

# Application of a $^{15}\text{N}$ tracer method to study the complementarity of bacteria versus yeast in nitrogen assimilation by *Artemia* nauplii

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**Abstract:** Using the  $^{15}\text{N}$  stable isotope technique, nitrogen (N) assimilation of *Artemia* was determined in feeding regimes where the two yeast strains wild-type (WT) and its mutant mnn9 were gradually replaced by HT3 or HT6, or where the bacterial strains were gradually replaced by each yeast strain. A trypsin assay was performed on *Artemia* fed the various replacement feeding regimes, in order to detect the effect of the presence of bacteria or of the yeasts on the protein digestive response in *Artemia*. HT6 fed *Artemia* showed a higher N assimilation than when using HT3, suggesting a better food value of HT6 for *Artemia*. The assimilation of nitrogen from bacteria was related to the presence of yeast strains. In the HT3 fed *Artemia* group, the presence of WT yeast showed higher N assimilation from HT3 than the presence of mnn9, suggesting an enhanced utilization of poor quality HT3 in the presence of WT yeast. When using the higher quality HT6 on the other hand, mnn9 acted in a very similar manner. This is the first observation where mnn9 improves the N assimilation from any bacteria. When N assimilation from yeast strains was tested in the presence of bacteria, both bacterial strains HT6 and HT3 showed a clear enhancement in utilization of N from mnn9, probably by providing exogenous enzymes, highlighting the additional importance of these strains of bacteria for *Artemia* in utilizing yeast. A significant reduction ( $p < 0.05$ ) of trypsin activity was observed in *Artemia* fed HT3 alone, as compared to all other feeding treatments, confirming the lower suitability of the HT3 isolate for sole feeding of *Artemia*. We conclude that HT6, being a better N source for *Artemia*, also favors the utilization of N derived from yeast in the food.

**Index term:** *Artemia*,  $^{15}\text{N}$ , bacteria, yeast, assimilation

## I. INTRODUCTION

Annually, 2500-3000 tonnes of dry *Artemia* cysts, mainly harvested in inland salt lakes such as Great Salt Lake, Utah, USA, are used for aquaculture hatcheries worldwide (FAO, 2011). *Artemia* cysts are stored easily over long periods and are obtained within 24 h by incubating cysts in seawater. *Artemia* are the best sources of live food cultured fish and shellfish species (Sorgeloos *et al.*, 2001). Due to these convenient characteristics, the demand cysts for aquaculture hatcheries has continuously increased over the past few decades. However, the production of *Artemia* cysts from inland salt lakes is not constant and the demand of this product for hatcheries sometimes exceeds the supply. Trying to meet the demand, the technique of *Artemia* culture in salt ponds has been introduced throughout the world such as in Thailand (Vos and Tunsutapanich, 1979) the Philippines in 1977 (De Los Santos *et al.*, 1980), Vietnam in the 1980's (Sivagnanam *et al.*, 2011), and Pakistan in 2007 (Sultana *et al.*, 2011). In typical pond culture, *Artemia* are fed with microalgae, chicken manure and rice bran thanks to their no-selective filter feeding characteristics (Sorgeloos *et al.*, 2001), and bacteria in the water are also play a role in *Artemia* nutrition. In indoor culture, bacteria grow in culture medium have proven as food for *Artemia* (Toi *et al.*, 2013). In addition, bacteria are also believed as the enzyme source to break down large particles and make them easy to absorb by *Artemia* (Intriago and Jones, 1993). However, these study did not prove how bacteria can improve the nutrient assimilation in *Artemia*, and it also extremely difficult to evaluate the contribution of bacteria nutrient to *Artemia* among variety food sources in the pond condition culture.

The present study was therefore conducted to elucidate the contribution of two known bacterial isolates from a laboratory *Artemia* culture to nitrogen assimilation in *Artemia* when they were used in a controlled laboratory culture system alongside two strains of

baker’s yeast *Saccharomyces cerevisiae* (wild type and mnn9), assumed to have different digestibility by *Artemia*, resulting in different *Artemia* growth. The latter strain has reduced mannose and increased glucan content in the cell wall as compared to the wild type strain. Coutteau *et al.* (1990) detected  $\beta$ -glucanase activity in the *Artemia* gut, but no mannase activity. In addition, trypsin activity was measured to evaluate the digestive response in *Artemia* (Rojas-García *et al.*, 2009) when bacteria and yeast were offered together as food. The  $^{15}\text{N}$  tracer method was used as a tool to detect the exact nitrogen source of the protein assimilated.

## II. MATERIALS AND METHODS

### Experimental design

Two experiments were performed under gnotobiotic culture conditions; the first to determine the effect of the presence of yeasts on nitrogen derived from bacteria in *Artemia* nauplii, and the second to determine the effect of the presence of bacteria on nitrogen derived from yeasts.

Hatched bacteria-free *Artemia* nauplii (second instar) were manipulated under the laminar flow hood for the experimental set-up. The nauplii were first harvested on a sterile sieve and washed thoroughly with FAASW to get rid of hatching waste products. *Artemia* nauplii were then diluted by FAASW in a sterile 500 mL beaker. Sub-samples of *Artemia* were collected (n = 4) to determine their density in 1 mL. Afterwards, the *Artemia* nauplii were distributed in 1 L sterile screw-cap bottles containing 1 L of FAASW at a stocking density of 20 nauplii/mL, and 9.1 mg (dry matter intake) of food was offered to each bottle, based on the feeding regime for *Artemia* as described by Coutteau *et al.* (1990). The proportion of each food in the mixed diets was prepared according to Table 1.

In experiment 1, *Artemia* were fed mixed diets containing the  $^{15}\text{N}$  labeled bacterial strain HT3 together with unlabeled WT or mnn9 yeast. In another set of treatments *Artemia* were fed mixed diets containing the  $^{15}\text{N}$  labeled bacterial strain HT6 and unlabeled yeast WT or mnn9. In experiment 2, labeled WT and mnn9 yeast were fed to *Artemia* together with the unlabeled bacterial strains HT3 or HT6. In both experiments, yeasts and bacteria were offered to *Artemia* in different proportions (90/10, 50/50 and 10/90), and two treatments with the pure diet (100% yeasts or 100% bacteria) were used as controls. After feeding manipulation, the culture bottles were tightly closed by special screw caps supplied with an air inlet and outlet. Three replications for each treatment and for the control were run under gnotobiotic conditions for 24 h.

Table 1: Feeding regime for *Artemia* nauplii during 24 h (adapted from Coutteau *et al.* 1990) expressing different portions (%) of each food type (bacteria versus yeast). based on dry matter. Experiment 1: *Artemia* fed mixed diets consisting of unlabeled yeast (WT or mnn9) and  $^{15}\text{N}$  labeled bacteria ( $^{15}\text{N}$  HT3 or  $^{15}\text{N}$  HT6). Experiment 2: *Artemia* fed mixed diets consisting of  $^{15}\text{N}$  labeled yeast ( $^{15}\text{N}$  WT or  $^{15}\text{N}$  mnn9) and unlabeled bacteria (HT3 or HT6)

Treatment code	$^{15}\text{N}$ yeasts	Bacteria	$^{15}\text{N}$ bacteria	Yeasts
<b>Experiment 1</b>				
E1-FR1 (control 1)	0	0	100	0
E1-FR2	0	0	90	10
E1-FR3	0	0	50	50
E1-FR4	0	0	10	90
E1-FR5 (control 2)*	0	0	0	100
<b>Experiment 2</b>				
E2-FR1 (control 1)	100	0	0	0
E2-FR2	90	10	0	0
E2-FR3	50	50	0	0
E2-FR4	10	90	0	0
E2-FR5 (control 2)*	0	100	0	0

\*:  $^{15}\text{N}$  natural abundance of *Artemia* fed solely unlabeled yeasts (Experiment 1) or unlabeled bacteria (Experiment 2) was used as a background to calculate the excess of  $^{15}\text{N}$  and N assimilation in *Artemia*.

### Preparation of *Artemia* nauplii

For preparation of bacteria-free nauplii, cysts of *Artemia franciscana* Kellogg 1906 (EG<sup>®</sup> Type, INVE Aquaculture, Belgium) were decapsulated according to the protocol as described by Sorgeloos *et al.* (1977) and Marques *et al.* (2006a). Briefly, the dry cysts were first soaked in tap water for 1 h and then transferred to a laminar flow hood for decapsulation. The *Artemia* cyst shell was removed by reaction with sodium hypochlorite (NaOCl). Decapsulated cysts were harvested on a sterile sieve, and washed with FAASW as much as possible to remove all residual bleach. Decapsulated cysts were transferred into 1 L sterile bottles containing 1 L FAASW (Marques *et al.*, 2006a) for hatching at 28 °C for 24 – 30 h under standardized hatching conditions (Sorgeloos *et al.*, 1986).

### Diet preparation

#### Culturing, labeling and harvesting of yeast

Two baker's yeast strains i.e. wild-type (WT) and its mutant mnn9, originated from the European *Saccharomyces cerevisiae* archive for functional analysis EUROSCARF, University of Frankfurt, Germany, were used in the study. WT was used for its low digestibility and the strain mnn9 for its improved digestibility by *Artemia* (Marques *et al.*, 2004b).

Before mass culturing, yeast was streaked on a Yeast Extract Peptone Dextrose (YEPD) agar plate (n = 2) containing 10 g/L yeast extract (Sigma), 10 g/L peptone (Sigma), 20 g/L dextrose (Sigma), and 20 g/L agar. The medium was prepared in FAASW. Visible colonies appeared after three days incubation in the dark at 28 °C. A single colony forming unit (CFU) 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 (YNB, without ammonium sulfate and amino acids), and was supplemented with 5.0 g/L ammonium sulfate, 5.0 g/L D-glucose, 0.02 g/L L-histidine, 0.04 g/L L-methionine, and 0.04 g/L LD-tryptophan in FAASW. For  $^{15}\text{N}$  yeast enrichment, each strain of yeast was grown on sterile YNB without ammonium sulfate and amino acid medium, supplemented with 4.99 g/L ammonium sulfate (Sigma) and 0.01 g/L  $^{15}\text{N}$   $\text{NH}_4\text{Cl}$  (Sigma) and the concentration of each amino acid was similar to that of the unlabeled yeast cultures.

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 in the exponential growth phase, detected by optical density (Marques *et al.*, 2004a). 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 ( $\pm 2,000 \times g$ ; 5 min). The pellet was washed twice in FAASW (Marques *et al.*, 2006a; 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. After a 24–36 h growth period, the  $^{15}\text{N}$  signature increased from 0.3709% to 1.8832% and from 0.3707% to 1.3796% for WT and mnn9 yeast, respectively.

#### Culturing, labeling and harvesting of bacteria

For mass culturing, each strain of bacteria, stored at – 80 °C was streaked on MA (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 modified marine broth (M-MB). Briefly, M-MB was prepared using 0.1 g/L  $\text{NaNO}_3$ , 2.0 g/L yeast extract, 0.1 g/L Fe(III) citrate, 19.45 g/L NaCl, 5.9 g/L  $\text{MgCl}_2$  (anhydrous), 3.24 g/L  $\text{NaSO}_4$ , 1.8 g/L  $\text{CaCl}_2$ , 0.55 g KCl, 0.16 g/L  $\text{Na}_2\text{CO}_3$ , 0.08 g/L KBr, 0.034 g/L  $\text{SrCl}_2$ , 0.022 g/L  $\text{H}_3\text{BO}_3$ , 0.004 g/L Na-silicate, 0.0024 g/L NaF, 0.0016 g/L  $\text{NH}_4\text{NO}_3$ , 0.008 g/L  $\text{Na}_2\text{PO}_4$ , with a final pH of 7.6. For labeling the bacteria, the M-MB ingredients were identical but

0.01 g/L NaNO<sub>3</sub> was replaced by <sup>15</sup>N NaNO<sub>3</sub> (Sigma). After inoculation, the inoculated flasks were carefully closed by sterile cotton caps and incubated in a shaker (28 °C; 150 rpm) for 24–48 h. The labeling technique resulted in an increase of <sup>15</sup>N signature in bacterial cells from 0.3677% to 8.7586% and from 0.3670% to 9.1965% for HT6 and HT3 bacteria, respectively.

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 ( $\pm 4,400 \times g$ ; 15 min). Bacterial cell pellets were washed twice in FAASW. The bacterial cell pellets were then re-suspended in FAASW and 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  $1.2 \times 10^9$  cells/mL, according to the McFarland standard (BioMerieux, Marcy L'Etoile, France).

### Dry weight determination of yeast and bacteria

Dry weight (DW) of yeast and bacteria was determined according to the methodology described by Soltanian *et al.* (2007). Briefly, 50 mL of culture suspension of each yeast and bacteria strain was filtered through a pre-weighed 0.45 µm Whatman membrane using a Buchner funnel connected to a vacuum pump. The filter was then washed with ammonium formate solution (0.5 M) to remove the salt, placed into an aluminum pre-tared cup and dried in an oven at  $104 \pm 1$  °C for 4 h. The samples were then removed from the oven, placed in a desiccator for cooling down, and weighed with an analytical balance (0.0001 g accuracy)

### Sample collection and data analysis

#### Nitrogen accumulation in *Artemia*

To prevent interference of undigested <sup>15</sup>N labeled food in the digestive tract of *Artemia* with the results of <sup>15</sup>N accumulation in *Artemia* tissue, the undigested food was biologically evacuated from the gut before <sup>15</sup>N analysis. This was done by placing the *Artemia* in a 1 L beaker containing 500 mL of FAASW and cellulose particles (20 µm; Sigma). The cellulose powder had been diluted in FAASW and sieved through a 50 µm net to prevent particle clumping. Cellulose was provided to the beakers with *Artemia* at a concentration three times higher than the feeding ration. Aeration was provided continuously to ensure homogeneous distribution of the cellulose in the water. *Artemia* was regularly checked under a binocular microscope for observation of the ingestion status. *Artemia* were harvested when the digestive tract was completely filled with cellulose.

After the evacuation step, *Artemia* were harvested on a sieve and rinsed with FAASW to remove all uneaten cellulose and wastes. After washing, *Artemia* was first soaked in a benzocaine solution (Sigma, 0.1%) for 10 s and then transferred to a benzalkonium chloride solution (Sigma, 0.1%) for 10 s to kill all the attached bacteria on the exoskeleton (Chládková *et al.*, 2004). Afterwards, *Artemia* was washed as much as possible with de-ionized water (DEMI-water). *Artemia* were then quickly stored in a freezer (–20 °C) to prevent leaking of <sup>15</sup>N caused by the metabolism.

After thawing, *Artemia* were placed into a Petri dish with DEMI-water, and 200 to 600 *Artemia* nauplii from each replicate were sampled by a Pasteur pipette and rinsed on a metal mesh sieve (250 µm pore size). *Artemia* were then gently transferred using a small forceps onto a pre-weighed tin capsule cup of 8 x 5 mm. The wet samples were oven-dried at 70 °C for a day (De Troch *et al.*, 2007), placed in the desiccator for cooling and weighed with an analytical balance (precision 0.1 mg). Subsequently, the <sup>15</sup>N assimilation in *Artemia* was measured by using an elemental analyzer (ANCA-SL, PDZ Europa, UK) coupled to an *isotope-ratio mass spectrometer* (IRMS) (20-20, SerCon, UK) at the Department of Applied Analytical and Physical Chemistry, Ghent University, Belgium.

The amount of nitrogen assimilation from food in *Artemia* (ng N/individual) was calculated with the equation described by Burford *et al.* (2004).

$$N (\text{ng/individual}) = (e \times n) / (f \times \text{no. } Artemia)$$

where e:  $^{15}\text{N}$ -ratio (atom % excess) in *Artemia*, n: total amount of nitrogen content in *Artemia* (ng), f:  $^{15}\text{N}$ -ratio (atom % excess) in the food, no. *Artemia*: number of *Artemia*.

### Trypsin assay

To prepare crude enzyme extracts, *Artemia* samples (35 mg) from all treatments in experiment 2 were ground with a plastic pestle (25 s) in an eppendorf tube containing 1 mL of cold homogenizing mixture (1:1) of 0.85% NaCl-2.5 mM ethylenediaminetetraacetic acid (EDTA) and Triton-X 1%-10 mM  $\text{CaCl}_2$  (Rojas-García *et al.*, 2009). After 24 h in darkness at 4 °C to allow enzyme extraction, the homogenates were centrifuged at 12,000 x g for 30 min at 4 °C. The supernatant was used as crude enzyme extract. Trypsin-L activity was measured using N-alpha-benzoyl-DL-arginine-p-nitroanilide (BAPNA) (Tseng *et al.*, 1982) as the substrate in 50 mM Tris-HCl and 10 mM  $\text{CaCl}_2$  buffer, pH 7.1. The measurement and calculation of trypsin activity was done by the methodology as described by Rojas-García *et al.* (2009). Briefly, the p-nitroanilide (pNA) formation by enzymatic BAPNA breakdown was recorded spectrophotometrically after 24 h.  $\text{DO}_{410}$  increment ( $\Delta$ ) was calculated as the difference of  $\text{DO}_{410}$  at 24 h ( $\text{DO}_f$ ) and at time 0 h ( $\text{DO}_i$ ). Background  $\text{DO}_{410}$  (increment) due to non-enzymatic pNA production was assayed in tubes without crude enzyme extract using buffer and BAPNA.

$$\Delta_{\text{sample or background}} = \text{DO}_f - \text{DO}_i$$

The  $\text{DO}_{410}$  increments due to positive trypsin-L were calculated by subtraction as follows:

$$\text{Trypsin-L } (\Delta\text{DO}_{410}) = \Delta_{\text{sample}} - \Delta_{\text{background}}$$

### Method used to verify axenity

Axenity of food, *Artemia* nauplii and *Artemia* culture medium was checked by the methodology as described by Marques *et al.* (2004a; 2004b). Briefly, food, *Artemia*, hatching and culture water were checked for contamination by plating on MA 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.

### Statistical analysis

The data of nitrogen assimilation or enzyme digestive response from yeasts, bacteria and mixed yeast-bacteria diets in *Artemia* were first checked for homogeneity of variance and normality of distribution by the Levene's *F* test and P-P plot, respectively. As all data failed to meet these assumptions, the datasets were logarithmically and square root transformed. Statistical analysis was performed using Statistica 7.0 for Windows. All datasets were analyzed using one-way analysis of variance (ANOVA) followed by Tukey's honestly significant difference (HSD) at 0.05 level of probability.

## III. RESULTS

### Nitrogen assimilated by *Artemia* from bacterial strains HT3 and HT6

Assimilation of N from HT3 (Fig. 1) was gradually reduced when the proportion of bacteria in the mixed diets with yeast (either WT or mnn9) was reduced. However, there appeared to be differences in this trend between the different strains of yeast. When replacement of HT3 bacteria by mnn9 and WT was done, there was always more N assimilation in *Artemia* from HT3 when co-fed with WT than with mnn9. Replacement of HT3 up to 50% by yeast did not significantly change N assimilation compared to the control 1 ( $p > 0.05$ ), except in case of 50% replacement with mnn9 (Fig. 1). Similarly, N assimilation derived from HT6 bacteria was gradually reduced when the proportion of this bacterial strain in the mixed diets with either type of yeast was reduced, but co-fed with HT6 there was no consistent difference between the two types of yeast (Fig. 1). A significant reduction ( $p < 0.05$ ) of assimilation was already evident at 10% replacement with WT compared to the control. When the controls of the two bacterial strains HT3 and HT6 are compared, the latter allowed for more N assimilation in *Artemia* than the former.

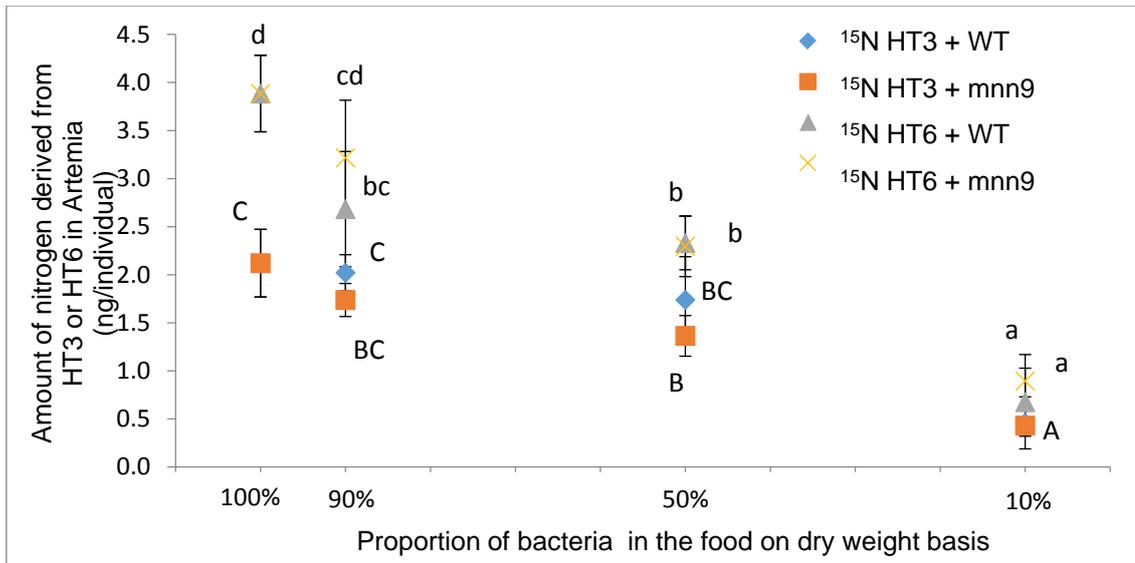


Figure 1: Effect of the presence of yeast on nitrogen assimilation from bacteria (ng/individual) in *Artemia*. The values are mean  $\pm$  standard deviation (n = 3). Different letter superscripts of HT3 treatments and HT6 treatments indicate significant difference ( $p < 0.05$ ) between the treatments. For the proportion of bacteria in the mixed diets, see Table 1.

**Nitrogen assimilated by *Artemia* from yeast strains WT and mnn9**

Nitrogen assimilation in *Artemia* from yeast strains WT and mnn9 (Fig. 2) was gradually reduced when the proportion of yeasts in the mixed diet with bacteria (either HT3 or HT6) was reduced. In wild type yeast (WT), a steady decline of N assimilation was evident when the proportion of WT in mixed diets was gradually replaced by bacteria. Except for 10% replacement of WT by HT6, N assimilation from WT in *Artemia* fed mixed diets was always significantly lower than the control ( $p < 0.05$ ). For mnn9, in contrast, significantly lower N assimilation than the control was only obtained at replacement levels as high as 50% in case of HT3 and 90% in case of HT6. The combination with HT6 always produced higher N assimilation from both yeast types than the one with HT3. All treatments with the WT strain, including the control, resulted in much lower yeast-derived N assimilation than those with mnn9.

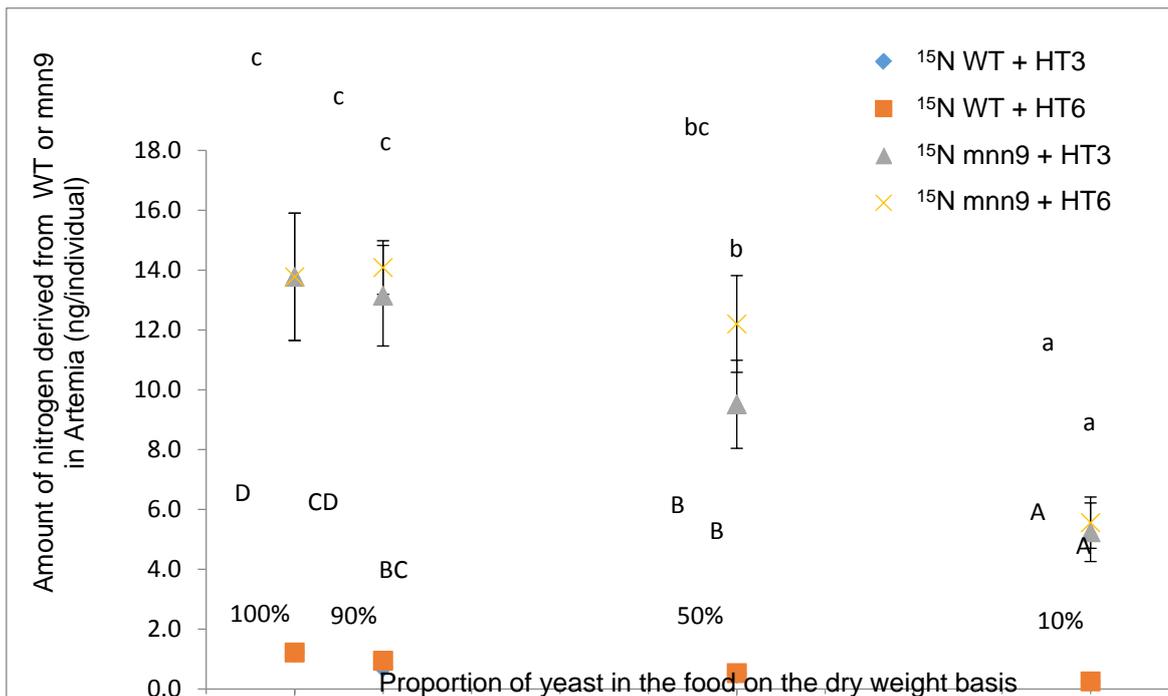


Figure 2: The effects of presence of bacteria on nitrogen assimilation from yeasts (ng/individual) in *Artemia*. The values are mean  $\pm$  standard deviation (n = 3). Different letter superscripts of WT treatments and mnn9 treatments indicate significant difference of  $p < 0.05$  between the treatments. For the proportion of yeast in the mixed diets, see Table 1.

**Trypsin activity of *Artemia* fed mixed diets of yeasts and bacteria**

No significant differences in trypsin-L activity were found among the treatments when replacing any of the two yeasts strains by either HT3 or HT6 bacteria (Fig. 3 A and 3 B, respectively), or when comparing the treatments with 100% yeast or 100% HT6 fed *Artemia*. In contrast, a significantly ( $p < 0.05$ ) lower trypsin-L activity was found in the 100% HT3 control compared to all mixed diets and to other controls.

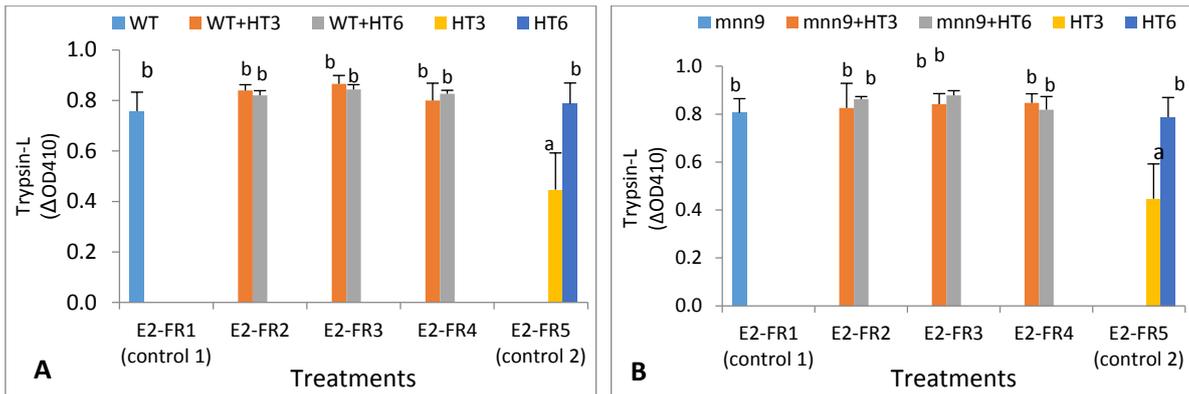


Figure 3: Effect of pure WT yeast and mixed WT yeast-bacteria diets (A) and of pure mnn9 yeast and mixed mnn9-bacteria diets (B) on trypsin-L activity in *Artemia* nauplii. Values are mean  $\pm$  standard deviation (n = 3). Different letter superscripts within each figure indicate statistical significant difference of  $p < 0.05$  between the treatments. For abbreviation of the treatments, see Table 1. For better comparison, control 2 (100% HT3 or HT 6) is shown on each graph.

IV. DISCUSSION

In previous study, bacterial isolates HT3 and HT6 associated with *Artemia* culture were demonstrated as being a suitable diet for *Artemia* nauplii (Toi *et al.*, 2014). In this study the nitrogen assimilation from the food was determined when these bacteria were offered to *Artemia* nauplii in a mixed diet together with wild type WT and mutant mnn9 yeast. The data illustrate that HT6 provides for higher N assimilation in *Artemia* than HT3 when equal amounts (DW) of bacteria are offered, as evident when the respective FR1 (controls) are compared. This is a confirmation of previous findings (Toi *et al.*, 2014) where N assimilation from HT6 in *Artemia* was also higher than from HT3.

When both bacterial strains in the food are gradually replaced by yeast (WT or mnn9), the assimilation of N from bacteria in *Artemia* is also gradually decreasing, probably due to the reduced availability of bacteria when yeast is added. There are, however, differential effects on N assimilation depending on the replacing yeast strain and the available bacterial strain. When HT3 is present, replacement by WT always provides higher assimilation from bacteria, though not statistically significant, than replacement by mnn9 (Fig. 1). A first possible explanation for this difference between both yeast types could be that the presence of mnn9 as replacement provides more digestible food than replacement by WT (Marques *et al.*, 2004b), hence producing lower N assimilation from bacteria. Alternatively, WT may contribute for more exogenous enzymes than mnn9 such as proteolytic enzymes and alkaline phosphatase, leading to better utilization of bacteria and hence resulting in less reduction of N assimilation from HT3. According to Lara-Flores *et al.* (2003) the addition of yeast into a formulated diet for 3-week old fish Nile tilapia (*Oreochromis niloticus*, Linnaeus 1758) triggered alkaline phosphatase activity. High activity of alkaline phosphatase is believed to be related to increased nutrients absorption into enterocytes of fish (Gawlicka *et al.*, 2000). Ozório *et al.* (2012) found the body weight of *O. niloticus* increasing 3 times at the end of the experiment (51 days), when yeast *S. cerevisiae* was added to formulated

diets up to 20%. These data therefore suggest a strong potential of wild type yeast to assist in utilization of bacteria by *Artemia*. This action seems to be less prominent in *mnn9* fed *Artemia*, co-fed with HT3, suggesting that *mnn9*, being a mutant, has not a similar positive effect on utilization of bacteria by *Artemia*.

However, these possibilities do not explain the interaction of HT6 and replacement by WT and *mnn9*, and its effect on N assimilation from the bacteria. In our experiment with HT6, *mnn9* replacement instead of WT generally caused less reduction of N assimilation from HT6, though the differences were not significant. Higher N assimilation from HT6 than from HT3 may interact with *mnn9* resulting into an increased N assimilation from bacteria (Fig. 3.1), though further studies are needed to prove or refute this hypothesis.

Our results further show that when yeast strains are replaced by HT3 and HT6, there is a gradual decline of N assimilation from yeast. This may be due to the reduced availability of yeast in the culture medium. Moreover, for both yeast types the N assimilation seems to be related to the bacterial strain used. With HT3, nitrogen assimilation from yeast is always less than with HT6 (Fig. 2). 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.*, 2004a). Significantly improved *Artemia* performance by the addition of bacteria as compared to axenic conditions has been reported by Douillet (1987) and Intriago and Jones (1993). Bacterial enzymes, such as N-acetyl- $\beta$ -glucosaminidase (chitinolytic activity) and  $\beta$ -glucosidase (cellulolytic activity),  $\beta$ -glucanase (glucalytic activity), and mannase were detected in selected bacterial strains (Fleet and Phaff, 1974; Araki *et al.*, 1992; Rombaut *et al.*, 1999) and their presence in the *Artemia* digestive tract may improve yeast digestibility, resulting in improved performance of *Artemia* (Marques *et al.*, 2006a) as well as rotifers (Tinh *et al.*, 2006).

Our study is the first to indicate that, when using the two yeast strains WT and *mnn9* as sole diet, the latter results into a remarkably high N assimilation when it is fed to *Artemia* nauplii. Even though the assimilation of N has never been analyzed before, similar findings are reported elsewhere as exemplified by increased growth and survival of *Artemia* nauplii fed with *mnn9* (Marques *et al.*, 2004b). The *mnn9* yeast mutant does not have a mannoprotein layer in the external cell wall (Marques *et al.*, 2004b), rendering it more digestible as *Artemia* nauplii do not have mannase activity (Coutteau *et al.*, 1990). Further, improved N assimilation in *mnn9* may also be related to the higher nutrient content of *mnn9*, as indicated by its higher ash-free dry weight (Marques *et al.*, 2004b; Soltanian *et al.*, 2007). Gunasekara *et al.* (2011) reported that the length of the *Artemia* nauplius mid gut increases when *Artemia* is fed with *mnn9* yeast compared to WT. The mid gut segment is more active in the absorbance of nutrients and the secretion of digestive fluids (Schrehardt, 1987). The experiment was performed for a short period (24 h) and as there were no obvious differences in *Artemia* length and survival among the treatments (results not shown) after 24 h, it was assumed that there was no difference in food consumption by *Artemia*, which might have affected N assimilation.

The enzyme assay of trypsin-L highlighted that whether *mnn9* or WT is used, there is no significant difference in trypsin-L activity in *Artemia*. When HT6 is fed solely, the activity of trypsin-L is similar to that in *Artemia* fed solely WT or *mnn9*, suggesting that, within the time duration of our experiment, WT, *mnn9* and HT6 stimulate similar levels of trypsin-L activity, be it fed solely or in combination. Only *Artemia* fed solely HT3 showed significantly lower levels of trypsin-L than all other feeding treatments. This effect is not present in treatments where HT3 is fed in combination with yeast. This finding is in agreement with our previous study where the presence of HT3 lead to lower N assimilation from microalgae in *Artemia* when compared to the presence of HT6 (Toi *et al.*, 2014). Similar negative effects of bacteria on trypsin-L activity in *Artemia* have been reported previously (Rojas-García *et al.*, 2008). The negative effects of HT3 on trypsin-L activity and its compensation by the yeast strains show that the yeast strains tested here can modulate digestive functions of *Artemia* in a favorable manner.

In conclusion, bacteria and yeast not only may play an important nutritional role in *Artemia* when fed as sole diet, but their joint presence in *Artemia* culture may also improve nitrogen assimilation in *Artemia*.

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