Aquaculture Studies, 25(3), 152-161, AQUAST2324
http://doi.org/10.4194/AQUAST2324

RESEARCH PAPER

Augmentation of *Apocyclops royi* Mass Production Using Live and Preserved *Chloroidium saccharophilum* for Marine Finfish Hatcheries

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How to Cite

Duraivel, P.K., Philip, T.R., Kareem, A., Radha, V. (2025). Augmentation of *Apocyclops royi* Mass Production Using Live and Preserved *Chloroidium saccharophilum* for Marine Finfish Hatcheries. *Aquaculture Studies*, *25(3), AQUAST2324*. http://doi.org/10.4194/AQUAST2324

Article History
Received 30 December 2024
Accepted 18 March 2025
First Online 19 March 2025

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Keywords
Apocyclops royi
Chloroidium saccharophilum
Live feed
Sustainable aquaculture

Abstract

This study aimed to explore sustainable feeding practices for the mass production of *Apocyclops royi* using *Chloroidium saccharophilum* in live, frozen, and lyophilized forms, combined with different feeding strategies. *A. royi*, collected from the Kovalam coastal area, was taxonomically identified. *C. saccharophilum* was cultured in Conway medium and processed into live, lyophilized, and frozen forms. Five experimental groups were established: Group 1 (A-CLi) fed live *C. saccharophilum*, Group 2 (A-CF) fed frozen *C. saccharophilum*, Group 3 (A-CLy) fed lyophilized *C. saccharophilum*, Group 4 (A-C-LFL) fed a mixed diet of all three forms, and Group 5 (A-C-LFLY) fed a mixed diet with yeast. The indoor mass production of *A. royi* was conducted under optimized conditions, and population density was monitored. Results showed that mixed diets (A-C-LFL and A-C-LFLY) significantly enhanced survival, growth, and reproduction. The fatty acid composition of *A. royi* was dominated by PUFAs, including EPA and DHA with *C. saccharophilum* combination diets. The amino acid profile revealed high levels of essential amino acids like Glutamine, Aspartic acid, and Leucine. This study demonstrates that mixed diets (A-C-LFLY) improve *A. royi* productivity, supporting year-round copepod culture and offering a sustainable solution for live feed production in marine aquaculture.

Introduction

The larval stage of most emerging finfish species in the marine aquaculture industry is sensitive and characterized by a small mouth, making larviculture highly challenging due to the lack of appropriate first-feeding protocols. Enhancing diversification and innovation in live feed production is crucial for advancing the fast-growing marine larviculture industry. As a result, live feed production technologies have become a focal point worldwide (Pan et al., 2022; El-Sayed et al., 2024; Xu et al., 2024). Copepods are the major prey item for early-stage marine fish larvae in natural habitats (Altaff, 2020), offering adequate nutritional value (Støttrup, 2003; van et al., 2008), stimulating feeding responses (Yen et al., 2015), and providing a wide size range (Turner, 2004). Feeding marine fish larvae with copepods has been shown to improve survival and growth rates (Azani and Rasdi, 2021; Vijayaraj et al., 2022), reduce deformities (Loufi et al., 2024), enhance pigmentation (Vijayaraj et al., 2022), and increase stress tolerance (Altaff and Vijayaraj, 2021; Saiz et al., 2022). These properties make copepods significantly more suitable for larval rearing compared to other commonly used live feeds.

Among copepod species, *Apocyclops royi* stands out as a euryhaline cyclopoid copepod widely cultured as live feed in the aquaculture industry. This species thrives across a broad range of salinity and temperature conditions, inhabiting environments such as

aquaculture ponds, estuaries, brackish waters, and marine ecosystems. With its small nauplii and short generation time (6–7 days), *A. royi* is well-suited for aquaculture and mass culture (Muthupriya and Altaff, 2009). Its ability to be cultivated at high densities makes it an excellent feed source for numerous fish larvae and juvenile stages in aquaculture, with its capacity to feed on microalgae thoroughly assessed (Dhanker and Hwang, 2013; Pan et al., 2018). Although several studies have demonstrated that *A. royi* supplements improve the growth and survival of finfish larvae (Pan et al., 2018; Nielsen et al., 2019; Abate et al., 2021; Nielsen et al., 2021), rotifers and *Artemia* nauplii remain the preferred live feeds in commercial hatcheries. This is largely because copepods are not yet produced at densities that are economically viable for large-scale use. The lack of standardized protocols for high-density copepod production continues to hinder their intensive cultivation (Santhanam et al., 2024).

To achieve higher density production of copepods, microalgae play an important role as a sustainable food source in their mass production (Rasdi and Qin, 2016; Ma et al., 2024). Microalgae are widely used as live feeds for various marine organisms, including bivalves and zooplankton including copepods (Altaff and Vijayaraj, 2021). They serve as a direct dietary source, providing essential nutrients to early developmental stages and acting as natural enrichment ingredients for zooplankton, including copepod live feed organisms (Altaff and Vijayaraj, 2021). Based on our earlier studies, the marine microalga, *C.saccharophilum* has proven to be the most suitable microalgal diet for the mass culture of *A. royi* (Vijayaraj et al., 2022). This is due to its ability to enhance productivity and improve the nutritional value of copepods as prey for finfish larvae (Vijayaraj et al., 2022). However, in recent advancements in aquaculture, particularly in larviculture, maintaining microalgal cultures remains challenging. It requires managing multiple environmental factors to achieve the desired biomass for marine copepod feeding, a gap that persists in recent research on larviculture and live feed production. Therefore, effective preservation techniques for marine microalgae are essential. In this regard, our hypothesis aims to explore lyophilization and freezing processes as viable methods for preserving microalgal cultures, potentially contributing to sustainable practices in live feed production. These approaches can provide an alternative means to meet copepod feeding requirements year-round and address challenges associated with insufficient microalgal biomass. Hence, the objective of this study is to evaluate the feeding strategies of marine copepods using live, frozen, and lyophilized microalgal diets to enhance the survival, population density, and nutritional quality of marine copepods, particularly *A. royi*. In this context, the present study aims to explore a novel approach for sustainable aquaculture practices by formulating *C. saccharophilum* diets with varying feeding strategies to support the mass production of *A. royi*.

Materials and Methods

Collection and Identification of *A. royi*

Zooplankton samples were collected from the coastal waters of Kovalam (13.0827° N, 80.2707° E), Chennai, using a Bongo net made of bolting silk (200 µm mesh size) was operated horizontally for approximately two hours from a mechanized boat at a depth of one meter during the early morning hours. Live zooplankton was immediately transferred into plastic containers filled with seawater and transported to the laboratory within two hours of collection. *A. royi* individuals were manually separated from the zooplankton samples and identified to species level based on the taxonomic key description provided by Loka et al. (2017).

For further confirmation, molecular characterization was performed using 18S rRNA sequencing techniques. Genomic DNA was extracted from specimens preserved in 95% ethanol. Before DNA extraction, the samples were rinsed with 500 µL of sterile distilled water and soaked overnight at room temperature. Individual copepods were placed in 2 mL PCR tubes for DNA extraction, which was conducted using the DNeasy Tissue Kit (QIAGEN), following the spin-column protocol as described by Nawaz et al. (2024).

PCR amplification of the 18S rRNA gene was carried out using universal invertebrate primers (forward: ACCTGGTTGATCCTGCCAG; reverse: GGGCATCACAGACCTG). The amplified PCR products were purified and sequenced. Sequence compilation and editing were performed using BioEdit version 7.2.5.0, and the sequences were aligned using ClustalX version 2.1. Phylogenetic analysis was conducted using the Maximum Likelihood (ML) method with the Kimura 2-parameter (K2P) model implemented in MEGA version 6.06. Bootstrapping with 1000 replicates was employed to assess the statistical reliability of the phylogenetic tree. The 18S rRNA gene sequences were cross-verified with existing sequences in the NCBI database for species-level identification. The validated sequences were submitted to NCBI, and accession numbers were obtained (PQ675701).

Mass Culture, Harvesting, and Preparation of Combination Microalgal Diets

For this study, the marine microalga, (*C. saccharophilum*) (GenBank accession number OM337702.1) were sub-cultured and scaled up using Conway medium. The cultures were maintained at a constant temperature of 29°C with vigorous aeration. A photoperiod of 12 hours light and 12 hours dark was set, with a light intensity of 8000 lux to optimize growth conditions. The microalgal biomass was harvested using centrifugation (Centrifuge: Remi-R-8C). The harvested biomass was processed into three forms: live, lyophilized, and frozen. The lyophilization was

performed using a freeze dryer (Mini Lyodel), while the frozen biomass was stored at -4°C in a deep freezer. These different forms of *C. saccharophilum* were prepared to evaluate their effects on the growth performance of *A. royi*. The processed *C. saccharophilum* cells were observed under a compound microscope to identify morphological differences between the cell surfaces across the three preparation methods. The harvested microalgae were subjected to further proximate composition analysis. The proximate composition, including carbohydrate (Dubois et al., 1956), protein (Lowry et al., 1951), lipid (Folch et al., 1957), and ash (AOAC, 1995) content, was estimated using standard methods.

Optimization of *A. royi* Culture with Different Feeding Regimes

The live *A. royi* collected from the wild were domesticated by rearing ovigerous females in 1 L beakers over several generations to establish a desirable stock. For survival and growth studies, 100 adults from this stock were used in the following experimental setups:

The experimental setups were divided into five groups. Group 1 (A-CLi) consisted of *A. royi* fed with live *C. saccharophilum* at a concentration of 30,000 cells/µL. Group 2 (A-CF) included *A. royi* fed with *C. saccharophilum* in a frozen form at the concentration of 30,000 cells/µL. Group 3 (A-CL) involved *A. royi* fed with *C. saccharophilum* in a lyophilized form at 30,000 cells/µL. In Group 4 (A-C-LFL), *A. royi* were fed with a mixed diet of *C. saccharophilum* in live, frozen, and lyophilized forms in a ratio of 4:3:3. And the Group 5 (A-C-LFLY) consisted of *A. royi* fed with *C. saccharophilum* in live, frozen, and lyophilized forms along with yeast in a ratio of 4:2:2:2.

Mass Production Experiment

For mass culture, active males and ovigerous females with a high number of eggs in their ovisacs were selected from the *A. royi* stock. The indoor culture was initiated with a density of approximately 100 adults per liter in a plastic CV tank containing 100 liters of filtered seawater. In the inoculum, male-to-female ratio at 1:4 was maintained. The culture was maintained under optimized environmental conditions, including a temperature of 29ºC, salinity of 30 psu, and pH of 7.8. Different combinations of microalgae diets were added daily to maintain the required dietary concentration for the copepods. Population density was monitored by collecting random samples from the culture tanks and counting the total number of nauplii, copepodites, and adults using a Sedgwick Rafter chamber.

Biochemical Analysis

At the end of the experiments, *A. royi* were harvested for further biochemical profile analysis. Amino acid profiles were analyzed using High-Performance Liquid Chromatography (HPLC, Shimadzu LC-10A), while fatty acid profiles were quantified using Gas Chromatography (Agilent 6890, Agilent Technologies, Santa Clara, CA). Triplicate samples were analyzed, and the data were recorded as mean±SD. An ANOVA and Tukey post-hoc test was performed on the fatty acid and amino acid profiles of the different feeding regimes of *A. royi* using PAST-3 software.

Results

Biochemical Composition of Different Microalgal Biomass

In this study, the morphological structure of live, freeze-dried, and lyophilized *C. saccharophilum* biomass was analyzed (Figure S1). No morphological changes or cell structure lysis were observed in *C. saccharophilum* during centrifugation, lyophilization and freezing processes. The size of microalgae ranged from 5 to 15 µm, making them an ideal food source for zooplankton, as microalgae within this size range are suitable for ingestion and digestion by the copepods. The lyophilized *C. saccharophilum* exhibited a spherical shape, dense chlorophyll content, and a mean diameter of 10 µm, indicating its suitability for filter-feeding and grazing copepod species. Similarly, the freeze-dried biomass

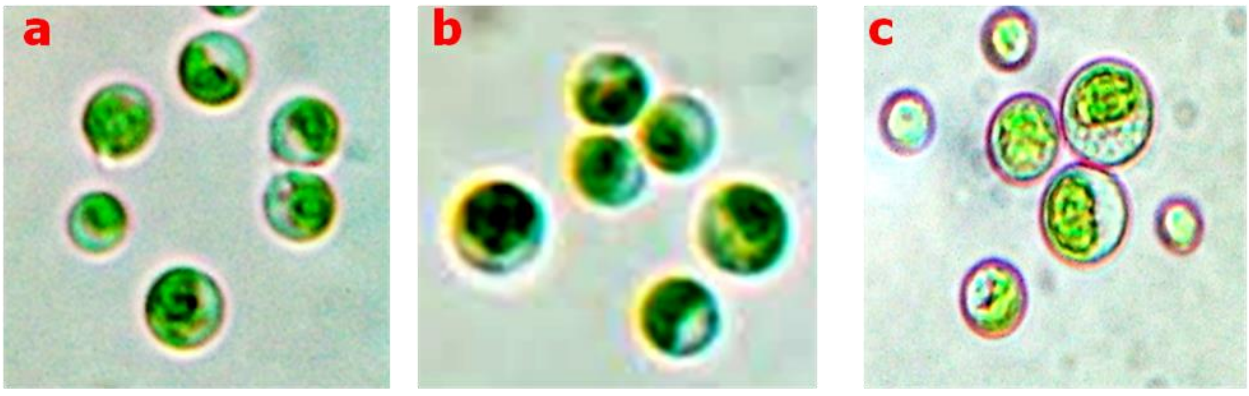

Figure S1. Morphology of different microalgae (a. Live; b. Freezed; c. Lyophilized).

consisted of spherical cells with a mean diameter of 10 µm, a smooth cell wall, and parietal chloroplasts. The proximate composition analysis revealed notable differences among the live, freeze-dried, and lyophilized biomass (Table 1). The live *C. saccharophilum* biomass yielded a dry cell weight of 0.85±0.05 g/L, with carbohydrate, protein, and lipid contents of 183.33±3.29 mg, 222±2.44 mg, and 7.13±0.28 mg, respectively. The freeze-dried biomass showed a slightly lower dry cell weight of 0.49±0.03 g/L, with carbohydrate, protein, and lipid contents of 139±2.44 mg, 208±3.26 mg, and 5.49±0.15 mg, respectively. The lyophilized biomass had a dry cell weight of 0.76±0.12 g/L and exhibited a carbohydrate content of 167±5.35mg, a protein content of 230.66±4.10mg, and a lipid content of 7.18±0.46 mg. These findings indicate that the lyophilized *C. saccharophilum* retains higher protein and lipid content compared to the freeze-dried biomass, making it a potentially better dietary option for feeding copepods.

Population Density, Number of Egg Production and Survival of *A. royi* with Different Diets

The total population density of *A. royi* under different diet is presented in Figure 1. Group 1 (A-CLi), fed with live *C. saccharophilum* at 30,000 cells/µL, exhibited a total density of 12,350±254.95 individuals per liter. In Group 2 (A-CF), where *A. royi* was fed with frozen *C. saccharophilum*, the total density was 10,100±216.03 individuals per liter. Group 3 (A-CLy), with *A. royi* fed on lyophilized *C. saccharophilum*, showed a slightly higher density at 12,400±163.30 individuals per liter. Group 4 (A-C-LFL), where a mixed diet of live, frozen, and lyophilized *C. saccharophilum* was provided, resulted in an increased density of 13,400±204.12 individuals per liter. The highest density, 14,966.67±124.72 individuals per liter, was observed in Group 5 (A-C-LFLY), where *A. royi* was fed with a mixed diet of live, frozen, lyophilized *C. saccharophilum* and yeast. Similarly, the highest percentage of nauplii population density of *A. royi* was observed when fed with a mixed diet of live, frozen, and lyophilized *C. saccharophilum* supplemented with yeast (Figure 2) and the same diet resulted in higher survival rates (Figure 3). These results suggest that the mixed diet, combined with yeast supplementation significantly enhances the total density, survival, and reproductive performance of *A. royi* in the culture system, indicating a more favorable nutritional environment for efficient culture of copepods for aquaculture applications.

Fatty Acid Profile

The fatty acid profile of A. royi varied significantly ($P<0.05$) among the different C. saccharophilum feeding regimes (Table 2). Among the saturated fatty acids, myristic acid (C14:0) was highest in the A-CF

Table 1. Proximate composition of different formulation of *C. saccharophilum*

Microalgae	Biomass (g/L of dry cell weight)	Carbohydrates (mg)	Protein (mg)	Lipid (mg)
Live *C. saccharophilum*	0.85±0.05	183.33±3.29	222±2.44	7.13±0.28
Freezed *C. saccharophilum*	0.49±0.03	139±2.44	208±3.26	5.49±0.15
Lyophilized *C. saccharophilum*	0.76±0.12	167±5.35	230.66±4.10	7.18±0.46

The values are given in Mean±SD

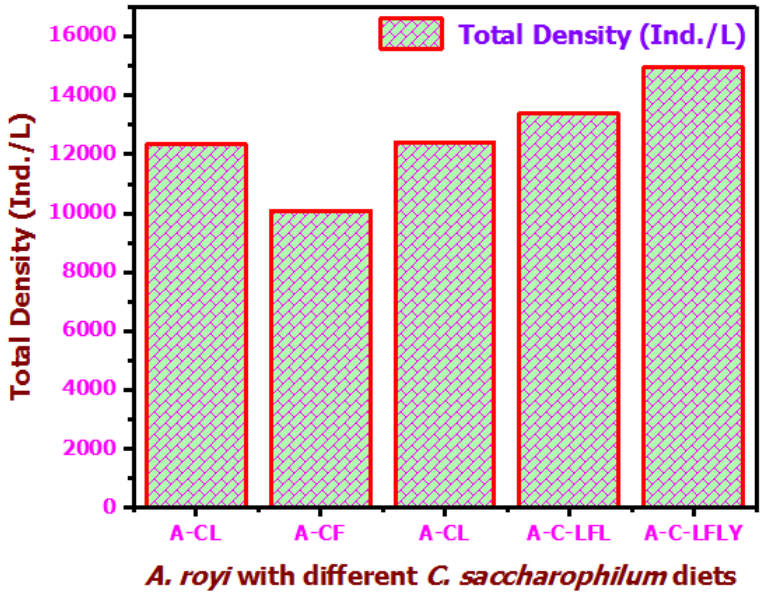

Figure 1. Total Density of *A. royi* with different *C. saccharophilum* feeding regimes.

(6.36±0.20%) and A-C-LFL (5.53±0.40%) treatments, whereas palmitic acid (C16:0) was significantly elevated in A-C-LFLY (20.86±0.57%). Stearic acid (C18:0) showed notable variation, with the highest concentration in A-CLy (4.03±0.69%) and A-C-LFL (5.37±0.16%). Among the monounsaturated fatty acids, palmitoleic acid (C16:1) was most abundant in A-C-LFLY (24.86±0.57%), followed by A-CF (8.43±0.12%) and A-C-LFL (8.96±0.44%). Oleic acid (C18:1 cis) was significantly higher in A-C-LFLY (6.89±0.82%) compared to the other treatments. Notably, trans-oleic acid (C18:1 trans) was highest in A-C-LFL (35.66±0.77%), followed by A-CLy (10.16±0.62%) and A-CF (2.5±0.08%). Among the polyunsaturated fatty acids, alpha-linolenic acid (C18:3n-3) was highest in A-C-LFL (5.36±0.36%), while eicosapentaenoic acid (EPA, C20:5n-3) showed significant variation, with the highest concentration in A-C-LFLY (28.13±0.32%) and A-CLy (22.23±0.75%). Docosahexaenoic acid (DHA, C22:6n-3) was notably higher in A-C-LFLY (8.56±0.28%) and A-CLy (8.5±0.40%). Arachidonic acid (C20:4n-6) was significantly elevated in A-C-LFLY (6.94±0.67%) compared to the other treatments. Overall, the study indicates that different C. saccharophilum feeding strategies influence the fatty acid composition of *A. royi*, with lyophilized and freeze-dried diets enhancing essential fatty acid content, particularly EPA and DHA, which are critical for marine aquaculture applications.

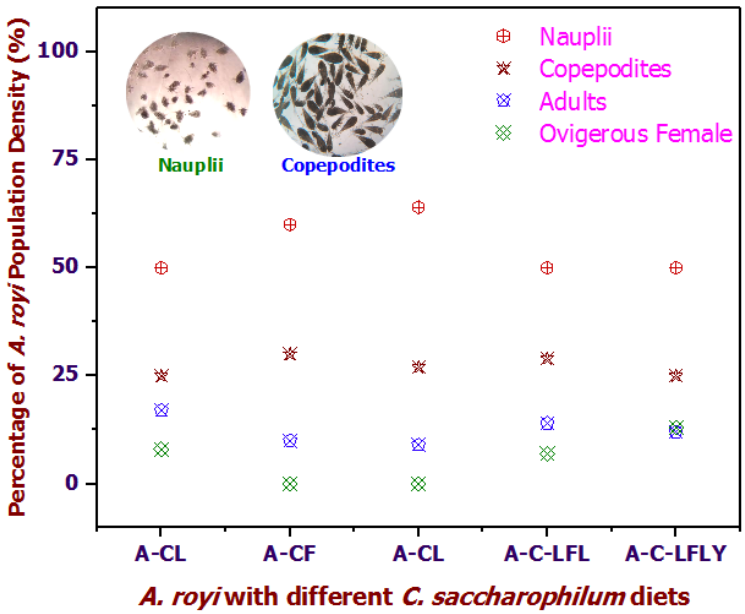

Figure 2. Population Density of *A. royi* with different *C. saccharophilum* feeding regimes.

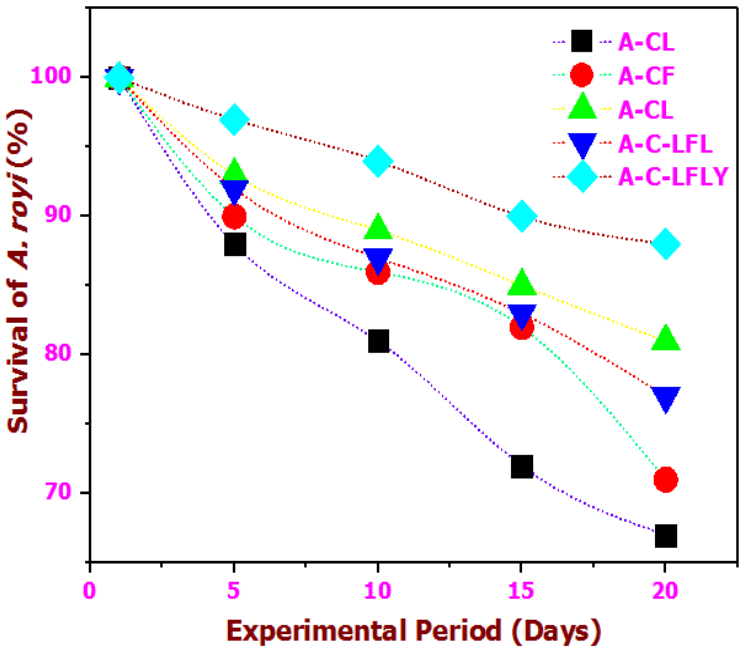

Figure 3. Survival of *A. royi* with feeding regimes.

Amino Acid Profile

The amino acid composition of A. royi varied significantly among the different *C. saccharophilum* feeding regimes (Table 3). Arginine was highest in A-CLy (9.87±0.37 nmol/mg), whereas threonine showed the highest concentration in A-C-LFLY (6.30±0.41 nmol/mg). Valine was also elevated in A-C-LFLY (6.43±0.37 nmol/mg), while histidine reached its peak in the same treatment (4.03±0.26 nmol/mg). Leucine was significantly higher in A-C-LFLY (8.33±0.37 nmol/mg), followed by A-CF (7.43±0.34 nmol/mg) and A-CLy (7.60±0.54 nmol/mg). Lysine was notably enriched in A-C-LFLY (9.47±0.37 nmol/mg), and isoleucine showed a similar trend, with the highest concentration in A-C-LFLY (6.33±0.49 nmol/mg). Phenylalanine was highest in A-C-LFLY (4.90±0.33 nmol/mg). Glutamic acid, the most abundant amino acid, reached its peak in A-C-LFLY (15.90±0.33 nmol/mg), while aspartic acid was also highest in the same treatment (11.20±0.45 nmol/mg). Alanine and glycine were significantly enriched in A-C-LFLY (8.40±0.37 nmol/mg and 11.20±0.45 nmol/mg, respectively). Serine and tyrosine were both highest in A-C-LFLY (6.67±0.45 nmol/mg and 6.90±0.43 nmol/mg,

Table 2. Fatty acid profile of *A. royi* with different *C. saccharophilum* feeding regimes (%)

Fatty acid profiles	A-CLi	A-CF	A-CLy	A-C-LFL	A-C-LFLY
C14:0	1.17±0.12^{a}	6.36±0.20^{c}	1.93±0.12^{a}	3.5±0.40^{b}	5.53±0.40^{c}
C14:1	0.17±0.04^{a}	1.73±0.12^{b}	0.50±0.03^{a}	1.9±0.24^{b}	0.12±0.06^{a}
C15:0	0.50±0.08	0.7±0.08	0.40±0.06	0.6±0.24	0.80±0.10
C16:0	11.40±0.14^{b}	11.6±0.16^{b}	10.6±0.29^{b}	7.73±0.20^{a}	20.86±0.57^{c}
C16:1	0.20±0.08^{a}	8.43±0.12^{c}	4.46±0.12^{b}	8.96±0.44^{c}	24.86±0.57^{d}
C17:0	1.77±0.12^{b}	0.67±0.13^{a}	1.04±0.16^{ab}	2.67±0.10^{c}	3.24±0.06^{c}
C18:0	3.37±0.16^{b}	0.53±0.04^{a}	4.03±0.69^{b}	5.37±0.16^{b}	0.77±0.11^{a}
C18:1 trans	0.20±0.08^{a}	2.5±0.08^{b}	10.16±0.62^{d}	35.66±0.77^{e}	4.14±0.47^{c}
C18:1 cis	2.27±0.12^{b}	1.7±0.08^{a}	1.27±0.56^{a}	4.75±0.34^{c}	6.89±0.82^{d}
C18:2 cis	2.83±0.09^{a}	2.83±0.04^{a}	2.7±0.29^{a}	4.3±0.32^{b}	3.06±0.33^{a}
C18:3 n-6	0.23±0.04^{a}	0.63±0.04^{a}	4.76±0.57^{c}	2±0.37^{b}	0.18±0.009^{a}
C18:4	7.30±0.08^{c}	6.7±0.16e	3.4±0.21^{a}	10.66±0.23d	4.78±0.01^{b}
C20:1	0.40±0.08^{a}	3.23±0.2^{c}	5.46±0.36d	2.5±0.24^{b}	0.17±0.01^{a}
C20:2	0.13±0.04^{a}	0.5±0.08^{ab}	0.16±0.02^{a}	0.63±0.20^{b}	1.27±0.52^{c}
C20:3 n-3	0.50±0.08^{ab}	0.22±0.08^{a}	1.7±0.16^{c}	0.66±0.20^{b}	0.70±0.14^{b}
C21:0	0.23±0.09^{ab}	0.11±0.06^{a}	0.3±0.11^{b}	0.53±0.08^{c}	0.76±0.16^{c}
C22:0	0.33±0.12^{ab}	0.23±0.04^{a}	0.48±0.08^{b}	0.55±0.11^{b}	0.84±0.18^{c}
C22:1	9.50±0.2^{d}	7.36±0.20^{c}	4±0.40^{a}	4.96±0.24^{ab}	5±0.36^{b}
C24:0	0.53±0.04^{b}	0.66±0.08^{bc}	0.84±0.16^{c}	0.72±0.06^{bc}	0.24±0.02^{a}
C24:1	0.23±0.04^{a}	1.46±0.09^{b}	2.93±0.09^{c}	0.56±0.24^{a}	1.12±0.10^{b}
C18:3n-3	3.2±0.40^{b}	2.6±0.08^{b}	3.93±0.49^{c}	5.36±0.36d	0.17±0.01^{a}
C20:5n-3	8.57±0.32^{b}	6.63±0.28^{a}	22.23±0.75^{d}	10.3±0.35^{c}	28.13±0.32^{e}
C22:6n-3	2.10±0.64d	2.53±0.28^{a}	8.5±0.40^{c}	6.33±0.36^{b}	8.56±0.28^{c}
C20:4n-6	0.43±0.04^{a}	0.4±0.08^{a}	0.63±0.16^{a}	0.8±0.08^{a}	6.94±0.67^{b}

Values represent mean±SD. Different superscripts (a, b, c, d, e) within a column indicate significant differences among feeding regimes (P<0.05, Tukey's HSD test). Feeding regimes with the same letter are not significantly different. (C14:0 (Myristic acid); C14:1 (Myristoleic acid); C15:0 (Pentadecanoic acid); C16:0 (Palmitic acid); C16:1 (Palmitoleic acid); C17:0 (Heptadecanoic acid); C18:0 (Stearic acid); C18:1 trans (TransOleic Acid (transvaccenic acid)); C18:1 cis (Oleic Acid); C18:2 cis (Linoleic Acid); C18:3 n-6 (GammaLinolenic Acid); C18:4 (Stearidonic Acid); C20:0 (Arachidic acid); C20:1 (Eicosenoic Acid); C20:2 (Eicosadienoic Acid); C20:3 n-3 (Eicosatrienoic Acid); C21:0 (Heneicosanoic Acid); C22:0 (Behenic Acid); C22:1 (Erucic Acid); C23:0 (Tricosanoic acid); C24:0 (Lignoceric acid); C24:1 (Nervonic Acid); C18:3n-3 (Alpha-linolenic acid); C20:5n-3 (Eicosapentaenoic acid); C22:6n-3 (Docosahexaenoic acid); C20:4n-6 (Arachidonic acid))

Table 3. Amino acid profile of *A. royi* with different *C. saccharophilum* feeding regimes (nmol/mg)

Amino acid	A-CLi	A-CF	A-CLy	A-C-LFL	A-C-LFLY
ARG	5.95±0.45^{a}	8.50±0.33^{b}	9.87±0.37^{c}	8.20±0.54^{b}	7.60±0.29^{b}
THR	3.80±0.53^{a}	5.4±0.24^{bc}	4.93±0.37^{b}	5.53±0.21^{bc}	6.30±0.41^{c}
VAL	4.56±0.80^{a}	5.37±0.31^{ab}	5.87±0.41^{ab}	5.93±0.29^{ab}	6.43±0.37^{b}
HIS	3.28±0.24^{b}	2.57±0.17^{a}	2.73±0.12^{ab}	2.67±0.26^{ab}	4.03±0.26^{c}
LEU	6.11±1.32^{a}	7.43±0.34^{ab}	7.60±0.54^{ab}	6.53±0.25^{a}	8.33±0.37^{b}
LYS	5.49±0.51^{a}	7.83±0.45^{b}	7.50±0.41^{b}	7.30±0.22^{b}	9.47±0.37^{c}
ILE	2.95±0.55^{a}	4.37±0.31^{b}	5.37±0.41^{b}	5.17±0.53^{b}	6.33±0.49^{c}
PHE	1.45±0.28^{a}	3.93±0.29^{b}	3.37±0.25^{b}	3.37±0.31^{bc}	4.90±0.33^{c}
GLU	11.73±0.45^{a}	14.43±0.25^{b}	14.23±0.26^{b}	14.33±0.45^{b}	15.90±0.33^{c}
ASP	6.47±0.73^{a}	9.97±0.37^{bc}	9.30±0.33^{b}	10.50±0.41^{bc}	11.20±0.45^{c}
ALA	6.46±0.73^{a}	7.50±0.08^{ab}	7.57±0.25^{ab}	8.30±0.34^{b}	8.40±0.37^{b}
GLY	4.65±0.47^{a}	8.87±0.37^{c}	6.33±0.21^{b}	7.50±0.33^{bc}	11.20±0.45d
SER	4.93±1.03^{ab}	4.43±0.25^{a}	4.90±0.33^{ab}	5.43±0.26^{ab}	6.67±0.45^{b}
TYR	5.28±0.16^{a}	5.83±0.57^{ab}	7.33±0.37^{b}	6.43±0.29^{ab}	6.90±0.43^{ab}
PRO	4.84±0.26^{a}	5.10±0.51^{a}	6.23±0.29^{b}	6.23±0.12^{b}	7.50±0.37^{c}

Values represent mean±SD. Means with different superscripts (a, b, c, d) within a column are significantly different (P<0.05, Tukey's HSD test). Feeding regimes with the same letter do not differ significantly. (ARG - Arginine; THR - Threonine; VAL - Valine; HIS - Histidine; LEU - Leucine; LYS - Lysine; ILE - Isoleucine; PHE - Phenylalanine; GLU - Glutamic acid; ASP - Aspartic acid; ALA - Alanine; GLY - Glycine; SER - Serine; TYR - Tyrosine; PRO - Proline)

respectively). Proline followed a similar pattern, with its highest concentration in A-C-LFLY (7.50±0.37 nmol/mg). Overall, the results suggest that the lyophilized and freeze-dried *C. saccharophilum* diets significantly enhance the essential and non-essential amino acid content of *A. royi*, potentially improving its nutritional quality for larviculture applications.

Discussion

In this study, a novel approach was adopted to enhance the high-density production of the marine copepod *A. royi* using the microalgal diet *C. saccharophilum*. To address the dietary and storage challenges, methodologies such as lyophilization and freezing were implemented. Various feeding regimes for *A. royi* were explored, including: Feeding with live *C. saccharophilum*; Feeding with frozen *C. saccharophilum*; Feeding with lyophilized *C. saccharophilum*; Feeding with a mixed diet of live, frozen, and lyophilized *C. saccharophilum*; Feeding with live, frozen, and lyophilized *C. saccharophilum* supplemented with yeast; Among these, *A. royi* fed with a mixed diet of live, frozen, lyophilized *C. saccharophilum* and yeast demonstrated significantly better growth, survival, and population density. Additionally, a higher nauplii production rate was recorded, indicating that this mixed diet is a suitable option for achieving high-density production of marine copepods in mass culture system. Similar findings have been reported in earlier studies. For instance, Jeyaraj and Santhanam (2013) observed that *Paracalanus parvus* achieved high densities when fed with various microalgal diets, such as *Chlorella marina* (1013±65.35 ind./L), *Dunaliella sp*. (431±34.8 ind./L), *Isochrysis galbana* (1374±76.30 ind./L), *Nannochloropsis* (769±52.50 ind./L), and *Tetraselmis* (990.3±86.55 ind./L) at a concentration of 20,000 cells/mL. Similarly, Chourpagar et al. (2021) reported high-density production of *Mesocyclops leuckarti* when fed with yeast. Furthermore, Nadiah et al. (2018) established that a combination diet of *Tetraselmis sp*. and *Nannochloropsis sp*. resulted in a maximum density of 1041±2.05 ind./L. In line with these studies, the findings of the present study indicate that *A. royi* fed with a mixed diet of live, frozen, lyophilized *C. saccharophilum* and yeast can serve as a reliable dietary combination for achieving high-density production of marine copepods, particularly *A. royi*.

The growth and development of early-stage of marine finfish larvae heavily rely on the nutritional value of their feed, particularly the presence of fatty acids (Dey et al., 2022; Zidan et al., 2024). Fatty acids serve as an essential source of energy, contribute to the formation of critical cellular components, and regulate various physiological processes (Samovski et al., 2023). They are also a vital metabolic energy source during embryonic development (Huang et al., 2022). From the fertilized egg stage to the open-mouthed larval stage, fatty acids such as C16:0 and C18:1 are primarily utilized as energy sources (Huang et al., 2022). Similarly, in our findings, *A. royi* fed with a mixture of diets like live, frozen, and lyophilized *C. saccharophilum* supplemented with yeast exhibited higher concentrations of these fatty acids. Although n-3 polyunsaturated fatty acids (PUFAs) constitute a significant proportion of the lipids in the cell membranes of marine fish, prostaglandin synthesis in marine fish predominantly arises from arachidonic acid (ARA) rather than eicosapentaenoic acid (EPA) (Chang et al., 2007; Siriwardhana et al., 2012). Docosahexaenoic acid (DHA) is crucial for the development of the brain and optic nerve in marine fish (Mourente et al., 1991; Garg et al., 2017). A deficiency in DHA can impair vision and neural functions, potentially leading to abnormal behavior (Forsyth et al., 2017; Garg et al., 2017). In our study, higher levels of DHA were observed in *A. royi* fed with the mixed diet, making it a highly suitable first feed for marine finfish larvae to meet their nutritional requirements. Regarding the amino acid profile required by marine finfish, amino acids play a critical role in normal metabolic processes, particularly during the early developmental stages (Sikorski et al., 2020). Amino acids perform two main functions during larval ontogeny: they are involved in protein synthesis and serve as a source of energy through decomposition and utilization (Li et al., 2021). Our study revealed higher concentrations of amino acids in *A. royi* fed with the mixed diet of live, frozen, and lyophilized *C. saccharophilum* supplemented with yeast. This nutrient-rich profile supports energy metabolism (Li et al., 2009; Finn and Fyhn, 2010; Tocher, 2010). The yeast *Saccharomyces cerevisiae* is a highly suitable feed for live food organisms, including copepods, significantly enhancing their growth up to ten times higher than when fed marine microalgae alone. It serves as an effective growth enhancer in aquahatcheries. Additionally, *S. cerevisiae* is a rich source of essential fatty acids and amino acids, making it a valuable nutritional supplement for live food organisms. However, sudden high mortality in live food cultures can occur due to insufficient nutrient availability when relying solely on yeast. In contrast, a diet combining yeast with marine Chlorella has been shown to support higher population densities and contribute to the successful mass production of marine finfish larvae during hatchery rearing (Watanabe and Ahmed Ali, 1987). Supporting earlier studies, the elevated levels of glutamic acid, glycine, and lysine in the A-C-LFLY group suggest that yeast plays a crucial role in boosting amino acid content. This enhancement contributes to improved seed production, further highlighting yeast's significance in aquaculture nutrition.

Earlier studies have indicated that yeast serves as a universal feed for enhancing the high-density production of live food organisms, including copepods (Winding et al., 2022; Sharma et al., 2023). Our previous study demonstrated that *C. saccharophilum* enhances egg production, improves survival rates, and provides

rich nutrients to meet the nutritional requirements of marine Copepod, *A. royi* (Vijayaraj et al., 2022). Furthermore, lyophilization of *C. saccharophilum* preserves its nutrient content without significant loss, as reported in earlier studies (Madhubalaji et al., 2021). Additionally, live microalgae remain active and floating within the *A. royi* culture system (Vijayaraj et al., 2022; Ma and Hu, 2024), while different preserved forms of *C. saccharophilum*, including live microalgae, distribute evenly throughout the culture system, ensuring continuous nutritional availability for *A. royi*. This approach offers a sustainable alternative for enhancing copepod culture to support marine finfish seed production. Regarding nutrient retention, lyophilization removes water content while preserving cellular integrity, resulting in a more nutrient-dense product. Unlike freezing, which can cause some degradation of cellular components, lyophilization minimizes nutrient loss and preserves essential biomolecules, including proteins and fatty acids.

The results indicate that *A. royi* fed with this mixed diet provides superior nutritional value and energy requirements for marine finfish larvae, making it an excellent choice for use in aquaculture hatcheries. This approach aligns with sustainable aquaculture practices by addressing the nutritional needs of early-stage fish larvae and promoting their higher survival and growth rates.

Conclusion

This study demonstrates that a mixed diet of live, frozen, and lyophilized *C. saccharophilum* (A-C-LFLY) enhances *A. royi* productivity by increasing population density, improving survival rates, and providing a rich nutritional profile, particularly in fatty acids and amino acids, to meet the dietary needs of marine fish larvae. The lyophilization techniques employed in this study support year-round copepod culture, offering a sustainable solution for live feed production in marine aquaculture. Additionally, this standardized protocol developed for high-density copepod production presents a cost-effective approach for marine aquaculture, promoting the adoption of copepods as a primary live feed in aquahatcheries.

Ethical Statement

Ethical review and approval were waived for this study as it complied with the regulations of the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Department of Animal Husbandry and Dairying, Ministry of Fisheries, Animal Husbandry and Dairying, Government of India (2021). Additionally, the guidelines of the Indian National Science Academy were followed. Ethical approval by an Animal Welfare Committee is not required for studies involving Planktons. The Copepods and Microalgae used in this study were maintained in a separate laboratory facility under optimized conditions designed to mimic natural environments.

Funding Information

Science and Engineering Research Board, Department of Science and Technology, Government of India for funding a major research project (EEQ/2023/000185).

Author Contribution

Conceptualization: VR, PKD, Data Curation: PKD, TRP, Formal Analysis: PKD, TRP Funding Acquisition: VR, Investigation: PKD, TRP, Methodology: AK, VR, Project Administration: VR, Resources: VR, AK, Supervision: VR, AK, Visualization: VR, PKD, TRP, Writing -original draft: VR, PKD, Writing -review and editing: VR, AK.

Conflict of Interest

The authors have no conflict of interest.

Acknowledgements

The authors are thankful to the Science and Engineering Research Board, Department of Science and Technology, Government of India for funding a major research project (EEQ/2023/000185). The authors express their gratitude to the Management of AMET University for providing research facilities to carry out this work.

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