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Research Article

Live Microalgae-Based Diets as Enrichment to Improve the Nutritional Profile of the Calanoid Copepod *Acartia tonsa* (Dana, 1849) Nauplii

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Most emerging species in marine aquaculture and the aquarium industry have a sensitive and small-mouthed larval stage, and their culture is very challenging due to a lack of appropriate early feeding protocols. The use of copepods has been widely recognized as a good alternative to traditional fish live feeds (rotifers and *Artemia* spp.), especially for new species targeted for the multibillion-dollar marine aquarium trade. However, most copepod culture trials are focused on biomass production, while their nutritional requirements and enrichment protocols remain scarcely known. The main objective of this study was to determine the dietary contribution of three microalgae-based diets, provided as a mono R (*Rhodomonas salina*) or binary I–R (*Isochrysis galbana* + *R. salina*) and T–R (*Tetraselmis suecica* + *R. salina*), on the biochemical composition (proximate and fatty acids (FAs)) and survival of the calanoid copepod *Acartia tonsa* (nauplii) versus the conventional rotifer *Brachionus plicatilis* throughout 96 hr exposure. Both live preys were characterized by the highest protein and total n-3 long-chain polyunsaturated FAs (LC-PUFAs) content when fed with the binary diet I–R compared to other treatments, although the nutritional value of *A. tonsa* nauplii was always much higher compared to *B. plicatilis*. In general, there was a wide difference in proximate and FAs composition between the two preys, denoting *A. tonsa* as better live feed in terms of capacity to retain n-3 LC-PUFAs, especially eicosapentanoic acid (20:5n-3) and docosahexanoic acid (22:6n-3). The present study highlights the nutritional profile of enriched *A. tonsa* nauplii and contributes to the production of high-quality live feeds, which may be able to reach the nutritional needs of new marine fish species during their larval development, as well as contributing to the improvement of *A. tonsa* culture.

1. Introduction

1.1. Marine Fish Larvae Production and Conventional Live Feeds. In the last decades, the production of high-quality marine fish species and its diversification were constrained by a bottleneck of nonoptimized nutritional food sources for larval stages, inducing low growth and survival [1, 2]. The

success in the larval breeding of new marine species is determined mainly during the early life stages of development, during the transition from endogenous to exogenous nutrition [3, 4]. At this stage, the live feed should be adapted to the morphological characteristics of the larvae and their needs in terms of nutritional requirements. In contrast to their natural environment, where larvae have access to a large variety

of feeding regimes, the main live preys employed in hatcheries consists of rotifers (*Brachionus* spp.) and brine shrimps (*Artemia* spp.). Rotifers have small sizes, suitable as first feeding for the earliest stages of several fish and shrimp larvae species [5]. Meanwhile, *Artemia* shows larger size variability compared to rotifers and can be hatched from dormant cysts, which can be easily distributed and stored for prolonged periods of time [5]. However, both preys have a limited ability to synthesize important long-chain polyunsaturated fatty acids (LC-PUFAs) from shorter FAs [6–8]. The rate of synthesis of these FAs is rather low, especially for docosahexaenoic acid (DHA: 22:6 n-3), and to supply large amounts of PUFAs to marine fish larvae, live preys must be enriched with PUFA-rich food [7]. Several techniques have been developed to improve the biochemical composition of rotifers and *Artemia* (e.g., oil emulsions, phytoplankton, etc.) or even to seek new types of live preys whose composition may fulfill the nutritional requirements of certain fish species [9].

1.2. Copepods as Live Feed. The use of copepods has been widely recognized as a good alternative to traditional live feeds [10–12]. Copepods can improve the survival and growth for a variety of fish larvae due to their small naupliar stages, swimming behavior, and FAs profile, which includes a high content of LC-PUFAs [9, 12]. Therefore, there is a considerable interest in studying the mechanisms responsible for its production [13, 14]. New techniques allow producing large quantities of calanoid copepods using cold-stored subitaneous eggs [14, 15–20]. However, cold-stored copepod embryos still retain a physiological activity during this period, and their nutritional quality may change over this time [14–17]. In particular, the major biochemical differences that occur during the storage period are related to the embryo FAs content [17]. Previous studies demonstrated that after 6 months of cold storage, the quality of copepods obtained from those eggs was suboptimal for the Saddleback clownfish (*Amphiprion polymnus*) larval rearing [21]. Therefore, unless proper enrichment can be assured, these changes in FAs may affect larval survival and development. Most calanoid copepods, which constitute the natural food source for many marine fish larvae [22], are unable to elongate and desaturate the 18:3n-3 FA to produce significant amounts of the LC-PUFAs, eicosapentanoic acid (EPA: 20:5n-3) and DHA FAs [23]. Their high content in n-3 LC-PUFAs is incorporated directly through the phytoplankton, exhibiting reduced growth and egg production when, in opposition, fed diets deficient in EPA and DHA [24, 25]. Moreover, food quantity and quality affect copepod ingestion, survival, growth, and fecundity [9, 14, 26–28]. The use of a mixed diet has been proven to sustain large copepod cultures for many generations [29]. Otherwise, monospecific diets may cause nutritional deficiencies because of the inadequate content of one or more essential nutrients [30].

1.3. *Acartia tonsa* Enrichment Study. Since most copepod culture trials are focussed on the quantity of mass production, the nutritional requirement and enrichment potential of copepod remain scarcely known [9, 14]. The nutritional

TABLE 1: Concentration of microalgae cultures expressed as mean value with standard deviation ($n \times 10^6$ cells/mL \pm SD) during the 4-day experiment.

Microalgae	Day 1	Day 2	Day 3	Day 4
<i>Tetraselmis suecica</i>	2.1 \pm 0.1	2.5 \pm 0.0	2.8 \pm 0.1	3.1 \pm 0.1
<i>Isochrysis galbana</i>	5.2 \pm 0.1	5.9 \pm 0.1	6.4 \pm 0.2	6.8 \pm 0.2
<i>Rhodomonas salina</i>	2.4 \pm 0.1	2.6 \pm 0.1	2.9 \pm 0.0	3.2 \pm 0.0

value of copepods is less predictable due to the variety of algae species available as feed [9, 31]. Little information to date is available on adequate protocols to enrich copepods with nutritional and trace elements required for marine fish larvae [32, 33]. The copepod *Acartia tonsa* (Dana, 1848) is a ubiquitous cosmopolitan estuarine species that is often used in biology and ecotoxicology studies, among others [34], but also as live prey for aquaculture [14, 22, 35]. Indeed, many studies have already highlighted the relevance of *A. tonsa* as live feed, favoring fish larval survival and growth in comparison to rotifers and *Artemia* nauplii [13, 31]. In this study, we examined the dietary contribution of three microalgae species (*Rhodomonas salina*, *Isochrysis galbana*, and *Tetraselmis suecica*), provided as a mono- or binary-diets, on the biochemical composition (proximate and FAs) and survival of *A. tonsa* nauplii versus the conventional live feed *Brachionus plicatilis*. Specifically, we focus on the relationship between the changes of FAs compositions depending on food source, in order to produce high-quality live feeds [2], able to reach the nutritional needs of new marine fish larvae species. Therefore, the identification of an adequate microalgal enrichment for *A. tonsa* may enhance the production of high-quality marine fish as also contribute to the culture of this copepod species.

2. Materials and Methods

The experiment was accomplished in accordance with the EU Directive 2010/63/EU for animal experiments or the National Research Council's Guide for the Care and Use of Laboratory Animals.

2.1. Facilities. The entire experiment was held in the quarantine areas of the Aquarium of Genoa (Genoa, Liguria, Italy), using the facilities described below.

2.1.1. Phytoplankton Cultures. The marine microalgae *T. suecica* (Kylin) Butcher 1959, *I. galbana* (Parke 1949), and *R. salina* (strain number MCCV118) (Wislouch) D.R.A. Hill & R. Wetherbee 1989 were used in the present study as a food source for copepods and rotifers. They were grown in 80-L polycarbonate cylinders, inoculated into 1 μ m filtered seawater (salinity 30%), UV sterilized, and mixed with filtered air. All algae species were grown on a F/2 medium [36]. Microalgae cultures were maintained at 20 \pm 1°C with rear illumination based on 10,000 K fluorescent tubes with a 24-hr light regime. The algae were harvested during the log phase. The concentration of algal cultures (cells/mL) (Table 1) was determined daily, using a Neubauer chamber (Marienfeld

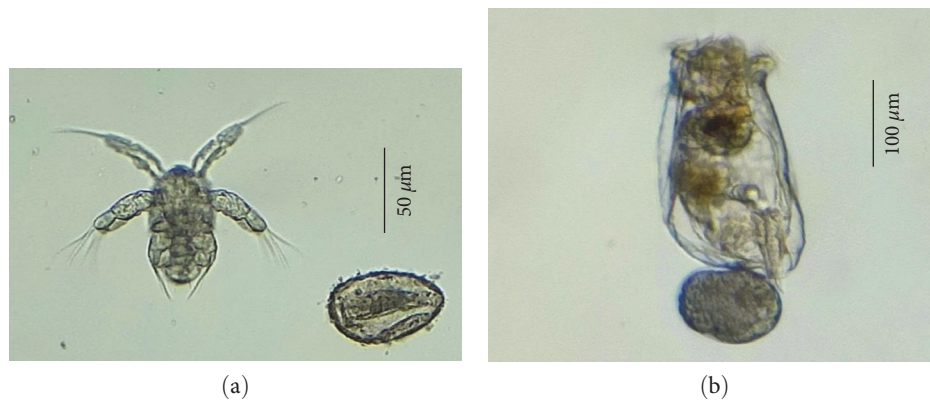


FIGURE 1: Naupliar stage of *Acartia tonsa* 24 hr post-hatch (a), and female of the rotifer *Brachionus plicatilis* (b).

Superior, Germany) under a microscope (DME, LEICA, Germany).

2.1.2. Zooplankton Cultures. Rotifers (*B. plicatilis*), with a size range of 180–250 μm (L size), were cultured on S.parkle[®] (INVE Aquaculture, Belgium) at 30% \pm 1% salinity and 20°C in two transparent cylindrical tanks of 80 L each. Cultures were renewed weekly with a 100% water change, keeping a mean culture density of 200 individuals/mL moved by a gentle airflow. The culture was 12 L/12 D (light/dark) illuminated by two 30 W fluorescent tubes. Density estimations of rotifer, with a mean size of 220 μm (Figure 1(b)), were done daily by averaging three 1 mL⁻¹ samples, and each of the samples was checked under a stereomicroscope (EZ4, LEICA, Germany). Nauplii of the calanoid copepod (*A. tonsa*) with a mean size of 70 μm (Figure 1(a)) were obtained by cold stored eggs distributed by Algova[®] (Germany).

2.1.3. Modular Rearing System (MoRS). The experiment was conducted inside the life support system named MoRS. This was connected to six experimental cylindrical tanks, with a volume of 20 L, and equipped with air line. MoRS filtration system includes a 200 L sump supported by a biological tower, 5 μm mechanical filtration, protein skimmer (Aqua Medic, Germany), and UV sterilization (Panaque, Italy). Water temperature inside MoRS tanks was on average 20 \pm 1°C (mean \pm standard deviation (SD)) with minimum variation and controlled by a heating device coupled with an automatic thermostat (Panaque, Italy). The whole system was illuminated 12 L/12 D by two 40 W fluorescent tubes, oxygen level ranged from 6 to 7 mg/L, and salinity was established at 30% \pm 1%.

2.2. Experimental Design. Cold-stored eggs of *A. tonsa* preserved at 4°C distributed by Algova[®] (Germany) were hatched following the instructions provided by the producer, while the rotifers *B. plicatilis* were collected from the culture tanks (see Section 2.1.2). Copepods eggs were washed from the buffer solution with 50 μm mesh sieve, inoculated in marine water, and incubated during 24 hr at 24 \pm 1°C in a glass flask at the density of 40,000 eggs/L with air bubbling. The newly hatched nauplii of *A. tonsa* at N I stage and the rotifer

B. plicatilis were immediately concentrated using a 50 μm mesh sieve and counted under a stereomicroscope in triplicate (EZ4, Leica, Germany). The experiment was launched after splitting live preys into three experimental feeding treatments ($n = 5$) as follows: Treatment R (control): mono algal diet based on *R. salina*; Treatment T–R: binary diet (1:1 ratio) based on *T. suecica* and *R. salina*; Treatment I–R: binary diet (1:1 ratio) based on *I. galbana* and *R. salina* (Figure 2). The three species of algae used in this study were chosen because of their significant differences in LC-PUFAs composition, which allowed a comparison of the effects of the diets on copepods and rotifers FAs composition. *R. salina* was selected as the control diet due to its good nutritional profile and suitable cell size for *A. tonsa* and *B. plicatilis* culture [16, 37–39]. While *T. suecica* and *I. galbana* were selected to generate the binary diets, combined with the control, since they are among the most frequently used species in the commercial mariculture industry [40]. All microalgae treatments were formulated with a ratio of 1:1 based on cell count, chosen to facilitate the application of the enrichment and the reintegration of the consumed algae. Each treatment was split into five replicates, incorporated in five tanks of 20 L ($n = 5$), configured without water exchange with the MoRS facility to standardize the culture conditions. Starting culture density was established in 10 ind./mL for *A. tonsa* and 280 ind./mL for *B. plicatilis* by counting individuals as described for zooplankton cultures (see Section 2.1.2). Respectively, there were an estimated 200,000 specimens in each copepod tank and 5,600,000 specimens in each rotifer tank, ensuring a sufficient final sample size for the subsequent biochemical analysis, avoiding overpopulation. During 96 hr, rotifers and copepods were fed with the designated diets after 10% daily water exchange supplied by MoRS connected to the culture tanks. Tanks were kept at 20 \pm 1°C, 30% salinity, and 14 L/10 D photoperiod [34], checking water quality parameters daily. Microalgae concentration was checked daily and kept at 1.5 $\times 10^5$ cells/mL (1:1) in each tank following an identical protocol applied for phytoplankton cultures (see Section 2.1.1), ensuring enough feed for live preys and avoiding starvation. High microalgae concentration was chosen as it has been described to support

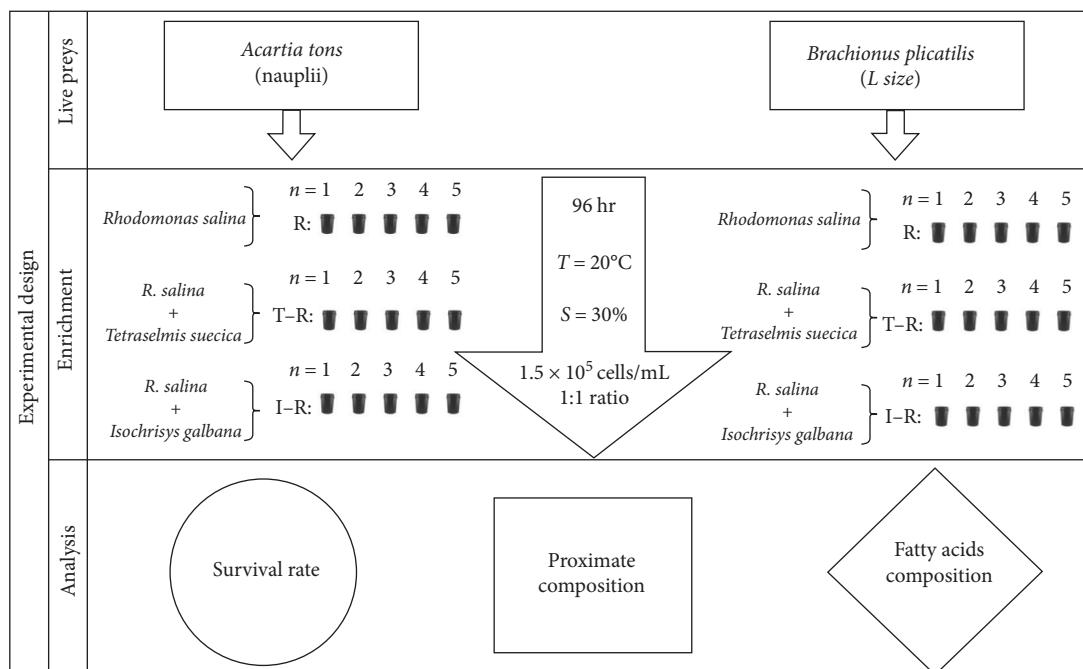


FIGURE 2: Experimental design flow chart: the copepod *A. tonsa* and the rotifer *B. plicatilis* were divided into three experimental feeding treatments ($n = 5$) and fed with the designated diets during 96 hr. Effects on survival rate, proximate, and fatty acids composition were studied.

TABLE 2: Concentration of rotifers (*Brachionus plicatilis*) and the number of females, as well as nauplii of calanoid copepods (*Acartia tonsa*) measured in culture tanks during the feeding experimentation using three microalgae treatments (R, *Rhodomonas salina*; I-R, *Isochrysis galbana* + *Rhodomonas salina*; T-R, *Tetraselmis suecica* + *Rhodomonas salina*).

Live feed	Treatment	Day 1		Day 2		Day 3		Day 4	
		Individuals	Females	Individuals	Females	Individuals	Females	Individuals	Females
<i>B. plicatilis</i>	R	280 ± 0.1	13.3 ± 0.8	278.2 ± 0.8	10.2 ± 0.5	277 ± 0.7	15.6 ± 1.1	276.6 ± 0.9	12.3 ± 1.6
	I-R	280 ± 0.1	14 ± 0.2	277.8 ± 1.6	12.6 ± 0.8	277.4 ± 1.5	14.8 ± 0.8	276.6 ± 1.1	18.5 ± 0.8
	T-R	280 ± 0.1	13.8 ± 0.6	274.6 ± 4.7	8.4 ± 0.3	272.2 ± 2.7	8.1 ± 0.6	267.8 ± 1.6	10.4 ± 1.1
		Nauplii		Nauplii		Nauplii		Nauplii	
<i>A. tonsa</i>	R	10 ± 0.1	–	8.8 ± 0.1	–	8.4 ± 0.1	–	8.0 ± 0.1	–
	I-R	10 ± 0.1	–	9.6 ± 0.1	–	9.2 ± 0.1	–	8.8 ± 0.1	–
	T-R	10 ± 0.1	–	9.6 ± 0.1	–	9.0 ± 0.1	–	8.7 ± 0.1	–

All values are expressed as mean values with standard deviation (mean value/mL ± SD).

high survival rates and increase the culture performance of *A. tonsa* and *B. plicatilis* [28, 39, 41, 42]. To estimate the survival rate of each experimental treatment, a daily counting of copepods nauplii and rotifers (Figure 1) was carried out by triplicate in each tank following an identical protocol applied for zooplankton cultures (see Section 2.1.2) (Table 2).

2.3. Samples Collection. Samples of each microalgae species (20 L) employed for live prey diets were collected at the beginning of the experiment from their respective phytoplankton culture tanks. The resulting samples were centrifuged (Rotofix 32°, Hettich, Germany), split in vials per triplicate, lyophilized (Alpha 1-2 LD plus, Christ; Germany), and stored at -20°C for subsequent analysis. The feeding trials were stopped on day 4 (96 hr) when nauplii of *A. tonsa* were molting from the fourth (N IV) to the fifth (N V)

naupliar stage, preventing metamorphosis to the copepodite stage [43]. The exogenous feeding was observed starting from 24 hr post-hatch at N II stage. The duration of the experiment (96 hr) ensured to avoid any influence that could come from the endogenous feeding of the first naupliar stage (N I) to the final biochemical composition of *A. tonsa* nauplii. At the end of the feeding trials, each experimental culture tank of copepods and rotifers ($n = 5$) generated a sample (Table 2). Each tank was fully filtered through a $50\ \mu\text{m}$ mesh sieve, the content rinsed with deionized water to remove residual salts, lyophilized (Alpha 1-2 LD plus, Christ; Germany), and stored in the vial at -20°C for subsequent analysis. The collected samples were preserved at the Regional Agency for Environmental Protection-Liguria (ARPAL), Department of Organic Chemistry and Physical Analysis (Genoa, Liguria, Italy).

2.4. Samples Analysis. Samples analysis was carried out at the laboratories of the Highly Specialized Aquaculture and Biotechnology Service (SABE) located at the University Institute of Sustainable Aquaculture and Marine Ecosystems (IU ECOAQUA), belonging to the University of Las Palmas de Gran Canaria (Canary Islands, Spain). Proximate composition of microalgae diets, *A. tonsa* nauplii, and *B. plicatilis* were conducted following standard procedures [44]. Moisture was determined by thermal dehydration until constant mass at 105°C. Ash content was determined by combustion at 600°C for 12 hr. Crude protein content (N · 6.25) was determined by the Kjeldahl method, and crude lipid was extracted following the Folch method [45]. Carbohydrate was estimated by difference (percentage carbohydrate content = 100 – (% moisture + % ash + protein + % fat)). The analysis of FAs was carried out by transmethylation of the total lipids in FA methyl ester as described by Christie and Han [46], separated, and identified by gas chromatography (7820A GC System, Agilent Technologies, United States) following the conditions described by Izquierdo [47]. All gross composition data were expressed as percentages of dry weight (DW ± SD), while the FAs content was expressed as a percentage of the total lipid (DW ± SD). All analyses were conducted in triplicate.

2.5. Data Analysis. All data were analyzed with Levene's test to assess the homogeneity of variances and a Shapiro–Wilk test to assess normality, applying log-transformed percent data when assumptions were not accomplished. Differences in proximate and FAs composition were assessed by one-way analysis of variance (ANOVA). Comparison between groups was calculated by Tukey post hoc test for pairwise comparisons and Games–Howell. To study the effect of diet on the survival of the copepods and the rotifers, a two-way repeated measures ANOVA with two fixed factors, time and diet, was used. The significance level of 0.05 (*P* value) was tested using the SPSS statistical software package (IBM SPSS for Windows V27.0; SPSS Inc., Chicago, IL, USA). Unless stated otherwise, measurements are presented as mean ± SD.

3. Results

3.1. Analysis of Biochemical Composition of Microalgae

3.1.1. Proximal Composition. All data of microalgae gross composition were expressed as percentages of dry weight (DW ± SD, Table 3). *T. suecica* showed significantly higher protein (29.0% ± 0.7%) and lipid (25.1% ± 0.5%) content in comparison to the other two microalgae tested (ANOVA, *P* < 0.05, Table 3). While *R. salina* showed the highest percentages of carbohydrates (16.1% ± 0.5%) and ash (52.5% ± 0.5%) (ANOVA, *P* < 0.05, Table 3). Moisture content was significantly higher in *I. galbana* (10.6% ± 0.7%) (ANOVA, *P* < 0.05, Table 3); meanwhile, there were no differences (ANOVA, *P* ≥ 0.05) between *R. salina* (8.12% ± 0.50%) and *T. suecica* (8.63% ± 0.55%) species.

3.1.2. FA Composition. The FA composition of microalgae was expressed as a percentage of the total lipid DW ± SD (Table 3). In comparison to other algae, *I. galbana* showed

the highest levels of total n-3 LC-PUFAs, total monounsaturated (MUFAs), total n-9 FAs, oleic acid (OA; 18:1n-9), and DHA (ANOVA, *P* < 0.05, Table 3), while EPA and total saturated FAs (SAFAs) were significantly lower (ANOVA, *P* < 0.05, Table 3). *T. suecica* showed the highest percentage of myristoleic acid (14:1n-5), palmitic acid (16:0), vaccenic acid (18:1n-7), linoleic acid (LA; 18:2n-6), gamma-linoleic acid (18:3n-6), alpha-linolenic acid (ALA; 18:3n-3), stearidonic acid (SA; 18:4n-3), arachidonic (ARA; 20:4n-6), eicosatetraenoic acid (20:4n-3), total n-3 and total n-6 FAs (ANOVA, *P* < 0.05, Table 3). In the same way, high contents in myristic acid (14:0) were detected in *R. salina* (ANOVA, *P* < 0.05, Table 3). Finally, all the studied FAs ratios were significantly higher in *R. salina* (ANOVA, *P* < 0.05, Table 3), except for the DHA/EPA and DHA/ARA ratio, which levels were significantly higher in *I. galbana* (ANOVA, *P* < 0.05, Table 3).

3.2. Analysis of Biochemical Composition of Rotifers

3.2.1. Proximal Composition. The gross composition of rotifers was expressed as a percentage of DW ± SD (Table 4). The group fed with treatment R showed the highest lipid content (17.3% ± 1.1%), followed by I–R (15.4% ± 0.8%) and T–R (13.1% ± 1.0%) groups (ANOVA, *P* < 0.05, Table 4). Protein was the major constituent in all groups, but I–R treatment showed the highest content (58.7% ± 1.6%) (ANOVA, *P* < 0.05, Table 4). Carbohydrate did not show any significant difference among all treatments (ANOVA, *P* ≥ 0.05, Table 4). While the group T–R showed the higher ash and moisture content (ANOVA, *P* < 0.05, Table 4), no difference was observed between I–R and R treatments (ANOVA, *P* ≥ 0.05, Table 4).

3.2.2. FA Composition. The FA composition of the rotifer *B. plicatilis* fed with the three experimental diets was expressed as a percentage of the total lipid DW ± SD (Table 4). Rotifers fed on diet I–R showed significantly higher content in total n-3 LC-PUFAs, total n-6 FAs, OA, LA, and DHA FAs, in comparison to other groups (ANOVA, *P* < 0.05, Table 4). Group fed on diet R showed higher percentage in 14:0, 16:0, palmitoleic acid (16:1n-7), stearic acid (18:0), vaccenic acid, ALA, as also total SAFAs and MUFAs series than other groups (ANOVA, *P* < 0.05, Table 4). FAs ratios n-6/n-9, DHA/EPA, and DHA/ARA were significantly higher in rotifers fed on I–R treatment, while n-3/n-6 ratio was higher with R treatment. (ANOVA, *P* < 0.05, Table 4).

3.3. Analysis of Biochemical Composition of Copepods

3.3.1. Proximal Composition. Data of copepods gross composition were expressed as a percentage of DW ± SD in Table 5, where protein represented the major constituent in all groups. The highest value for protein was found in copepods fed with binary diets T–R (71.8% ± 0.7%) and I–R (70.7% ± 0.7%), which showed significantly higher content compared to the group fed on R diet (ANOVA, *P* < 0.05, Table 5). Copepod fed with T–R also showed significantly higher lipid content compared to other diets (18.0% ± 0.3%) (ANOVA, *P* < 0.05, Table 5); meanwhile, no differences were observed in groups fed on I–R and R treatments (ANOVA,

TABLE 3: Proximal composition (lipid, protein, carbohydrate, ash, and moisture content; g/100 g dry weight) and fatty acid composition (mg/g dry weight) of microalgae used as food for live preys, $n = 3$.

Microalgae	<i>Tetraselmis suecica</i>	<i>Isochrysis galbana</i>	<i>Rhodomonas salina</i>
Lipids	25.1 ± 0.5 ^c	18.8 ± 0.2 ^b	14.2 ± 0.6 ^a
Proteins	29.0 ± 0.7 ^c	22.6 ± 0.4 ^b	17.3 ± 0.5 ^a
Carbohydrates	9.4 ± 0.6 ^a	11.3 ± 0.7 ^b	16.1 ± 0.5 ^c
Ash	36.5 ± 0.7 ^a	47.3 ± 0.5 ^b	52.5 ± 0.5 ^c
Moisture	8.6 ± 0.6 ^a	10.6 ± 0.7 ^b	8.1 ± 0.5 ^a
14:0	0.1 ± 0.0 ^a	1.4 ± 0.1 ^b	1.8 ± 0.2 ^c
14:1n-5	0.3 ± 0.1 ^b	0.1 ± 0.0 ^a	0.1 ± 0.0 ^a
16:0	3.2 ± 0.2 ^b	0.7 ± 0.0 ^a	0.8 ± 0.2 ^a
16:1n-7	0.2 ± 0.1 ^a	0.7 ± 0.1 ^b	0.9 ± 0.2 ^b
16:3n-1	3.4 ± 0.1	–	–
18:0	0.1 ± 0.0	0.1 ± 0.0	0.6 ± 0.5
18:1n-9	1.9 ± 0.5 ^b	3.4 ± 0.2 ^c	0.5 ± 0.0 ^a
18:1n-7	0.8 ± 0.1 ^b	0.5 ± 0.1 ^a	0.3 ± 0.2 ^a
18:2n-6	1.8 ± 0.1 ^c	1.0 ± 0.0 ^b	0.7 ± 0.1 ^a
18:3n-6	0.2 ± 0.0 ^b	0.1 ± 0.0 ^a	–
18:3n-3	4.8 ± 0.0 ^c	1.7 ± 0.2 ^a	3.7 ± 0.4 ^b
18:4n-3	3.7 ± 0.2 ^c	2.8 ± 0.3 ^b	0.7 ± 0.1 ^a
20:1n-5	0.4 ± 0.1	–	–
20:2n-6	0.1 ± 0.0	–	–
20:4n-6 ARA	0.3 ± 0.0 ^b	0.1 ± 0.0 ^a	–
20:3n-3	0.1 ± 0.0	–	–
20:4n-3	0.2 ± 0.0 ^b	0.1 ± 0.0 ^a	0.1 ± 0.0 ^a
20:5n-3 EPA	2.2 ± 0.2 ^b	0.6 ± 0.1 ^a	2.1 ± 0.2 ^b
22:1n-11	–	0.1 ± 0.0	–
22:5n-3	–	0.1 ± 0.0	–
22:6n-3 DHA	0.1 ± 0.1 ^a	4.5 ± 0.2 ^c	1.3 ± 0.1 ^b
Σn-3 ¹	11.3 ± 0.3 ^c	9.8 ± 0.2 ^b	8.0 ± 0.7 ^a
Σn-6 ²	2.4 ± 0.1 ^c	1.2 ± 0.1 ^b	0.7 ± 0.1 ^a
Σn-9 ³	2.3 ± 0.2 ^b	3.9 ± 0.2 ^c	0.5 ± 0.0 ^a
ΣSAFAs ⁴	3.5 ± 0.2 ^b	2.3 ± 0.1 ^a	3.5 ± 0.4 ^b
ΣMUFAs ⁵	4.2 ± 0.2 ^b	5.4 ± 0.2 ^c	1.9 ± 0.3 ^a
Σn-3 LC-PUFAs ⁶	2.6 ± 0.2 ^a	5.3 ± 0.3 ^c	3.6 ± 0.3 ^b
ratio n-3/n-6	4.7 ± 0.4 ^a	8.2 ± 0.5 ^b	10.8 ± 1.5 ^c
ratio n-3/n-9	4.9 ± 0.4 ^b	2.5 ± 0.2 ^a	15.7 ± 0.8 ^c
ratio n-6/n-9	1.1 ± 0.1 ^b	0.3 ± 0.0 ^a	1.5 ± 0.3 ^b
ratio DHA/EPA	0.1 ± 0.1 ^a	7.6 ± 0.5 ^c	0.6 ± 0.0 ^b
ratio DHA/ARA	0.3 ± 0.4 ^a	71.1 ± 1.2 ^c	32.6 ± 2.0 ^b
ratio EPA/ARA	6.6 ± 0.0 ^a	9.4 ± 0.6 ^b	52.6 ± 2.5 ^c

Values (mean ± SD) followed by different superscripts within a row were ($P < 0.05$) significantly different among the three treatments. n , number of replicates; SAFA, saturated fatty acid; MUFA, monounsaturated fatty acid; LC-PUFA, long-chain polyunsaturated fatty acid; DHA, docosahexanoic acid; EPA, eicosapentanoic acid; ARA, arachidonic acid. ¹Includes 16:3n-3, 16:4n-3, 18:3n-3, 18:4n-3, 20:3n-3, 20:4n-3, 20:5n-3, 22:5n-3, and 22:6n-3. ²Includes 16:2n-6, 18:2n-6, 18:3n-6, 20:2n-6, 20:3n-6, 20:4n-6, 22:4n-6 and 22:5n-6. ³Includes 18:1n-9, 18:2n-9, 20:1n-9, 20:2n-9, 20:3n-9 and 22:1n-9. ⁴Includes 14:0, 15:0, 16:0, 17:0, 18:0, and 20:0. ⁵Includes 14:1n-5, 14:1n-7, 15:1n-5, 16:1n-7, 16:1n-9, 18:1n-9, 18:1n-7, 18:1n-5, 20:1n-9, 20:1n-7, 20:1n-5, 22:1n-9 and 22:1n-11. ⁶Includes 20:3n-3, 20:4n-3, 20:5n-3, 22:5n-3, and 22:6n-3.

$P \geq 0.05$, Table 5). Carbohydrates constituted the smallest portion of the nauplii proximate composition, with values ranged between $4\% \pm 0.4\%$ in T–R to $9.1\% \pm 0.6\%$ for treatment R. Data of ash and moisture content showed no statistical difference among all treatments (ANOVA, $P \geq 0.05$, Table 5).

3.3.2. FAs Composition. The FA composition of *A. tonsa* nauplii fed with the three experimental diets was expressed

as a percentage of the total lipid DW ± SD (Table 5). Total n-3 FAs and total n-3 LC-PUFAs, as well as EPA and DHA, were significantly higher in copepods fed on I–R compared with the other two diets (ANOVA, $P < 0.05$, Table 5). Group fed on diet T–R gave copepod higher percentage of 16:0, OA, LA, ALA, vaccenic acid, ARA, as also MUFAs, total n-6 and n-9 FAs series (ANOVA, $P < 0.05$, Table 5). FAs ratios n-3/n-6 and n-3/n-9 were significantly lower (ANOVA, $P < 0.05$, Table 5) in group fed on T–R, while DHA/EPA

TABLE 4: Proximal composition (lipid, protein, carbohydrate, ash, and moisture content; g/100 g dry weight) and fatty acid composition (mg/g dry weight) of rotifers (*Brachionus plicatilis*) fed with three microalgae diets (R, *Rhodomonas salina*; I–R, *Isochrysis galbana* + *Rhodomonas salina*; T–R, *Tetraselmis suecica* + *Rhodomonas salina*), $n = 5$.

<i>Brachionus plicatilis</i>	T–R	I–R	R
Lipids	13.1 ± 1.0 ^a	15.4 ± 0.8 ^b	17.3 ± 1.1 ^c
Proteins	57.4 ± 0.8 ^{ab}	58.7 ± 1.6 ^b	56.2 ± 1.7 ^a
Carbohydrates	7.8 ± 0.6	8.7 ± 0.9	7.2 ± 1.0
Ash	21.7 ± 0.4 ^b	17.2 ± 1.7 ^a	19.4 ± 0.6 ^a
Moisture	18.6 ± 2.1 ^b	14.6 ± 1.5 ^a	13.6 ± 2.1 ^a
14:0	0.2 ± 0.1 ^a	0.2 ± 0.1 ^a	0.9 ± 0.1 ^b
14:1n-5	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.1
16:0	1.6 ± 0.4 ^a	1.6 ± 0.2 ^a	2.6 ± 0.3 ^b
16:1n-7	0.3 ± 0.1 ^a	0.5 ± 0.1 ^{ab}	0.7 ± 0.1 ^b
16:3n-1	0.2 ± 0.0	–	–
18:0	0.4 ± 0.1 ^a	0.5 ± 0.0 ^a	0.8 ± 0.1 ^b
18:1n-9	1.3 ± 0.4 ^{ab}	1.7 ± 0.2 ^b	1.0 ± 0.1 ^a
18:1n-7	0.6 ± 0.1 ^a	0.5 ± 0.1 ^a	0.9 ± 0.1 ^b
18:2n-6	0.8 ± 0.2 ^{ab}	1.2 ± 0.3 ^b	0.6 ± 0.0 ^a
18:3n-6	0.1 ± 0.0	–	–
18:3n-3	1.7 ± 0.5 ^a	1.4 ± 0.2 ^a	2.7 ± 0.3 ^b
18:4n-3	0.7 ± 0.3	0.5 ± 0.1	0.7 ± 0.1
20:1n-5	0.6 ± 0.2	0.5 ± 0.1	0.7 ± 0.1
20:2n-6	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0
20:4n-6 ARA	0.2 ± 0.0	0.2 ± 0.0	0.2 ± 0.0
20:3n-3	0.2 ± 0.1	0.2 ± 0.0	0.2 ± 0.1
20:4n-3	1.0 ± 0.3	0.9 ± 0.1	0.9 ± 0.0
20:5n-3 EPA	1.1 ± 0.3	1.4 ± 0.1	1.2 ± 0.1
22:1n-11	0.2 ± 0.1	0.1 ± 0.1	0.2 ± 0.1
22:5n-3	0.3 ± 0.0	0.3 ± 0.1	0.4 ± 0.1
22:6n-3 DHA	0.5 ± 0.1 ^a	1.4 ± 0.1 ^c	0.8 ± 0.1 ^b
Σn-3 ¹	5.7 ± 1.3	6.1 ± 0.6	7.0 ± 0.5
Σn-6 ²	1.3 ± 0.2 ^{ab}	1.7 ± 0.3 ^b	1.2 ± 0.1 ^a
Σn-9 ³	2.0 ± 0.4	2.0 ± 0.2	2.0 ± 0.3
ΣSAFAs ⁴	2.4 ± 0.5 ^a	2.5 ± 0.3 ^a	4.4 ± 0.5 ^b
ΣMUFAs ⁵	4.0 ± 0.8 ^{ab}	3.9 ± 0.4 ^a	4.9 ± 0.3 ^b
Σn-3 LC-PUFAs ⁶	3.0 ± 0.6 ^a	4.1 ± 0.3 ^b	3.4 ± 0.2 ^a
ratio n-3/n-6	4.4 ± 1.1 ^{ab}	3.7 ± 0.4 ^a	6.1 ± 0.8 ^b
ratio n-3/n-9	2.9 ± 0.8	3.0 ± 0.2	3.6 ± 0.7
ratio n-6/n-9	0.7 ± 0.2 ^{ab}	0.8 ± 0.1 ^b	0.6 ± 0.0 ^a
ratio DHA/EPA	0.5 ± 0.1 ^a	1.0 ± 0.1 ^c	0.7 ± 0.0 ^b
ratio DHA/ARA	2.8 ± 0.5 ^a	7.4 ± 2.2 ^b	3.7 ± 0.6 ^a
ratio EPA/ARA	5.7 ± 2.0	7.2 ± 1.7	5.5 ± 1.1

Values (mean ± SD) followed by different superscripts within a row were ($P < 0.05$) significantly different among the three treatments. n , number of replicates; SAFA, saturated fatty acid; MUFA, monounsaturated fatty acid; LC-PUFA, long-chain polyunsaturated fatty acid; DHA, docosahexanoic acid; EPA, eicosapentanoic acid; ARA, arachidonic acid. ¹Includes 16:3n-3, 16:4n-3, 18:3n-3, 18:4n-3, 20:3n-3, 20:4n-3, 20:5n-3, 22:5n-3, and 22:6n-3. ²Includes 16:2n-6, 18:2n-6, 18:3n-6, 20:2n-6, 20:3n-6, 20:4n-6, 22:4n-6 and 22:5n-6. ³Includes 18:1n-9, 18:2n-9, 20:1n-9, 20:2n-9, 20:3n-9 and 22:1n-9. ⁴Includes 14:0, 15:0, 16:0, 17:0, 18:0, and 20:0. ⁵Includes 14:1n-5, 14:1n-7, 15:1n-5, 16:1n-7, 16:1n-5, 18:1n-9, 18:1n-7, 18:1n-5, 20:1n-9, 20:1n-7, 20:1n-5, 22:1n-9 and 22:1n-11. ⁶Includes 20:3n-3, 20:4n-3, 20:5n-3, 22:5n-3, and 22:6n-3.

and DHA/ARA ratio levels were significantly higher with I–R treatment (ANOVA, $P < 0.05$, Table 5). Finally, copepods fed on R and I–R did not show any significant differences in EPA/ARA ratio (ANOVA, $P \geq 0.05$, Table 5); meanwhile, treatment T–R was significantly lower (ANOVA, $P < 0.05$, Table 5) compared to the other diets.

3.4. Survival. During the feeding trials, survival rates were different among preys. While *B. plicatilis* survival did not change significantly after exposure to each treatment, *A. tonsa* showed different survival peaks over time depending on the type of diet administered (Figure 3, “diet · day”, $F_{6,54} = 117.28$, $P < 0.05$). Thus, from day 2, survival of nauplii

TABLE 5: Proximal composition (lipid, protein, carbohydrate, ash, and moisture content; g/100 g dry weight) and fatty acid composition (mg/g dry weight) of copepods (*A. tonsa*) nauplii, fed with three microalgae diets (R, *Rhodomonas salina*; I–R, *Isochrysis galbana* + *Rhodomonas salina*; T–R, *Tetraselmis suecica* + *Rhodomonas salina*), $n = 5$.

<i>Acartia tonsa</i>	T–R	I–R	R
Lipids	18.0 ± 0.3 ^b	16.4 ± 0.5 ^a	15.9 ± 0.3 ^a
Proteins	71.8 ± 0.7 ^b	70.7 ± 0.7 ^b	68.1 ± 0.6 ^a
Carbohydrates	4.0 ± 0.4 ^a	6.4 ± 0.6 ^b	9.1 ± 0.6 ^c
Ash	6.3 ± 0.4	6.6 ± 0.6	6.9 ± 0.2
Moisture	7.2 ± 0.7	7.6 ± 0.6	8.4 ± 0.7
14:0	0.4 ± 0.1	0.5 ± 0.1	0.5 ± 0.2
14:1n-5	0.1 ± 0.0	0.1 ± 0.1	0.1 ± 0.1
16:0	3.8 ± 0.4 ^c	1.2 ± 0.2 ^a	2.5 ± 0.3 ^b
16:1n-7	0.6 ± 0.5	0.2 ± 0.0	0.2 ± 0.0
16:3n-1	0.2 ± 0.0	–	–
18:0	1.3 ± 0.1 ^b	0.7 ± 0.1 ^a	1.4 ± 0.2 ^b
18:1n-9	1.3 ± 0.3 ^b	0.7 ± 0.1 ^a	0.6 ± 0.2 ^a
18:1n-7	0.6 ± 0.1 ^b	0.3 ± 0.0 ^a	0.4 ± 0.1 ^a
18:2n-6	0.7 ± 0.1 ^b	0.4 ± 0.1 ^a	0.2 ± 0.1 ^a
18:3n-6	0.1 ± 0.0	–	–
18:3n-3	1.8 ± 0.2 ^b	1.0 ± 0.1 ^a	1.2 ± 0.1 ^a
18:4n-3	0.7 ± 0.2	0.5 ± 0.1	0.4 ± 0.1
20:1n-5	0.2 ± 0.1	0.1 ± 0.0	–
20:2n-6	0.1 ± 0.0	0.1 ± 0.0	–
20:4n-6 ARA	0.2 ± 0.0 ^b	0.1 ± 0.0 ^a	0.1 ± 0.0 ^a
20:3n-3	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0
20:4n-3	0.2 ± 0.1	0.1 ± 0.0	0.1 ± 0.1
20:5n-3 EPA	2.9 ± 0.3 ^{ab}	2.9 ± 0.1 ^b	2.5 ± 0.1 ^a
22:1n-11	0.1 ± 0.0	–	–
22:5n-3	0.1 ± 0.0	0.1 ± 0.0	0.2 ± 0.1
22:6n-3 DHA	2.1 ± 0.3 ^a	6.7 ± 0.2 ^c	4.6 ± 0.2 ^b
Σn-3 ¹	7.8 ± 0.8 ^a	11.5 ± 0.3 ^c	9.2 ± 0.4 ^b
Σn-6 ²	1.1 ± 0.2 ^c	0.7 ± 0.2 ^b	0.4 ± 0.1 ^a
Σn-9 ³	1.5 ± 0.3 ^b	0.8 ± 0.1 ^a	0.8 ± 0.1 ^a
ΣSAFAs ⁴	5.6 ± 0.5 ^b	2.5 ± 0.1 ^a	4.6 ± 0.4 ^b
ΣMUFAs ⁵	3.2 ± 0.6 ^b	1.5 ± 0.1 ^a	1.7 ± 0.2 ^a
Σn-3 LC-PUFAs ⁶	5.3 ± 0.5 ^a	9.9 ± 0.2 ^c	7.6 ± 0.4 ^b
ratio n-3/n-6	7.2 ± 1.7 ^a	17.2 ± 3.7 ^b	24.5 ± 5.0 ^b
ratio n-3/n-9	5.5 ± 0.9 ^a	14.7 ± 2.0 ^b	12.2 ± 2.1 ^b
ratio n-6/n-9	0.8 ± 0.2	0.9 ± 0.4	0.5 ± 0.1
ratio DHA/EPA	0.7 ± 0.1 ^a	2.3 ± 0.1 ^c	1.9 ± 0.1 ^b
ratio DHA/ARA	12.2 ± 3.2 ^a	96.8 ± 5.0 ^c	80.9 ± 6.7 ^b
ratio EPA/ARA	16.7 ± 3.6 ^a	42.0 ± 2.7 ^b	43.6 ± 3.2 ^b

Values (mean ± SD) followed by different superscripts within a row were ($P < 0.05$) significantly different among the three treatments. n , number of replicates; SAFA, saturated fatty acid; MUFA, monounsaturated fatty acid; LC-PUFA, long-chain polyunsaturated fatty acid; DHA, docosahexanoic acid; EPA, eicosapentanoic acid; ARA, arachidonic acid. ¹Includes 16:3n-3, 16:4n-3, 18:3n-3, 18:4n-3, 20:3n-3, 20:4n-3, 20:5n-3, 22:5n-3, and 22:6n-3. ²Includes 16:2n-6, 18:2n-6, 18:3n-6, 20:2n-6, 20:3n-6, 20:4n-6, 22:4n-6 and 22:5n-6. ³Includes 18:1n-9, 18:2n-9, 20:1n-9, 20:2n-9, 20:3n-9 and 22:1n-9. ⁴Includes 14:0, 15:0, 16:0, 17:0, 18:0, and 20:0. ⁵Includes 14:1n-5, 14:1n-7, 15:1n-5, 16:1n-7, 16:1n-5, 18:1n-9, 18:1n-7, 18:1n-5, 20:1n-9, 20:1n-7, 20:1n-5, 22:1n-9 and 22:1n-11. ⁶Includes 20:3n-3, 20:4n-3, 20:5n-3, 22:5n-3, and 22:6n-3.

fed with binary diets I–R (96.2% ± 0.8%) and T–R (95.8% ± 0.9%) was significantly higher than nauplii fed on R treatment (87.9% ± 0.7%) (ANOVA, $P < 0.05$, Figure 3). At the end of the experiment, survival was significantly higher in binary diets, especially with I–R treatment. (88.1% ± 0.7%) (ANOVA, $P < 0.05$).

4. Discussion

4.1. Biochemical Composition of Live Preys. The present study showed how different mono and binary diets based on microalgae (T–R, I–R, and R) affect the survival and biochemical composition of *A. tonsa* nauplii and the rotifer

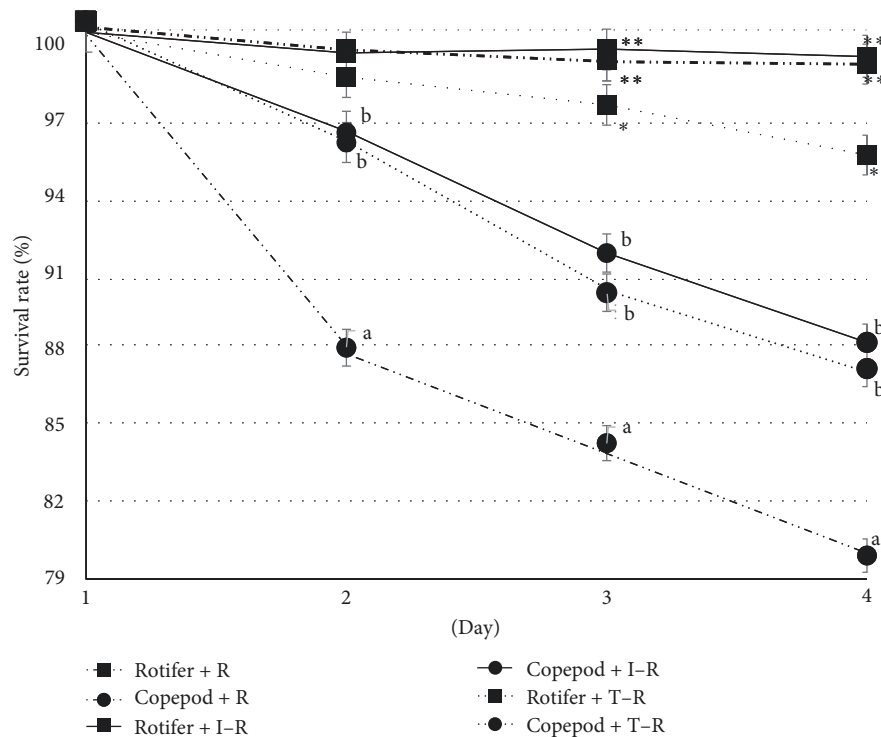


FIGURE 3: Survival (% \pm SD) of *Brachionus plicatilis* (rotifer) and *Acartia tonsa* (copepod) nauplii fed with three experimental diets. Values (mean \pm SD) followed by different superscripts were ($P < 0.05$) significantly different among the three treatments. R, *Rhodomonas salina*; I-R, *Isochrysis galbana* + *Rhodomonas salina*; T-R, *Tetraselmis suecica* + *Rhodomonas salina*.

B. plicatilis. In both preys, the use of binary diets based on the combination of *I. galbana* and *R. salina* (I-R treatment) improved their protein and LC-PUFAs content as well as the survival of *A. tonsa* nauplii, compared to control and T-R treatment. Indeed, among all microalgae employed in this study, the treatment I-R showed the highest nutritional value, especially in LC-PUFAs. It is plausible that this binary formula took advantage of the content in EPA of *R. salina* and the DHA content of *I. galbana*, potentially generating an LC-PUFAs boosted diet. Although these microalgae strains naturally produce high amounts of PUFAs, their FA content and composition can vary with environmental parameters such as the composition of the growth medium, nutrient availability, or illumination [48]. Therefore, it was important to standardize the microalgae culture conditions, in order to ensure the same FA enrichment day by day. In general, the biochemical composition of *A. tonsa* nauplii fed on I-R treatment was the richest in terms of protein, lipids, total LC-PUFAs, EPA, and DHA, also compared to *B. plicatilis* fed with the same treatment. Despite being a monoalgal diet, *R. salina* (control) has been shown to better improve the nutritional value of *A. tonsa*, especially in EPA and DHA, compared to the binary diet T-R. Indeed, this was probably due to the composition of *T. suecica*, which showed the lowest content in LC-PUFAs, especially in DHA, compared to the other microalgae employed to generate treatments (Table 3). These results agreed with previous studies reporting the excellence of *Rhodomonas* spp. as a diet for calanoid copepods [37, 39, 49, 50], suggesting that *R. salina* is an

outstanding candidate to generate binary diet for the calanoid copepod *A. tonsa*. On the contrary, the rotifer *B. plicatilis* was characterized by a lower content in proximate and FAs composition, even after enrichment with I-R treatment, compared to *A. tonsa* nauplii. The retention ability of LC-PUFAs was clearly species-specific [8]. Although *B. plicatilis* is able to synthesize LC-PUFAs, it showed a limited ability to retain EPA and DHA compared to *A. tonsa* [7]. On the contrary, *A. tonsa* nauplii were able to retain a high quantity of n-3 LC-PUFAs acquired exclusively from their phytoplankton diet, especially EPA and DHA. These results are consistent with those obtained by [15], where *A. tonsa* nauplii fed with *Rhodomonas baltica* until 12 days post-hatch reached the highest levels of EPA and DHA after 3 days of culture. The high percentage of DHA and EPA observed in copepods nauplii after 4 days fed with I-R suggested that the combination between *R. salina* and *I. galbana* could be an optimal food source for *A. tonsa*, as diet or enrichment. Besides, results also suggest that *A. tonsa* could retain the portion of saturated FAs (14:0, 16:0, and 18:0), and PUFAs (18:3n-3, 18:4n-3, 20:4n-6, 20:5n-3, and 22:6n-3), particularly EPA and DHA, which may be catabolized by the copepod as important growth factors [28, 51]. The ability to retain LC PUFAs could be related to the growth and reproduction of calanoid copepods, providing them with a considerable energy store and enabling their growth also during the food-scarce seasons [27, 28, 52]. This high LC-PUFAs retention capacity makes *A. tonsa* an optimal live feed for marine larval cultures, which may replace totally

or partially both rotifers and even *Artemia* throughout all marine fish larvae development. Compared to conventional live preys, *A. tonsa* presents not only a high content in protein, DHA, and EPA but also an attractive swimming behavior and wide size range (ca. 70 μm of newly hatched nauplii till 1.5 mm of adult females) [5, 9, 14, 17, 19, 53]. On the other hand, the latest cold storing techniques of *A. tonsa* eggs, resisting at 4°C for several months [35], represent to date the easiest way to manage high quantities of copepods nauplii, although it implies the inevitable decrease in viability and nutritional content [14]. In this sense, the enrichment exposure of *A. tonsa* nauplii to microalgae rich in LC-PUFAs, such as *I. galbana* and *R. salina*, arrange this decrease in nutritional content, improving their nutritional value before being administrated as a live feed to marine fish larvae. Indeed, the increased importance of LC-PUFAs (e.g., EPA and DHA) for marine fish larvae has been reviewed for many fish species [8, 54–56].

4.2. Survival. Finally, none of the diets fed to *B. plicatilis* produced significant mortality; only the group fed on T–R treatment showed reduced survival. That difference could be related to the size and motility of the microalgae employed as enrichment. Indeed, *T. suecica* presented larger cell size and higher motility, around 17 μm in cell length and four flagella, compared to *R. salina* and *I. galbana*, which showed, respectively, 11 and 5 μm cell length, both with two flagella. In contrast, the calanoid copepod *A. tonsa* showed significant differences in survival depending on the treatment supplied, with the highest survival rate when fed on I–R treatment. The mortality observed in all culture trials of *A. tonsa* nauplii could be explained since they pass through the delicate phase of molting where mortality is due not only to the food source but also to many other multifactorial causes such as energy reserves, parents nutrition, eggs storage conditions, storage time, etc. [14, 17]. Despite all treatments showing mortality, these results highlight the importance of using live microalgae binary diets as food sources for *A. tonsa* naupliar stages.

5. Conclusions

The present study provides evidence of the importance of enriching *A. tonsa* nauplii with a binary microalgal diet based on the combination of *I. galbana* and *R. salina* (I–R) in order to supply an adequate content of n-3 LC-PUFAs. Moreover, it showed how the biochemical composition of *A. tonsa* and *B. plicatilis* depended on their FAs retention ability and the diet supplied. The wide differences in nutritional composition observed between both preys revealed *A. tonsa* as a superior live feed, with higher protein content and improved ability to retain LC-PUFAs (EPA and DHA), compared to *B. plicatilis*. In conclusion, this study highlights the biochemical profile of enriched *A. tonsa* nauplii and contributes to the production of high-quality live feeds, which may be able to reach the nutritional needs and enhance the production of new marine fish species, as well as contributing to the improvement of *A. tonsa* culture.

Data Availability

The data in this study are available from the corresponding author upon reasonable request.

Disclosure

This research is part of a doctoral thesis belonging to the PhD program in Sustainable Aquaculture and Marine Ecosystems (ACUISEMAR) of the University of Las Palmas de Gran Canaria (ULPGC).

Conflicts of Interest

The authors declare that they have no conflicts of interest.

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References

- [1] K. Hamre, M. Yúfera, I. Rønnestad, C. Boglione, L. E. C. Conceição, and M. Izquierdo, “Fish larval nutrition and feed formulation: knowledge gaps and bottlenecks for advances in larval rearing,” *Reviews in Aquaculture*, vol. 5, no. s1, pp. S26–S58, 2013.
- [2] B. W. Hansen and S. Möller, “Review: a bibliometric survey of live feed for marine finfish and shrimp larval production,” *Aquaculture Research*, vol. 52, no. 11, pp. 5124–5135, 2021.
- [3] I. Olivotto, M. Planas, N. Simões, G. J. Holt, M. A. Avella, and R. Calado, “Advances in breeding and rearing marine ornamentals,” *Journal of the World Aquaculture Society*, vol. 42, no. 2, pp. 135–166, 2011.
- [4] I. Olivotto, G. Chemello, A. Vargas, B. Randazzo, C. C. Piccinetti, and O. Carnevali, “Marine ornamental species culture: from the past to “Finding Dory”,” *General and Comparative Endocrinology*, vol. 245, pp. 116–121, 2017.
- [5] J. Dhont, K. Dierckens, J. Støttrup, G. Van Stappen, M. Wille, and P. Sorgeloos, “Rotifers, *Artemia* and copepods as live feeds for fish larvae in aquaculture,” in *Advances in Aquaculture Hatchery Technology*, pp. 157–202, Woodhead Publishing Series in Food Science, Technology and Nutrition, 2013.
- [6] E. Lubzens, A. Marko, and A. Tietz, “De novo synthesis of fatty acids in the rotifer, *Brachionus plicatilis*,” *Aquaculture*, vol. 47, no. 1, pp. 27–37, 1985.
- [7] M. Birkou, D. Bokas, and G. Aggelis, “Improving fatty acid composition of lipids synthesized by *Brachionus plicatilis* in large scale experiments,” *Journal of the American Oil Chemists’ Society*, vol. 89, no. 11, pp. 2047–2055, 2012.
- [8] T. Dey, P. K. Ghosh, S. K. Nandi, G. Chowdhury, S. Mian, and M. S. Uddin, “A review on n-3 HUFA and live food organism for marine fish larvae nutrition,” *American Journal of Agricultural Science, Engineering, and Technology*, vol. 6, no. 3, pp. 88–102, 2022.

- [9] N. W. Rasdi and J. G. Qin, "Improvement of copepod nutritional quality as live food for aquaculture: a review," *Aquaculture Research*, vol. 47, no. 1, pp. 1–20, 2016.
- [10] O. O. Ajiboye, A. F. Yakubu, T. E. Adams, E. D. Olaji, and N. A. Nwogu, "A review of the use of copepods in marine fish larviculture," *Reviews in Fish Biology and Fisheries*, vol. 21, pp. 225–246, 2011.
- [11] T. Camus, *The improvement of copepods intensive culture protocols as live feeds for aquaculture hatcheries*, 2012, James Cook University, PhD Thesis, <https://researchonline.jcu.edu.au/29905/>.
- [12] C. Zeng, L. Shao, A. Ricketts, and J. A. Moorhead, "The importance of copepods as live feed for larval rearing of the green mandarin fish *Synchropus splendidus*," *Aquaculture*, vol. 491, pp. 65–71, 2018.
- [13] R. J. Shields, J. G. Bell, F. S. Luizi, B. Gara, N. R. Bromage, and J. R. Sargent, "Natural copepods are superior to enriched *Artemia* Nauplii as feed for Halibut Larvae (*Hippoglossus hippoglossus*) in terms of survival, pigmentation and retinal morphology: relation to dietary essential fatty acids," *The Journal of Nutrition*, vol. 129, no. 6, pp. 1186–1194, 1999.
- [14] G. Drillet, S. Frouel, M. H. Sichlau et al., "Status and recommendations on marine copepod cultivation for use as live feed," *Aquaculture*, vol. 315, no. 3-4, pp. 155–166, 2011.
- [15] J. G. Støttrup, J. G. Bell, and J. R. Sargent, "The fate of lipids during development and cold-storage of eggs in the laboratory-reared calanoid copepod, *Acartia tonsa* Dana, and in response to different algal diets," *Aquaculture*, vol. 176, no. 3-4, pp. 257–269, 1999.
- [16] G. Drillet, N. O. G. Jørgensen, T. F. Sørensen, H. Ramløv, and B. W. Hansen, "Biochemical and technical observations supporting the use of copepods as live feed organisms in marine larviculture," *Aquaculture Research*, vol. 37, no. 8, pp. 756–772, 2006.
- [17] G. Drillet, M. H. Iversen, T. F. Sørensen, H. Ramløv, T. Lund, and B. W. Hansen, "Effect of cold storage upon eggs of a calanoid copepod, *Acartia tonsa* (Dana) and their offspring," *Aquaculture*, vol. 254, no. 1–4, pp. 714–729, 2006.
- [18] M. A. Peck and L. Holste, "Effects of salinity, photoperiod and adult stocking density on egg production and egg hatching success in *Acartia tonsa* (Calanoida: Copepoda): optimizing intensive cultures," *Aquaculture*, vol. 255, no. 1–4, pp. 341–350, 2006.
- [19] G. Drillet, L. C. Lindley, A. Michels, J. Wilcox, and N. H. Marcus, "Improving cold storage of subitaneous eggs of the copepod *Acartia tonsa* Dana from the Gulf of Mexico (Florida–USA)," *Aquaculture Research*, vol. 38, no. 5, pp. 457–466, 2007.
- [20] M. A. Peck, B. Ewest, L. Holste, P. Kanstinger, and M. Martin, "Impacts of light regime on egg harvests and 48-h egg hatching success of *Acartia tonsa* (Copepoda: Calanoida) within intensive culture," *Aquaculture*, vol. 275, no. 1–4, pp. 102–107, 2008.
- [21] I. Olivotto, G. Gaiot, L. Holste et al., "Are *Acartia tonsa* cold-stored eggs a suitable food source for the marine ornamental species *Amphiprion polymnus*? A feeding study," *Aquaculture Nutrition*, vol. 18, no. 6, pp. 685–696, 2012.
- [22] J. G. Støttrup, "The elusive copepods: their production and suitability in marine aquaculture," *Aquaculture Research*, vol. 31, no. 8-9, pp. 703–711, 2000.
- [23] Ó. Monroig, A. C. Shu-Chien, N. Kabeya, D. R. Tocher, and L. F. C. Castro, "Desaturases and elongases involved in long-chain polyunsaturated fatty acid biosynthesis in aquatic animals: from genes to functions," *Progress in Lipid Research*, vol. 86, Article ID 101157, 2022.
- [24] J. G. Støttrup and J. Jensen, "Influence of algal diet on feeding and egg-production of the calanoid copepod *Acartia tonsa* Dana," *Journal of Experimental Marine Biology and Ecology*, vol. 141, no. 2-3, pp. 87–105, 1990.
- [25] S. H. Jónasdóttir, D. Fields, and S. Pantoja, "Copepod egg production in Long Island Sound, USA, as a function of the chemical composition of seston," *Marine Ecology Progress Series*, vol. 119, pp. 87–98, 1995.
- [26] A. N. S. Siuda and H. G. Dam, "Effects of omnivory and predator-prey elemental stoichiometry on planktonic trophic interactions," *Limnology and Oceanography*, vol. 55, no. 5, pp. 2107–2116, 2010.
- [27] M. Chen, H. Liu, and B. Chen, "Effects of dietary essential fatty acids on reproduction rates of a subtropical calanoid copepod, *Acartia erythroa*," *Marine Ecology Progress Series*, vol. 455, pp. 95–110, 2012.
- [28] S. Besiktepe and H. G. Dam, "Effect of diet on the coupling of ingestion and egg production in the ubiquitous copepod, *Acartia tonsa*," *Progress in Oceanography*, vol. 186, Article ID 102346, 2020.
- [29] F. Alajmi and C. Zeng, "Evaluation of microalgal diets for the intensive cultivation of the tropical calanoid copepod, *Parvocalanus crassirostris*," *Aquaculture Research*, vol. 46, no. 5, pp. 1025–1038, 2015.
- [30] A. C. Puello-Cruz, S. Mezo-Villalobos, B. González-Rodríguez, and D. Voltolina, "Culture of the calanoid copepod *Pseudodiaptomus euryhalinus* (Johnson 1939) with different microalgal diets," *Aquaculture*, vol. 290, no. 3-4, pp. 317–319, 2009.
- [31] A. M. Malzahn, N. Aberle-Malzahn, K. Schoo, and M. Boersma, "Culture conditions affect the nutritional value of the copepod *Acartia tonsa*," *Journal of Agricultural and Marine Sciences [JAMS]*, vol. 20, pp. 40–46, 2015.
- [32] M. F. Payne and R. J. Rippingale, "Effects of salinity, cold storage and enrichment on the calanoid copepod *Gladioferens imparipes*," *Aquaculture*, vol. 201, no. 3-4, pp. 251–262, 2001.
- [33] S. Sun, L. Chen, X. Ge, and J. Qin, "Examination of a practical method for copper enrichment of euryhaline rotifers (*Brachionus plicatilis*) as diet of *Eriocheir sinensis* zoea larvae," *Aquaculture Nutrition*, vol. 19, no. 5, pp. 809–817, 2013.
- [34] G. Gorbi, M. Invidia, F. Savorelli et al., "Standardized methods for acute and semichronic toxicity tests with the copepod *Acartia tonsa*," *Environmental Toxicology and Chemistry*, vol. 31, no. 9, pp. 2023–2028, 2012.
- [35] J. G. Støttrup, "A Review on the status and progress in rearing copepods for marine larviculture. Advantages and Disadvantages. Among calanoid, harpacticoid and cyclopoid copepods," in *Avances En Nutrición Acuicola*, pp. 62–83, Universidad Autónoma de Nuevo León, Monterrey, 2006.
- [36] R. R. L. Guillard and J. H. Ryther, "Studies of marine planktonic diatoms: I. *Cyclotella nana* Hustedt, and *Detonula confervacea* (Cleve) Gran," *Canadian Journal of Microbiology*, vol. 8, no. 2, pp. 229–239, 1962.
- [37] S. M. H. Ismar, T. Hansen, and U. Sommer, "Effect of food concentration and type of diet on *Acartia* survival and naupliar development," *Marine Biology*, vol. 154, pp. 335–343, 2008.
- [38] M. Guevara, L. Bastardo, R. Cortez, B. Arredondo-Vega, L. Romero, and P. Gómez, "Pastas de *Rhodomonas salina* (Cryptophyta) como alimento para *Brachionus plicatilis* (Rotifera)," *Revista De Biología Tropical*, vol. 59, no. 4, pp. 1503–1515, 2011.

- [39] J. Zhang, C. Wu, D. Pellegrini et al., "Effects of different monoalgal diets on egg production, hatching success and apoptosis induction in a Mediterranean population of the calanoid copepod *Acartia tonsa* (Dana)," *Aquaculture*, vol. 400-401, pp. 65-72, 2013.
- [40] I. Sirakov, K. Velichkova, S. Stoyanova, and Y. Staykov, "The importance of microalgae for aquaculture industry. Review," *International Journal of Fisheries and Aquatic*, vol. 2, no. 4, 2015.
- [41] A. R. Abd Rahman, Z. C. Cob, Z. Jamari, A. R. Mohamed, T. Toda, and O. B. H. Ross, "The effects of microalgae as live food for *Brachionus plicatilis* (Rotifer) in intensive culture system," *Tropical Life Sciences Research*, vol. 29, no. 1, pp. 127-138, 2018.
- [42] G. Sew, A. Calbet, G. Drillet, and P. A. Todd, "Effects of concentration and size of suspended particles on the ingestion, reproduction and mortality rates of the copepod, *Acartia tonsa*," *Marine Environmental Research*, vol. 140, pp. 251-264, 2018.
- [43] S. M. Leandro, P. Tiselius, and H. Queiroga, "Growth and development of nauplii and copepodites of the estuarine copepod *Acartia tonsa* from southern Europe (Ria de Aveiro, Portugal) under saturating food conditions," *Marine Biology*, vol. 150, pp. 121-129, 2006.
- [44] AOAC, *Official Methods of Analysis of the Association of Official Analytical Chemists: Official Methods of Analysis of AOAC International*, AOAC, 2019.
- [45] J. Folch, M. Lees, and G. H. S. Stanley, "A simple method for the isolation and purification of total lipides from animal tissues," *Journal of Biological Chemistry*, vol. 226, no. 1, pp. 497-509, 1957.
- [46] W. H. M. Christie and X. Han, "Lipid analysis: isolation, separation, identification and lipidomic analysis," 2010, <https://ci.nii.ac.jp/ncid/BB04922608>.
- [47] M. S. Izquierdo, T. Watanabe, T. Takeuchi, T. Arakawa, and C. Kitajima, "Optimum EFA levels in Anemia to meet the EFA requirements of red seabream (*Pagrus major*)," in *Status of Fish Nutrition in Aquaculture Proceedings Third International Symposium on Feeding and Nutrition in Fish*, M. Takeda and T. Watanabe, Eds., pp. 221-232, Japan Translation Center Ltd., 1990.
- [48] T. Fernandes, I. Fernandes, C. A. P. Andrade, and N. Cordeiro, "Changes in fatty acid biosynthesis in marine microalgae as a response to medium nutrient availability," *Algal Research*, vol. 18, pp. 314-320, 2016.
- [49] I. Buttino, A. Ianora, S. Buono, V. Vitello, G. Sansone, and A. Miralto, "Are monoalgal diets inferior to pluralgal diets to maximize cultivation of the calanoid copepod *Temora stylifera*?" *Marine Biology*, vol. 156, pp. 1171-1182, 2009.
- [50] U. Dahl, C. R. Lind, E. Gorokhova, B. Eklund, and M. Breitholtz, "Food quality effects on copepod growth and development: implications for bioassays in ecotoxicological testing," *Ecotoxicology and Environmental Safety*, vol. 72, no. 2, pp. 351-357, 2009.
- [51] A. J. Veloza, F.-L. E. Chu, and K. W. Tang, "Trophic modification of essential fatty acids by heterotrophic protists and its effects on the fatty acid composition of the copepod *Acartia tonsa*," *Marine Biology*, vol. 148, pp. 779-788, 2006.
- [52] G. K. Saba, D. K. Steinberg, and D. A. Bronk, "Effects of diet on release of dissolved organic and inorganic nutrients by the copepod *Acartia tonsa*," *Marine Ecology Progress Series*, vol. 386, pp. 147-161, 2009.
- [53] B. W. Hansen, "Advances using copepods in aquaculture," *Journal of Plankton Research*, vol. 39, no. 6, pp. 972-974, 2017.
- [54] I. Olivotto, M. Cardinali, L. Barbaresi, F. Maradonna, and O. Carnevali, "Coral reef fish breeding: the secrets of each species," *Aquaculture*, vol. 224, no. 1-4, pp. 69-78, 2003.
- [55] C. K. Faulk and G. J. Holt, "Advances in rearing cobia *Rachycentron canadum* larvae in recirculating aquaculture systems: live prey enrichment and greenwater culture," *Aquaculture*, vol. 249, no. 1-4, pp. 231-243, 2005.
- [56] I. Olivotto, A. Rollo, R. Sulpizio, M. Avella, L. Tosti, and O. Carnevali, "Breeding and rearing the Sunrise Dottyback *Pseudochromis flavivertex*: the importance of live prey enrichment during larval development," *Aquaculture*, vol. 255, no. 1-4, pp. 480-487, 2006.