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The effect of feeding with different microalgae on survival, growth, and fatty acid composition of *Artemia franciscana* metanauplii and on predominant bacterial species of the rearing water

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Abstract
Artemia franciscana metanauplii widely is being used in cultured marine species. The aim of this study was to evaluate the effect of feeding with two marine and two freshwater microalgae as feed alone or in combination on survival, growth, and fatty acid composition of *A. franciscana* metanauplii and on predominant bacterial species of the rearing water. Five microalgae diets were used for feeding: *Amphora viridis* (AV), *Chlamydomonas reinhardtii* (CR), *Chlorella vulgaris* (CV), and *Dunaliella salina* (DS) and a combination of four microalgae (MX diet). *Artemia franciscana* fed AV, DS, and MX diets showed higher survival than that fed CR and CV diets. MX group showed higher total length among groups ($P < 0.05$). Total n-3 fatty acid content was higher in the *Artemia franciscana* fed MX group, whereas total n-3 HUFA levels were found significantly higher in *Artemia franciscana* fed DS and AV diet ($P < 0.05$). The bacterial load of the rearing water was significantly decreased with the use of CV; therefore, the CV diet might be suggested to be used in *Artemia franciscana* grow-out for reducing bacterial proliferation. According to 16S rRNA gene sequencing results, four different bacterial species including *Carnobacterium maltaromaticum*, *Pseudomonas stutzeri*, *Pseudoalteromonas* sp., and *Vibrio* sp. species were found predominantly in the rearing water of *Artemia franciscana*.

Keywords *Artemia* · Microalgae · Growth · Essential fatty acids · Bacterial load

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Introduction

Artemia nauplii are the most common live prey utilized in cultured marine and freshwater fish species (Sorgeloos et al. 2001). Aquaculture hatcheries require live feeds similar to natural live preys larger than 1 mm, such as copepods, for further larval fish feeding. In this point of view, *Artemia franciscana* metanauplii are of appropriate size for crustacean (Nelson et al. 2002), fish (Lim et al. 2001), seahorse (Souza-Santos et al. 2013; Vite-Garcia et al. 2014), clownfish (Chen et al. 2020), anemones (Leal et al. 2012), soft corals (Petersen et al. 2008; Tsounis et al. 2010), and cephalopods culture (Okumura et al. 2005; Seixas et al. 2008; Guinot et al. 2013b). Therefore, juvenile or adult forms of *Artemia* sp. are also largely in demand in the new candidate marine cultured species. *Artemia* sp. is a filter-feeder fast-growing brine shrimp, which mostly consumes various small-sized particles such as bacteria (Toi et al. 2013), microalgae (Zhukova et al. 1998), and organic manure in the water column (Maldonado-Montiel et al. 2003). Among them, *Artemia* sp. mostly consumes various microalgae for efficient growth.

Microalgae are the first step of the marine food chain/web and directly utilized in marine fish larvae tank, which is called the green water technique (Faulk and Holt 2005; Neori 2011). They are also used as alternative feed ingredients replacing fish oil and fishmeal (Miller et al. 2007; Ganuza et al. 2008; Eryalçın et al. 2013, 2015), filter-feeder bivalve (Arney et al. 2015), rotifer (Ma and Qin 2014; Rehberg-Haas et al. 2015), *Artemia* sp. (Lora-Vilchis et al. 2004; Makridis et al. 2006), and copepod feeding (Rasdi et al. 2016). Different forms of microalgae biomass such as spray-dried, freeze-dried, and freshly cultured microalgae have been studied for *Artemia* sp. nutrition (Thinh et al. 1999; Olsen et al. 2000; Lora-Vilchis et al. 2004). Additionally, bacteria, yeast, and other suspended organic compounds and commercial feeds have been also investigated (Marques et al. 2004; Toi et al. 2014; Ahmadi et al. 2017; Planas et al. 2017; Vahdat et al. 2018). Ingestion, digestion, and chemical conversion of essential compounds of feeds are also main issues in *Artemia* sp. grow-out stages (Glencross et al. 2007). Based on the recent findings, the best mono-algal diet, microalgae *Rhodomonas lens*, was suggested for *Artemia* sp. (Seixas et al. 2009). However, combination of different microalgae has also been recommended for *Artemia* sp. nutrition in order to sustain higher growth and better nutritional quality (Pacheco-Vega et al. 2015; El-Kassas et al. 2016).

The successful culture of marine fish larvae mainly depends on high-quality live prey containing appropriate levels of essential nutrients such as highly unsaturated fatty acids (HUFA), amino acids, vitamins, and minerals. On the other hand, the bacterial load of the live feed is a concern in fish larviculture and the "content" of this load has been found to be highly correlated with the feed consumed by live feed (Hache and Plante 2011; Avila-Villa et al. 2011). The live feeds are known to have numerous pathogenic species in their microbiota and they may affect the health of the larvae by being vectors of viruses and bacteria (Skjermo and Vadstein 1993; Dhert et al. 2001; Da Silva et al. 2015). Olsen et al. (1999) found that the percentages of hemolytic bacteria and *Vibrios* in the intestines of halibut larvae fed with 2-day-old *A. franciscana* were significantly higher than those fed only short-term enriched *A. franciscana* nauplii.

Rearing water of marine fish larvae includes high nutrient salts and organic wastes together with feed added into the rearing water column and this leads to high proliferation of bacterial flora (Zarkasi et al. 2016). Therefore, live feeds should be also evaluated for bacterial community before given into the larvae culture tanks (Planas and Cunha 1999). Microalgae may alter the bacterial microbiota of the environment they exist in, particularly because of their

ability to inhibit bacterial proliferation by secreting certain antibacterial and antiviral chemicals. Mostly, small-sized microalgae (2–6 μm) such as *Nannochloropsis* sp. are preferred for this purpose (Muller-Feuga 2000; Spolaore et al. 2006).

Biochemical composition of *Artemia* sp. is directly affected by consumed microalgae diets (Fabregas et al. 2001), and microalgae species are known to significantly differ in their specific biocompounds such as different essential fatty acids (EFA). Most importantly, crustaceans and other invertebrates are not capable of synthesizing n-3 and n-6 polyunsaturated fatty acids (PUFA) efficiently. For that reason, enrichment process or feeding with high-quality feeds is desired. The four microalgae species *A. viridis*, *D. salina*, *C. vulgaris*, and *C. reinhardtii* have not been evaluated for *Artemia franciscana* nauplii feeding for long-term grow-out culture so far. The aim of this study was to determine the effect of feeding with these two marine and two freshwater microalgae alone or in combination on survival, growth, and fatty acid composition of *A. franciscana* metanauplii and on predominant bacterial species of the rearing water.

Materials and methods

Microalgae culture

Two marine microalgae *A. viridis* (strain number: CCAP1001/1) and *D. salina* (strain number: CCAP19/12) and two freshwater microalgae *C. vulgaris* (strain number: CCAP211/12) and *C. reinhardtii* (strain number: CCAP 11/32A) were obtained from CCAP (Culture Collection Algae and Protozoa). For marine microalgae, artificial seawater was prepared as 32 ppt in a polyethylene conic bottom tank with the following inside dimensions: 50 cm × 100 cm × 80 cm by addition of artificial sea salt (Instant Ocean®) with tap water. Prepared seawater was chemically sterilized. For that purpose, 3 mL L^{-1} sodium hypochlorite was added to the total tank volume and aerated for 24 h. After 24 h, neutralization was carried out by adding 150 mg L^{-1} sodium thiosulfate (Merck, Germany) and then aerated for an additional 24 h to the desired seawater pH. Marine microalgae *A. viridis* and *D. salina* were cultured in medium f/2+Si, f/2 (Guillard and Ryther 1962), and freshwater microalgae *C. vulgaris* and *C. reinhardtii* were cultured in 3N-BBM+V medium. All sub-cultures were maintained at 27 °C and salinity of 32 ppt under an initially 12L:12D photoperiod. Microalgae culture volumes were increased from 50-mL test tubes to 250-mL Erlenmeyer flasks. The contents of 250-mL flasks were transferred to 1-L, 5-L, and then 30-L volume culture vessels. The population growth of microalgae was determined by using Neubauer hemocytometer counter daily. Inoculation was performed by reaching population to late log-phase into the larger volume.

Experimental design and diets

A. franciscana nauplii were obtained from the hatching of Great Salt Lake *A. franciscana* cysts (INVE Aquaculture Nutrition, Dendermonde, Belgium). Cysts were decapsulated as described by Bruggeman et al. (1980) and then incubated for 24 h in a 5-L cylindrical conical glass tank containing seawater (38 g L^{-1} salinity) at 28°C. Gentle aeration was placed in the bottom of the glass tank with continuous illumination. After hatching, instar I nauplii were moved into cylindrical containers at 23 °C and rinsed with UV-treated seawater for 20 min. Initial *A. franciscana* stock density was 60 nauplii mL^{-1} in all experimental feeding culture tanks. *A. franciscana* nauplii groups were fed every day selected microalgae during 15 days of the

experiment. Microalgae diets were (a) *A. viridis* (AV) (b) *D. salina* (DS), (c) *C. vulgaris* (CV), (d) *C. reinhardtii* (CR), and (e) mix (MX) (1:1:1:1) due to the high nutritional values. Concentration of microalgal density was 10^7 cell/mL^{-1} for 10^6 *A. franciscana* constant during the culture period. Each group was tested in triplicate. Water exchange renewal rate in all vessels was adjusted as 10% of the total volume per day.

Growth performance and sampling

Dry weight was determined by sampling 5 mL of microalgal culture filtered on previously weighed pre-combusted Whatman GF/C (Maidstone, England; diameter 47 mm) fiberglass filters. Microalgal biomass was washed three times with 5 mL of 0.5-M ammonium formate in order to remove salts (Zhu and Lee 1997). Filters were dried overnight at 80 °C and dry weight was determined gravimetrically. Microalgal biomass (25–50 mL) were sampled by centrifugation and immediately frozen at −80°C. One hundred fifty milliliters of *A. franciscana* culture was filtered on a sieve (45-μm mesh size), rinsed with distilled water, and frozen at −80 °C for proximate and fatty acid composition at the end of the experiment. During the experiment, ten *A. franciscana* from each culture vessel were sampled in 5% formalin solution every 3 days. *A. franciscana* growth was determined by measuring the total length of 10 *A. franciscana* at 3, 6, 9, 12, and 15 days after hatching using an Olympus BX-51 microscope. At the end of the experiment, survival rate was determined by counting all live *A. franciscana* in each culture vessel.

Proximate composition and fatty acid analysis

Moisture, protein, and crude lipid contents of microalgae and *A. franciscana* were analyzed according to Folch et al. (1957) and AOAC (1990) (Table 1, Table 3). Fatty acid methyl esters in microalgae, diets, and experimental fish were obtained by transmethylation with 1% sulfuric acid in methanol (Christie 1989). Fatty acid methyl esters were separated by GC (GC-14A; Shimadzu, Tokyo, Japan) in a Supercolvax-10 fused silica capillary column (constant pressure with 100KPa, length: 30 m; internal diameter: 0.32 mm; 0.25 i.d (Ref.: 24080-U) Supelco, Bellefonte, PA, USA) using helium as a carrier gas. Column temperature was 180 °C for the first 10 min, increasing to 220 °C at a rate of 2 °C min^{-1} and then held at 220 °C for 15 min. Fatty acid methyl esters were quantified by FID following the conditions described to Izquierdo et al. (1990) and identified by comparison with external standards and well-characterized fish oils (EPA 28, Nippai, Ltd Tokyo, Japan).

Table 1 Proximate composition of experimental microalgae (% dw)

Proximate composition	AV	DS	CV	CR	MX
Crude protein	49.1 ± 0.4^{d}	48.5 ± 0.4^{d}	50.7 ± 0.2^{c}	53.6 ± 0.8^{a}	52.2 ± 0.5^{b}
Crude lipid	13.2 ± 0.6^{b}	7.6 ± 0.7^{d}	12.1 ± 0.8^{c}	15.3 ± 0.1^{a}	14.9 ± 0.2^{a}
Crude ash	19.4 ± 0.1^{a}	2.2 ± 0.5^{c}	3.1 ± 0.4^{c}	3.1 ± 0.3^{c}	7.3 ± 0.6^{b}
Moisture	8 ± 0.4^{a}	3.8 ± 0.5^{d}	3.75 ± 0.7^{d}	4.7 ± 0.4^{c}	6.7 ± 0.9^{b}

Values expressed in mean ± standard deviation (SD) (n = 3 tanks/diet), lower case letters denote significant variable differences between means using Duncan's multiple range test ($P < 0.05$)

Determination of the heterotrophic plate count and identification of the predominant bacterial species in the rearing water

The rearing water of *A. franciscana* was sampled three times during the experiment (day 0, 2, and 15), and these samples were inoculated onto Marine Agar 2216 and TCBS agar to determine the heterotrophic plate counts (HPC) and total *Vibrio* sp. counts (Austin 1988; Makridis et al. 2006). The morphological and physiological characteristics of predominant bacterial colonies from each plate were determined by using conventional biochemical and physiological tests and rapid identification kits (API 20E) according to Buller (2004) and Austin and Austin (2007). For molecular identification, the isolates displaying the same morphological and biochemical characteristics were selected and inoculated into Marine Broth 2216 (Difco) and incubated overnight at 22 °C in a shaking incubator. Genomic DNA was then extracted by the PureLink Genomic DNA Mini Kit (Invitrogen) according to the manufacturer's instructions and used as a template for PCR. An approximately 540-bp-long fragment of the 16S rRNA gene was amplified using the universal bacteria primer set; primer S-20 (5' AGA GTT TGA TCC TGG CTC AG 3') and primer A-18 (5' GWA TTA CCG CGG CKG CTG 3') (Suau et al. 1999). Amplification was performed using a thermal cycler (Biometra, TPersonal) with the following parameters: initial denaturation at 95 °C for 3 min, followed by 30 cycles of amplification (denaturation at 95 °C for 30 s, annealing at 56 °C for 1 min, extension at 72 °C for 1 min) and a final extension step of 72 °C for 4 min. PCR products were purified and sequenced bidirectionally and sequence editing and analysis were performed in Bioedit v7.0.0 (Hall 1999) using the ClustalX 2.1 (Larkin et al. 2007) and BLASTN 2.2.20 algorithm (Zhang et al. 2000).

Statistical analysis

All data were tested for normality with the Shapiro-Wilk test and homogeneity of variances by Levene's test and treated using one-way ANOVA. Means were compared by the two-tailed Student's t test ($P < 0.05$) and Duncan's multiple range test (DMRT) using SPSS software (SPSS for Windows 11.5; SPSS Inc., Chicago, IL, USA). Duncan's multiple range test (DMRT) is a post-hoc test to measure differences between pairs of means (which are shown as a, b, c, d, etc. in relevant figures below). In this test, whilst the same letters mean no significant difference, different letters indicate significant differences among pairs of means. Values presented in the figures and tables are means ± standard deviations SD. All values presented as percentages were arc cosine transformed before performing any statistical test.

Results

Growth performance of *Artemia franciscana*

In our study, *A. franciscana* fed CR (1760±63μm), MX (1725±25μm), and AV (1650±36μm) diet showed significantly higher total body length than *A. franciscana* fed DS (1196±38μm) and CV (1310±32μm) at the 12th day of the experiment ($P < 0.05$) (Fig. 1). However, feeding *A. franciscana* nauplii with a CR diet provided significantly higher body length (2520±45μm) among other treatments at the end of the experiment ($P < 0.05$).

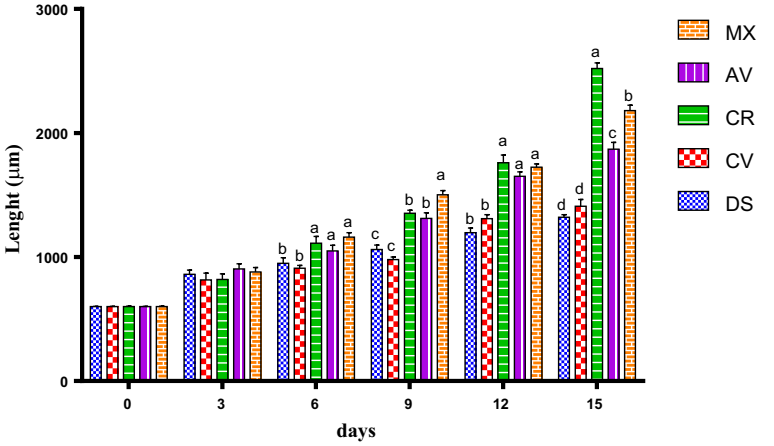

Fig. 1 Total length of *A. franciscana* groups fed experimental microalgae (CV, *C. vulgaris*; CR, *C. reinhardtii*; DS, *D. salina*; AV, *A. viridis*; MX, mix. Lower case letters (a, b, c) denote significant variable differences between groups using Duncan's multiple range test, $P < 0.05$)

Survival of *Artemia franciscana*

Final survival was found similar in CV, AV, and MX groups and significantly higher than DS and CR groups ($P < 0.05$). *A. franciscana* fed DS diet showed a moderate level of survival, whereas the lowest survival rate was observed in *A. franciscana* fed CR diet (Fig. 2).

Proximate composition and fatty acid profile of *Artemia franciscana* metanauplii

CR and MX microalgae diets contained higher crude lipid levels than other diets. CR diet contained higher crude protein level among experimental microalgae (Table 1). Fatty acid profiles of microalgae are shown in Table 2

At the end of the study, crude protein content of *A. franciscana* metanauplii was found similar in CR and MX groups and these two groups were significantly higher than other groups ($P < 0.05$). Similarly, lipid and ash contents of the *A. franciscana* fed MX diet showed significantly higher values than those of other groups ($P < 0.05$) (Table 3).

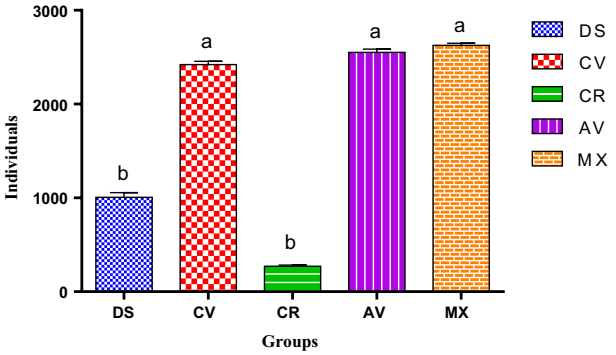

Fig. 2 Survival of *A. franciscana* groups fed experimental microalgae diet at the end of the experiment (CV, *C. vulgaris*; CR, *C. reinhardtii*; DS, *D. salina*; AV, *A. viridis*; MX, mix. Lower case letters (a, b, c) denote significant variable differences between groups using Duncan's multiple range test, $P < 0.05$)

Table 2 The fatty acid compositions of cultured microalgae (% total fatty acids)

Fatty acid (%)	AV	DS	CV	CR	MX
6:0	n.d.	n.d.	n.d.	n.d.	0.02 ± 0.00
8:0	n.d.	n.d.	n.d.	n.d.	0.07 ± 0.01
10:0	n.d.	n.d.	0.03 ± 0.00	n.d.	0.04 ± 0.00
12:0	0.22 ± 0.00^{a}	0.23 ± 0.01^{a}	0.16 ± 0.00^{b}	0.05 ± 0.00^{c}	0.16 ± 0.00^{b}
13:0	0.04 ± 0.00^{c}	0.22 ± 0.01^{b}	n.d.	n.d.	0.32 ± 0.01^{a}
14:0	6.07 ± 0.00^{b}	8.94 ± 0.01^{a}	0.60 ± 0.02^{d}	1.05 ± 0.02^{c}	1.43 ± 0.00^{c}
14:1	3.32 ± 0.00^{a}	0.53 ± 0.01^{b}	0.11 ± 0.00^{c}	n.d	0.32 ± 0.01^{b}
15:0	2.60 ± 0.01^{b}	4.34 ± 0.00^{a}	0.26 ± 0.01^{c}	0.50 ± 0.00^{c}	0.49 ± 0.02^{c}
16:0	31.02 ± 0.04^{b}	35.61 ± 0.04^{a}	18.80 ± 0.21^{e}	28.45±0.08^{c}	25.94 ± 0.08^{d}
16:1	33.6 ± 0.01^{a}	0.24 ± 0.01^{c}	28.76 ± 0.18^{b}	1.65 ± 0.02^{c}	0.95 ± 0.03^{c}
17:0	0.82 ± 0.00^{a}	0.08 ± 0.01^{d}	0.23 ± 0.02^{b}	0.11 ± 0.01^{d}	0.17 ± 0.00^{c}
18:0	5.22 ± 0.01^{b}	14.75 ± 0.01^{a}	14.38 ± 0.05^{a}	2.11 ± 0.02^{c}	4.51 ± 0.00^{b}
18:1n-9	4.55 ± 0.00^{d}	7.95 ± 0.00^{c}	28.21 ± 0.13^{a}	24.83±0.02^{a}	15.71 ± 0.02^{b}
18:2n-6	3.20 ± 0.01^{e}	5.88 ± 0.01^{d}	7.46 ± 0.01^{c}	19.71±0.03^{b}	23.98 ± 0.05^{a}
18:3n-3	2.08 ± 0.00^{d}	3.36 ± 0.00^{c}	n.d.	17.29 ± 0.06^{a}	10.97 ± 0.03^{b}
18:3n-6	3.03 ± 0.00^{b}	7.12 ± 0.21^{a}	n.d.	2.29 ± 0.01^{b}	1.12 ± 0.01^{c}
20:0	0.32 ± 0.01^{b}	0.09 ± 0.01^{c}	0.14 ± 0.01^{c}	0.14 ± 0.01^{c}	0.95 ± 0.01^{a}
20:1	0.22 ± 0.01^{b}	2.2 ± 0.01^{a}	0.09 ± 0.01^{b}	0.18 ± 0.02^{b}	0.42 ± 0.01^{b}
20:2	0.10 ± 0.01^{b}	0.23 ± 0.00^{a}	n.d.	0.10 ± 0.01^{b}	0.09 ± 0.00^{b}
20:1n-9	1.12 ± 0.03^{c}	2.53 ± 0.01^{b}	n.d.	0.98 ± 0.04^{c}	3.43 ± 0.01^{a}
20:3n-3	0.30 ± 0.00^{c}	0.99 ± 0.01^{a}	0.24 ± 0.00^{b}	n.d	0.03 ± 0.01^{c}
20:3n-6	0.06 ± 0.00^{b}	0.69 ± 0.00^{a}	n.d.	n.d.	0.7 ± 0.01^{a}
20:4n-6	0.04 ± 0.01^{b}	0.28 ± 0.01^{a}	n.d.	n.d.	0.10 ± 0.00^{b}
20:5n-3	0.24 ± 0.01^{b}	0.74 ± 0.00^{a}	n.d.	n.d.	0.05 ± 0.01^{b}
22:0	0.05 ± 0.01^{b}	0.06 ± 0.01^{b}	0.06 ± 0.01^{b}	0.08 ± 0.00^{a}	0.30 ± 0.01^{c}
22:2	0.27 ± 0.00^{b}	0.68 ± 0.02^{a}	n.d.	n.d.	n.d.
22:5n-6	0.52 ± 0.01^{b}	0.21 ± 0.01^{b}	n.d	0.09 ± 0.01^{a}	0.08 ± 0.01^{a}
22:6n-3	0.88 ± 0.01^{b}	0.98 ± 0.01^{b}	0.09 ± 0.02^{c}	0.10 ± 0.08^{c}	7.04 ± 0.00^{a}
23:0	n.d.	n.d.	n.d.	n.d.	0.11 ± 0.01
24:0	0.04 ± 0.00^{c}	0.22 ± 0.01^{b}	0.16 ± 0.01^{b}	0.24 ± 0.00^{b}	0.46 ± 0.00^{a}
24:1	0.04 ± 0.00^{b}	0.17 ± 0.01^{a}	n.d.	n.d.	n.d.
Σ saturated	46.4 ± 0.05^{b}	64.5 ± 0.05^{a}	34.8 ± 0.18^{c}	32.71 ± 0.07^{c}	34.8 ± 0.04^{c}
Σ monounsaturated	37.2 ± 0.00^{a}	3.1 ± 0.02^{c}	29.0 ± 0.16^{b}	1.80 ± 0.04^{c}	1.7 ± 0.04^{c}
Σ n-3	3.5 ± 0.01^{c}	6.1 ± 0.00^{b}	0.3 ± 0.02^{d}	17.4 ± 0.02^{a}	18.1 ± 0.03^{a}
Σ n-6	6.9 ± 0.02^{d}	14.2 ± 0.05^{c}	7.5 ± 0.01^{d}	22.1 ± 0.04^{b}	26.0 ± 0.06^{a}
Σ n-9	5.7 ± 0.00^{e}	10.5 ± 0.00^{d}	28.2 ± 013^{a}	25.8 ± 0.02^{b}	19.1 ± 0.02^{c}
Σn-3 HUFA	1.40 ± 0.01^{c}	2.70 ± 0.00^{b}	0.30 ± 0.02^{d}	0.10 ± 0.08^{d}	7.1 ± 0.00^{a}
EPA/ARA	6.0 ± 1.18^{a}	2.6 ± 0.13^{b}	n.d.	n.d.	0.50 ± 0.07^{c}
DHA/EPA	3.7 ± 3.15^{b}	1.3 ± 0.01^{c}	n.d.	n.d.	14.8 ± 0.14^{a}
DHA/ARA	22.0 ± 1.46^{b}	3.5 ± 6.27^{c}	n.d.	n.d.	70.4 ± 0.00^{a}
n-3/n-6	0.51 ± 0.04^{b}	0.43 ± 1.41^{b}	0.04 ± 0.02^{c}	0.79 ± 0.00^{a}	0.70 ± 0.00^{a}

AV, *A. viridis*; *DS*, *D. salina*; *CV*, *C. vulgaris*; *CR*, *C. reinhardtii*; *MX*, mix; *n.d.*, not detected. Values expressed in mean ± standard deviation (SD) (n = 3 tanks/diet), lower case letters denote significant variable differences between means using Duncan's multiple range test ($P < 0.05$)

A. franciscana fed CR diet presented significantly ($P < 0.05$) higher oleic acid (OA; 18:1n-9) content than other *A. franciscana* groups. Linoleic acid (LA; 18:2n-6) was found also higher in *A. franciscana* fed CR diet. α-linoleic acid (ALA; 18:3n-3) level was increased by utilization of MX diet ($P < 0.05$). Arachidonic acid (ARA; 20:4n-6) level was found significantly higher in both DS and AV groups ($P < 0.05$). Additionally, *Artemia* fed AV diet showed a significantly higher level of docosahexaenoic acid (DHA; 22:6n-3) ($P < 0.05$). Eicosapentaenoic acid (EPA; 20:5n-3) level was enhanced by utilization of DS diet. Total n-3

Table 3 Proximate composition of *Artemia franciscana* metanauplii at the end of the experiment (% dw)

Proximate composition	Initial	AV	DS	CV	CR	MX
Crude protein	45.8 ± 0.04	54.4 ± 0.41^{b}	53.3 ± 0.74^{b}	55.3 ± 0.42^{b}	57.51 ± 0.55^{a}	58.45 ± 0.98^{a}
Crude lipid	4.4 ± 0.08	14.60 ± 0.13^{e}	10.3 ± 0.18^{c}	8.9 ± 0.12^{d}	12.9 ± 0.48^{b}	18.71 ± 0.05^{a}
Crude ash	18.15 ± 0.01	21.67 ± 0.03^{b}	21.21 ± 0.02^{c}	21.23 ± 0.01^{c}	21.33 ± 0.03^{c}	23.94 ± 0.05^{a}

AV, *A. viridis*; *DS*, *D. salina*; *CV*, *C. vulgaris*; *CR*, *C. reinhardtii*; *MX*, mix. Values expressed in mean ± standard deviation (SD) (n = 3 tanks/diet), lower case letters denote significant variable differences between means using Duncan's multiple range test ($P < 0.05$)

content was higher in the MX group, whereas total n-3 HUFA levels were found significantly ($P < 0.05$) higher in *A. franciscana* fed DS and AV diet (Table 4).

HPC, total *Vibrio* count, and predominant bacterial species

Bacterial load of the rearing water of *A. franciscana* changed significantly during the experiment (Fig. 3). HPC and *Vibrio* sp. counts decreased gradually in all rearing water of *A. franciscana* during the experiment regardless of the algal species used. However, at the end of the experiment, we found that rearing water containing *D. salina*, *C. reinhardtii*, and *A. viridis* and MX groups had the highest number of HPC (Fig. 3A). The rearing water containing MX diet showed the highest *Vibrio* sp. counts at the beginning of the experiment, whereas *C. vulgaris* had the lowest number of both counts among the algal species on day 2 and at the end of the experiment (Fig. 3B). A total of 40 bacterial isolates were obtained from the rearing water samples. According to both conventional identification and 16S rRNA gene sequencing results, four different bacterial species were found predominantly in the rearing water of *A. franciscana* including *Carnobacterium maltaromaticum* (IUET-ME1), *Pseudomonas stutzeri* (IUET-ME2), *Pseudoalteromonas* sp. (IUET-ME3), and *Vibrio* sp. (IUET-ME4) (accession numbers: MF503245-48) (Table 5; Fig. 4).

Discussion

Microalgae is the first step of the marine and freshwater food chain/web (Spolaore et al. 2006; Hemaiswarya et al. 2011). Several microalgae diets have been evaluated for rotifer (Rehberg-Haas et al. 2015), *Artemia* sp. (Pacheco-Vega et al. 2015), *Daphnia* sp. (Taipale et al. 2011), and copepods (Rasdi et al. 2016) in order to improve growth performance and nutritional value of cultured species. In this study, two marine and two freshwater microalgae alone or in combination were used for long-term feeding of *A. franciscana.* Nutritional composition of microalgae can directly reflect to live prey. Vismara et al. (2003) showed *Artemia* sp. fed *Euglena gracilis* contained high α-tocopherol levels which is a very important antioxidant source for larval fish (Betancor et al. 2011). In another study, Sánchez-Saavedra and Paniagua-Chávez (2017) used refrigerated cyanobacteria *Synechococcus elongatus* and compared it to fresh microalgae *Chaetoceros muelleri* for *A. franciscana* culture and refrigerated *S. elongatus* performed similarly as fresh microalgae maintaining growth and proximate composition. This result shows that frozen

Table 4 The fatty acid compositions of *Artemia* after 15 days of culture (% total fatty acids)

Fatty acid (%)	Initial	AV	DS	CV	CR	MX
10:0	0.09 ± 0.01	n.d.	n.d.	0.11 ± 0.01^{a}	n.d.	0.04 ± 0.00^{c}
12:0	1.37 ± 0.03	0.21 ± 0.01^{b}	0.22 ± 0.00^{b}	0.60 ± 0.01^{a}	0.10 ± 0.00^{c}	n.d.
14:0	1.95 ± 0.02	4.48 ± 0.04^{b}	5.38 ± 0.01^{a}	3.02 ± 0.06^{c}	2.49 ± 0.07^{d}	3.22 ± 0.01^{c}
15:0	0.37 ± 0.02	1.89 ± 0.02^{a}	0.62 ± 0.00^{e}	0.90 ± 0.01^{d}	1.21 ± 0.04^{b}	1.14 ± 0.03^{c}
16:0	10.99 ± 0.04	22.74 ± 0.12^{a}	20.16 ± 0.00^{b}	19.08 ± 0.02^{c}	16.10 ± 0.06^{e}	17.30 ± 0.01^{d}
16:1	16.68 ± 0.06	6.26 ± 0.04^{b}	2.37 ± 0.02^{c}	13.61 ± 0.06^{a}	2.07 ± 0.02^{d}	1.87 ± 0.01^{e}
17:0	0.23 ± 0.01	0.62 ± 0.03^{a}	0.28 ± 0.01^{c}	0.58 ± 0.00^{a}	0.29 ± 0.01^{c}	0.33 ± 0.01^{b}
18:0	5.04 ± 0.07	4.17 ± 0.01^{b}	2.49 ± 0.01^{d}	5.58 ± 0.02^{a}	2.92 ± 0.02^{c}	2.73 ± 0.01^{c}
18:1n-9	33.03 ± 0.13	25.34 ± 0.00^{b}	24.26 ± 0.10^{c}	23.76 ± 0.08^{d}	26.41 ± 0.01^{a}	24.82 ± 0.01^{c}
18:2n-6	13.45 ± 0.08	14.20 ± 0.07^{d}	16.67 ± 0.05^{c}	12.76 ± 0.10^{e}	20.56 ± 0.16^{a}	17.76 ± 0.01^{b}
18:3n-3	0.51 ± 0.02	0.26 ± 0.07^{e}	1.21 ± 0.01^{d}	4.20 ± 0.01^{c}	8.49 ± 0.03^{b}	12.40 ± 0.03^{a}
18:3n-6	1.10 ± 0.01	1.64 ± 0.01^{d}	1.13 ± 0.03^{e}	5.20 ± 0.01^{a}	3.50 ± 0.01^{b}	2.50 ± 0.01^{c}
20:0	0.14 ± 0.01	0.20 ± 0.00^{a}	0.13 ± 0.00^{c}	0.20 ± 0.00^{a}	0.12 ± 0.01^{c}	0.17 ± 0.02^{b}
20:1	2.66 ± 0.03	0.54 ± 0.06^{d}	0.63 ± 0.01^{d}	2.25 ± 0.02^{a}	1.11 ± 0.02^{c}	1.43 ± 0.01^{b}
20:2	0.53 ± 0.01	0.30 ± 0.01^{d}	1.35 ± 0.07^{c}	n.d.	1.93 ± 0.01^{a}	1.72 ± 0.06^{b}
20:1n-9	2.50 ± 0.01	n.d.	3.20 ± 0.01	n.d.	n.d.	n.d.
20:3n-3	3.50 ± 0.01	2.15 ± 0.04^{a}	0.88 ± 0.00^{c}	0.10 ± 0.00^{d}	1.13 ± 0.01^{b}	n.d.
20:3n-6	0.31 ± 0.02	0.33 ± 0.00^{c}	1.51 ± 0.00^{a}	n.d.	0.89 ± 0.01^{b}	0.91 ± 0.01^{b}
20:4n-6	0.70 ± 0.04	1.78 ± 0.30^{a}	1.75 ± 0.01^{a}	1.10 ± 0.01^{c}	1.01 ± 0.01^{c}	1.34 ± 0.04^{b}
20:5n-3	0.32 ± 0.00	2.02 ± 0.01^{b}	2.54 ± 0.01^{a}	0.21 ± 0.05^{d}	0.15 ± 0.01^{e}	0.28 ± 0.01^{c}
21:0	2.10 ± 0.01	n.d.	n.d.	1.10 ± 0.02	n.d	n.d.
22:0	0.13 ± 0.01	0.23 ± 0.00^{b}	1.15 ± 0.01^{a}	0.20 ± 0.01^{b}	0.12 ± 0.01^{c}	0.11 ± 0.00^{c}
22:2	0.33 ± 0.01	0.52 ± 0.05^{d}	0.94 ± 0.01^{c}	0.17 ± 0.01^{e}	1.51 ± 0.01^{b}	1.75 ± 0.01^{a}
22:5n-3	n.d.	n.d.	2.90 ± 0.01	n.d.	n.d.	n.d.
22:1n-9	0.92 ± 0.01	0.23 ± 0.01^{d}	1.28 ± 0.00 ^{a}	0.70 ± 0.01^{b}	0.32 ± 0.04^{c}	n.d.
22:5n-6	n.d.	1.13 ± 0.08^{a}	1 ± 0.00^{b}	n.d.	n.d.	n.d.
22:6n-3	1.44 ± 0.01	7.78 ± 0.27^{a}	5.74 ± 0.19^{d}	4.23 ± 0.21^{e}	7.21 ± 0.01^{b}	6.22 ± 0.03^{c}
24:0	n.d.	0.28 ± 0.01^{a}	0.18 ± 0.01^{d}	0.24 ± 0.00^{b}	0.21 ± 0.01^{c}	0.14 ± 0.00^{e}
24:1	0.59 ± 0.04	n.d.	n.d.	0.67 ± 0.11^{a}	0.42 ± 0.35^{b}	0.31 ± 0.03^{c}
Σ saturated	22.41 ± 0.00	34.82 ± 0.17^{a}	30.61 ± 0.02^{b}	31.61 ± 0.04^{b}	23.56 ± 0.16^{c}	25.18 ± 0.01^{c}
Σ monounsaturated	53.72 ± 0.01	31.83 ± 0.03^{b}	31.11 ± 0.01^{b}	38.74 ± 0.04^{a}	29.22 ± 0.40^{c}	27.01 ± 0.05^{c}
Σ n-3	5.77 ± 0.02	12.21 ± 0.17^{c}	13.27 ± 0.21^{c}	8.74 ± 0.05^{d}	16.98 ± 0.01^{b}	18.90 ± 0.04^{a}
Σ n-6	15.56 ± 0.10	19.08 ± 0.16^{c}	22.06 ± 0.03^{b}	19.06 ± 0.10^{c}	25.96 ± 0.18^{a}	22.51 ± 0.02^{b}
Σ n-9	36.45 ± 0.15	25.57 ± 0.01^{c}	28.74 ± 0.10^{a}	24.46 ± 0.08^{d}	26.73 ± 0.02^{b}	24.82 ± 0.01^{d}
Σ n-3 HUFA	5.26 ± 0.00	11.95 ± 0.24^{a}	12.06 ± 0.21^{a}	4.54 ± 0.05^{d}	8.49 ± 0.02^{b}	6.50 ± 0.01^{c}
EPA/ARA	0.46 ± 0.11	1.13 ± 1.10^{b}	1.45 ± 0.00^{a}	0.19 ± 0.05^{c}	0.15 ± 0.01^{d}	0.21 ± 0.05^{c}
DHA/EPA	4.50 ± 0.09	3.85 ± 0.12^{c}	2.26 ± 0.03^{d}	20.14 ± 0.05^{b}	48.07 ± 0.10^{a}	22.21 ± 0.04^{b}
DHA/ARA	2.06 ± 0.02	4.37 ± 2.09^{b}	3.28 ± 0.06^{c}	3.85 ± 0.05^{c}	7.24 ± 0.10^{a}	4.64 ± 0.01^{b}
n-3/n-6	0.37 ± 0.00	0.64 ± 0.01^{b}	0.60 ± 0.01^{b}	0.46 ± 0.02^{c}	0.65 ± 0.00^{b}	0.84 ± 0.00^{a}

AV, *A. viridis*; *DS*, *D. salina*; *CV*, *C. vulgaris*; *CR*, *C. reinhardtii*; *MX*, mix; *n.d.*, not detected. Values expressed in mean ± standard deviation (SD) (n = 3 tanks/diet), lower case letters denote significant variable differences between means using Duncan's multiple range test ($P < 0.05$)

microalgae can also be efficiently utilized for *Artemia* sp. feeding and enhancing growth performance in advanced.

Different microalgae such as *Isochrysis* sp. and *Chaetoceros muelleri* have been tested for *A. franciscana*, and *Chaetoceros muelleri* has been selected as a better microalgae diet due to the high EFA conversion rate and EPA content (Lora-Vilchis et al. 2004). More recently, endemic microalgae species *Grammatophora* sp., *Rhandonema* sp., *Navicula* sp., and *Schizochytrium* sp. have been selected as potential food for *A. franciscana* and performed better than commonly used microalgae *Chaetoceros muelleri* (Pacheco-Vega et al. 2015). This result was also similar to other combination of different microalgae for *Artemia* sp. grow-out experiments (El-Kassas et al. 2016).

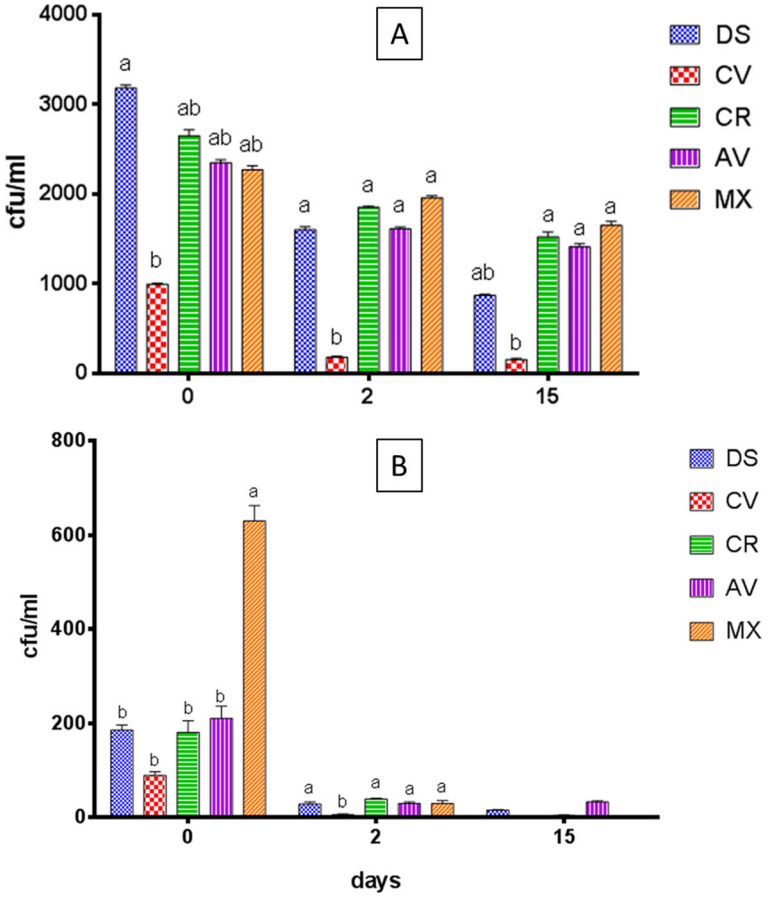

Fig. 3 **A** Total aerobic mesophilic bacteria of *A. franciscana* culture water and **B** total *Vibrio* sp. count of *A. franciscana* culture water (CV, *C. vulgaris*; CR, *C. reinhardtii*; DS, *D. salina*; AV, *A. viridis*; MX: mix. Lower case letters (a, b) denote significant variable differences between groups using Duncan's multiple range test, $P < 0.05$)

Concentration, ingestion, and assimilation rates of microalgae in crustacean feeding have vital importance (Evjemo and Olsen 1999). *Artemia* sp. growth is related to the size of the ingested amount of feeds and these diets should be at a certain size (Chaoruangrit et al. 2018). Suitable size of microalgae is reported between 7 and 28 μm, and optimum size is described as 16 μm for *Artemia* sp. feeding. *A. viridis* is a diatom with a diameter of 14.7 μm, and its beneficial effects have been reported in the *Haliotis asinina* metamorphosis (Jing Ding et al. 2017). In another trial, fairy shrimp (*B. thailandensis*) fed *Chlorella vulgaris* was recorded as 19.13 mm in total length for 15 days of culture period and lower concentration of microalgae lead to reduce growth (Chaoruangrit et al. 2018). Moreover, not only size but also nutritional properties of microalgae play an important role in growth (Fabregas et al. 1996). In our study, the highest total length of *A. franciscana* was achieved by utilization of *C. reinhardtii* (CR) which resulted in a 25.20-mm total length of *A. franciscana.* This result could be related to the CR diet containing high protein level (see Table 1). Additionally, these psychrophilic microalgae can contain a higher amount of polyunsaturated fatty acids (Giroud et al. 1988;

Table 5 Morphological and phenotypic characteristics of the strains isolates

Characteristics	Isolate			
	IUET-ME1	IUET-ME2	IUET-ME3	IUET-ME4
Cell morphology	R	R	R	R
Gram reaction	+	−	−	−
Motility	−	−	+	+
Oxidase	−	+	+	+
Catalase	−	+	+	+
O/F (glucose)	F	F	O	F
O/129	R	R	S	S
Indol formation	−	−	+	+
Voges-Proskauer	+	−	−	+
Methyl red	+	−	+	+
Nitrate reduction	+	−	+	+
ONPG	+		−	+
Citrate utilization	−	+	−	+
Starch hydrolysis	+	+	+	−
H_2S production	−	−	−	−
Esculine	+	+	+	+
Arginine dihydrolase	+	−	−	+
Lysine decarboxylase	−	+	+	−
Ornithine decarboxylase	−	+	+	−
Lactose	+	−	+	+
Glucose	+	+	+	+
Sucrose	+	−	+	+
Sorbitol	−	−	−	−
Arabinose	−	−	−	−
Xylose	−	−	−	−
TCBS	+	+	−	+
MacConkey	−	−	−	+
Accession number	MF503248	MF503247	MF503246	MF503245

R, rods; +, positive; −, negative; *S*, sensitive; *R*, resistant *F*, fermentative; O, oxidative

Siaut et al. 2011; Kim et al. 2016). Nutritional quality of *Artemia* sp. is also related to the application time of the feeds or enrichers. For instance, short-time enrichment with *Chlorella marina* supported the growth and nutritional value of *Artemia* sp. However, it has been reported that combination of different *Chlorella* species could be better for long-time enrichment (El-Kassas et al. 2016).

Protein level of *A. franciscana* (five days old) fed *Tetraselmis suecica* was recorded as 63.6% and *A. franciscana* fed *Rhodomonas lens* was 67.7% of the dry weight of 5-day-old *A. franciscana* (Seixas et al. 2009). Our protein values were lower than those values; one of the reasons could be the age of the *A. franciscana* culture. Lipid levels of *A. franciscana* fed *Tetraselmis suecica* and *Rhodomonas lens* were recorded as 15.2% and 12.3%, respectively (Seixas et al. 2009). However, in comparison to the lipid levels in our study, *A. franciscana* fed MX diet showed the highest lipid value (18.71%) than that obtained by Seixas et al. (2009). In the same study, protein and lipid levels were found similar to that obtained by *Artemia* fed *Chaetoceros muelleri* comparing to *A. franciscana* fed 8 weeks of refrigerated cyanobacteria *Synechococcus elongatus* (Sánchez-Saavedra and Paniagua-Chávez 2017). In our study, protein levels of *A. franciscana* fed experimental diets ranged between 53.3 and 58.45% and these values were found higher than those of *Artemia* sp. fed refrigerated *Synechococcus elongatus* and *Chaetoceros muelleri* by Sánchez-Saavedra and Paniagua-Chávez (2017).

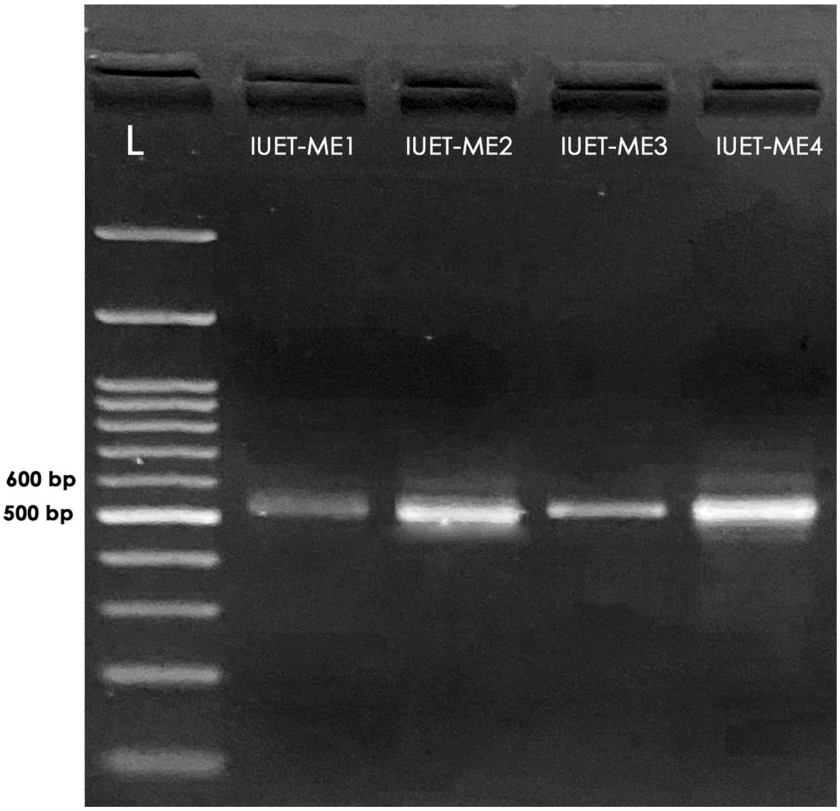

Fig. 4 Agarose gel electrophoresis image of the PCR products. Lane 1: (L) GoldBio 100 bp PLUS™ DNA Ladder. Lanes 2–6: Partially amplified 16S rRNA gene of the isolates found predominantly in the rearing water

Enhancement of protein value can be attributed to the combined utilization of microalgae. *Chlorella* species and *C. reinhardtii* have a thick cell wall, and this feature results in low digestibility of these microalgae (Dhont and Lavens 1996). On the other hand, these two species contain highly active biocompounds such as lutein, α-carotene, ß-carotene, ascorbic acid, and α-tocopherol which are valuable biocompounds for lipid preservation and utilization (Hashimoto et al. 1982; Shibata et al. 2001, 2003). *Artemia* is strongly recommended to be enriched with astaxanthine and lutein (Hemaiswarya et al. 2011). In this study, one of the highest survivals was found in the *Artemia* group fed *C. vulgaris*. This could be related to the antioxidant effect of these microalgae (see Fig. 2). Additionally, recent studies showed that *C. vulgaris* extracts can prevent lipid peroxidation induced by some toxic effects (Vijayavel et al. 2007). Similar survival level was maintained by *Chlorococcum humicola* in fairy shrimp (*B. thailandensis*) which resulted in 70% rate of survival.

Marine fish larvae require sufficient amount of essential fatty acids for high growth and survival (Izquierdo and Koven 2010). These fatty acids should be delivered by rotifer, *Artemia* sp. and/or copepods to fish larvae. *Artemia* sp. is known to be able to incorporate and concentrate some dietary lipids. However, they are limited to elongate and desaturate 18C to 22C fatty acids and deposition and utilization of fatty acids. Feeding of *Nannochloropsis oculata* and *Phaedactylum tricornatum* have resulted in low levels of EPA *Artemia* sp. fed *Nannochloropsis oculata* and *Phaedactylum tricornatum* (Zhukova et al. 1998). Recent

studies showed that on-growing *Artemia* sp. prefers DHA being preferentially transferred from dietary phospholipids to triacylglycerols (Guinot et al. 2013a). Therefore, after feeding process with qualified diets, HUFA enrichment should be applied at the metanauplii stage. In our trial, experimental microalgae were grown under phototrophic conditions, which lead to lower long-chain fatty acid conversion and accumulation in their body cell, whereas *A. viridis* enhanced higher ARA, DHA, and Σn-3 HUFA in *A. franciscana* at the end of the experiment. *Amphora* sp. can contain high amount of lipid deposition under stress conditions (Chtourou et al. 2015), and AV has been reported to have the highest EFA level among other diatoms (Huang et al. 2007a; Huang et al. 2007b). This feature of AV supported the post-larval growth of *Haliotis asinina* (Jing Ding et al. 2017). Moreover, AV is also used as a feed supplement for *Penaeus monodon* post larvae production (Khatoon et al. 2009). Monospecific diets may result in nutritional deficiencies due to the inadequate nutritional content of several essential compounds. Combination of more than one microalga could meet the nutritional requirements of the live prey (Brown et al. 1989; Smith et al. 1992). Similar positive results were observed in *A. franciscana* fed MX diet in our study. Recent studies on copepods also showed combination of different microalgae could be the optimum diet for survival and growth (Milione et al. 2007).

Utilization of feeds affects distinctively bacterial community of rearing water and gut of live feeds. In order to grow out *Artemia* sp. nauplii, several commercial algae powders (Claus et al. 1979; Griffiths et al. 2016) and fresh culture of microalgae have been studied so far (Hemaiswarya et al. 2011). Especially, in algae-limited conditions, bacterial microbiota may significantly contribute as a dietary source for *Artemia* sp. production (Toi et al. 2013). According to this study, higher survival of *A. franciscana* can be enhanced by utilization of *C. vulgaris*, *A. viridis*, and combination of four microalgae. *A. franciscana* nutritional requirement may change in further development stages. It is possible that this may be one of the reasons why the combination of the four microalgae diets works best in terms of growth. This study suggests the combined utilization of marine and freshwater microalgae species could promote survival and total length of *A. franciscana*.

Since many studies have shown that there are many pathogenic species in the microbiota of live feeds, various algal species including *Skeletonema* sp., *Chaetoceros* sp., *Nannochloropsis* sp., *Isochrysis* sp., *Pavlova* sp., *Tetraselmis* sp., and *Chlorella* sp. have been investigated to reduce the bacterial load of *Artemia* sp. or to decontaminate these feeds and successful results have been reported in various degrees (Salvesen et al. 2000; Tolomei et al. 2004; Makridis et al. 2006; Interaminense et al. 2014). Among these, the closest study to our work was made by Makridis et al. (2006) by using another *Chlorella* species (*Chlorella minutissima*) and they found this species was very successful in reducing both the bacterial load and *Vibrio* sp. counts as we determined in our study with *C. vulgaris*. We think that this microalgae species caused the highest decrease in the number of bacterial counts due to having strong antimicrobial properties (Falaise et al. 2016). It was also found that the use of different algal species might play a role in altering the total number of *Vibrio* sp. members that are known to contain disease-causing strains in marine fish. The predominant species *Vibrio* sp., *Pseudomonas* sp., and *Pseudoalteromonas* sp. identified in *Artemia* sp. rearing water are compatible with some other studies (Hameed and Balasubramanian 2000; McIntosh et al. 2008), but we also identified *Carnobacterium maltaromaticum* as lactic acid bacteria (LAB). *Carnobacterium* species are commonly found in various environments including humans, healthy and diseased fish, seafood, meat, and poultry and dairy products both in fresh and vacuum-packed products and in probiotics (Laursen et al. 2005; Afzal et al. 2010).

Future studies should be focused on different forms of microalgae biomass such as spray-dried or lyophilized microalgae powder for *Artemia* sp. grow-out feeding. Additionally, the current knowledge about the microbiota of the live feeds used in aquaculture is very limited and in order to detect microorganisms introduced in the aquaculture systems by live feeds and to better understand the effects of these microorganisms on cultured species, community profiling must be done by the latest and more powerful methodologies such as next-generation sequencing in future works.

Abbreviations *AA,* Amino acid; *ARA,* Arachidonic acid; *AV, Amphora viridis*; *CR, Chlamydomonas reinhardtii*; *CV, Chlorella vulgaris*; *DS, Dunaliella salina*; *EFA,* Essential fatty acids; *EPA,* Eicosapentaenoic acid; *HPC,* Heterotrophic plate counts; *HUFA,* Highly unsaturated fatty acids; *LAB,* Lactic acid bacteria; *MX,* Combination of four microalgae; *PUFA,* Polyunsaturated fatty acids; *WSSV,* White spot syndrome virus

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Author contribution GT and KME conducted feeding experiment. REY and ET performed bacterial load analysis. KME and GT drafted the manuscript.

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Declarations

Ethics approval All applicable international, national, and/or institutional guidelines for the care and use of animals were followed by the authors.

Conflict of interest The authors declare no competing interests.

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