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# Impact of arachidonic acid enrichment of live rotifer prey on bacterial communities in rotifer and larval fish cultures

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**Abstract:**

Rotifers (*Brachionus plicatilis*), commonly used at first feeding in commercial fish hatcheries, carry a large bacteria load. Because they are relatively poor in essential fatty acids, it is common practice to enrich them with fatty acids, including arachidonic acid (AA). This study aims to determine whether prey enrichment with AA may act as a prebiotic and modify the microbial community composition either in AA-enriched rotifer cultures or in larval-rearing water using winter flounder (*Pseudopleuronectes americanus*) as a larval fish model. AA enrichment modified the bacterial community composition in both the rotifer culture tanks and the larval-rearing tanks. We observed an increase in the number of cultivable bacteria on TCBS (thiosulfate–citrate–bile salts–sucrose) agar, used as a proxy for the abundance of *Vibrio* sp. The results suggest that AA may also play an indirect role in larval health.

**Keywords:** bacterial community ; rotifers ; arachidonic acid enrichment ; *Vibrio* sp. ; prebiotic

**Résumé:**

Les rotifères (*Brachionus plicatilis*), fréquemment utilisés comme première source alimentaire dans les pisciculturescommerciales, portent une charge bactérienne importante. Parce qu'ils sont relativement pauvres en acides gras essentiels, il est de pratique courante de les enrichir en acides gras, notamment en acide arachidonique (AA). Cette étude vise à déterminer si l'enrichissement des proies à l'AA peut agir comme prébiotique et modifier la composition de la communauté microbienne non seulement dans les cultures de rotifères enrichies à l'AA, mais aussi dans l'eau d'élevage des larves, en utilisant la plie rouge (*Pseudopleuronectes americanus*) comme modèle de larves de poisson. L'enrichissement en AA a modifié la composition de la communauté microbienne tant dans les bassins de culture de rotifères que dans les bassins d'élevage des larves. Un accroissement du nombre de bactéries cultivables sur la gélose TCBS (thiosulfate – citrate – sels biliaires – sucrose), utilisée pour estimer l'abondance de *Vibrio* sp., a également été observé. Les résultats suggèrent que l'AA peut aussi jouer un rôle indirect dans la santé des larves.

**Mots-clés :** communauté bactérienne ; rotifères ; enrichissement en acide arachidonique ; *Vibrio* sp. ; prébiotique

## 1. Introduction

The aquatic environment contains beneficial and neutral bacterial strains as well as a plethora of obligate and opportunistic bacterial pathogens that could represent a significant constraint on the commercial production of fish and shellfish. Over the last decade, the aquaculture industry has greatly increased its productivity and is now a major economic activity in many countries. The intensive rearing of marine larvae requires the addition of natural or artificial food sources to seawater. This activity could easily introduce numerous bacteria and, as a consequence, modify the microbial communities in the rearing environment or in organisms (Munro et al. 1994). Moreover, some production facilities use disinfection

processes that may lead to the proliferation of opportunistic bacteria (Olafsen 2001), including pathogens such as *Vibrio* (Schulze et al. 2006; Skjermo and Vadstein 1993). The gut microbial communities of adult fish and fish resistance to infections will be greatly influenced by pioneer bacteria in the diet and ambient water of developing larvae (Lubzens et al. 1985; Munro et al. 1994; Nicolas 1989; Ringø and Birkbeck 1999).

In commercial marine fish hatcheries, the rotifers *Brachionus plicatilis* and *Brachionus rotundiformis* are commonly used at first feeding (Haché and Plante 2011). Without the proper enrichment, rotifers are not suitable as live prey in intensive aquaculture since they do not contain all the essential nutrients required to sustain larval growth and promote survival (Castell et al. 2003). Because rotifer cultures carry a large bacterial load (Haché and Plante 2011; Skjermo and Vadstein 1993), they represent a significant vector for bacterial transmission in larval cultures. A direct relationship has been demonstrated between the bacterial flora found in the larvae and that in the live food (Munro et al. 1994). Commercial rotifer enrichments have also been shown to impact the bacterial load and the bacterial community composition in live prey (Haché and Plante 2011; Høj et al. 2009) as well as in larval fish cultures (Seychelles et al. 2011).

In a previous study, we demonstrated that arachidonic acid (AA) plays a crucial role in larval winter flounder *Pseudopleuronectes americanus* development and that AA enrichment could modify bacterial colonization of the intestinal lumen in this species (Seychelles et al. 2011). The present study focuses on the effect of AA enrichment in rotifer cultures on the bacterial communities present in the hatchery environment, especially in terms of total bacterial abundance, community composition, and the

66 occurrence of specific pathogens such as *Vibrio* sp. in the rearing seawater of both rotifers and winter
67 flounder larvae fed with this live prey.
68
69 ## Materials and methods
70
71 **Rotifer culture**
72 Experiments were conducted at the UQAR–ISMER aquaculture facility (Pointe-au-Père, 48° 27' N; 68°
73 32' W, Quebec, Canada) from April to August 2006. Rotifers (*Brachionus plicatilis*) were cultured in six
74 replicate 18 L tanks at 20–25°C using aerated, filtered (0.2 μm) seawater at a salinity of 27 ± 1. Rotifers
75 were fed daily with a microalgal paste (final concentration: $3 \times 10^6$ cell.ml$^{-1}$) composed of a mixture of
76 three non-viable microalgae (*Nannochloropsis occulata*, *Pavlova lutheri*, and *Isochrysis galbana*, v/v/v)
77 and a commercial enrichment (Culture Selco Plus, INVE Aquaculture, Belgium) as a complementary
78 protein source. Of the six replicates, three received an additional artificial supplement of AA (Sigma-
79 Aldrich #10931-1G) at a ratio of 1 μg for $10^6$ phytoplankton cells after dilution in ethanol (Seguineau et
80 al. 2005). The flagellates *I. galbana* and *P. lutheri* are good sources of docosahexaenoic DHA; *P. lutheri*
81 contains a high relative proportion of eicosapentaenoic acid (EPA); and the chlorophyceae *N. occulata* is
82 a good source of EPA and AA (Brown et al. 1997). Microalga culture conditions are described in
83 Seychelles et al. (2011). Rotifer culture tanks were sampled on days 4, 15, and 26 to determine the fatty
84 acid composition of rotifers and to describe the bacterial community present in rotifer-rearing seawater.
85
86 **Larval culture**
87 Detailed protocols for larval culture and sampling are given in Seychelles et al. (2011). Briefly, newly
88 hatched (day 0) winter flounder larvae were reared in three replicate 57 L cylindro-conical polyethylene

tanks for each dietary treatment. Flounder larvae were fed rotifers from day 4 to 26 post hatching. After day 26, the fish larvae needed larger-sized food and the experiment ended.

Rotifers were given in excess, and their density was adjusted three times a day to 5 rotifers.ml$^{-1}$. Larval tanks were supplied with filtered (10 µm) seawater (salinity 27 ± 1) and maintained at 10°C under a 12h:12h light:dark photoperiod; aeration provided upwelling water circulation. During the day, the water intake was closed and the same microalgal paste used for rotifer enrichment was added to the larval rearing tanks (final concentration: $0.7 \times 10^6$ cells.ml$^{-1}$) to provide the pseudo-green water conditions required for larval rearing. During night, water flow resumed and allowed for the complete renewal of tank water. Flounder larvae were sampled just before lights-on and the first meal to ensure that stomachs were empty. Sampling was done at mouth opening (day 4) and on days 15 and 26 for dry weight determination. At the end of the rotifer-feeding period (day 26), 10 larvae per larval tank (total of 30 in the AA-enriched treatment and 20 in the control treatment) were fixed with 10% formaldehyde (≥ 24 h at ambient temperature) for light microscopy observations. Fixed larvae were dehydrated in an ascending series of ethanol solutions and embedded in methacrylate resin for histological observation, as described in Seychelles et al. (2011). The same sampling periods were used for bacterial analyses in larval-rearing seawater.

**Rotifer fatty acid composition**

At days 4, 15, and 26, two samples of 20 000 rotifers were collected from each tank and pre-rinsed with filtered seawater (0.2 µm) on a 50 µm net before being filtered onto pre-combusted (450°C) GF/C filters (25 mm) for fatty acid analysis. As described in Seychelles et al. (2011), fatty acid profiles were determined on a Varian CP3900 gas chromatograph (Varian, Canada) equipped with a ZB-wax fused

112 silica capillary column (20 m × 0.18 mm internal diameter × 0.18 µm film thickness; Supelco) after
113 extraction following Folch et al. (1957); fatty acid methyl esters were obtained by acid catalyzed
114 transesterification with 2% (v/v) $H_2SO_4$ in methanol at 100°C.
115
116 **Total bacteria abundance**
117 Total bacteria abundance was determined in the rotifer-rearing seawater and in the larval-rearing
118 seawater on days 4, 15, and 26. All samplings were done early in the morning, before the addition of
119 enrichment to the rotifer tanks or before the addition of green water and food to the larval tanks.
120 Additional samples were taken of seawater before it entered the rearing tanks ("source seawater") at the
121 beginning of the experiment (day 0) and on days 4, 15, and 26 to determine the natural bacterial
122 abundance. Each sampling included two 4 ml aliquots of seawater fixed in 2% formaldehyde (final
123 concentration; pH 7). Samples were frozen at -80°C until further analyses. Total free bacteria (TB) were
124 enumerated using an EPICS ALTRA™ cell sorting flow cytometer (Beckman-Coulter Inc., Mississauga,
125 ON, Canada) equipped with a laser emitting at 488 nm. Fluorescent beads (Fluoresbrite YG 1 µm
126 microspheres, Polysciences™) were systematically added to each sample as an internal standard to
127 normalize cell fluorescence emission and light scatter values. For the analysis of bacterial abundance,
128 frozen samples were thawed and two subsamples of 1 ml were half-diluted in TE 10X buffer (100 mM
129 Tris-HCl, 10 mM EDTA, pH 8). A 1 ml volume of the resulting diluted sample was stained with 0.25 µl
130 of SYBR Green I nucleic acid gel stain (Ci = 10,000X, Invitrogen, Inc.), incubated for 10 min at room
131 temperature in the dark, and analyzed for 180s. To calculate bacterial cell abundances, the volume
132 analyzed was calculated by weighing samples before and after each run. This volume was corrected for
133 a dead volume of 50 µl (the water volume taken from the sample tube but not counted when data
134 acquisition is stopped).

TB were detected in a plot of green fluorescence recorded at 530 ± 30 nm (FL1) versus side angle light scatter (SSC). Bacteria with high, very high, and low nucleic acid content (HNA, VHNA, and LNA subgroups, respectively) were discriminated by gating the FL1-versus-SSC plot, and the abundances of all subgroups were determined (Lebaron et al. 2001). For the purpose of this study, TB abundance was used to describe the bacterial community distribution, and %HNA (the ratio of HNA cells to TB) was used to describe the physiological structure of the bacterial community, as has been suggested by different studies (Gasol and del Giorgio 2000; Gasol et al. 1999; Lebaron et al. 2001)

**Bacteria cultivable on TCBS**

Bacteria forming colonies (colony forming units, CFU) were enumerated in triplicate from day 4 to 26 after 24 to 48h of incubation at room temperature in the dark on TCBS agar (thiosulfate–citrate–bile salts–sucrose; Merck KgaA, Germany). Cultures on TCBS agar were used to estimate the number of *Vibrio* sp. in the samples (Buller 2004). Only colonies with the characteristics of *Vibrio* sp. (good growth, yellow colonies with halo) were counted, but because no definite identification was made of the colonies, the expression "CFU on TCBS" will be used to refer these counts.

**Bacteria colonizing larvae**

Fixed larvae were dehydrated in an ascending series of ethanol solutions and embedded in methacrylate resin. Tissues were sectioned (3 μm thickness) with a Supercut Reichert-Jung model 2050 (Cambridge Instruments GMbH, Germany). Sections were mounted onto glass slides, stained with the Gram Staining kit (Sigma #77730), and photographed at 1000X (Olympus BX41, Japan). The occurrence of bacteria was determined and quantified in gut lumen, gills, and skin. Bacterial density was randomly calculated

158 within the intestinal lumen (number of bacteria.mm$^{-2}$), and the ratio "area occupied by bacteria/total
159 tissue area" was calculated on three histological gill and fin sections for each larva using the Image Pro
160 Plus software (Media Cybernetics, Canada).
161
162 **Bacterial community characterization**
163 Bacterial community composition was analyzed from larva- and rotifer-rearing seawater and from
164 source seawater. Samples (200 ml) were filtered on polycarbonate membranes (0.2 µm pore size, 25 mm
165 diameter); the filters were then cut and transferred to sterile 1.5 ml tubes containing 840 µl of lysis
166 buffer (40 mM EDTA, 50 mM Tris, pH 8, 0.75 M sucrose) and 50 µl of lysozyme (20 mg.ml$^{-1}$), and
167 incubated for 45 min at 37°C (Ghiglione et al. 2005). Next, 100 µl of sodium dodecyl sulfate solution
168 (10%) and 10 µl of proteinase K (20 mg.ml$^{-1}$) were added to each sample and incubated at 55°C for 60
169 min. Total DNA extraction was then performed using a classic phenol–chloroform–isoamyl alcohol
170 (25/24/1) protocol. PCR amplification of the 16S rDNA gene was then performed using a Mastercycler
171 epS (Eppendorf) thermal cycler following the method proposed by Schäfer and Muyzer (2001). Three
172 PCR amplifications were performed on each DNA sample to overcome the effect of PCR biases
173 (Perreault et al. 2007). Amplicons were then purified with MinElute (QIAGEN) columns according to
174 the manufacturer's instructions and stored at -20°C prior to analysis by denaturing gradient gel
175 electrophoresis (DGGE). DGGE was performed using a DGGE-4001-Rev-B (C.B.S. Scientific
176 Company, CA, USA) system according to Schäfer and Muyzer (2001). Gels were stained with a half-
177 diluted solution of SYBR Green I (10,000X, Molecular Probes, Oregon) for 1 h according to the
178 manufacturer's instructions. Gels were photographed under UV light, and DGGE profiles were analyzed
179 using an AlphaImager HP (Alpha-Innotech). The number of bands, corresponding to different
180 operational taxonomic units (OTU), was determined, and the comparison between DGGE fingerprints

was performed using the Phoretix 1D Pro software (TotalLab Limited, Nonlinear Dynamics, Newcastle upon Tyne, UK) on the basis of a similarity matrix using Jaccard's index (Bourne et al. 2006; Clarke 1993).

### Statistical analyses

All statistical analyses were done using STATISTICA software version 6.0 (Statsoft, USA) with $\alpha = 0.05$. Data normality was examined using the Kolmogorov-Smirnov test and homoscedasticity tested with the Brown-Forsythe test (Zar 1999). Differences between treatments were tested using a one-way ANOVA (rotifer diet, D) or a two-way repeated ANOVA (rotifer diet and sampling time, T). For subsequent multiple comparisons, Tukey tests or Tukey tests for unequal n were performed when appropriate. Data related to CFU counts on TCBS in source seawater were square-root transformed. The Games & Howell test was used when heteroscedasticity was observed.

## Results

### Rotifer enrichment

The level of AA in enriched rotifers was three-fold higher than that in control rotifers. Other essential fatty acids (EPA and DHA) and total fatty acids were similar for the two diets (Table 1).

### Bacterial communities

In source seawater, TB abundance was similar from day 0 to 15 and was 1.3-fold higher on day 26 ($T: p < 0.001$, $F = 11.12$, $df = 3$) (Table 2). TB abundance was 10,000-fold higher in rotifer cultures than in larval-rearing seawater on days 4 and 15 and 1,000-fold higher on day 26. TB abundance in larval tanks

followed the same pattern as that observed in source seawater, with similar values from day 4 to 15 and a significant increase (1.2-fold higher) on day 26 (T: $p < 0.001$, $F = 9.17$, $df = 3$) (Table 2). Conversely, TB abundance in rotifer tanks reached a higher level and dropped significantly on day 26 (T: $p < 0.001$, $F = 9.32$, $df = 2$) in the two treatments (D, D × T: $p > 0.05$) (Table 3).

Bacterial communities in rotifer-rearing seawater and in larval-rearing seawater had different proportions of HNA, LNA, and VHNA subpopulations (Fig. 1-2). HNA and LNA bacterial subpopulations were observed in the rearing seawater of all tanks during the experiment (Fig. 1-2), but the percentage of HNA cells was lower in rotifer-rearing seawater than in larval-rearing seawater (Tables 2-3). On day 26, the VHNA subpopulation was four-fold higher in the control larval tanks than in the AA-enriched tanks (Table 2). No VHNA subpopulation was detected in the source seawater.

DGGE fingerprint patterns indicated marked differences in the bacterial community composition in larval- and rotifer-rearing seawater. The number of OTUs in source seawater ranged between 19 on day 4 and 12 on day 26. OTUs were more numerous in the larval-rearing seawater, with 25 on day 4 and 16 on day 26, independent of the diet. In AA-enriched rotifer rearing seawater, only 9 OTUs were observed on day 4 and 10 on day 26. In the rearing seawater of control rotifers (no AA enrichment), the number of OTUs was slightly higher, with 12 on day 4 and 13 on day 26.

The cluster analysis of DGGE fingerprints (Fig. 3) indicated that control and AA-enriched rotifer-rearing seawater samples were clustered according to the AA-enrichment procedure and that bacterial community composition in the rotifer-rearing seawater was poorly correlated with the bacterial community composition in the corresponding larval-rearing seawater, as indicated by the relatively large

227 distance (> 0.75). The bacterial community composition was similar in the source seawater and in larval
228 tanks receiving non-enriched rotifers throughout the experiment. On day 4, the bacterial community
229 composition was similar in larval-rearing seawater for the two different larval diets (control and AA-
230 enriched), and the bacterial community composition in the larval tanks receiving the AA-enriched diet
231 was very similar to that of the source seawater (Jaccard's distance 0.31). On day 26, the distance
232 increased (0.68) between AA-enriched and control samples, whereas control and seawater source
233 samples had a similar bacterial community composition (0.44).
234
235 CFU counts on TCBS in source seawater were low and similar from day 0 to 15 ($0.6 \pm 0.7$ CFU.ml$^{-1}$)
236 but slightly higher on day 26 ($2.2 \pm 0.3$ CFU.ml$^{-1}$) (D: $p < 0.001$, $F = 20.35$, $df = 3$). A similar pattern
237 was observed in AA-enriched larval tanks, where CFU counts peaked on day 26 (D × T: $p < 0.001$, $F =$
238 943.77, $df = 3$) at a level 35-fold higher than observed in source seawater. CFU counts in control larval
239 tanks slightly increased on day 26 and was 5-fold higher than the count observed in source seawater on
240 days 0 and 4 (Fig. 4A). CFU counts were 3- to 14-fold higher in rotifer cultures than the highest count
241 observed in larval tanks. In AA-enriched rotifer tanks, the count peaked on day 15 and was more than
242 twice as high (D × T: $p < 0.001$, $F = 24.44$, $df = 2$) on days 15 and 26 compared to control rotifer tanks
243 (Fig. 4B).
244
245 Histological observations in larval intestinal lumen, gills, and skin revealed no significant difference in
246 bacterial colonization between larval groups at the end of the experiment (day 26). Bacterial densities in
247 control larvae and larvae fed AA-enriched rotifers were respectively $2.5\times10^3 \pm 2.3\times10^3$ and
248 $2.3\times10^3 \pm 2.5\times10^3$ bacteria mm$^{-2}$ in intestinal lumen, $28.5 \pm 35.8\%$ and $34.5 \pm 48.6\%$ of the gill area,
249 and $16.3 \pm 16.3\%$ and $11.7 \pm 8.5\%$ of the fin area. Fish larvae from both treatments exhibited similar

250 total lengths (5.36 ± 0.55 mm), widths (1.07 ± 0.15 mm), and dry weights (0.083 ± 0.035 mg.larva$^{-1}$) at
251 the end of the experiment.
252
253 ## Discussion
254
255 The AA enrichment process did not affect TB abundance in either rotifer or larval tanks during the 26
256 days of the experiment. Bacterial concentrations in rotifer tanks were four orders of magnitude greater
257 than in larval tanks, where TB abundances (~$10^5$ cells.ml$^{-1}$) corresponded to concentrations generally
258 reported in seawater from the St. Lawrence Estuary. In this experiment, TB abundances were expressed
259 as total counts and not as CFU counts, but considering that a maximum of 10% of total marine bacteria
260 are cultivable, our results are similar to those reported by Skerjmo and Valdstein (1993) and Haché and
261 Plante (2011) in their commercially enriched rotifer cultures. The differences observed between
262 seawater in larval tanks and rotifer culture tanks could be attributed to the dissolved organic matter
263 (DOM) supply (Nagata 2000) associated with the grazing of algae by rotifers, which may enhance
264 bacterial degradation and as a consequence promote bacterial multiplication. This is confirmed by the
265 higher percentage of VHNA bacteria cells in rotifer cultures than in source seawater. VHNA cells are
266 identified by their high fluorescence and their high amount of nucleic acids. These results indicate that
267 around 50% of bacterial cells are highly productive in these cultures. In larval fish cultures, VHNA
268 bacteria cells were only detected after day 15 (~ 10% of total cells) while they were observed in rotifer
269 cultures throughout the experiment. Nishimura et al. (2005) found a negative correlation between
270 %VHNA and chlorophyll *a* concentration. The late appearance of VHNA bacteria in larval tanks could
271 be explained by the daily water renewal, which reduced the availability of DOM at the beginning of the
272 larval rearing. Since VHNA cells were not observed in source seawater, these cells were probably

273 transferred from rotifer to larval cultures and actively contributed to changes in the larval tank
274 microflora. Nevertheless, considering only TB abundances and community structure, AA enrichment
275 produced no significant modifications of the bacterial community in rotifer- or larval-rearing seawater.
276
277 In contrast, CFU counts on TCBS agar sharply increased in both rotifer and larval-rearing seawater with
278 AA enrichment. The TCBS medium is used to identify bacteria of the genus *Vibrio*, which are
279 ubiquitous in the marine environment and potentially important pathogens of marine and brackish-water
280 fish (Reed and Francis-Floyd 1996). Since only low concentrations of these bacteria were observed in
281 the source seawater during the experiment, the increase in CFU counts on the TCBS medium in our
282 cultures was probably due to our experimental culture conditions, which included the addition of live
283 food in the tanks. Although the microbial communities associated with live food can be very different
284 between marine fish hatcheries (Skjermo and Vadstein 1993), most bacteria identified in rotifer cultures
285 have been from the Vibrionaceae family, which includes two important genera of fish pathogens,
286 *Aeromonas* and *Vibrio*. Other notable fish pathogens identified were *Moraxella* and *Flavobacterium*
287 (Rombaut et al. 2001; Verdonck et al. 1994). Because larvae were not uniformly distributed in rearing
288 tanks (patchy distribution) and the tiny dead larvae decomposed very rapidly in sea water, we were not
289 able to compare mortality rates in the different larval treatments and to correlate them with the increase
290 in bacteria cultivable on TCBS agar. However, the increase in bacteria cultivable on TCBS agar did not
291 alter larval development: both groups (AA-enriched and non- enriched food sources) had similar growth
292 (used as proxy for larval health) and similar bacterial colonization in their tissues, despite strong inter-
293 individual variations. This has also been observed in the intestines of goldfish *Carassius auratus* (Asfie
294 et al. 2003; Sugita et al. 1988), carp *Cyprinus carpio,* and tilapia *Oreochromis mossambicus* (Asfie et al.
295 2003). Thus though AA enrichment may enhance the development of bacteria that are cultivable on

296 TCBS agar, these bacteria did not seem pathogenic for winter flounder larvae under our culture
297 conditions.
298
299 The cluster analysis of the DGGE fingerprint patterns of the larval rearing water over 26 days confirms
300 that winter flounder larvae are exposed to different bacteria before feeding on day 4 and after the onset
301 of exogenous feeding on days 15 and 26. Since bacterial populations representing at least 1% of the total
302 community can be detected by PCR-DGGE (Muyzer and Smalla 1998), this method is a good tool for
303 characterizing bacterial populations present during early life stages of fish larvae in hatcheries
304 (Brunvold et al. 2007). Our results demonstrated that the bacterial community composition developed
305 differently in rotifer and larval-rearing seawater through the experiment. Furthermore, we observed a
306 change in the bacterial community composition between the beginning and the end of the experiment
307 (day 4 and 26). Brunvold et al. (2007) demonstrated a similar change in the bacterial community
308 associated with hatchery cod larvae, corresponding to the onset of exogenous feeding and a relatively
309 stable bacterial community during larval feeding on rotifers. In the present experiment, the number of
310 OTUs decreased in all larval treatments, with a reduction of 9 OTUs from day 4 to day 16 without any
311 effect related to the diet. Since a reduction of 7 OTUs was observed in the source seawater, the decrease
312 in the larval-rearing seawater may be partly explained by the source seawater. There were fewer OTUs
313 in rotifer seawater samples than in source seawater: only 9 and 12 OTUs were observed on day 4 in AA-
314 enriched and control rotifer tanks, respectively. While the number of OTUs did not change in rotifer
315 tanks, DGGE fingerprints indicate a modification of the community composition: only 6 and 7 OTUs
316 were common to the day 4 and day 16 samples in AA-enriched and control rotifer tanks, respectively
317 (data not shown). At the end of the experiment, only 6 OTUs were common to AA-enriched and control
318 rotifer tanks out of the 23 OTUs that were present. Thus even though slight modifications were observed

319 in the larval rearing seawater of larvae fed enriched rotifers, the AA enrichment seems to interact with
320 the bacterial community in rotifer tanks. The DGGE fingerprint cluster analysis revealed a marked
321 distance between water samples from rotifer and larval tanks, which suggests that even if some bacteria
322 were introduced with rotifers, these bacteria did not become dominant in the bacterial community of the
323 larval tanks.
324
325 The proliferation of bacteria in intensive aquaculture systems may be responsible for poor growth and
326 mass mortality of marine fish larvae. Essential fatty acids provided in the diet could protect larvae by
327 modulating the immune response via AA and EPA. The antibacterial effect of a given fatty acid is
328 influenced by its structure and shape, with unsaturated fatty acids tending to have greater potency at low
329 concentrations (Kabara 1978; Kanai and Kondo 1979; Nieman 1954) than saturated fatty acids with the
330 same carbon chain length (Desbois and Smith 2010; Knapp and Melly 1986; Kodicek 1949). For
331 example, AA is more toxic for gram-positive bacteria than for gram-negative species, such as bacteria of
332 the genus *Vibrio*. The gram-negative species sensitive to AA have a more permeable outer membrane
333 than other negative species (Knapp and Melly 1986). In our study, AA enrichment may have contributed
334 to the development of AA-resistant bacteria species cultivable on TCBS, especially in rotifer cultures.
335 Such bacterial resistance or growth inhibition has been observed *in vitro* on *Escherichia coli* and
336 *Pseudomonas aeruginosa* strains challenged with gamma-linoleic acid, DHA, and AA (Giamarellos-
337 Bourboulis et al. 1994, 1995). The gut microflora of adult fish and the fish's resistance to infections are
338 greatly influenced by the pioneer bacteria in the diet and in the ambient rearing water of the developing
339 larvae. However, we observed no difference in larval development with or without AA enrichment of
340 the rotifers after 26 days of experimentation, suggesting no positive or negative effects for larvae.
341

342 In conclusion, we previously demonstrated that AA plays a crucial role in winter flounder larval
343 development and that AA enrichment could modify bacterial colonization of the intestinal lumen in this
344 species (Seychelles et al. 2011). The present study demonstrates that AA also modifies the nature of the
345 bacterial communities present both in prey (rotifers) and in larval-rearing water. The next step would be
346 to precisely identify which bacteria are positively and negatively affected by the addition of AA and to
347 test if they can directly alter larval health or if they could act as prebiotics.
348

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355
356

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462

**Table 1** Selected fatty acid composition (mean ± SD) of rotifers fed control and AA-enriched diets. Asterisks indicate a significant difference between the two diets. AA: Arachidonic acid; DHA: docosahexaenoic acid; EPA: eicosapentaenoic acid; SFA: saturated fatty acids; MUFA: monounsaturated fatty acids; PUFA: polyunsaturated fatty acids.

| | Control rotifers | AA-enriched rotifers | Summary of ANOVA results |
|---|---|---|---|
| Fatty acid proportions (% TFA) and ratio | | | |
| AA | 0.9 ± 0.2 | 3.0 ± 0.9* | D: $p < 0.001$; D × T: $p > 0.05$ |
| EPA | 3.7 ± 0.6 | 3.7 ± 0.9 | All NS |
| DHA | 2.7 ± 0.5 | 2.7 ± 0.6 | T: $p < 0.01$; D × T: $p > 0.05$ |
| SFA | 21.6 ± 0.9 | 22.3 ± 1.4* | D, T: $p < 0.01$; D × T: $p > 0.05$ |
| MUFA | 55.5 ± 2.7* | 53.2 ± 3.5 | D, T: $p < 0.01$; D × T: $p > 0.05$ |
| PUFA | 22.9 ± 2.4 | 24.5 ± 2.8* | D, T: $p < 0.01$; D × T: $p > 0.05$ |
| DHA:EPA | 0.7 ± 0.1 | 0.8 ± 0.2 | All NS |
| Total fatty acids (mg $g^{-1}$) | 19.5 ± 6.8 | 25.8 ± 8.9 | All NS |

D: diet; T: sampling time; D × T: interaction between D and T; NS: not significant.

**Table 2** Concentrations of total free bacteria (TB) and bacteria with high (HNA) and very high (VHNA) nucleic acid contents in fish culture water and source seawater (sampled prior to use in the larval tanks).

| | **TB ($\times 10^5$ bacteria.ml$^{-1}$)** | | | **HNA ($\times 10^5$ bacteria.ml$^{-1}$) (%HNA)** | | | **VHNA ($\times 10^4$ bacteria.ml$^{-1}$) (%VHNA)** | | |
|---|---|---|---|---|---|---|---|---|---|
| **Sample** | *Day 4* | *Day 15* | *Day 26* | *Day 4* | *Day 15* | *Day 26* | *Day 4* | *Day 15* | *Day 26* |
| SW | 2.70±0.26 | 2.84±0.26 | 3.63±0.58 | 2.10±0.22 (77.52) | 2.15±0.18 (75.61) | 2.91±0.40 (80.27) | ND | ND | ND |
| CLT | 2.58±0.15 | 2.94±0.29 | 3.42±0.07 | 2.28±0.12 (78.53) | 2.20±0.38 (74.64) | 2.65±0.19 (72.21) | ND | 2.47±0.01 (8.38) | 5.71±0.20 (15.53) |
| AA-LT | 2.90±0.17 | 2.95±0.39 | 3.67±0.14 | 2.05±0.12 (79.23) | 2.16±0.22 (73.55) | 2.56±0.06 (74.96) | ND | ND | 1.41±0.12 (4.12) |

SW: Source seawater; CLT: Control larval tanks; AA-LT: AA-enriched larval tanks; ND: not detected

**Table 3** Concentrations of total free bacteria (TB) and bacteria with high (HNA) and very high (VHNA) nucleic acid contents in rotifer culture water.

| | **TB ($\times 10^9$ bacteria.ml$^{-1}$)** | | | **HNA ($\times 10^9$ bacteria.ml$^{-1}$) (%HNA)** | | | **VHNA ($\times 10^9$ bacteria.ml$^{-1}$) (%VHNA)** | | |
|---|---|---|---|---|---|---|---|---|---|
| **Sample** | *Day 4* | *Day 15* | *Day 26* | *Day 4* | *Day 15* | *Day 26* | *Day 4* | *Day 15* | *Day 26* |
| CR | 16.47±2.39 | 20.44±12.56 | 4.68±0.47 | 3.84±1.41 (23.32) | 8.16±9.69 (39.94) | 2.17±0.40 (46.36) | 10.25±3.48 (62.25) | 10.64±5.71 (52.08) | 1.03±0.79 (22.01) |
| AA-R | 13.46±6.36 | 15.52±8.79 | 3.77±0.20 | 2.85±2.21 (21.15) | 6.04±4.89 (38.93) | 1.72±0.29 (45.58) | 7.91±3.57 (58.74) | 6.89±2.63 (44.42) | 1.91±0.32 (50.67) |

CR: Control rotifers; AA-R: AA-enriched rotifers.

**Figure captions**

**Figure 1** Flow cytometric dot-plots (FL1/SSC) of source seawater and culture water sampled in larval tanks. Reference beads of 1 µm diameter (1), HNA subpopulation (2), LNA subpopulation (3), and VHNA subpopulation (4) are indicated.

**Figure 2** Flow cytometric dot-plots (FL1/SSC) of culture water sampled in rotifer tanks. Reference beads of 1 µm diameter (1), HNA subpopulation (2), LNA subpopulation (3), and VHNA subpopulation (4) are indicated.

**Figure 3** Dendrogram of the DGGE fingerprint patterns of the microbial community showing the distance between bacterial communities from day 0 to 26 in larval rearing water and rotifer cultures. SW: source seawater (sampled before being added to larval tanks); AA: water in AA-enriched larval tanks; AAR: water in AA-enriched rotifer tanks; CT: water in control larval tanks; and CTR: water in rotifer tanks; d: sampling day. The cluster analaysis was based on Jaccard coefficient similarity indices and constructed using the Phoretix 1D Pro software (Nonlinear Dynamics, Newcastle upon Tyne, UK).

**Figure 4** A) CFU counts on TCBS in control and AA-enriched larval tanks. B) CFU counts on TCBS in rotifer tanks. Counts are expressed as $CFU.ml^{-1}$ ± SD. Different letters indicate significant differences among sampling times or diets.

Figure 1

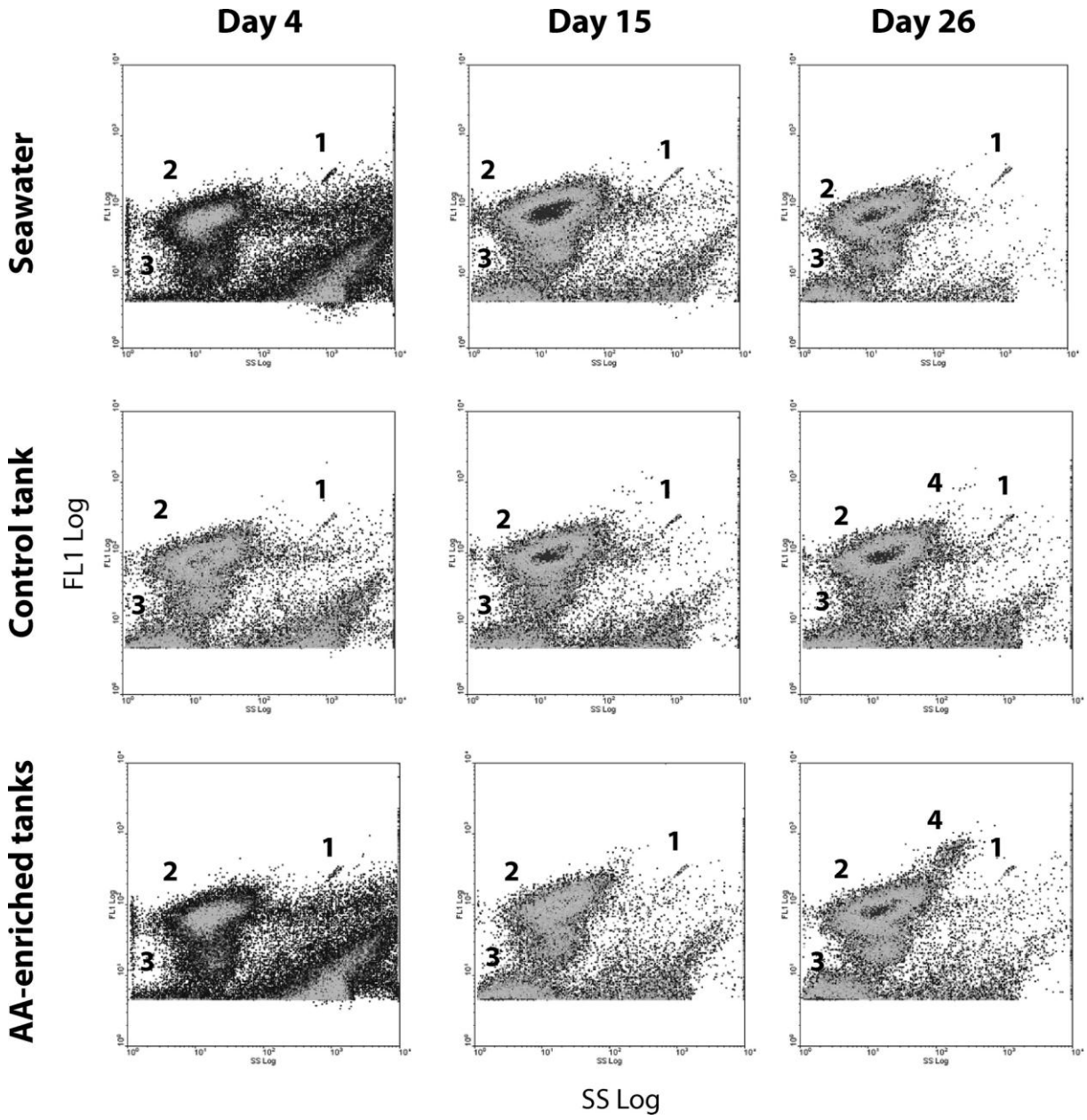

Day 4
Day 15
Day 26
Seawater
Control tank
AA-enriched tanks
FL1 Log
SS Log
1
2
3
4

Figure 2

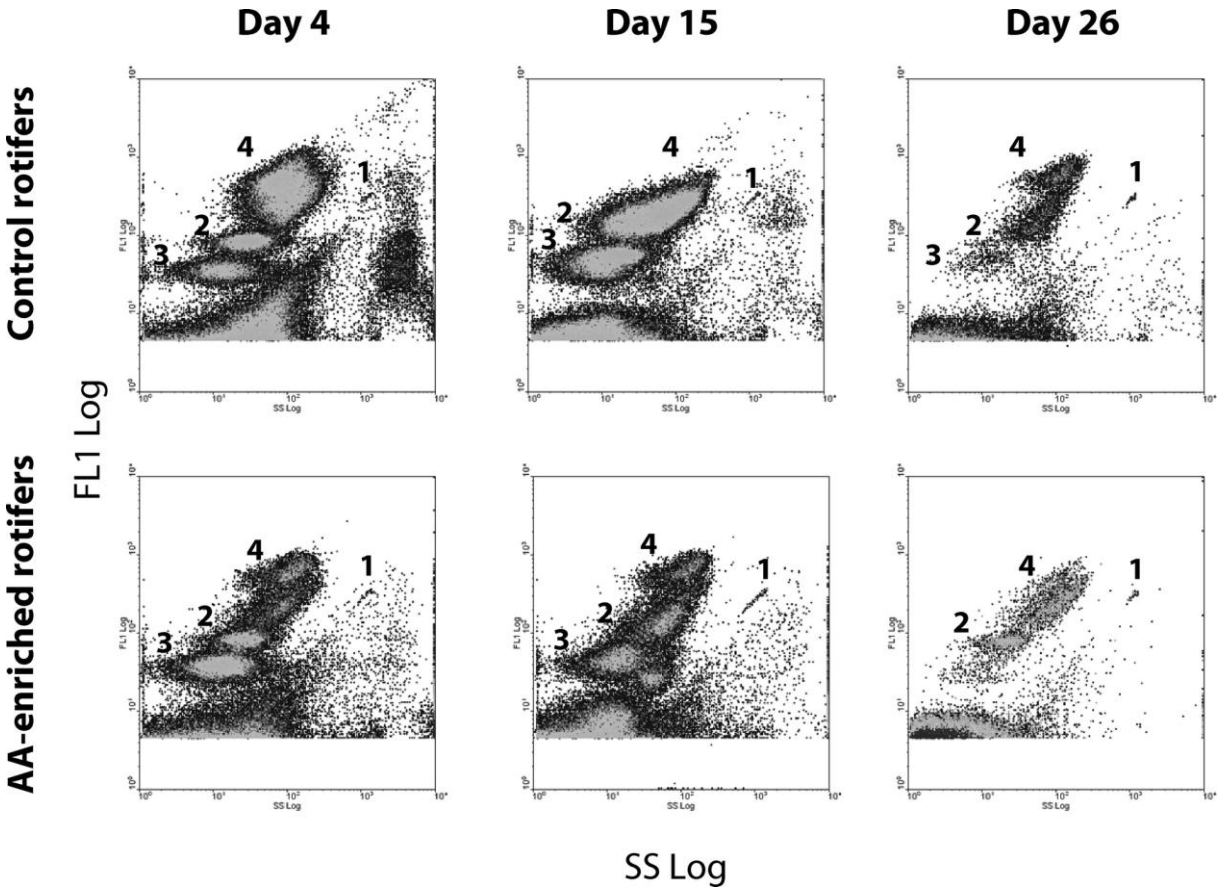



Figure 3

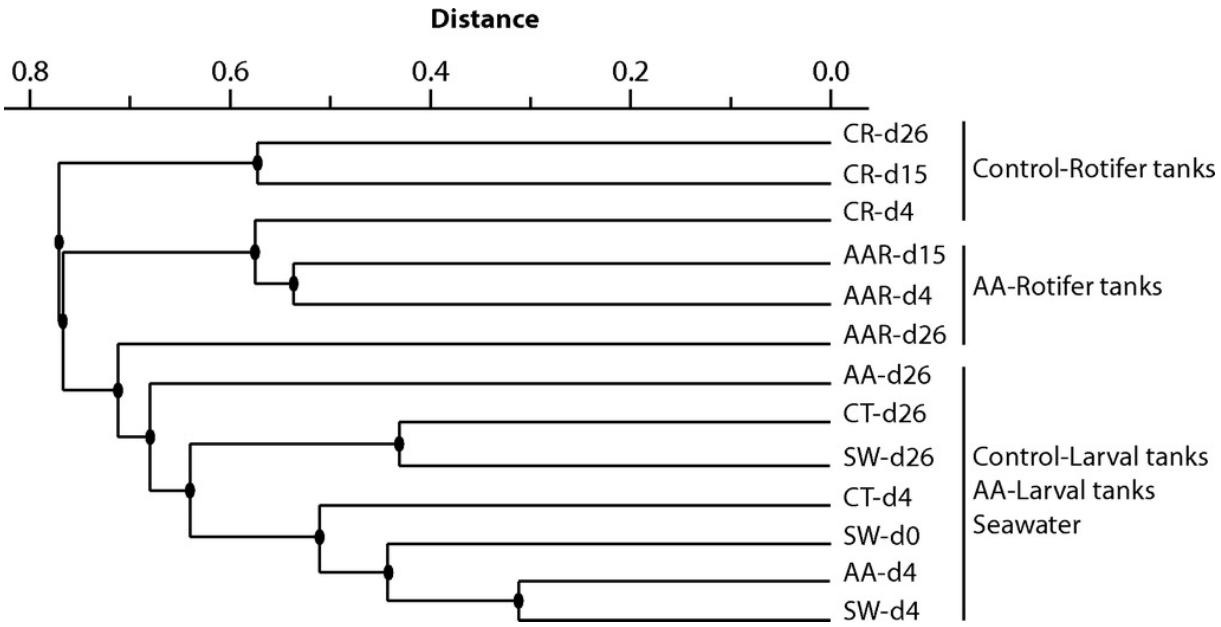
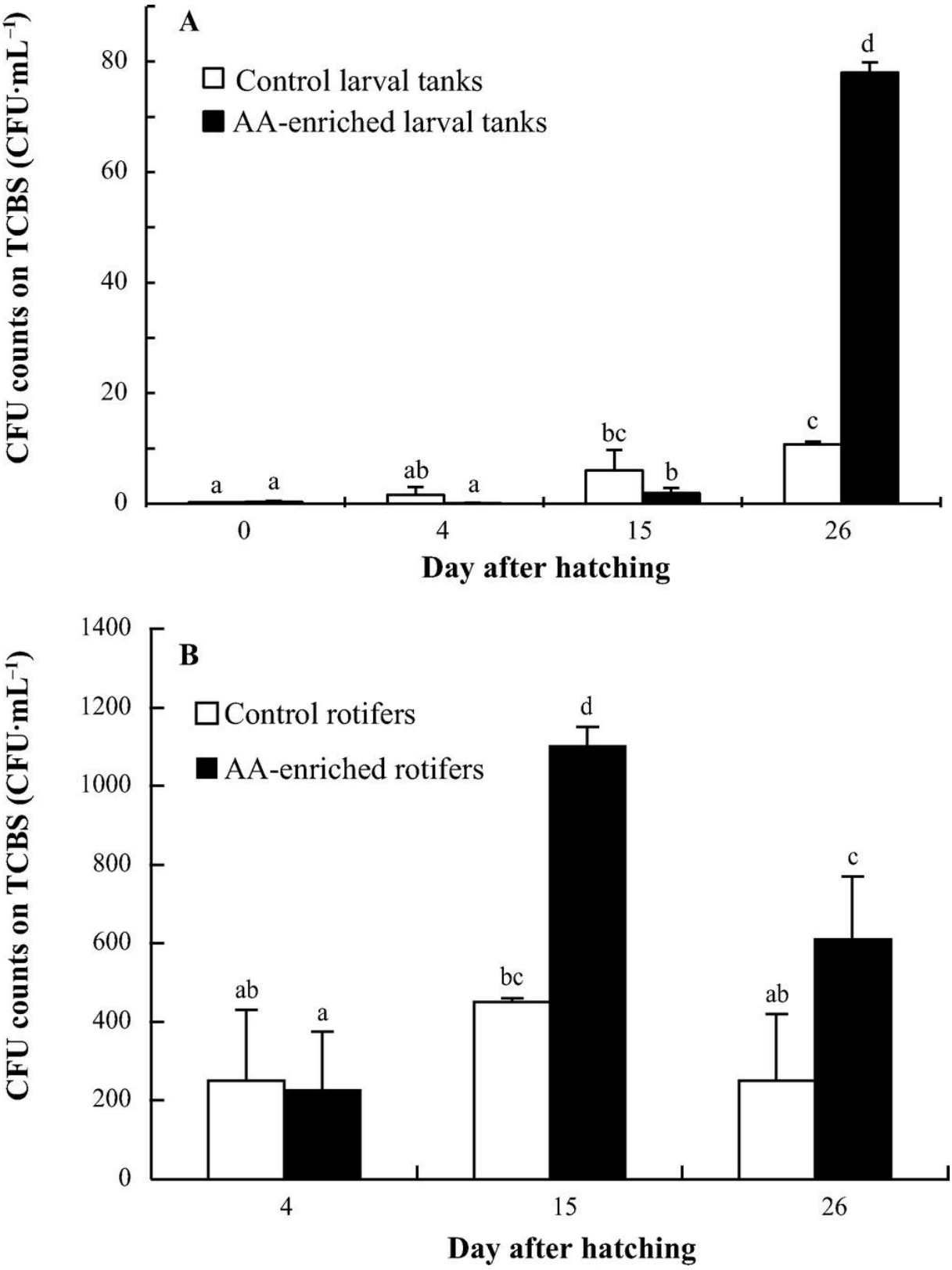


Figure 4


A
Control larval tanks
AA-enriched larval tanks
CFU counts on TCBS (CFU·mL⁻¹)
0
20
40
60
80
a
a
ab
a
bc
b
c
d
0
4
15
26
Day after hatching
B
Control rotifers
AA-enriched rotifers
CFU counts on TCBS (CFU·mL⁻¹)
0
200
400
600
800
1000
1200
1400
ab
a
bc
d
ab
c
4
15
26
Day after hatching