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Influence of the green microalga, *Chlorococcum* sp. on the growth of freshwater rotifer, *Brachionus calyciflorus* Pallas

Abstract

The success of the aquaculture sector relies on a consistent supply of healthy fish seeds. Rotifer has been identified as superior live food to artificial feed for nurturing fish larvae, the culture development of which largely depends on green microalgae. This study aimed to investigate the suitability of *Chlorococcum* sp. for enhancing the production of *Brachionus calyciflorus*. Two experiments were conducted to assess the effects of different food types and concentrations of *Chlorococcum* sp. on the growth of *B. calyciflorus*. In the first experiment, three food types were tested: live *Chlorococcum* sp. (1 x 10^5 cells/mL; T_1), baker's yeast (0.2 gm/L; T_2), and a combination of live *Chlorococcum* sp. and baker's yeast (0.5 x 10^5 cells/mL + 0.1 gm/L; T_3). The highest population density and growth rate (r) of rotifers were observed in the T_1 diet. In the second experiment, three concentrations of *Chlorococcum* sp. were tested: 0.5 x 10^6 cells/mL (T_1), 1 x 10^6 cells/mL (T_2), and 3 x 10^6 cells/mL (T_3). Both the population density and growth rate of *B. calyciflorus* were found highest in the T_3 diet. In conclusion, *Chlorococcum* sp. at a concentration of 3 x 10^6 cells/mL is suggested as the best food for the successful mass culture of the rotifer *B. calyciflorus*.

Keywords: *Brachionus calyciflorus*, *Chlorococcum* sp., culture, growth rate, population density

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Introduction

Aquaculture is currently one of the fast-growing food production industries. To ensure the food and nutrition security of the global population, there is a prime need to make strides in aquaculture, the progress of which largely depends upon the advancement of fish larval-rearing techniques. The availability of sufficient healthy fish seeds is a crucial requirement for a successful fish culture.[1] Nevertheless, the major impediment to fish seed production in hatcheries is ensuring a consistent supply of live food for fish larvae. Over the past few decades, rotifers have proven to be the most suitable species for the initial feeding of larvae for numerous commercially important fishes. The mass culture of *Brachionus* sp. is a key factor in the success of the global aquaculture sector.[2,3]

Rotifers belonging to the genus *Brachionus* are commonly used as live food in the rearing of larvae of both freshwater and marine species of fish, shrimps, crabs, prawns, and mollusks. The nutritional value, density, and body size of rotifers all play critical roles in the feeding, development, and survival of larval fish.[4,5] Various types of food, including naturally occurring ones like microalgae and chemically prepared diets such as micro-encapsulated pellets have been extensively used for the mass-production of *Brachionus* sp.[6,7] A higher proportion of *Brachionus* individuals bearing multiple amictic eggs is observed when more suitable food is available.[8,9] Baker's yeast is also utilized in the large-scale production of *Brachionus* spp.[10] which further unveiled the poor nutritional quality of yeast-fed rotifer.[11] Among various feed types, microalgae have been predominantly used as food in rotifer cultures.[12,13] Under both field and laboratory conditions, diverse microalgae are used for feeding planktonic rotifers because producing microalgae on a large scale is substantially less expensive than producing other prepared meals.[14, 15]

Microalgae are frequently used for zooplankton rearing because of their ability to produce higher biomass in a short period and their enriched nutritive profile.[16–18] One of the most often employed types of microalgae for feeding larval fish and zooplankton is the green microalgae from the Chlorophyceae family. Among them, *Chlorococcum* sp. is a green freshwater microalga that is considered one of the highest carotenoid producers including astaxanthin, lutein, zeaxanthin, neoxanthin, canthaxanthin, and violaxanthin that may have great significance in aquaculture industry.[19–21] *Chlorococcum* sp. was examined for its potential to inhibit cholinesterase and for its antioxidant properties.[22] *Chlorococcum* sp. can produce astaxanthin, which includes several critical metabolic capacities counting upgrade of safe reactions and security against infections such as cancer by rummaging oxygen-free radicals.[23] Astaxanthin-producing microalgae are also broadly utilized as a shade-added substance within the aquaculture industry.[23,24] Consequently, for the desirable growth of rotifers, the green microalga *Chlorococcum* sp. may be a viable live food source.

Numerous biotic and abiotic factors have significant effects on rotifer density.[25] The number of rotifers is significantly influenced by biotic elements such as phytoplankton diversity and concentration.[12] Laboratory feeding trials of *B. calyciflorus* exhibit increased algal intake with an increase in feed concentration until an asymptote is reached.[25,26] Microalgal species broadly used in rearing rotifers are namely *Chlorella ellipsoidea*,[27] *Chlorella* sp., *Scenedesmus obliquus*,[25,28,29] *Scenedesmus acutus*,[12] *Chlorella vulgaris*,[12–14,30] *Aurantiochytrium* sp., *Isochrysis* sp., *Nannochloropsis* sp.[31] and *Monoraphidium contortum*.[32] The effect of astaxanthin-producing green microalga *Chlorococcum* sp. on the culture and growth of *B. calyciflorus* has not been studied yet. With that in mind, the effects of green microalga *Chlorococcum* sp. on the growth rate and population density of rotifer *B. calyciflorus* were investigated.

Materials and methods

Collection and culture of the microalga and the rotifer

The previously maintained pure strain of the green microalga *Chlorococcum* sp. in the "Laboratory of Plankton Research, Department of Fisheries Management, Bangladesh Agricultural University, Mymensingh" was used for this study (Figure 1). *Chlorococcum* sp. was mass cultured in Bold Basal Medium (BBM) (Figure 2). Microalgal cells in the log phase of growth were harvested, centrifuged at 3000 rpm for 7 minutes, rinsed with distilled water, and resuspended in moderately hard water that was prepared by dissolving 96 mg of $NaHCO_3$, 60 mg of $CaSO_4$, 60 mg of $MgSO_4$, and 4 mg of KCl in one liter of distilled water (EPA medium).[33]

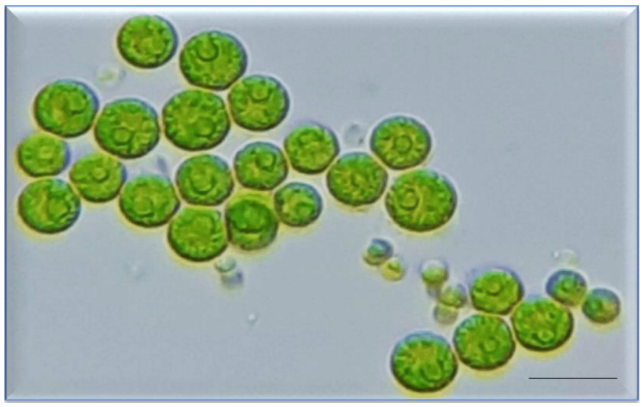

Figure 1 Pure cells of green microalga *Chlorococcum* sp. Scale bar: 10 µm.

Figure 2 Mass culture of *Chlorococcum* sp. in the Bold Basal Medium (BBM).

The rotifer species *Brachionus calyciflorus* was isolated from the backyard pond of the Faculty of Fisheries, Bangladesh Agricultural University, Mymensingh-2202, Bangladesh. *Chlorococcum* sp. was used as food to successfully culture the rotifer *Brachionus calyciflorus*.

Crude protein and crude lipid determination of *Chlorococcum* sp.

Crude protein and crude lipid content of *Chlorococcum* sp. were analyzed in triplicates by following the method of Ritu *et al.*[16]

Experimental design

Two experiments were conducted to investigate the effects of different food types and cell concentrations of *Chlorococcum* sp. on the population density and growth rate of *B. calyciflorus* (Figure 3). In the first experiment, the effects of live *Chlorococcum* sp. (1 x 10^5 cells/mL; T_1), baker's yeast (0.2 gm/L; T_2), and a combination diet of baker's yeast + *Chlorococcum* sp. (0.5 x 10^5 cells/mL + 0.1 gm/L; T_3) on the growth of the rotifer *B. calyciflorus* was examined. In the second experiment, the impact of three different concentrations of the green microalga *Chlorococcum* sp. on the growth of the rotifer *B. calyciflorus* was examined. The concentrations of the microalgae given as feed to the rotifers were 0.5 x 10^6 cells/mL (T_1), 1 x 10^6 cells/mL (T_2), and 3 x 10^6 cells/mL (T_3). In both tests, rotifers were grown under usual conditions in 20 L container using EPA medium with an initial density of 6 individuals/ mL, and the food was given two times a day – at 8:30 AM and at 8:30 PM. During the experiment, the room temperature was 25 ± 2°C. By obtaining 2-3 aliquot samples from each container the density of the rotifers was estimated every day under a microscope (B-510BT OPTIKA, Italy) at a magnification of ×10. After 12 days, the experiment was closed since the quantity of *B. calyciflorus* started to decrease. Based on the collected data, the rate of population increase (r) was calculated using the exponential growth equation as follows:

$$r = (\ln N_t - \ln N_0) / t$$

Where, N_0 = initial population density, and N_t = density of population after time t (days).[34] The value of r was obtained from a mean of 4-5 values during the exponential phase of the population growth in each treatment.

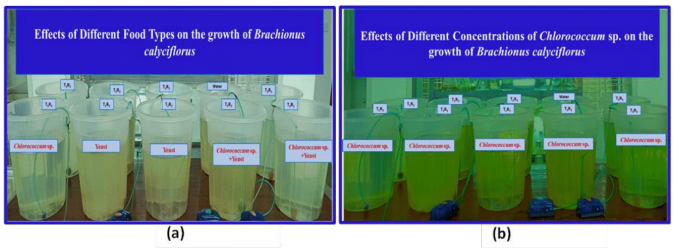

Figure 3 Experimental setups of *Brachionus calyciflorus* culture: (a) Feed given in Experiment I [T_1 = Live *Chlorococcum* sp. (1 x 10^5 cells/mL), T_2 = Baker's yeast (0.2 gm/L), and T_3 = Live *Chlorococcum* sp. 0.5 x 10^5 cells/mL + Baker's yeast 0.1 gm/L, and (b) Feed given in Experiment 2 [T_1 = 0.5 x 10^6 cells/mL, T_2 = 1 x 10^6 cells/mL, and T_3 = 3 x 10^6 cells/mL of live *Chlorococcum* sp.].

Statistical analysis

The data were analyzed using one-way ANOVA (SPSS 25). Significant differences among means were determined using Duncan's multiple range test (DMRT). Differences were considered significant at $P < 0.05$.

Results

Crude protein and crude lipid content of *Chlorococcum* sp.

On a dry matter basis, the crude protein and crude lipid content of *Chlorococcum* sp. were 30.5% and 18.2%.

Experiment I

The population growth curve of the rotifer, *B. calyciflorus* in response to three different feed treatments (live *Chlorococcum* sp., baker's yeast, and a combination diet of live *Chlorococcum* sp. + baker's yeast) is shown in Figure 4. The food types resulted substantial impact on the rotifer's maximal population density. When *Chlorococcum* sp., baker's yeast, and a combination diet of live *Chlorococcum* sp. + baker's yeast were fed, the highest population densities of *B. calyciflorus* were found as 255 ± 11, 182 ± 14, and 72 ± 4 individuals/mL in T_1, T_3, and T_2, respectively (Figure 4). In T_2

where yeast was used as feed, the rotifer population peaked on the 7th day of the culture period. The highest population densities of the rotifer were found on the 8th day of the culture period where the live *Chlorococcum* sp. (T_1) and a combination diet of live *Chlorococcum* sp. + baker's yeast (T_3) were fed. Among the three treatments, *B. calyciflorus* showed the best growth in terms of population density in T_1 where live *Chlorococcum* sp. was fed (Figure 5).

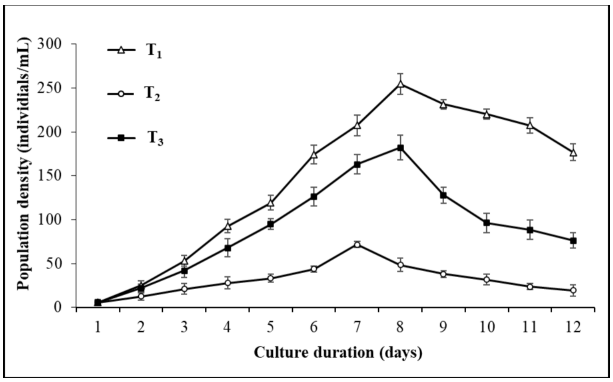

Figure 4 Population density of freshwater rotifer, *Brachionus calyciflorus* (individuals/mL) fed on different diets (T_1 = Live *Chlorococcum* sp. 1 x 10^5 cells/mL; T_2 = Baker's yeast 0.2 gm/L; T_3 = Live *Chlorococcum* sp. 0.5 x 10^5 cells/mL + Baker's yeast 0.1 gm/L). Each point and vertical line represents mean ± SD for three replicates.

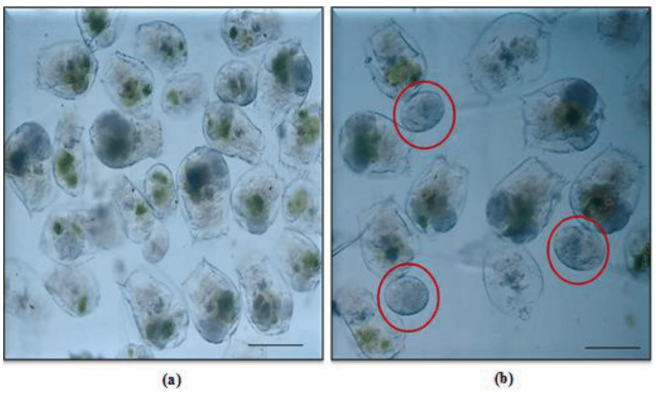

Figure 5 Micrographs showing (a) *Chlorococcum* fed individuals of *Brachionus calyciflorus*, and (b) Eggs of *B. calyciflorus* within red circles. Scale bar: 100 µm.

Food type showed a highly significant ($P< 0.05$) impact on the population growth rate (r) of *B. calyciflorus* (Figure 6). The food types considerably altered the population growth rate (r) for *B. calyciflorus,* which was 0.587 ± 0.01, 0.35 ± 0.02, and 0.53 ± 0.01 day^{-1} in T_1 (live *Chlorococcum* sp.), T_2 (baker's yeast), and T_3 (a combination diet of live *Chlorococcum* sp., + baker's yeast) respectively.

Experiment II

The population growths of the rotifer, *B. calyciflorus* raised in three different concentrations of *Chlorococcum* sp. are shown in Figure 7. The microalgal concentration significantly ($P< 0.05$) impacted the rotifer population density. The highest rotifer density of 128 ± 6 (in T_1), 255 ± 11 (in T_2), and 399 ± 5 individuals/mL (in T_3) were found on the 7th, 8th, and 10th day of the culture period respectively (Figure 7). Rotifer growth began to decrease after the 7th day of culture in treatment where a concentration of 0.5 x 10^6 cells/mL of *Chlorococcum* sp. was given as feed. At 1 x 10^6 cells/mL microalgal concentration, rotifer development began to decrease from the 9th day. In T_3 where 3 x 10^6 cells/mL of *Chlorococcum* sp. was given as feed, the maximum density of the rotifer was obtained on the 10th day and then it began to decrease from the 11th day.

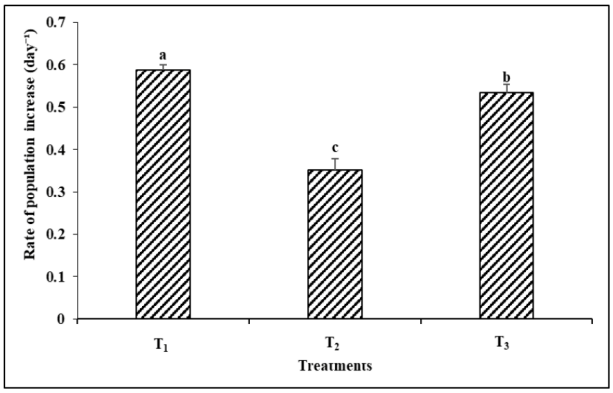

Figure 6 Rate of population increase (r) per day for *Brachionus calyciflorus* **in relation to**/ with different food types. (T_1 = Live *Chlorococcum* sp. 1 x 10^5 cells/mL; T_2 = Baker's yeast 0.2 gm/L; T_3 = Live *Chlorococcum* sp. 0.5 x 10^5 cells/mL + Baker's yeast 0.1 gm/L). Each bar and vertical line represent mean ± SD for three replicates. Means with different letters are significantly different from one another ($P< 0.05$).

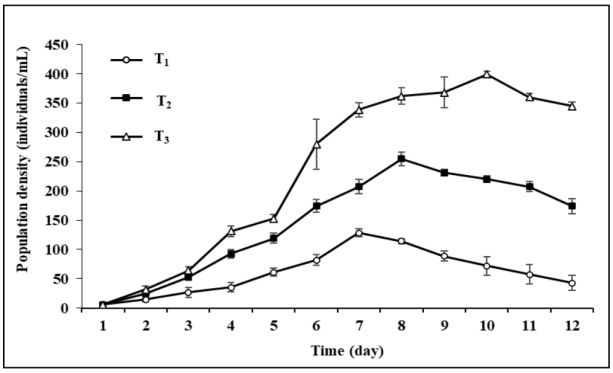

Figure 7 Population density of freshwater rotifer, *Brachionus calyciflorus* (individuals/mL) fed on different concentrations of *Chlorococcum* sp. (T_1 = 0.5 x 10^6 cells/mL; T_2 = 1 x 10^6 cells/mL; T_3 = 3 x 10^6 cells/mL). Each point and vertical line represent mean ± SD for three replicates.

The highest population growth rate (r) of *B. calyciflorus* was recorded as 0.66 ± 0.01 day^{-1} at the concentration of 3 x 10^6 cells/mL of *Chlorococcum* sp. which was significantly higher ($P<0.05$) than other food types (Figure 8). The growth rate of the rotifer was 0.59 ± 0.01 and 0.45 ± 0.03 day^{-1} when fed 1 x 10^6 cells/mL and 0.5 x 10^6 cells/mL of *Chlorococcum* sp. respectively.

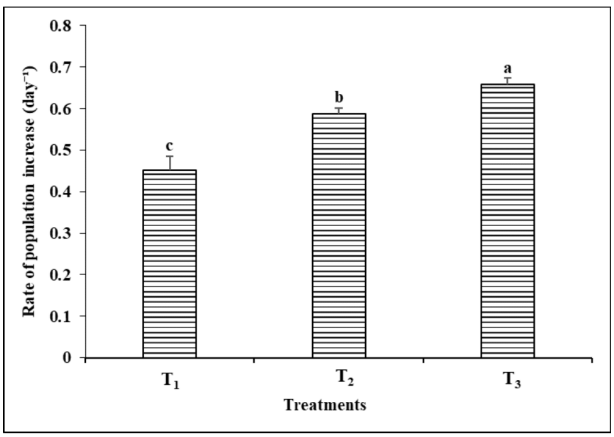

Figure 8 Rate of population increase (r) per day of *Brachionus calyciflorus* in relation to different cell concentrations of *Chlorococcum* sp. given as feed (T_1 = 0.5 x 10^6 cells/mL; T_2 = 1 x 10^6 cells/mL; T_3 = 3 x 10^6 cells/mL). Each bar and vertical line represent mean ± SD for three replicates. Means with different letters are significantly different from one another ($P< 0.05$).

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Discussion

Both feed type and feed concentrations have remarkable effects on the population growth rates of rotifers.[13] In the first experiment, three treatments including live *Chlorococcum* sp., baker's yeast, and a combination diet of live *Chlorococcum* sp. + baker's yeast were used in the culture of the rotifer *Brachionus calyciflorus*. The population growth of the rotifer *B. calyciflorus* was found higher (255 ± 11 individuals/mL) in T_1 where they were fed only live *Chlorococcum* sp. followed by 182 ± 14 individuals/mL in T_3 (a combination diet of *Chlorococcum* sp. and baker's yeast) and 72 ± 4 individuals/mL in T_2 (only baker's yeast). Compared to other diets the live *Chlorococcum* sp. was found best for the growth of the rotifer *B. calyciflorus* which is supportive of the findings of Akter et al.[27] The highest population density of *B. calyciflorus* was reported as 28.6 ± 5 individuals/mL when fresh live *Chlorella ellipsoidea* was fed[27] which was far lower than the present findings. Sarma *et al.*[13] also showed the maximum population density (103 ± 8 individuals/mL) and growth rate (0.63 ± 0.04 day^{-1}) of *B. calyciflorus* when fed *Chlorella vulgaris* which is also far lower than the present study. The growth patterns of *B. calyciflorus* might potentially be influenced by the nutritional value and digestibility of the microalgal cells.[14,35,36] On the other hand, in the present study baker's yeast didn't show satisfactory growth of the rotifer. Hirayama and Funamoto[11] have also stated that baker's yeast is unstable for zooplankton culture. But when baker's yeast was supplemented in a combination with live *Chlorococcum* sp., *B. calyciflorus* attained higher peak population abundance compared to the rotifer population found in the diet of only yeast. Khatun et al.[37] also got satisfactory production of *B. calyciflorus* (67 ± 8 individuals/mL) when fed a combination diet of yeast with fresh *Chlorella vulgaris* which is far lower than the population density (182 ± 14 individuals/mL) found in the treatment of combination diet of the present study. Hence, in the controlled laboratory conditions, whether served *Chlorococcum* sp. solely or as part of a diet that contains yeast, has a positive impact on the population growth of *B. calyciflorus*. Consequently, despite not being equivalent to *Chlorococcum* diet, yeast can be employed in rotifer growth systems at low concentrations to complement the needs of algae.

In the second experiment, the concentration of the microalga *Chlorococcum* sp. as food resulted in a direct impact on the increase of the population density of the rotifer *B. calyciflorus* agreeing with the findings of several earlier investigations, both from field collections and at laboratory studies.[38,39] Rotifers, reported as opportunistic species that respond more quickly to changes in microalgal food levels[40] are also confirmed in the present study. The suitability of a microalgal species as food for rotifers is influenced by several elements, including size, shape, motility, digestibility, and nutritional content.[41–44] In the present study, with the increasing rate of the concentration of *Chlorococcum* sp. as a feed from 0.5 x 10^6 cells/mL to 3 x 10^6 cells/mL, the population abundance of *B. calyciflorus* was also increased. A similar result was found by Nhi et al.[45] where the population density of *B. angularis* increased with elevated concentration of the cultured *Chlorella* sp. as feed. A similar increasing growth pattern with the rise in food levels has been documented in several rotifer genera of the family Brachionidae.[13,14,29,39,46] Depending on the amount of microalgal food concentration, the peak abundance of the rotifer *B. calyciflorus* in the current research varied from 128 ± 6 to 399 ± 5 individuals/mL. The difference in microalgal concentration also significantly changed the population growth rate (r) of *B. calyciflorus*. The population growth rate of *B. calyciflorus* rose with increased microalgal feed concentration. The growth rates of *B. calyciflorus* were 0.45 ± 0.03/day in T_1, 0.59 ± 0.01/day in T_2, and 0.66 ± 0.01/day in T_3 where *Chlorococcum* sp. was given as feed at the rate of 0.5 x 10^6 cells/mL, 1 x 10^6 cells/mL and 3 x 10^6 cells/mL respectively. The growth rate trends related to the concentration of the microalgal feed given in the present study are similar to the findings of Kenari et al.[25] who reported the growth rate of *B. calyciflorus* as 0.18, 0.42, and 0.51/d when fed *Chlorella* sp. at the cell concentration of 0.1 × 10^6, 1 × 10^6, and 10 × 10^6 cells/mL respectively, though the growth rates in the present study are higher than those reported by them. These may be due to the variation in the nutritional status of different species of microalgae given as feed to the rotifers or the environmental conditions during the culture period. Finally, it can be said that the green microalga *Chlorococcum* sp. is a nutritious species enriched with a higher content of crude protein (30.5%) and lipid (18.2%) that can be used for mass-scale commercial production of the rotifer *B. calyciflorus.* However, repeated outdoor mass culture trials are necessary before the dissemination of the culture technology to the rural fish farmers and hatchery operators.

Conclusion

The findings of the study revealed that the cultured live green microalga *Chlorococcum* sp. is very suitable for mass culture of the rotifer *Brachionus calyciflorus.* The population growth of the rotifer *B. calyciflorus* was found to increase with the increased concentration of the live microalgal feed. Outdoor large-scale mass culture of the microalga *Chlorococcum* sp. and subsequently the rotifer *B. calyciflorus* using the microalgae may play a significant role in the enhancement of aquaculture by using both of them as live feed in rearing fish larvae in hatcheries. Further outdoor research is needed before the implementation of the findings at the farm/hatchery level.

Author contributions

Md. Sayem Ahmed: Investigation, Formal Analysis and writing -Original Draft; **Jinnath Rehana Ritu:** Investigation, Formal Data Analysis, Graphical Abstract Preparation and Writing - Original Draft; **Saleha Khan:** Conceptualization, Methodology, Supervision, Resources, Project Administration, Fund Acquisition and Writing - Review and Editing; **Md Helal Uddin:** Writing - Review and Editing; **Sadiqul Awal:** Writing - Review and Editing; **Md. Mahfuzul Haque:** Supervision, Writing - Review and Editing; **Md Kowshik Ahmed:** Formal Data Analysis and Graphical Abstract Preparation, **Md. Shahin Alam:** Formal Data Analysis and Graphical Abstract Preparation.

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Conflicts of interest

The author(s) declare that they have no known competing financial or non-financial, professional, or personal conflicts that could have appeared to influence the work reported in this paper.

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