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Effect of associated bacteria on gnotobiotic *Artemia* performance

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ABSTRACT

Bacteria naturally occurring in Artemia culture systems act as direct food for the culture organism and also food digestion promoter. However, in the open culture conditions, that, Artemia consumes plenty of food sources available in the pond, makes it difficult to evaluate the role of associated bacteria in growth enhancement. Therefore, this study was conducted under gnotobiotic culture conditions to evaluate the effect of associated bacteria on growth and survival of Artemia. Bacteria-free baker´s yeast Saccharomyses cerevisiae strain wild-type (WT) was used as a main food source for bacteria-free Artemia nauplii in 6 days of culture. Nine unidentified bacteria strains (HT1-HT9) were isolated from culture water of good performance Artemia in our preliminary test, some of these isolates had proven as a diet for Artemia, bacteria were added to Artemia culture only one time at a pre-tested concentration of $5x10^6$ cells/mL. The results of this study showed that Artemia survival and growth mostly increased in cultures where isolate added as compared to that obtained in Artemia fed WT solely. Especially, the total length of Artemia/screw-cap glass tube was obtained nearly double in the culture where HT1, HT3, HT6 and HT7 added, compared to that obtained in solely WT-fed Artemia. The results of this study indicated that bacteria not only act as a direct food but the presence of these bacteria in the culture water also as water bio-remediator that result in improved survival of Artemia, and bacteria may also improve the availability of yeast nutrients to Artemia that result in the increased length of Artemia.

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1 INTRODUCTION

Newly hatched *Artemia* nauplii have been one of the most reliable live food organisms for fish and shellfish larviculture ever since their use was reported for the first time in many decades ago (Seale, 1933; Sorgeloos *et al*., 2001). The ability of *Artemia* to thrive well both in controlled indoor conditions and in exposed outdoor conditions is considered invaluable for a variety of its applications. *Artemia* is extensively cultured in typical outdoor conditions such as saltworks in the Mekong Delta, Vietnam. Animal waste material and agricultural byproducts have reportedly been used for this type of pond production, for the purpose of manuring the ponds to stimulate primary production and/or for direct feeding (Brands, 1996; Baert *et al*., 1997; Anh, 2009).

In both outdoor and indoor production systems, bacteria in the water play a role in *Artemia* nutrition. Bacteria in culture systems prove to be a source of food for the filter feeding *Artemia* (Yasuda and Taga, 1980; Douillet, 1987; Intriago and Jones, 1993; Gorospe *et al*., 1996), e.g. as a dietary

source of fatty acids and amino acids (Austin, 1988; Gorospe *et al*., 1996). Moreover, bacterial enzymes act as extrinsic enzymes in the *Artemia* gut, facilitating the digestive capability of the host (Intriago and Jones, 1993; Marques *et al*., 2004). Bacteria also remove toxic metabolites (Verschuere *et al*., 2000) by the bacterial nitrification process in which ammonium is converted to nitrite and then to the less toxic nitrate. However, literature data do not provide reliable information on the effect of the bacteria, associated with *Artemia* laboratory or pond culture, on *Artemia* performance. To optimize the biomass and cyst production in pond based *Artemia* culture systems, utilization of these associated bacteria may play a significant role. But in these open systems, uncontrollable variations in many parameters, such as in the availability of different food sources (bacteria or algae) make it extremely difficult to evaluate the contribution of single diet among a wide variety of foods available at the same time.

In previous study (Toi *et al*., 2014), some associated bacteria, which were isolated from the culture medium from a preliminary test run where *Artemia* had shown good performance in terms of survival and body length, were demonstrated as a diet for *Artemia* in gnotobiotic culture conditions. However, these isolates were offered to *Artemia* as a main food, and did not point out the improvement of biomass production when there was a presence of bacteria. This study was, therefore, to aim at elucidating the effect of bacterial strains, isolated from *Artemia* cultures in laboratory, on the performance of *Artemia* in terms of survival and body length. For this purpose, the isolated bacteria were added into a pre-test of the free-bacteria *Artemia* culture where bacteria-free baker´s yeast *Saccharomyses cerevisiae* strain wild-type (WT) was offered as a main food to *Artemia* under a controlled laboratory gnotobiotic culture system.

2 MATERIALS AND METHODS

2.1 Experimental designs

Nine unidentified bacteria strains (based on a difference in shape, size, and color of colony forming unit; and a difference in DNA band position of bacteria in the electrophoresis pattern) coded from HT1 to HT9 from previous study (Toi *et al*., 2014) were used in the experiment. These bacteria were isolated from the culture medium from a preliminary test run where *Artemia* had shown good performance in terms of survival and body length. In this preliminary, *Artemia* culture baker's yeast *Saccharomyces cerevisiae* strain WT was offered to *Artemia* (Toi *et al*., 2014). Some of isolates had proven as a diet for *Artemia* in the previous study under laboratory gnotobiotic culture conditions (Toi *et al*., 2014). The results of this study are presented in Table 1.

Table 1: Average survival (%), individual length (IL) (mm) and total length (TL) (mm)/tube of *Artemia* fed on bacteria isolated from *Artemia* cultures at the day 6th of culturing. The values are mean ± standard deviation (n = 4). Different superscripts in the same column denote significant differences (p < 0.05). IL values showed no significant differences (Toi *et al*., 2014)

Treatment	Survival (%)	IL (mm)	TL (mm)/tube
1. LVS3 (control)	35±11^{c}	0.9 ±0.2	6.4±2.2^{b}
2. No bacteria	0.0^{a}	-	-
3. HT1	0.0^{a}	-	-
4. HT2	0.0^{a}	-	-
5. HT3	35±4^{c}	1.1±0.2	7.5±1.3^{b}
6. HT4	0.0^{a}	-	-
7. HT5	0.0^{a}	-	-
8. HT6	48±14^{c}	1.0±0.2	9.7±3.4^{b}
9. HT7	6±5^{b}	0.9±0.1	1.1±0.9^{a}
10. HT8	13±10^{bc}	1.0±0.2	2.5±2.1^{a}
11. HT9	0.0^{a}	-	-

In order to evaluate the effect of associated bacteria on *Artemia* survival and growth, 10 treatments were carried out on germ-free *Artemia* nauplii with the nine isolates under gnotobiotic culture conditions. WT yeasts were offered to *Artemia* as a main feed, and bacteria was added as a supplement. As the control, *Artemia* was offered with bacteria-free WT yeast only. The performance of *Artemia* in terms of survival, individual body length and total length was used as criterion to assess the positive effect of bacteria on *Artemia*. Twenty bacteria-free *Artemia* nauplii were transferred under laminar flow conditions to a sterile 40 mL screw-cap glass tube containing 30 mL of filtered (0.2 µm) auto-

claved artificial seawater (FAASW) containing 30 g/L of Instant Ocean synthetic sea salt. There were four replicates for each treatment. *Artemia* were daily fed with WT yeast according to feeding schedule with adapted from Coutteau *et al*. (1990) (Table 2). Bacteria were added to *Artemia* culture only once at a pre-tested concentration of $5x10^6$ cells/mL (Marques *et al*., 2006) over 6 days of culture period.

After feeding, all the tubes were carefully closed and placed on a rotator at 4 cycles per minute in a room controlled temperature (28.0 ± 1.0^0C).

Table 2: Feeding schedule of *Artemia* fed on microalgae and baker's yeast added per tube (FT) (adapted from Coutteau *et al*., 1990)

Day	Baker's yeast (Cells/FT)
1	$9x10^6$
2	$18x10^6$
3	$18x10^6$
4	$24x10^6$
5	$36x10^6$

2.2 Diet preparation

2.2.1 Culturing and harvesting bacteria

For mass culture, each strain of bacteria stored at −80°C was streaked on marine agar (BD Difco™) plates (n=2). Visible colonies appeared after two days of incubation at 28°C. A single colony from each bacterial strain was picked by a sterile loop and transferred to a sterile Erlenmeyer containing autoclaved marine broth 2216 (MB Difco™). After inoculation, the inoculated flasks were carefully closed by sterile cotton caps and incubated in a shaker (28°C; 150 rpm) for 24−48 hours.

When visible growth appeared, the bacterial suspension in the incubated flasks was transferred to sterile 50 mL screw-cap falcon tubes under laminar flow hood conditions. The cells were harvested at the stationary growth phase by centrifugation (±4,400 × g; 15 minutes). Bacterial cell pellets were washed twice in FAASW and re-suspended in FAASW. The density of bacteria was determined by measuring its turbidity using a spectrophotometer set at 550 nm, assuming that an optical density of 1 corresponds to 1.2×10^9 cells/mL, according to the McFarland standard (BioMerieux, Marcy L'Etoile, France).

2.2.2 Culturing and harvesting yeast

Baker´s yeast strains WT, originated from the European *Saccharomyces cerevisiae* archived for functional analysis EUROSCARF, University of Frankfurt, Germany, were used in the study. WT was used for its low digestibility (Marques *et al*., 2004).

Before mass culturing, yeast was streaked on a Yeast Extract Peptone Dextrose agar plate (n = 2) containing 10 g/L yeast extract (Sigma), 10 g/L pep tone (Sigma), 20 g/L dextrose (Sigma), and 20 g/L agar. The medium was prepared in FAASW. Visible colonies appeared after three-day incubation in the dark at 28°C.

A single colony forming unit of each yeast strain was picked up from the agar plate by a sterile loop and transferred into 500 mL sterile Erlenmeyer flasks for inoculation. Each inoculated Erlenmeyer contained 300 mL of 0.2 µm filter-sterilized yeast nitrogen base medium (without ammonium sulfate and amino acids), and was supplemented with 5.0 g/L ammonium sulfate, 5.0 g/LD-glucose, 0.02 g/L L-histidine, 0.04 g/L L-methionine, and 0.04g/L LD-tryptophan in FAASW.

After inoculation, the inoculated flasks were carefully closed by sterile cotton caps and incubated in a shaker (28°C; 120 rpm). Yeast cultures were harvested at the exponential growth phase, detected by optical density (Marques *et al*., 2004). Yeast cultures from the Erlenmeyer were first transferred to 50 mL sterile screw-cap plastic falcon tubes, and the yeast cell pellet was obtained by centrifuging (± 2,000 x g; 5 minutes). The pellet was washed twice in FAASW (Marques *et al*., 2006; Soltanian *et al*., 2007) and then resuspended in FAASW. The yeast solution was stored at 4°C for subsequent use. Its concentration was determined based on cell counts using a Burker haemocytometer chamber. All the manipulations related to yeast harvesting were performed under a laminar flow hood to maintain sterility.

2.3 Preparation of *Artemia* nauplii

For preparation of bacteria-free nauplii, cysts of *Artemia franciscana* Kellogg1906 (EG® Type, INVE Aquaculture, Belgium) were decapsulated according to the protocol as described by Sorgeloos *et al*. (1977) and Marques *et al*. (2006). Briefly, the dry cysts were first soaked in tap water for 1 hour and then transferred to a laminar flow hood for decapsulation. The *Artemia* cyst shell was removed by reaction with sodium hypochlorite. Decapsulated cysts were harvested on a sterile sieve, and washed with FAASW as much as possible to remove all residual bleach and were transferred into 1 L sterile bottles containing 1 L of FAASW for hatching at 28°C for 24-30 hours under standardized hatching conditions (Sorgeloos *et al*., 1986).

2.4 Method used to verify axenity

The axenity of food, *Artemia* nauplii and *Artemia* culture medium was checked by the methodology as described by Marques *et al*. (2004). Briefly, food, *Artemia*, hatching and culture water were checked for contamination by plating on marine agar plates (n=2). The plates were checked for absence of bacteria after incubation at 28°C for 5 days. The experiment was discarded whenever the *Artemia* nauplii, water or food were found to be contaminated.

2.5 Sample collection and data analysis

Survival, individual length and total length

The data of survival, the IL of *Artemia* were recorded. At the end of the culture period, free-swimming *Artemia* were counted, and the survival of *Artemia* per treatment was calculated. Subsequently, the *Artemia* were fixed in Lugol´s solution to measure the IL, which was done using a dissecting microscope set with a drawing mirror, digital plan measure and the software *Artemia* 1.0 (courtesy of Marnix Van Damme). The TL of *Artemia*/crew-cap glass tube was calculated according to the following equation as reported by Marques *et al*. (2006):

TL of *Artemia* (mm/crew-cap glass tube) = number of survivors x mean IL (mm)

Statistical analysis

The datasets of survival, IL were checked for homogeneity of variance and normal distribution by Levene's F test and P-P plot by using Statistica 7.0 for Windows. The data failed to meet these assumptions were logarithmically transformed to satisfy normal distribution and to homogenize variance. For all datasets, one-way analysis of variance (ANOVA) followed by Tukey´s honestly significant difference post-hoc test at 0.05 probability level was employed.

3 RESULTS AND DISCUSSION

The survival of *Artemia* fed with WT combined with other nine bacterial strains and *Artemia* fed only WT is presented in Figure 1. The mean survival of *Artemia* ranged from 40% to nearly 100% at day 6th. The mean survival of *Artemia* fed only WT was around 40%. The survival increased in all treatments where isolated bacteria added, except for the culture where HT2 added. The survival of *Artemia* in the culture with HT4, HT5, HT8 and HT9 addition was nearly 70%, but the increase of survival in these cultures was not significantly different ($p > 0.05$) when compared to that obtained in the solely WT-fed *Artemia*. Specially, the addition of bacteria HT1, HT3, HT6, and HT7 strain yielded nearly double survival and higher significantly different ($p < 0.05$) as compared to that obtained in the control WT-fed *Artemia*.

In previous findings, Toi *et al*. (2014), under gnotobiotic culture conditions, have proven that some isolates associated with *Artemia* culture were being as suitable diet for *Artemia* nauplii (Table 1). However, some isolates were not a good diet for *Artemia*, survival was zero at day 6th. No attempt was made to identify these bacteria. Maybe they sustained *Artemia* growth for a period shorter than 6 days.

In this study, the isolates (HT1 to HT9) were used as supplement for WT-fed *Artemia*, experiments were aimed to elucidate the effect of bacterial presence on survival and TL of *Artemia*. Marques *et al*. (2004) found that the low survival was obtained in *Artemia* fed on WT yeast because this strain contains mannoprotein cell wall which was poor digestibility to *Artemia*. According to Coutteau *et al*. (1990), mannase activity is absent in the digestive tract of *Artemia*. The findings may explain for poor survival in WT-fed *Artemia*. But the addition of isolates only one time in the pre-test and survival of *Artemia* mostly increased. Bacteria may be as a supplement diet for *Artemia* (Intriago and Jones, 1993; Gorospe *et al*., 1996; Toi *et al*., 2014), playing an important role in environmental improvement regard to nitrifying bacteria control the ammonia level in the culture (Verschuere *et al*., 2000; Loka *et al*., 2016) and also enzymes producers (Intriago and Jones, 1993; Titapoka *et al*., 2008; Asad *et al*., 2011). Gorospe *et al*. (1996) reported that the survival of *Artemia* was improved when bacterial *Pseudomonas* strain was added to rice bran-fed *Artemia*. In addition, the supplement of *Bacillus* sp. LT3 bacteria to the *Artemia* culture improved survival of *Artemia* (Niu *et al*., 2014). This may explain the survival of *Artemia* in this study mostly increased where isolates added. In the previous study (Toi *et al*., 2014), some isolates (HT1-HT6) had proven as a diet for *Artemia*, but *Artemia* could not survive up to day 6th with some isolates. In this study, the improved survival was obtained in most of culture where these isolates were added with small amount in the pre-test to WT-fed *Artemia*, this result proves that the associated bacteria are not only a diet for *Artemia* but their association in the culture water also as water bio-remediator that results in improved survival of *Artemia*.

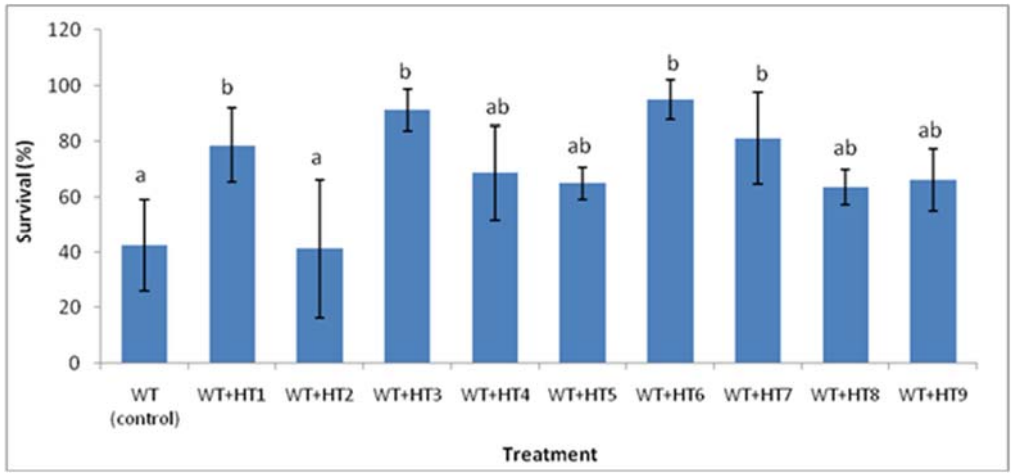

Fig. 1: Average survival (%) of *Artemia* fed on isolated bacteria only and of *Artemia* fed WT yeast with bacteria addition

The values are mean ± standard deviation (n = 4). Mean denoted by different letters are significantly different (p < 0.05)

The IL of *Artemia* also increased when isolates added (Figure 2), except for the addition of HT2 which had nearly similar IL compared to that obtained in *Artemia* fed only WT. The mean IL ranged 1.2-1.4 mm. The highest value (nearly 1.4 mm) was recorded in HT1, HT3 and HT7 addition, but the addition of these three bacteria was not significantly different ($p > 0.05$) in IL as compared to obtained in WT-fed *Artemia*. In the previous study, WT had been reported as poor diet for *Artemia* (Marques *et al*., 2005), they may still lack of some particular nutrient for *Artemia*. The length of WT-fed *Artemia* in this study was around 1.2 mm after 6 days of culture. The IL mostly increased when small amount of isolates were added in the pre-test. According to Toi *et al*. (2013) bacteria can be used as nutrient source for *Artemia* compensating for suboptimal food conditions. Moreover, literature data suggest that, other than being a dietary component, bacteria can also improve the availability of yeast nutrients to *Artemia*. The potential of bacteria as a source of digestive enzymes for *Artemia* has been reported previously (Intriago and Jones, 1993; Marques *et al*., 2004). The improvement of IL by bacteria addition in this study was similar to that obtained in previous studies carried out in *Artemia* culture where bacteria were added. Intriago and Jones (1993) reported that the addition of *Flexibacter* strain Inp3 to algae *Rhodomonas*-fed *Artemia* resulted in improvement of *Artemia* performance in term of body length. Moreover, Gorospe *et al*. (1996) also demonstrated that the improved *Artemia* body length in the rice bran-fed *Artemia* was obtained in the culture with bacteria *Pseudomonas* sp. addition.

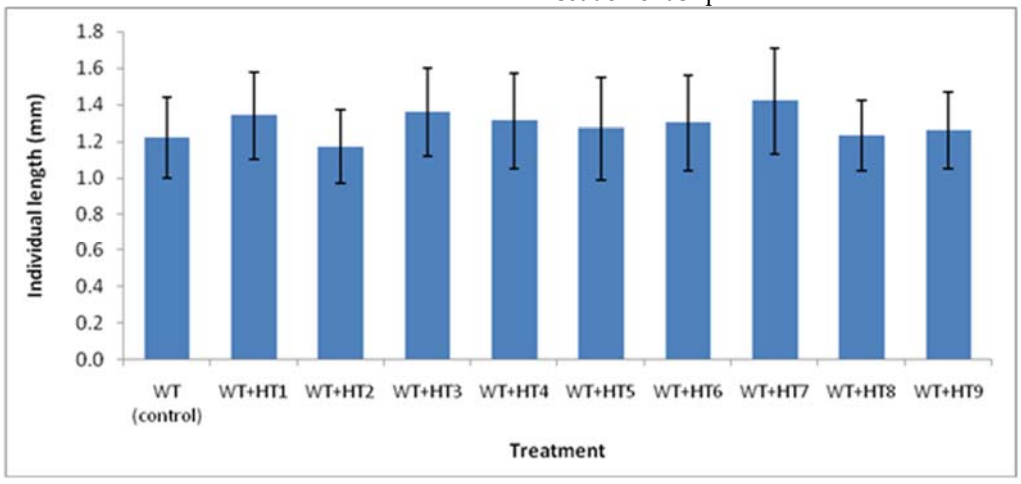

Fig. 2: IL (mm) of *Artemia* fed on isolated bacteria only and of *Artemia* fed WT yeast with bacteria addition

IL values showed no significant difference (p > 0.05). The values are mean ± standard deviation (n=4)

Similarly, the TL of *Artemia* also increased in all treatments where isolates addition (Figure 3), except for HT2 addition which did not increase TL of *Artemia*. The mean of TL ranged from nearly 10.0 mm to nearly 25.0 mm. The lowest TL was obtained at nearly 10.0 mm in solely WT-fed *Artemia* and the culture with HT2 addition. For the cultures where HT4, HT5, HT8 and HT9 were added, TL was nearly 16.0 mm, but no significant difference ($p > 0.05$) was found when compared to that obtained in solely WT-fed *Artemia*. The highest TL was recorded in the culture where HT1, HT3, HT6 and HT7 were added, TL was nearly double and was significantly higher than that obtained in WT-fed *Artemia* ($p < 0.05$), which was due to a significantly better survival and IL. There was no significant difference when compared any TL of *Artemia* in the group of the highest TL to TL of *Artemia* in the culture where HT4, HT5, HT8 and HT9 added ($p > 0.05$).

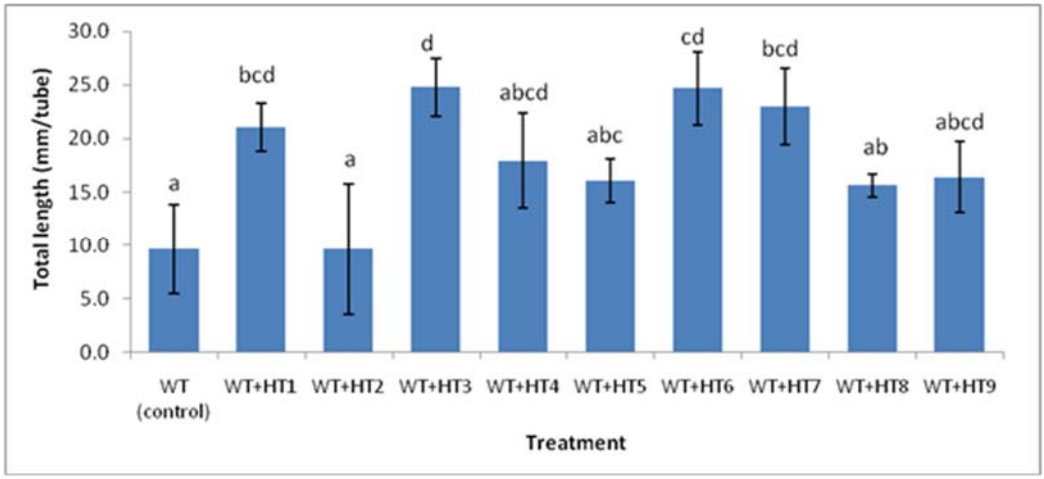

Fig. 3: TL/crew-cap glass tube (mm) of *Artemia* fed on isolated bacteria only and of *Artemia* fed WT yeast with bacteria addition

The values are mean ± standard deviation (n=4). Mean denoted by different letters are significantly different ($p < 0.05$)

4 CONCLUSIONS AND RECOMMENDATIONS

The results of this study elucidated that the addition of bacteria into the pre-tested WT-fed *Artemia* cultures increased the survival and length of *Artemia* as compared to that obtained in *Artemia* fed bacteria-free WT solely. However, these results were obtained under gnotobiotic culture conditions, these results remain to be validated in xenic culture conditions.

Further research is needed to evaluate the preemptive colonization of probiotics in *Artemia* cultures or application of bioflocs technique in *Artemia* biomass production in tank cultures to improve biomass production.

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