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Use of *Microbacterium* sp. and *Exiguobacterium mexicanum* to improve the survival and development of *Artemia* under xenic conditions

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Abstract The effect of *Microbacterium* sp. strain 8L and *Exiguobacterium mexicanum* strain 8N was evaluated in the diet of *Artemia* under xenic conditions. Viable cultures of bacteria were provided to xenic cultures of *Artemia* in combination with *Sacharomyces cerevisiae*, cornflour or *Spirulina*, and the effect on the survival and growth was recorded. The use of these bacterial strains improves significantly the survival of *Artemia* independently of the used food ($P < 0.05$), and variable results were observed in the growth.

Keywords *Artemia* · Bacteria · *Microbacterium* · *Exiguobacterium* · Culture

Abbreviations

DI	Development index
A	Instar stage
N	Number of organisms
ANOVA	Analysis of variance
P	Significance level
Sc	*Sacharomyces cerevisiae* yeast
CF	Cornflour
Spr	*Spirulina*

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Introduction

Sacharomyces cerevisiae has been used as a partial or total substitute for microalgae in *Artemia* production (Coutteau et al. 1990, 1992; Kyun and Chung 2001; Marques et al. 2004), but generally a low survival rate and variable results in the growth have been reported, which suggests that the bread yeast is inadequate as a food for *Artemia* (Coutteau et al. 1990).

Most studies on the use of yeast in *Artemia* have been carried out under xenic conditions and the role of the bacteria has not been considered. During a meticulous experiment, Orozco-Medina et al. (2002) found improvements in the survival and development of *Artemia* when bread yeast was used in combination with cultures of *Microbacterium* sp. (strain 8L) and *Exiguobacterium mexicanum* (strain 8N). Those improvements could be useful for aquaculture by enabling the elimination of microalgae from the diet of *Artemia*. However, their experiments were carried out under gnotobiotic conditions, which are impractical for the production at commercial scale. In the present study, we analyze if the beneficial effect attributable to these strains also occurs under xenic conditions. Furthermore, we compare the effect of *Microbacterium* sp. and *E. mexicanum* with cornflour and *Spirulina*.

Material and methods

Bacteria

The strains 8L (*Microbacterium* sp.) and 8N (*Exiguobacterium mexicanum*) used by Orozco-Medina et al. (2002) were provided by Dr. Alejandro López-Cortéz (Centro de Investigaciones Biológicas del Noroeste, México) from a cryopreservated stock. Originally these strains were isolated from *Artemia* cysts. The strains were cultured in marine agar 2216 (Difco) at 27°C during 24 h, the cells were transferred to 3.6 l of liquid media and incubated during 8 days at 27°C in an orbital shaker. The biomass was harvested by centrifugation at 10,000 rpm and 4°C by 10 min. The supernatant was discarded and the cells were dried at 45°C under sterile conditions. The numbers of viable bacteria were determined using standard microbiological procedures.

Artemia

Artemia cysts (Argentemia, San Francisco Bay, California, USA) were placed to hatch in 1.0-μm-filtered seawater at 27°C under continuous illumination and aeration, according the procedures described by Sorgeloos et al. (1986). The nauplii (instar I) were harvested and distributed in 3-liter aquariums with 500 ml of seawater at a density of 100 nauplii by aquarium. All aquaria were placed in a water bath at 28°C. The larvae were fed twice a day at 08:00 h and 16:00 h with 1 ml of experimental food. The dosage of food was increased at 2 ml from day 5 of culture.

Experimental design

Three experiments were carried out. In the first experiment we evaluate the effect of 8L and 8N alone and in combination during the culture of *Artemia* nauplii fed with yeast

during a 4-day trial; in the second experiment we evaluate the effect of cornflour alone and with yeast in combination of 8N and 8L during a 6-day trial; in the third experiment we evaluated the effect of yeast complemented with *Spirulina* powder (Prot-Alga, México) and the combination of 8L and 8N during a 12-day trial. Four replicates per treatment and controls without bacteria were included in each experiment.

In all cases the foods were prepared at 5 g l^{-1} of yeast, cornflour, and *Spirulina*, respectively, in artificial seawater (Salt commercial Instant Ocean) and autoclaved for 40 min. Treatments with bacteria were inoculated with 5 g l^{-1} of dried bacteria and were incubated at 27°C during 48 h. The bacteria in the prepared food after incubation were estimated at 8×10^7 CFU ml^{-1} for 8L, and 3×10^7 CFU ml^{-1} for 8N.

The number of survivals and their development stages (as described by Scherhardt 1987) was recorded at the end of each experiment. The development in each replicate was evaluated by the mean development index (DI), which was calculated according Villegas and Kanazawa (1979):

$$DI = \sum A/N$$

where A is the instar stage of each organism and N is the number of organisms in the sample.

The values for A were 1–17, considering four metanauplii stages (I–IV), seven post-metanauplii stages (I–VII), five postlarvae stages (I–V), and the adult stage.

Statistical analysis

The data for survival (percentage) were normalized by the arcsin function according to Zar (1996) and analyzed by the analysis of variance (ANOVA) test followed by a Tukey multiple test. Because the DI were normally distributed they were compared by ANOVA and Tukey. All analysis were performed using Statistica version 7.0 (Stasoft®).

Results

During our experiments, significant differences were found in survival and growth ($P < 0.05$). Consistently the best results in survival and growth of *Artemia* were obtained in the treatments with 8L and 8N (Fig. 1).

During the first experiment high percentage survival (99%) was obtained with the mixture of both strains. However, when compared with the 8N treatment (survival 93%), no statistical difference was found ($P > 0.05$). The lowest survival percentage (44%) was recorded in the treatment with only yeast ($P > 0.05$) (Fig. 1a).

Similar results were found in the *Artemia* development (DI), where the higher values were found with the mixture of both bacteria (Fig. 1b) and a significantly lower value was found in the treatment with only yeast ($P < 0.05$). Statistical differences were not found when comparing development between the individual treatments of 8L and 8N ($P > 0.2$) (Fig. 1b).

In the second experiment the lowest survival was recorded with cornflour only, but this was clearly improved with the addition of bacteria ($P < 0.05$) (Fig. 1c). No differences were found with treatments that include yeast ($P > 0.05$). The development (DI) was the same in the treatments that contained bacteria ($P > 0.8$) (Fig. 1d), and was higher than that recorded in the cornflour-only treatment ($P < 0.001$).

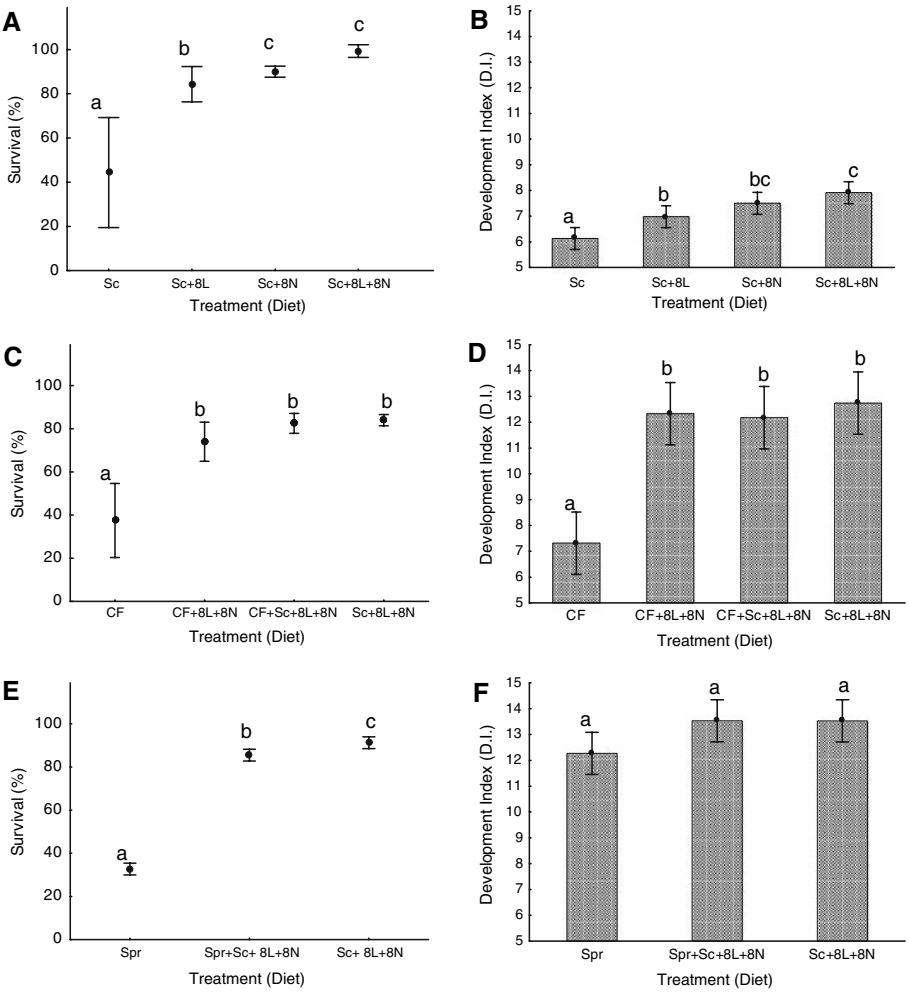

Fig. 1 Effect of *Microbacterium* sp. (8L) and *Exiguobacterium mexicanum* (8N) on survival (**a**, **c**, and **e**) and development (**b**, **d**, and **f**) of *Artemia* fed with yeast (Sc), cornflour (CF) or Spirulina (Spr) under xenic conditions (see text for details). Data are the average and standard deviations; different letters indicate significant differences at $P < 0.05$

Similar results were found during the third experiment; the use of 8L and 8N improved significantly the survival and the development of *Artemia* compared with the treatment with *Spirulina* only (Fig. 1e), in which the survival only reached 32%. No significant differences were found between the development index ($P > 0.05$) in this experiment (Fig. 1f).

Discussion

In the present study we found that *Microbacterium* sp. and *Exiguobacterium mexicanum* (strains 8L and 8N, respectively) induced significant improvements in the development and survival of *Artemia* under xenic conditions.

Improvements were previously reported by Orozco-Medina et al. (2002) under gnotobiotic conditions. However, in general our results were better than those reported by Orozco-Medina et al. (2002) in terms of survival and growth, which suggests that the normal microbiota does not interfere with the beneficial effects of 8L and 8N in the *Artemia* cultures.

The xenic conditions were also favorable to promote improvements in growth; during our study we obtain postmetanauplii VII at 4 days after hatch (dah) whereas Orozco-Medina et al. (2002) obtained postmetanauplii I at 6 dah. We did not evaluate the actual cause of that improvements, however we suppose that the normal microbiota is a synergistic component to 8N and 8L, serve as a nutritive complement, or produce components that promote the development (e.g., vitamins).

During our study we obtain a low survival rate (44%) in cultures of *Artemia* fed with yeast only; similar results were reported by Coutteau et al. (1990). However, when the 8L and 8N strains were included in the feed, survival increased to 99%. This beneficial effect was also observed when each strain was assayed separately (Fig. 1a) and survival of 85% and 93%, respectively, was recorded.

Marques et al. (2005) found variable effects of different bacteria on the survival of *Artemia*, apparently associated with food quality. Significant improvements in survival were only recorded with a yeast of poor nutritional quality and their best results were obtained with *Aeromonas hydrophila* (76%) followed by *Vibrio* sp. (66%) and *Roseobacter* sp. (60%). During our study we found a greater improvement in survival (>90%) than that reported by those authors and, although we did not evaluate the nutritional value of the yeast strain, the survival recorded in our treatment with yeast only was comparable to that reported by Marques et al. (2005), with their medium-quality yeast strain (about 45%) in which no difference were found with the use of bacteria. These differences could be attributed to the specific characteristics of 8L and 8N or to the effect of xenic conditions.

Some diets based on cereal flour have been evaluated in *Artemia* production (Dobbeleir et al. 1980; Sorgeloos et al. 1986; Rosowski 1989; Lavens and Sorgeloos 2000) producing a middle survival rate. During our experiment, the survival in the treatments with cornflour was 37% but was improved when the cornflour was inoculated with 8L and 8N, inducing a significant increase in the survival rate to 74% ($P < 0.05$) (Fig. 1c). However, those results were inferior to those obtained with controls of yeast-bacteria (84%).

The use of *Spirulina* in the diet of *Artemia* was previously evaluated by Johnson (1980) with acceptable results in terms of growth and survival. However, Castro et al. (1995) and Doulliet (1987) reported 18% and 32.5% survival in *Artemia* fed with *Spirulina*, respectively. Also Doulliet found that the use of selected bacteria together with *Spirulina* increased survival to 92.5%. During our experiments, similar result were found with *Spirulina* alone and complemented with bacteria, with survival of 32% and 85%, respectively. However, during our experiments the diet of *Spirulina* was also complemented with yeast and, considering that the yeast-bacteria combination induced a significant increase in survival to 91%, we think that the beneficial effect in the *Spirulina* bacteria treatment could be due to the combined effect of bacteria and yeast in the diet. The advantageous use of yeast in the diet of *Artemia* has been previously reported, and Marques et al. (2006) demonstrated a protective effect against pathogenic bacteria; more studies are necessary to evaluate their contribution to the beneficial effect observed in the present study.

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