± 1.53^{B}	40.82 ± 3.01^{A}	43.20 ± 2.43^{A}	48.35 ± 1.89^{A}	0.046	0.017	0.018	0.021
LVS8	−	*Vibrio* sp.	$42.21 \pm 12.09^{A,B}$	62.62 ± 6.15^{A}	35.71 ± 2.11^{B}	55.56 ± 10.85^{A}	0.059	0.021	0.023	0.028
GR 8	−	*Cytophaga* sp.	59.40 ± 3.23^{A}	37.19 ± 4.09^{B}	61.65 ± 1.29^{A}	$46.11 \pm 7.46^{A,B}$	0.058	0.021	0.023	0.027
GR 10	−	*Roseobacter* sp.	51.67 ± 1.30^{A}	53.48 ± 15.35^{A}	50.79 ± 7.93^{A}	45.05 ± 6.27^{A}	0.051	0.018	0.020	0.024
GR 11	−	*Ruegeria atlantica*	46.67 ± 5.31^{A}	32.41 ± 2.73^{B}	45.90 ± 1.39^{A}	27.13 ± 3.48^{B}	0.044	0.016	0.017	0.021
GR 12	−	*Paracoccus* sp.	32.50 ± 1.50^{B}	25.27 ± 0.32^{C}	38.40 ± 0.91^{A}	23.79 ± 2.34^{C}	0.036	0.013	0.014	0.017
Cluster A	−	α-*Proteobacteria*	36.29 ± 0.61^{A}	23.94 ± 0.74^{B}	32.15 ± 1.26^{A}	15.49 ± 2.69^{C}	0.034	0.012	0.013	0.016
LMG21363	−	*Vibrio campbellii*	37.98 ± 0.14^{A}		33.56 ± 0.56^{A}		0.036	0.013	0.014	0.017
CW8T2	−	*Vibrio proteolyticus*	36.02 ± 0.54^{A}		31.91 ± 3.04^{B}		0.034	0.012	0.013	0.016
AFDW (mg/FT)							1.06 ± 0.08	2.92 ± 0.04	2.71 ± 0.05	2.26 ± 0.07

[a] The AFDW of the feed supplied to *Artemia* during the experiments (WT yeast, mnn9 yeast, DT CCAP 19/6B, and DT, CCAP 19/27) is also presented (mg/FT), as well as the ratio between the maximum average amount of bacteria added and the average amount of feed added, in terms of AFDW. Mean values of AFDW are presented with the respective standard deviations (mean ± standard deviation [SD]). Values in the same row showing the same superscript letter (A, B, or C) are not significantly different ($P > 0.05$).

tigate whether dead or live bacteria have any beneficial effect towards *Artemia* and if live bacteria can have additional effects beyond those observed with dead bacteria. For that purpose, 10 different bacterial strains were tested dead or alive in a model system of gnotobiotic *Artemia* culture. These bacteria were combined with four different axenic live feeds with different nutritional values for *Artemia*: poor-quality feed (wild-type [WT] yeast), medium-quality feed (mnn9 yeast and the microalga DT CCAP 19/27), and good-quality feed (microalga DT CCAP 19/6B) (29, 30).

MATERIALS AND METHODS

Axenic cultures of yeast and algae. Two strains of axenic baker's yeast (*Saccharomyces cerevisiae*) were used as feed for *Artemia*: the WT strain (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 (2, 27, 29). Both strains were provided by the European *Saccharomyces cerevisiae* Archive for Functional Analysis, University of Frankfurt, Frankfurt, Germany. Yeast cultures were grown in sterile Erlenmeyer flasks with a cotton cap placed on a shaker in the dark (30°C, 150 rpm). Both strains were cultured in a complete yeast extract-peptone-dextrose medium containing yeast extract (composed of 1% [1% wt/vol] yeast extract, 1% [wt/vol] bacteriological-grade peptone, and 2% [wt/vol] D-glucose; Sigma), bacteriological-grade peptone (1%, wt/vol; Sigma), and D-glucose (2%, wt/vol; Sigma). This medium was prepared in natural seawater (35 g/liter) previously filtered (0.22-μm filter) and sterilized by autoclaving at 120°C for 20 min. The growth curve of each yeast strain was established by regularly measuring the absorbance at 600 nm with a spectrophotometer. Both strains were harvested by centrifugation ($\pm 800 \times g$ for 10 min) in the stationary growth phase (after 3 days of culture, starting from a single colony). Cells were resuspended twice in sterile Falcon tubes (FT) (TRP) (γ-irradiated) with 20 ml of filtered and autoclaved seawater (FASW) (0.22 μm). All handlings were performed in a laminar flow hood to maintain sterility.

Axenic cultures of two strains of the microalga *Dunaliella tertiolecta* (strains DT CCAP 19/6B and DT CCAP 19/27) were obtained from the Culture Collection of Algae and Protozoa Department (CCAP), Dunstaffnage Marine Laboratory, Scotland. According to Marques et al. (30), both *D. tertiolecta* strains are identical in terms of the DNA sequence using the nuclear rRNA gene internal transcribed spacer region 2, indicating that they are phylogenetically very close. Each strain was grown in sterile autoclaved 500-ml bottles (10% inocula) with 0.22-μm-filtered aeration at 19°C and continuous light (± 41 μEm^{-2}), using a standard Walne medium (54) and FASW. The growth curve of each algal strain was obtained by measuring the cell density of each culture daily using a Bürker hemocytometer, complemented with daily measurements of the optical density using a spectrophotometer (λ = 600 nm). Algal strains were harvested in the exponential growth phase by centrifuging the culture ($\pm 800 \times g$ for 5 min) in the middle of the exponential growth curve. Cultures were resuspended in 20 ml FASW in sterile Falcon tubes.

Yeast and microalga densities were determined by measuring the cell concentration twice using a Bürker hemocytometer. The suspensions were stored at 4°C and used to feed *Artemia* until the end of each experiment.

Bacterial strains and growth conditions. A selection of 10 bacterial strains was examined: strains LVS 2, LVS 3, and LVS 8, for their positive effect towards *Artemia* (50, 51); strains GR 8, GR 10, GR 11, and GR 12, for their positive effect towards *Brachionus plicatilis* (42); a representative of cluster A, for its positive effect in turbot larviculture (22); *Vibrio proteolyticus* strain CW8T2, for its negative effect towards *Artemia* (50, 51); and *Vibrio campbellii* strain LMG21363, for its negative effect towards *Artemia* and shrimp (16, 46). Pure cultures of the 10 bacterial strains, stored at −80°C, were grown overnight at 28°C on marine agar (MA) containing Difco marine broth 2216 (37.4 g/liter; BD Biosciences) and bacteriological-grade agar (20 g/liter; 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, $\pm 2{,}200 \times g$), the supernatant was discarded, and the pellet was resuspended in 20 ml FASW. The densities of the new bacterial suspensions were determined by measuring their optical density at 550 nm with a spectrophotometer, 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).

Characterization of the bacterial strains. Five beneficial bacterial strains were previously characterized (Table 1) based on their 16S rRNA gene sequencing (LVS 3, GR 8, GR 10, GR 11, and GR 12) by Makridis et al. (28). In the present work, the remaining strains, LVS 2, LVS 8, and a representative of cluster A, were characterized by 16S rRNA gene sequencing. For this purpose, genomic DNA was prepared according to the protocol of Pitcher et al. (37). 16S rRNA gene amplification, purification, and sequencing were performed as previously described (48), with the following modifications. 16S rRNA gene amplicons were purified by using a NucleoFast 96 PCR Clean-up kit (Macherey-Nagel). Sequencing reactions were performed by using a BigDye Terminator Cycle Sequencing kit (Applied Biosystems) and purified using a Montage SEQ_{96} Sequencing Reaction Clean-up kit (Millipore). Electrophoresis of sequence reaction products was performed by using an ABI Prism 3100 genetic analyzer (Applied Biosystems). Sequence assembly was performed using the program AutoAssembler (Applied Biosystems). Online similarity searches were performed with the BLAST (Basic Local Alignment Search Tool) family of programs in GenBank.

Killing of the bacteria. Dead bacteria offered to *Artemia* were previously killed by using either 10-kGy γ-irradiation (provided by a 15-MeV 20-kW linear electron accelerator), 40% norvanol D (composed of 90% [vol/vol] ethanol, 2.9% [vol/vol] ether, and 9% [mass/vol] H_2O; Merck Eurolab) (incubated for 30 min at 28°C, centrifuged twice to remove norvanol residues, and replaced with FASW), or high temperature (autoclaving at 120°C for 20 min). For the two pathogenic strains (*V. campbellii* and *V. proteolyticus*), only autoclaving was used to obtain dead bacteria. In order to check if the bacteria were effectively killed by the three methods, all strains were plated after being exposed to each method by transferring 100 μl of the culture medium to MA ($n = 3$). Absence of bacterial growth was monitored after incubating plates for 5 days at 28°C. Autoclaving and γ-irradiation treatments were 100% effective, since no bacterial growth was observed on the MA after 5 days of incubation. As to the norvanol treatment, it was not effective in the gram-positive strain LVS 2. For this reason, strain LVS 2 treated with norvanol was not used in the four experiments. Dead and live bacterial suspensions were stored at 4°C until the end of each experiment (being supplied only once to *Artemia* at the beginning of the experiment).

Ash-free dry weight content. To determine the ash-free dry weight (AFDW) content of live and dead bacteria (killed by either autoclaving, norvanol treatment, or γ-irradiation) and of the four feeds added to *Artemia* (yeast and algae), 50 ml of each culture sample was filtered on predried nitrocellulose filters (0.22-μm pore size; $n = 2$). Filters were subsequently dried at 60°C for 48 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 the dry weight and the ash weight. The dry weight and AFDW of the control (filter only with the culture medium; $n = 2$) were subtracted from all samples.

***Artemia* gnotobiotic culture.** Experiments were performed with *Artemia franciscana* cysts, originating from Great Salt Lake, Utah (EG type; INVE Aquaculture NV, Belgium). Bacterium-free cysts and nauplii were obtained via decapsulation according to the procedure described previously by Sorgeloos et al. (45). 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 containing 30 ml of FASW. The tube was capped and placed on a rotator at 4 cycles per min and exposed to constant incandescent light (± 41 μEm^{-2}) at 28°C for 18 to 20 h. After this period, the hatching tube was taken to the laminar flow hood, and 20 hatched nauplii (Instar II) were transferred to new sterile 50-ml Falcon tubes containing 30 ml of FASW, together with the amount of feed scheduled for day 1. Each treatment consisted of four Falcon tubes (replicates). After feeding, the bacterial suspension (dead or live) was added only at day 1 at a density of approximately 5×10^6 cells/ml in treatments where bacteria were used. All Falcon tubes were put back on the rotator and were transferred to the laminar flow hood just once per day for feeding. The daily feeding schedule was adapted from methods described previously by Coutteau et al. (11) and Marques et al. (30), who optimized the feeding schedule of *Artemia* using baker's yeast and *D. tertiolecta* as feed. The feeding schedule is intended to provide ad libitum ratios while avoiding excessive overfeeding in order not to affect the water quality in the test tubes. As control treatments, *Artemia* cultures were fed only yeast or algae without the addition of bacteria.

Methods 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 and live counting according to the procedures of Marques et al. (29, 30). 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] (0.5%, wt/vol; Sigma) in a sterile recipient (1 part MTT to 9 parts sample) and incubated at 30°C for 30 min. Under a light microscope (×1,000 magnification), live bacterial detection was performed. The MTT blue stains all viable/living cells remaining in a culture (44), making the detection of bacterial contaminations in a culture medium easier. A combination of plating and live counting was also used to detect contaminations in treatments where live bacteria were supplied to *Artemia*. The characteristics of the live bacteria present in these treatments were compared to the known characteristics of the bacterial strains used in order to verify the similarity between them. Whenever a culture tube was found to be contaminated, data were rejected and the treatment was repeated, as well as its axenic control.

Experimental design. In experiment 1, 10 bacterial strains were tested dead or live on *Artemia* fed with the poor-quality WT yeast. In experiments 2 and 3, the medium-quality feeds (mnn9 yeast and the microalga DT CCAP 19/27, respectively) were used as feed for *Artemia* and inoculated with the same 10 bacterial strains added dead or live. Finally, in experiment 4, the same 10 bacterial strains were added dead or live to *Artemia* fed with the good-quality microalga DT CCAP 19/6B. In each experiment, performance of *Artemia* in the control treatments was compared to results previously obtained by Marques et al. (29, 30) to evaluate reproducibility. If significant differences were detected, data were not considered for further analysis and the experiment was repeated.

Survival and growth of *Artemia*. At the end of each experiment (day 6 after hatching), the number of swimming larvae was determined and the survival percentage was calculated. Living larvae 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 of Marnix Van Damme). As a criterion that combines both the effects of survival and IL, the total biomass production (TBP or total length) was determined according to the following equation: TBP (millimeters per FT) = number of survivors × mean IL.

Statistics. Values of larval survival (percentage) were arc sine transformed, while values of IL, TBP, and AFDW were logarithmic or square root transformed to satisfy normality and homocedasticity requirements. Differences between AFDW of dead and live bacteria and differences between survival, IL, and TBP of *Artemia* fed with different feeds and inoculated with different dead or live bacterial strains were investigated with analysis of variances and multiple comparisons of Tukey's range. All statistical analysis was tested at a 0.05 level of probability using the software Statistica 5.5 (Statsoft, Inc.).

RESULTS

Characterization of bacterial strains. Three strains included in the present study, i.e., LVS 2, LVS 8, and a representative of cluster A, were characterized by blasting their complete 16S rRNA gene sequences in GenBank (Table 1). Strain LVS 2 was found to belong to the genus *Bacillus*, possibly representing a currently undescribed species that is phylogenetically closest to the marine species *Bacillus aquimaris* and *Bacillus marisflavi*. Strain LVS 8 was allocated in the *Vibrio splendidus* group and was most closely positioned to the species *V. tasmaniensis*, *V. lentus*, and *V. cyclitrophicus* of this group. The representative of cluster A was classified as a member of the α-*Proteobacteria* but could not be clearly assigned a particular genus in this phylogenetic group. Its closest phylogenetic neighbors included the genera *Ruegeria* and *Silicibacter*.

***Artemia* fed with poor-quality feed.** *Artemia* fed with the poor-quality WT yeast was inoculated with 10 bacterial strains added either dead or live and compared with nauplii fed with the same yeast in the absence of bacteria. Results presented in Table 2 (experiment 1) show that the addition of small amounts of dead bacteria (Table 1) could strongly improve *Artemia* TBP in comparison to the bacterium-free control, mostly due to an increase in the survival rate rather than a better individual length (IL). LVS 2 was the only strain with which no significant improvements in *Artemia* performance

TABLE 2. Average survival, IL, and TBP of *Artemia* fed with poor-quality WT yeast and inoculated with 10 bacterial strains[a]

Bacterial strain or parameter	Treatment	Survival (%)	IL (mm)	TBP (mm/FT)
LVS 2	No bacteria	14 ± 10^{B}	1.91 ± 0.23^{A}	5.26 ± 3.72^{B}
	Autoclaved	31 ± 8^{B}	1.86 ± 0.20^{A}	11.63 ± 2.79^{B}
	Norvanol			
	γ-Irradiated	$40 \pm 24^{A,B}$	1.91 ± 0.27^{A}	$15.28 \pm 9.09^{A,B}$
	Live	55 ± 7^{A}	2.04 ± 0.30^{A}	22.44 ± 2.88^{A}
LVS 3	No bacteria	14 ± 10^{A}	$1.91 \pm 0.23^{A,B}$	5.26 ± 3.72^{C}
	Autoclaved	50 ± 25^{B}	1.47 ± 0.24^{B}	$14.70 \pm 7.30^{B,C}$
	Norvanol	55 ± 14^{B}	$1.94 \pm 0.25^{A,B}$	21.34 ± 5.49^{B}
	γ-Irradiated	91 ± 9^{A}	$1.90 \pm 0.27^{A,B}$	34.68 ± 3.24^{A}
	Live	$76 \pm 14^{A,B}$	2.32 ± 0.26^{A}	35.38 ± 6.39^{A}
LVS 8	No bacteria	14 ± 10^{B}	1.91 ± 0.23^{A}	5.26 ± 3.72^{C}
	Autoclaved	71 ± 8^{A}	1.50 ± 0.25^{A}	21.38 ± 2.25^{B}
	Norvanol	85 ± 9^{A}	1.98 ± 0.30^{A}	33.66 ± 3.61^{A}
	γ-Irradiated	88 ± 10^{A}	1.94 ± 0.27^{A}	33.95 ± 3.71^{A}
	Live	66 ± 16^{A}	2.00 ± 0.37^{A}	$26.56 \pm 6.32^{A,B}$
GR 8	No bacteria	12 ± 10^{B}	$1.29 \pm 0.22^{B,C}$	3.25 ± 1.88^{C}
	Autoclaved	50 ± 18^{A}	$1.86 \pm 0.37^{A,B}$	$18.60 \pm 6.79^{A,B}$
	Norvanol	$44 \pm 21^{A,B}$	$1.75 \pm 0.40^{A,B,C}$	$15.31 \pm 7.34^{A,B}$
	γ-Irradiated	43 ± 9^{A}	1.17 ± 0.23^{C}	9.95 ± 2.03^{B}
	Live	53 ± 9^{A}	1.88 ± 0.27^{A}	19.74 ± 3.26^{A}
GR 10	No bacteria	12 ± 10^{C}	$1.29 \pm 0.22^{A,B}$	3.25 ± 1.88^{B}
	Autoclaved	11 ± 9^{C}	$1.45 \pm 0.32^{A,B}$	3.26 ± 2.74^{B}
	Norvanol	68 ± 21^{A}	1.85 ± 0.37^{A}	24.98 ± 7.78^{A}
	γ-Irradiated	34 ± 11^{B}	1.11 ± 0.29^{B}	7.44 ± 2.49^{B}
	Live	60 ± 7^{A}	1.92 ± 0.39^{A}	23.04 ± 2.72^{A}
GR 11	No bacteria	12 ± 10^{B}	$1.29 \pm 0.22^{A,B}$	3.25 ± 1.88^{C}
	Autoclaved	56 ± 8^{A}	1.79 ± 0.35^{A}	20.14 ± 2.69^{A}
	Norvanol	48 ± 17^{A}	1.78 ± 0.35^{A}	$16.91 \pm 6.08^{A,B}$
	γ-Irradiated	45 ± 13^{A}	1.09 ± 0.17^{B}	9.76 ± 2.76^{B}
	Live	44 ± 11^{A}	1.65 ± 0.33^{A}	$14.44 \pm 3.66^{A,B}$
GR 12	No bacteria	12 ± 10^{B}	$1.29 \pm 0.22^{B,C}$	3.25 ± 1.88^{B}
	Autoclaved	49 ± 14^{A}	$1.87 \pm 0.36^{A,B}$	18.23 ± 5.15^{A}
	Norvanol	19 ± 13^{B}	$1.87 \pm 0.34^{A,B}$	7.01 ± 4.92^{B}
	γ-Irradiated	$29 \pm 9^{A,B}$	1.00 ± 0.30^{C}	5.75 ± 1.89^{B}
	Live	16 ± 13^{B}	1.99 ± 0.31^{A}	6.47 ± 4.98^{B}
Cluster A	No bacteria	12 ± 10^{B}	$1.29 \pm 0.22^{A,B}$	3.25 ± 1.88^{B}
	Autoclaved	39 ± 12^{A}	1.84 ± 0.34^{A}	14.26 ± 4.35^{A}
	Norvanol	$29 \pm 34^{A,B}$	1.78 ± 0.24^{A}	$10.24 \pm 12.28^{A,B}$
	γ-Irradiated	$25 \pm 4^{A,B}$	1.11 ± 0.20^{B}	5.55 ± 0.91^{B}
	Live	$24 \pm 25^{A,B}$	1.66 ± 0.30^{A}	$7.89 \pm 8.29^{A,B}$
LMG21363 (*V. campbellii*)	No bacteria	12 ± 10^{B}	1.29 ± 0.22^{A}	3.25 ± 1.88^{B}
	Autoclaved	49 ± 13^{A}	1.76 ± 0.29^{A}	17.16 ± 4.40^{A}
	Live	0 ± 0^{C}		0 ± 0^{C}
CW8T2 (*V. proteolyticus*)	No bacteria	12 ± 10^{B}	1.29 ± 0.22^{A}	3.25 ± 1.88^{B}
	Autoclaved	65 ± 7^{A}	1.83 ± 0.31^{A}	23.79 ± 2.59^{A}
	Live	16 ± 13^{B}	1.57 ± 0.38^{A}	5.10 ± 4.13^{B}
Significant improvements (no.)	Autoclaved	8/10	0/10	7/10
	Norvanol	4/7	0/7	5/7
	γ-Irradiated	5/8	0/8	4/8
	Live	6/10	2/10	6/10

[a] Shown are average survival (percent), IL (millimeters), and total length or TBP (millimeters per FT) of *Artemia* fed with poor-quality WT yeast and inoculated with 10 bacterial strains added dead (obtained via autoclaving, norvanol treatment, or γ-irradiation) or live and compared with a control treatment in axenic conditions (no bacteria) (experiment 1). The number of bacterial strains providing significant improvements of *Artemia* TBP, survival, and IL, when added dead or live, in comparison to the bacterium-free control is also presented. Means and standard deviations were placed together (mean ± SD). For each bacterial strain, values in the same column showing the same superscript letter (A, B, or C) are not significantly different ($P > 0.05$).

were observed. The enhanced *Artemia* performance was, however, highly dependent on the procedure used to kill bacteria. The most remarkable increase in TBP was obtained with γ-irradiated preparations of strains LVS 3 and LVS 8 and with a norvanol-treated preparation of LVS 8 and GR 10. With other bacteria (i.e., GR 8, GR 11, GR 12, and cluster A), autoclaving supported a higher increase in TBP in comparison to γ-irradiation, mainly due to significantly higher survival.

In terms of live bacteria, significant improvements in *Artemia* TBP were observed in nauplii inoculated with six strains (LVS 2, LVS 3, LVS 8, GR 8, GR 10, and GR 11) in comparison to bacteria-free nauplii, while the opposite occurred when live *Vibrio campbellii* strain LMG21363 was added. All differences were due especially to significant variations in survival and to a lesser extent in IL of nauplii. For GR 12, cluster A, and *Vibrio proteolyticus* strain CW8T2, no significant differences were observed in *Artemia* TBP. Only two bacterial strains (i.e., LVS 2 and GR 8) increased *Artemia* performance (although not always significantly) in all tested parameters (survival, IL, and TBP) when live bacteria were added instead of

TABLE 3. Average survival, IL, and TBP of *Artemia* fed with medium-quality mnn9 yeast and inoculated with 10 bacterial strains[a]

Bacterial strain or parameter	Treatment	Survival (%)	IL (mm)	TBP (mm/FT)
LVS 2	No bacteria	$32 \pm 30^{A,B}$	2.23 ± 0.31^{A}	$14.23 \pm 13.31^{A,B}$
	Autoclaved	28 ± 3^{B}	2.77 ± 0.30^{A}	15.24 ± 1.60^{B}
	Norvanol			
	γ-Irradiated	49 ± 13^{A}	2.76 ± 0.39^{A}	26.91 ± 6.90^{A}
	Live	59 ± 14^{A}	2.97 ± 0.46^{A}	34.90 ± 8.53^{A}
LVS 3	No bacteria	$32 \pm 30^{A,B}$	2.23 ± 0.31^{B}	$14.23 \pm 13.31^{B,C}$
	Autoclaved	69 ± 16^{A}	$2.79 \pm 0.54^{A,B}$	38.36 ± 8.93^{A}
	Norvanol	29 ± 5^{B}	2.09 ± 0.27^{B}	12.02 ± 2.00^{C}
	γ-Irradiated	49 ± 9^{A}	$2.42 \pm 0.23^{A,B}$	23.60 ± 4.13^{B}
	Live	48 ± 9^{A}	3.03 ± 0.37^{A}	$28.79 \pm 5.25^{A,B}$
LVS 8	No bacteria	$32 \pm 30^{A,B}$	2.23 ± 0.31^{B}	14.23 ± 13.31^{B}
	Autoclaved	73 ± 15^{A}	$2.64 \pm 0.51^{A,B}$	38.70 ± 8.12^{A}
	Norvanol	56 ± 5^{A}	3.04 ± 0.33^{A}	34.20 ± 2.91^{A}
	γ-Irradiated	$54 \pm 24^{A,B}$	$2.52 \pm 0.43^{A,B}$	$27.09 \pm 12.06^{A,B}$
	Live	33 ± 6^{B}	$2.61 \pm 0.40^{A,B}$	16.97 ± 3.37^{B}
GR 8	No bacteria	58 ± 14^{A}	2.76 ± 0.45^{B}	32.23 ± 8.22^{B}
	Autoclaved	74 ± 15^{A}	$3.00 \pm 0.34^{A,B}$	$44.36 \pm 8.73^{A,B}$
	Norvanol	71 ± 8^{A}	$3.01 \pm 0.40^{A,B}$	$42.89 \pm 4.52^{A,B}$
	γ-Irradiated	65 ± 7^{A}	$3.51 \pm 0.55^{A,B}$	$45.63 \pm 4.96^{A,B}$
	Live	60 ± 9^{A}	4.23 ± 0.74^{A}	50.76 ± 7.72^{A}
GR 10	No bacteria	58 ± 14^{A}	2.76 ± 0.45^{B}	32.23 ± 8.22^{B}
	Autoclaved	70 ± 11^{A}	$3.07 \pm 0.48^{A,B}$	$42.98 \pm 6.63^{A,B}$
	Norvanol	70 ± 11^{A}	$3.09 \pm 0.48^{A,B}$	$43.26 \pm 6.68^{A,B}$
	γ-Irradiated	58 ± 15^{A}	$3.17 \pm 0.45^{A,B}$	$36.46 \pm 9.51^{A,B}$
	Live	63 ± 10^{A}	4.10 ± 0.78^{A}	51.25 ± 8.53^{A}
GR 11	No bacteria	58 ± 14^{A}	2.76 ± 0.45^{A}	32.23 ± 8.22^{A}
	Autoclaved	74 ± 13^{A}	2.86 ± 0.46^{A}	42.19 ± 7.52^{A}
	Norvanol	46 ± 19^{A}	3.10 ± 0.39^{A}	24.80 ± 11.60^{A}
	γ-Irradiated	63 ± 6^{A}	3.08 ± 0.51^{A}	38.50 ± 3.98^{A}
	Live	53 ± 12^{A}	3.36 ± 0.72^{A}	35.28 ± 8.00^{A}
GR 12	No bacteria	$58 \pm 14^{A,B}$	2.76 ± 0.45^{B}	32.23 ± 8.22^{B}
	Autoclaved	71 ± 17^{A}	2.87 ± 0.40^{B}	$40.90 \pm 9.48^{A,B}$
	Norvanol	29 ± 13^{B}	2.83 ± 0.29^{B}	12.74 ± 7.49^{C}
	γ-Irradiated	$55 \pm 11^{A,B}$	$3.09 \pm 0.59^{A,B}$	33.99 ± 6.68^{B}
	Live	$59 \pm 8^{A,B}$	4.22 ± 0.55^{A}	49.59 ± 6.33^{A}
Cluster A	No bacteria	$58 \pm 14^{A,B}$	2.76 ± 0.45^{A}	$32.23 \pm 8.22^{A,B}$
	Autoclaved	68 ± 10^{A}	2.99 ± 0.56^{A}	40.37 ± 6.22^{A}
	Norvanol	41 ± 9^{B}	3.22 ± 0.54^{A}	26.57 ± 6.10^{B}
	γ-Irradiated	$64 \pm 17^{A,B}$	3.20 ± 0.54^{A}	$40.80 \pm 10.89^{A,B}$
	Live	66 ± 13^{A}	2.86 ± 0.54^{A}	$37.90 \pm 7.52^{A,B}$
LMG21363 (*V. campbellii*)	No bacteria	58 ± 14^{A}	2.76 ± 0.45^{B}	32.23 ± 8.22^{B}
	Autoclaved	73 ± 21^{A}	4.37 ± 0.76^{A}	64.09 ± 18.19^{A}
	Live	50 ± 10^{A}	2.76 ± 0.35^{B}	27.60 ± 5.52^{B}
CW8T2 (*V. proteolyticus*)	No bacteria	58 ± 14^{A}	2.76 ± 0.45^{B}	32.23 ± 8.22^{B}
	Autoclaved	69 ± 16^{A}	4.31 ± 0.94^{A}	59.26 ± 13.80^{A}
	Live	63 ± 13^{A}	$3.15 \pm 0.58^{A,B}$	$39.38 \pm 8.33^{A,B}$
Significant improvements (no.)	Autoclaved	0/10	2/10	4/10
	Norvanol	0/7	1/7	1/7
	γ-Irradiated	0/8	0/8	0/8
	Live	0/10	4/10	3/10

[a] Shown are average survival (percent), IL (millimeters), and total length or TBP (millimeters per FT) of *Artemia* fed with medium-quality mnn9 yeast and inoculated with 10 bacterial strains added dead (obtained via autoclaving, norvanol treatment, or γ-irradiation) or live and compared with a control treatment in axenic conditions (no bacteria) (experiment 2). The number of bacterial strains providing significant improvements of *Artemia* TBP, survival, and IL, when added dead or live, in comparison to the bacterium-free control is also presented. Means and standard deviations were placed together (mean ± SD). For each bacterial strain, values in the same column showing the same superscript letter (A, B, or C) are not significantly different ($P > 0.05$).

dead bacteria. Yet overall, none of the tested bacterial strains yielded significantly better *Artemia* TBP when added live in comparison to the equivalent treatments with inactivated bacteria.

***Artemia* fed with medium-quality feeds.** Results of survival, IL, and TBP with *Artemia* fed with the medium-quality feeds (mnn9 yeast and the microalga DT CCAP 19/27) and inoculated with 10 bacterial strains added dead or live are presented in Tables 3 (experiment 2) and 4 (experiment 3), respectively. With both feeds, when dead bacteria were provided to nauplii, higher values of TBP were observed in comparison to the bacterium-free control. However, significantly higher performances were observed only in *Artemia* fed mnn9 yeast and inoculated with dead preparations of LVS 3, LVS 8, *Vibrio campbellii* strain LMG21363, and *Vibrio proteolyticus* strain CW8T2, mainly due to significantly higher IL. Nauplii fed with the microalga DT CCAP 19/27 presented significantly higher TBP when supplied with dead LVS 3, LVS 8, GR 8, GR 10, GR 11, GR 12, and cluster A. *Artemia* fed mnn9 yeast and inoculated with dead bacteria killed with different methods

TABLE 4. Average survival, IL, and TBP of *Artemia* fed with medium-quality microalga *Dunaliella tertiolecta* (strain DT CCAP 19/27) and inoculated with 10 bacterial strains[a]

Bacterial strain or parameter	Treatment	Survival (%)	IL (mm)	TBP (mm/FT)
LVS 2	No bacteria	65 ± 5^{B}	1.70 ± 0.29^{A}	22.16 ± 1.87^{B}
	Autoclaved	50 ± 4^{B}	2.01 ± 0.28^{A}	20.10 ± 1.64^{B}
	Norvanol			
	γ-Irradiated	60 ± 9^{B}	2.02 ± 0.44^{A}	24.24 ± 3.69^{B}
	Live	93 ± 6^{A}	1.97 ± 0.29^{A}	36.45 ± 2.54^{A}
LVS 3	No bacteria	65 ± 5^{B}	1.70 ± 0.29^{A}	22.16 ± 1.87^{B}
	Autoclaved	88 ± 12^{A}	1.68 ± 0.28^{A}	29.44 ± 4.03^{A}
	Norvanol	43 ± 6^{C}	1.89 ± 0.25^{A}	16.07 ± 2.44^{C}
	γ-Irradiated	$63 \pm 15^{A,B,C}$	1.84 ± 0.23^{A}	$23.00 \pm 5.52^{B,C}$
	Live	79 ± 5^{A}	1.92 ± 0.26^{A}	30.37 ± 2.07^{A}
LVS 8	No bacteria	$65 \pm 5^{B,C}$	1.70 ± 0.29^{A}	22.16 ± 1.87^{C}
	Autoclaved	91 ± 9^{A}	1.64 ± 0.24^{A}	29.93 ± 2.80^{B}
	Norvanol	94 ± 6^{A}	1.99 ± 0.29^{A}	37.33 ± 2.51^{A}
	γ-Irradiated	55 ± 4^{C}	2.01 ± 0.41^{A}	22.11 ± 1.64^{C}
	Live	$78 \pm 12^{A,B}$	1.91 ± 0.26^{A}	29.61 ± 4.55^{B}
GR 8	No bacteria	67 ± 8^{C}	$2.07 \pm 0.62^{A,B}$	27.74 ± 8.31^{B}
	Autoclaved	83 ± 6^{B}	$1.60 \pm 0.31^{A,B}$	26.40 ± 2.07^{B}
	Norvanol	88 ± 10^{B}	1.48 ± 0.28^{B}	25.94 ± 3.09^{B}
	γ-Irradiated	85 ± 12^{B}	3.11 ± 1.17^{A}	52.87 ± 7.62^{A}
	Live	100 ± 10^{A}	2.35 ± 0.40^{A}	47.00 ± 8.00^{A}
GR 10	No bacteria	67 ± 8^{B}	$2.07 \pm 0.62^{A,B}$	$27.74 \pm 8.31^{B,C}$
	Autoclaved	$81 \pm 17^{A,B}$	1.66 ± 0.30^{B}	$26.98 \pm 5.48^{B,C}$
	Norvanol	69 ± 9^{B}	1.58 ± 0.29^{B}	21.73 ± 2.70^{C}
	γ-Irradiated	$84 \pm 11^{A,B}$	3.21 ± 0.99^{A}	53.93 ± 7.34^{A}
	Live	94 ± 9^{A}	1.80 ± 0.33^{B}	33.75 ± 3.41^{B}
GR 11	No bacteria	67 ± 8^{C}	2.07 ± 0.62^{A}	$27.74 \pm 8.31^{B,C,D}$
	Autoclaved	86 ± 3^{B}	1.57 ± 0.22^{A}	27.08 ± 0.79^{C}
	Norvanol	64 ± 13^{C}	1.53 ± 0.24^{A}	19.62 ± 4.10^{D}
	γ-Irradiated	84 ± 3^{B}	2.94 ± 1.28^{A}	49.25 ± 1.47^{A}
	Live	98 ± 3^{A}	1.78 ± 0.32^{A}	34.71 ± 1.03^{B}
GR 12	No bacteria	67 ± 8^{B}	2.07 ± 0.62^{A}	$27.74 \pm 8.31^{B,C}$
	Autoclaved	70 ± 11^{B}	1.54 ± 0.22^{A}	21.65 ± 3.39^{C}
	Norvanol	75 ± 6^{B}	1.57 ± 0.26^{A}	23.55 ± 1.81^{C}
	γ-Irradiated	89 ± 6^{A}	2.82 ± 1.28^{A}	50.06 ± 3.55^{A}
	Live	96 ± 5^{A}	1.92 ± 0.34^{A}	36.96 ± 1.84^{B}
Cluster A	No bacteria	67 ± 8^{B}	$2.07 \pm 0.62^{A,B}$	$27.74 \pm 8.31^{B,C}$
	Autoclaved	84 ± 5^{A}	1.57 ± 0.23^{B}	26.32 ± 1.68^{C}
	Norvanol	83 ± 12^{A}	1.55 ± 0.24^{B}	25.67 ± 3.62^{C}
	γ-Irradiated	83 ± 9^{A}	3.27 ± 0.94^{A}	54.12 ± 5.61^{A}
	Live	93 ± 6^{A}	1.73 ± 0.25^{B}	32.01 ± 2.23^{B}
LMG21363 (*V. campbellii*)	No bacteria	67 ± 8^{A}	2.07 ± 0.62^{A}	27.74 ± 8.31^{A}
	Autoclaved	76 ± 14^{A}	1.83 ± 0.42^{A}	27.91 ± 5.04^{A}
	Live	64 ± 15^{A}	2.13 ± 0.39^{A}	27.16 ± 6.36^{A}
CW8T2 (*V. proteolyticus*)	No bacteria	67 ± 8^{A}	2.07 ± 0.62^{A}	27.74 ± 8.31^{A}
	Autoclaved	80 ± 15^{A}	1.85 ± 0.35^{A}	29.60 ± 5.45^{A}
	Live	60 ± 14^{A}	1.89 ± 0.40^{A}	22.68 ± 5.12^{A}
Significant improvements (no.)	Autoclaved	5/10	0/10	2/10
	Norvanol	3/7	0/7	1/7
	γ-Irradiated	4/8	0/8	5/8
	Live	7/10	0/10	4/10

[a] Shown are average survival (percent), IL (millimeters), and total length or TBP (millimeters per FT) of *Artemia* fed with the medium-quality microalga *Dunaliella tertiolecta* (strain DT CCAP 19/27) and inoculated with 10 bacterial strains added dead (obtained via autoclaving, norvanol treatment, or γ-irradiation) or live and compared with a control treatment in axenic conditions (no bacteria) (experiment 3). The number of bacterial strains providing significant improvements of *Artemia* TBP, survival, and IL, when added dead or live, in comparison to the bacterium-free control is also presented. Means and standard deviations were placed together (mean ± SD). For each bacterial strain, values in the same column showing the same superscript letter (A, B, C, or D) are not significantly different ($P > 0.05$).

revealed almost no differences in TBP. The only exceptions occurred with the strain LVS 2 (significantly lower performances in nauplii inoculated with autoclaved bacteria) and LVS 3, GR 12, and cluster A (significantly lower performances in nauplii inoculated with norvanol-killed bacteria). Yet nauplii fed with the microalga DT CCAP 19/27 presented, in most cases, higher variability in TBP depending on the procedure used to kill bacteria, except with strain LVS 2. γ-Irradiated preparations of GR 8, GR 10, GR 11, GR 12, and cluster A enhanced the performance of *Artemia* in comparison to the other two killing methods. For strain LVS 3, the highest nauplius TBP was achieved with autoclaved bacteria, while for strain LVS 8, the highest values were obtained with a norvanol-killed preparation. All differences were due to a combination of higher nauplius survival and IL but were not always statistically significant.

With live bacteria and mnn9 yeast as the main feed, significant improvements in *Artemia* TBP were observed only in

nauplii inoculated with three live strains, i.e., GR 8, GR 10, and GR 12, in comparison to bacterium-free nauplii, mainly due to the significantly higher IL of nauplii. For all other bacterial strains, no significant differences were observed in *Artemia* TBP. In contrast, nauplii fed with the microalga DT CCAP 19/27 showed significant improvements in *Artemia* TBP when supplied with four live bacterial strains (i.e., LVS 2, LVS 3, LVS 8, and GR 8). These differences were mainly due to both higher nauplius survival (often significantly) and/or IL (not significantly). When mnn9 yeast was used as feed, no live bacterial strains significantly enhanced the performance of *Artemia* beyond those registered with inactivated bacteria irrespective of the method used. The opposite occurred for LVS 8 and *Vibrio campbellii* strain LMG21363, due to decreased survival (LVS 8) or to a combination of both IL and survival (*Vibrio campbellii* strain LMG21363). In contrast, *Artemia* fed with the microalga DT CCAP 19/27 and inoculated with live cells of strain LVS 2 grew significantly better (higher values of TBP in comparison to nauplii inoculated with inactivated bacteria), mainly due to improvements in survival. The opposite occurred for strains LVS 8, GR 10, GR 11, and GR 12 and cluster A, due to decreased IL.

***Artemia* fed with good-quality feed.** Ten bacterial strains were tested dead and live in the gnotobiotic *Artemia* culture fed with a well-performing strain of the microalga *D. tertiolecta* (DT CCAP 19/6B). According to Table 5, (experiment 4), significant improvements in *Artemia* TBP were observed for nauplii inoculated with seven dead bacterial strains (i.e., LVS 3, LVS 8, GR 11, GR 12, cluster A, *Vibrio campbellii* strain LMG21363, and *Vibrio proteolyticus* strain CW8T2) in comparison to nauplii in bacterium-free conditions, mostly due to a statistically insignificant increase in IL. The comparison of *Artemia* fed with dead bacteria killed with different methods revealed significant differences in TBP of nauplii (except for LVS 2). γ-Irradiated bacteria (GR 8, GR 10, GR 11, GR 12, and cluster A) produced the highest *Artemia* performances, followed by the other two treatments, while for strains LVS 3 and LVS 8, nauplii performed better with autoclaved and norvanol-killed bacteria. These differences were mainly due to increases in IL of nauplii, which were not always statistically significant.

Significant improvements in *Artemia* TBP were observed only in nauplii inoculated with five live bacterial strains (i.e., LVS 3, LVS 8, GR 8, cluster A, and *V. proteolyticus*), in comparison to bacterium-free nauplii. All differences were mostly due to significantly higher IL. Living cells of strain GR 8 and cluster A enhanced significantly the performance of *Artemia* beyond those registered with inactivated bacteria irrespective of the method used, while the opposite occurred for strains GR 11 and GR 12, in all cases mostly due to differences in IL.

Bacteria ash-free dry weight content. In general, cells of live bacteria had higher AFDW contents than dead bacteria (Table 1) (although not always statistically significant). This might have contributed to the considerably higher TBP observed on *Artemia* fed microalgae DT CCAP 19/27 (supplied with the live strain LVS 2) and DT CCAP 19/6B (supplied with the live strain GR 8 and live cluster A) in comparison to nauplii inoculated with the same dead bacteria. The highest ratio obtained between the amounts of bacteria and yeast or microalgae added to *Artemia* in the experiments (in terms of AFDW) occurred in nauplii fed WT yeast (0.034 to 0.059), followed by DT CCAP 19/27 (0.016 to 0.028), DT CCAP 19/6B (0.013 to 0.023), and the mnn9 yeast (0.012 to 0.021). These ratios could explain, especially for WT yeast, the significant improvements registered in survival of nauplii when inoculated with bacteria, although the amount of bacterial biomass given was insufficient to significantly enhance the IL. For the other feeds, even with such low ratios, the addition of bacteria could sometimes significantly enhance *Artemia* performance (Tables 3 to 5).

DISCUSSION

Although WT and mnn9 yeast were cultured under identical conditions, *Artemia* fed with mnn9 yeast always performed better (Tables 2 and 3), even when bacteria were added. Similar results were obtained by Marques et al. (29, 30) for axenic *Artemia* fed with the same yeast strains and using a similar experimental setup. This difference can be due to an improvement in yeast digestibility to *Artemia* caused by the mnn9 mutation. As postulated by Coutteau et al. (11), the digestive tract of *Artemia* displays low mannase and high β-glucanase activities, complicating the digestion of yeast cells rich in cell wall-associated mannoproteins, such as WT yeast, and contributing to the proper digestion of yeast cells with reduced mannoprotein content, such as mnn9. As for the two microalga strains cultured in the same conditions, DT CCAP 19/6B always appeared to be a better feed for *Artemia* (Tables 4 and 5), even when bacteria were added. This difference was previously reported by Marques et al. (30) for axenic *Artemia* fed with the same algae using a similar experimental setup and is probably related to differences in the nutritional values of the two strains. Although the reason for the difference in feed quality between the four types of feed is still unclear, the experimental setup of the gnotobiotic environment was found to be very suitable for verifying in which way *Artemia* can benefit from the presence of bacteria against four nutritionally different backgrounds.

Bacteria can play an important role as a direct feed source for herbivorous zooplankton (40) and are reported to contribute to the nutritional value of *Artemia* feeds by being a major source (directly or indirectly) of proteins, vitamins (e.g., B_{12}), essential amino acids, fatty acids, polyamines, enzymes, and inorganic nutrients (17, 21). Lipids, as well as proteins, are the main nutrients required during the early developmental stages of *Artemia*, whereas carbohydrates, together with proteins, are more important for juveniles and adults (12). Levels of myristic, palmitic, stearic, oleic, linoleic, and linolenic fatty acids seem to stimulate growth and fertility of *Artemia* when added to the culture medium (12, 39), while the lack of some vitamins creates severe deficiencies in the metabolism of *Artemia* (20, 38). The efficacy of *Artemia* in bioencapsulating bacteria is dependent on the type of bacteria used, time of exposure, and status (live or dead) of the bacteria (14).

It is obvious from the data that dead bacteria were able to promote TBP of *Artemia* (Tables 2 to 5) independently from the quality of the feed provided. Especially when the major feed type was of low quality (WT yeast), almost all tested bacteria had a significant positive effect on the TBP (Table 6). However, the AFDW data indicate that when WT yeast is the major feed source, the relative proportion of bacteria in the

TABLE 5. Average survival, IL, and TBP of *Artemia* fed with good-quality microalga *Dunaliella tertiolecta* (strain DT CCAP 19/6B) and inoculated with 10 bacterial strains[a]

Bacterial strain or parameter	Treatment	Survival (%)	IL (mm)	TBP (mm/FT)
LVS 2	No bacteria	90 ± 7^{A}	2.91 ± 0.62^{A}	52.61 ± 3.79^{A}
	Autoclaved	89 ± 8^{A}	2.92 ± 0.54^{A}	51.83 ± 4.38^{A}
	Norvanol			
	γ-Irradiated	96 ± 8^{A}	2.89 ± 0.49^{A}	55.63 ± 4.33^{A}
	Live	91 ± 8^{A}	3.12 ± 0.61^{A}	56.94 ± 4.68^{A}
LVS 3	No bacteria	90 ± 7^{A}	2.91 ± 0.62^{A}	52.61 ± 3.79^{B}
	Autoclaved	100 ± 0^{A}	3.27 ± 0.61^{A}	65.30 ± 5.10^{A}
	Norvanol	99 ± 3^{A}	3.01 ± 0.61^{A}	59.45 ± 1.51^{A}
	γ-Irradiated	96 ± 5^{A}	2.68 ± 0.52^{A}	51.59 ± 2.57^{B}
	Live	98 ± 3^{A}	3.20 ± 0.69^{A}	62.43 ± 1.81^{A}
LVS 8	No bacteria	90 ± 7^{A}	2.91 ± 0.62^{A}	52.61 ± 3.79^{B}
	Autoclaved	93 ± 15^{A}	3.28 ± 0.63^{A}	60.68 ± 9.84^{A,B}
	Norvanol	100 ± 0^{A}	3.34 ± 0.77^{A}	66.80 ± 5.40^{A}
	γ-Irradiated	99 ± 3^{A}	2.93 ± 0.62^{A}	57.87 ± 1.47^{B}
	Live	99 ± 3^{A}	3.19 ± 0.62^{A}	63.00 ± 1.59^{A}
GR 8	No bacteria	92 ± 5^{A}	2.21 ± 0.43^{A}	40.97 ± 2.41^{B,C}
	Autoclaved	96 ± 5^{A}	1.91 ± 0.31^{A}	36.81 ± 1.75^{C}
	Norvanol	98 ± 3^{A}	1.87 ± 0.31^{A}	36.47 ± 1.08^{C}
	γ-Irradiated	92 ± 6^{A}	2.52 ± 0.61^{A}	46.12 ± 3.01^{B}
	Live	96 ± 3^{A}	2.84 ± 0.66^{A}	54.67 ± 1.42^{A}
GR 10	No bacteria	92 ± 5^{A}	2.21 ± 0.43^{A}	40.97 ± 2.41^{A}
	Autoclaved	98 ± 3^{A}	1.82 ± 0.30^{A}	35.53 ± 1.01^{B}
	Norvanol	100 ± 0^{A}	1.71 ± 0.31^{A}	34.20 ± 6.20^{A,B}
	γ-Irradiated	100 ± 0^{A}	2.47 ± 0.49^{A}	49.40 ± 9.80^{A}
	Live	98 ± 5^{A}	2.10 ± 0.54^{A}	40.95 ± 2.10^{A}
GR 11	No bacteria	92 ± 5^{A}	2.21 ± 0.43^{A}	40.97 ± 2.41^{B}
	Autoclaved	95 ± 4^{A}	1.84 ± 0.29^{A}	34.96 ± 1.50^{C}
	Norvanol	99 ± 2^{A}	1.75 ± 0.33^{A}	34.58 ± 0.83^{C}
	γ-Irradiated	95 ± 4^{A}	2.47 ± 0.55^{A}	47.05 ± 2.03^{A}
	Live	98 ± 5^{A}	2.02 ± 0.53^{A}	39.39 ± 2.02^{B}
GR 12	No bacteria	92 ± 5^{A}	2.21 ± 0.43^{A,B}	40.97 ± 2.41^{B}
	Autoclaved	96 ± 8^{A}	1.62 ± 0.27^{B}	31.19 ± 2.43^{C}
	Norvanol	99 ± 3^{A}	1.74 ± 0.30^{A,B}	34.37 ± 0.87^{C}
	γ-Irradiated	96 ± 5^{A}	2.47 ± 0.50^{A}	47.55 ± 2.36^{A}
	Live	100 ± 0^{A}	1.79 ± 0.40^{A,B}	35.80 ± 8.00^{B,C}
Cluster A	No bacteria	92 ± 5^{A}	2.21 ± 0.43^{A,B}	40.97 ± 2.41^{C}
	Autoclaved	96 ± 2^{A}	1.64 ± 0.27^{B}	31.63 ± 0.78^{D}
	Norvanol	96 ± 2^{A}	1.67 ± 0.28^{B}	32.17 ± 0.82^{D}
	γ-Irradiated	98 ± 3^{A}	2.53 ± 0.57^{A,B}	49.34 ± 1.46^{B}
	Live	98 ± 3^{A}	3.21 ± 0.70^{A}	62.60 ± 1.85^{A}
LMG21363 (*V. campbellii*)	No bacteria	92 ± 5^{A}	2.21 ± 0.43^{A}	40.97 ± 2.41^{B}
	Autoclaved	86 ± 9^{A}	3.18 ± 0.59^{A}	54.86 ± 5.43^{A}
	Live	79 ± 12^{A}	3.05 ± 0.62^{A}	48.04 ± 7.21^{A,B}
CW8T2 (*V. proteolyticus*)	No bacteria	92 ± 5^{A}	2.21 ± 0.43^{A}	40.97 ± 2.41^{B}
	Autoclaved	88 ± 12^{A}	3.30 ± 0.59^{A}	57.75 ± 7.86^{A}
	Live	95 ± 7^{A}	3.07 ± 0.64^{A}	58.33 ± 4.34^{A}
Significant improvements (no.)	Autoclaved	0/10	0/10	3/10
	Norvanol	0/7	0/7	2/7
	γ-Irradiated	0/8	0/8	3/8
	Live	0/10	0/10	5/10

[a] Shown are average survival (percent), IL (millimeters), and total length or TBP (millimeters per FT) of *Artemia* fed with the good-quality microalga *Dunaliella tertiolecta* (strain DT CCAP 19/6B) and inoculated with 10 bacterial strains added dead (obtained via autoclaving, norvanol treatment, or γ-irradiation) or live and compared with a control treatment in axenic conditions (no bacteria) (experiment 4). The number of bacterial strains providing significant improvements of *Artemia* TBP, survival, and IL, when added dead or live, in comparison to the bacterium-free control is also presented. Means and standard deviations were placed together (mean ± SD). For each bacterial strain, values in the same column showing the same superscript letter (A, B, C, or D) are not significantly different ($P > 0.05$).

feed offered was high (Table 1), suggesting that the TBP increase was partly due to the amount of bacterial biomass offered. Yet, when the dead autoclaved *Vibrio proteolyticus* strain CW8T2 and the *Vibrio campbellii* strain LMG21363 were added, this ratio was rather low (Table 1), while the relative increase in TBP was among the highest. This suggests that in some cases, the increase in *Artemia* TBP could not be attributed to the microbial biomass added. Further analysis of data in Tables 2 to 5 suggests that dead bacterial biomass affects *Artemia* survival to a larger extent, particularly when poor- and medium-quality feeds were used. However, dead bacteria do not seem to supply *Artemia* with enough essential nutrients to improve IL, since only in rare occasions was a significant increase in this parameter observed. This argues against the idea that dead bacteria can be suppliers of essential nutrients, especially when added in small amounts.

Performances of *Artemia* inoculated with bacteria killed with the three different methods revealed significant differences

TABLE 6. Effects of supplying *Artemia* fed yeast (WT or mnn9) or microalgae (DT CCAP 19/27 or DT CCAP 19/6B) with dead or live bacterial strains[a]

Feed	Effects of bacterial strain									
	LVS 2	LVS 3	LVS 8	GR 8	GR 10	GR 11	GR 12	Cluster A	LMG21363	CW8T2
Poor-quality										
WT	i/***L***/l	***I***/***L***/l	***I***/***L***/d	***A***/***L***/l	***N***/***L***/d	***A***/***L***/d	***A***/l/***D***	***A***/l/d	***A***/***B***/***D***	***A***/l/***D***
Medium-quality										
mnn9	i/l/l	***A***/l/d	***A***/l/***D***	i/***L***/l	n/***L***/l	a/l/d	a/***L***/l	i/l/d	***A***/b/***D***	***A***/l/d
DT CCAP 19/27	i/***L***/***L***	***A***/***L***/l	***N***/***L***/***D***	***I***/***L***/d	***I***/l/***D***	***I***/l/***D***	***I***/l/***D***	***I***/l/***D***	a/b/d	a/b/d
Good-quality										
DT CCAP 19/6B	i/l/l	***A***/***L***/d	***N***/***L***/d	i/***L***/***L***	i/b/d	***I***/b/***D***	***I***/b/***D***	***I***/***L***/***L***	***A***/l/d	***A***/***L***/l

[a] Three effects were distinguished (separated by shill [/]): (i) the killing method (represented by the letters I [irradiated], N [norvanol treated], and A [autoclaved]) that gave the highest TBP above the blank (no bacteria), (ii) the treatment with live bacteria (l) that gave higher TBP than the blank (b), and (iii) live bacteria (l) that gave higher TBP than the best treatment with dead bacteria (d), independently of the method used. Capital boldface and italic letters represent significant differences in TBP.

(Table 6). Overall, two distinct patterns could be observed in the present study. In the first pattern, higher performance of nauplii occurred when γ-irradiated bacteria were added, followed by norvanol- and autoclave-killed bacteria. According to Marques et al. (29), autoclaving destroys more "sensitive molecules" than less destructive methods such as γ-irradiation. This fact could explain the results obtained in the first pattern. In the second pattern, autoclaved bacteria supported the highest *Artemia* performance, followed by norvanol-killed and γ-irradiated bacteria. Despite destroying "sensitive molecules," autoclaving also damages the cell wall of an organism (including bacteria) (33) by thermal denaturation of proteins essential for cell wall rigidity. In this way, the cell wall becomes weaker (24) and digestion of whole autoclaved bacterial cells by *Artemia* enzymes is improved. The denaturation of proteins also occurs with norvanol (26) and γ-irradiation, but to a lesser extent (36).

Previous experiments by Verschuere et al. (50, 51) characterized live cells of strains LVS 2, LVS 3, and LVS 8 as performance enhancing, supporting significant improvements in *Artemia* TBP compared to the axenic control, while the *Vibrio proteolyticus* strain CW8T2 was typed as a strain with a negative influence on *Artemia* performance. Similar results were obtained in the present study when low-quality feed (WT yeast) was used to feed nauplii (Tables 2 and 6), except for live *Vibrio proteolyticus* strain CW8T2, which acted as a neutral bacterium, thus not influencing *Artemia* performance. It thus seems that this strain is not a primary pathogen to *Artemia* but rather an opportunistic organism that expresses its virulence only when *Artemia* is cultured in suboptimal conditions. In fact, in *Artemia* fed with the good-quality microalga DT CCAP 19/6B, *Vibrio proteolyticus* strain CW8T2 even behaved as a performance-enhancing bacterium. Likewise, Verschuere et al. (50, 51) used γ-irradiation to obtain bacterium-free inert feed that was provided to axenic nauplii. However, γ-irradiation causes some negative effects on the feed quality (e.g., by destruction of essential nutrients) (29), in this way weakening nauplii provided with such feeds.

Live cells of strains GR 8, GR 10, and GR 11 were considered as performance-enhancing bacteria to nauplii fed poor-quality WT yeast, while the live *Vibrio campbellii* strain LMG21363 was characterized as a bacterium with a negative influence on the performance of *Artemia* (Table 6), confirming the findings of Soto-Rodriguez et al. (46) that this bacterium is pathogenic to *Artemia*. When better-quality feeds were provided to *Artemia*, different live bacterial strains were considered performance-enhancing bacteria. Yet, independently of the feed added to cultures of nauplii, the strain GR 8 was always a performance-enhancing bacterium in a significant way (Table 6), and thus, this strain can be considered as a candidate for further tests on its quality as a probiont. The two pathogens (the *Vibrio campbellii* strain LMG21363 and *Vibrio proteolyticus* strain CW8T2) did not express any detrimental effect when added to medium- and good-quality feeds. Previous findings of Burgents et al. (8) and Patra and Mohamed (35) reported protection of shrimp and *Artemia*, respectively, by yeast supplements against pathogenic *Vibrio*.

Beneficial or pathogenic effects caused by a bacterial strain in *Artemia* were reduced or even disappeared when medium/good-quality feeds were used. This could be due to improvements in the *Artemia* status (health condition) caused by specific characteristics of the feeds either of a nutritional nature, due to the induction of digestive enzymes secreted by the feed, in the same way as previously described for European sea bass supplied with microalgae (9) and baker's yeast (47), or eventually as a result of nonspecific stimulation of the brine shrimp immune response against the bacteria. Mnn9 yeast possesses high levels of β-glucans and chitin in the cell wall, while the microalga *D. tertiolecta* is reported to contain high levels of β-carotene in its cells (1). β-Carotene (6, 25), chitin (4, 43), and β-glucans (10, 32) are involved in the optimal function of the immune system in terrestrial and aquatic animals and are known to enhance overall disease resistance and improved health and performance in aquatic organisms (e.g., shrimp and fish). However, further studies are still needed to verify these hypotheses using the present gnotobiotic system.

Previous studies on the effects of bacteria in *Artemia* (13, 34, 50, 51) did not clarify the mode of action of live bacteria in *Artemia* assays. In order to separate the nutritional effects of a bacterial strain from any probiotic effect, the performance of *Artemia* inoculated with γ-irradiated, norvanol-treated, or autoclaved bacteria (depending on which treatment had the highest effect) was compared to that of nauplii cultured with the same live bacterium. Significant improvements in *Artemia* TBP were detected when live cells of LVS 2 (DT CCAP 19/27), GR 8, and cluster A (DT CCAP 19/6B) were added in comparison

to dead bacteria. The AFDW was generally lower in γ-irradiated and norvanol- and autoclave-killed bacterial cells than in the same live bacterium (Table 1). Hence, these enhancements could be partly due to a reduction of the bacterial quality and/or the amount of nutrients by γ-irradiation. In addition, live bacteria are likely to grow in the gnotobiotic environment during the 6-day test, resulting in the recycling of nutrients which are channeled towards *Artemia* and in an improvement of the overall water quality (51), or, more specifically, in removing toxic metabolic substances that can adversely affect the growth and survival of *Artemia*, especially when cultured under suboptimal conditions (50). However, improvements observed in *Artemia* performance when live bacteria were added could also be related to other beneficial effects of bacteria, such as supplying active bacterial enzymes allowing additional digestive abilities in the intestine of *Artemia* (23, 50) and inducing digestive enzyme secretion of *Artemia*.

In contrast, *Artemia* supplied with the other live beneficial bacterial strains did not profit from the extra amount of nutrients delivered by these bacteria in comparison to nauplii provided with the same amount of dead bacterium. However, these bacteria should not necessarily be discarded as probionts, since their probiotic nature may eventually be expressed only in the presence of pathogens, as these bacteria can enhance the nauplius immune response (52) or prevent the proliferation of opportunistic pathogens by competing for available resources (nutrients, space, adhesion sites in the gut or on the surface of *Artemia*, etc.) or through antagonism (production of toxic or inhibitory substances) (51, 52).

It became clear that in the above-described gnotobiotic environment, the tested microorganisms can be catalogued in different classes: probionts (which have a beneficial and active effect on an organism and/or on the culture medium), pathogens (including opportunistic bacteria that can cause diseases or any other disturbance in an organism), and neutral microorganisms (not causing any marked effects on an organism). In parallel, many microorganisms can be considered as potential sources of nutrients for *Artemia* (depending on the nutritional requirements and on the accessibility of the nutrients, e.g., thickness or smoothness of the cell wall), improving its general condition in this way. Yet, further research is still ongoing to elucidate the exact mode of action of the observed beneficial and pathogenic effects of bacteria, combining the present approach with challenge tests and other types of analysis (e.g., transcriptome and proteonomic analyses). Finally, experimental trials in all possible host-target combinations are also required before making any critical assessment.

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