Indian J. Fish., 58(2) : 59-65, 2011

Nutritional composition of rotifer (*Brachionus plicatilis* Muller) cultured using selected natural diets

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ABSTRACT

The rotifer (*Brachionus plicatilis* Muller) was cultured by feeding on phytoplankton monocultures (*Nannochloropsis* sp. and *Isochrysis galbana),* mixed algal cultures (*Nannochloropsis* sp. + *Isochrysis galbana*; at 1:1 ratio), and a probiotic culture (*Nannochloropsis* sp. + *Bacillus licheniformis* MTCC 6824 at 1:1 ratio). Biochemical compositions of rotifers fed on different diets were determined on day 4 of the culture. Protein and total lipid levels were the maximum when fed with *I. galbana* (46.02 ± 0.226% and 36.1 ± 0.07% respectively). Though dry matter content was the highest in the probiotic culture (5.4 ± 0.1%), ash free dry weight was the highest for those fed with *Nannochloropsis* sp. Carbohydrate was the highest (14.62 ± 0.1%) in rotifers fed with *Nannochloropsis* sp. Total amino acids excluding tryptophan was found to be 28.93 g 100 g rotifer^{-1} when fed with *Nannochloropsis* sp. The maximum energy content per gram dry rotifer sample was 17.61 K calories when fed with *Nannochloropsis* sp. Rotifer density, lipid, carbohydrate and protein content of rotifers fed with *I. galbana* and *Nannochloropsis* sp. indicate that these are the most suitable diets for the rotifer.

Keywords: *Bacillus licheniformis, Brachionus plicatilis, Isochrysis galbana, Nannochloropsis* sp., Natural diets

Introduction

The rotifer *Brachionus plicatilis* is indispensable for aquaculture, as it is widely used as a primary food organism for the initial larval stages of many finfish and crustacean larvae (Ando *et al.*, 2004; Cheng *et al*., 2004). Rotifer forms an excellent initial food because of its appropriate size (130-320 µm), planktonic nature, rapid production rate, suitability for mass culture under controlled conditions, ability to grow and reproduce in high density cultures and the possibility of artificially manipulating its nutritional qualities along with the euryhaline nature (Fielder *et al*., 2000; Dhert *et al*., 2001).

B. plicatilis feeds on microalgae, protozoa, bacteria and dead organic materials (Rezeq and James, 1987; Oie and Olsen, 1997) in addition to artificial feeds. Diet is regarded as the most important criterion that could affect growth as well as nutritive quality of rotifers (Lubzens, 1987; Nhu, 2004). The lipid content and fatty acid composition of marine microalgae vary among species and culture conditions (Reitan *et al.*, 1994), and the algae fed to rotifer cultures or larval tanks will alter the lipid and fatty acid composition of the rotifers (Watanabe *et al*., 1983; Ben-Amotz *et al*., 1987).

Larviculture is the major bottleneck for industrial scaling up of the culture of marine finfish and shellfish. Research on live food production for marine fish larvae is essential for successful mariculture operations. The objective of the study was to study the nutritional composition of *B. plicatilis* grown using microalgae and bacteria as diets. A detailed data on the nutritive value of *B. plicatilis* fed on different natural diets, if made available, would help the entrepreneurs and hatchery operators to identify a cost effective and nutritionally complete rotifer culture system, especially when enrichment media available are only imported, expensive and the process is cumbersome.

Materials and methods

Algal culture

The algal species used in the study were the chlorophycean algae *Nannochloropsis* sp. and the haptophycean flagellate *Isochrysis galbana*. The stock cultures of the algae were obtained from the algal culture collection of Central Marine Fisheries Research Institute (CMFRI), Cochin. The stock cultures were maintained in Guillard and Ryther's modified F/2 medium in 10 ml culture tubes under axenic conditions (Droop, 1967).

The stock cultures were sub-cultured into 3-10 l batch cultures that were maintained under controlled conditions

at 25 °C and constant illumination. Mild aeration using diffuser stones was provided. Algae were harvested during the log phase of growth period by centrifugation (5000 X g) and used to feed the rotifers. The number of cells was determined in diluted aliquots of suspended concentrate by counting in a haemocytometer. Guillard and Ryther's modified F/2 medium was used for mass culture also.

Bacterial supplement for B. plicatilis culture

Bacillus licheniformis MTCC 6824 maintained in 50% peptone water (Himedia, Mumbai) was used as a bacterial supplement in combination with the *Nannochloropsis* sp. (1:1) for rotifer culture.

Diets and feeding

In the first two trials, phytoplankters *Nannochloropsis* sp. and *I. galbana* were used individually as feed. Individual algae were fed at the rate of $7x10^6$ cells ml^{-1} to the rotifer (Arajuo and Hagiwara, 2005; Zhang *et al*., 2005). In the third trial, the algae (*Nannochloropsis* sp. and *I. galbana*) were mixed (1:1) and fed to the rotifer. The fourth trial was carried out with a bacterial supplementation using *B. licheniformis* MTCC 6824 along with *Nannochloropsis* sp in 1:1 ratio. Whenever, the culture water got cleared of algae, freshly harvested quantity of algae was fed to the rotifer tanks.

Culture of B. plicatilis

The inoculum of *B. plicatilis* obtained from the Marine Research Hatchery of CMFRI, was maintained in 50 ml culture tubes as stock culture. Sterile seawater (30 ppt and pH above 7.5) was used for the culture. Rotifers were inoculated at 2 nos. ml^{-1}, and 4 ml of *Nannochloropsis* sp. (36×10^6 cells ml^{-1}) was added daily to the culture tubes. Culture tubes were kept 20 cm away from continuous light source of 3000 lux illumination and the temperature was maintained at 28 ± 1 °C. The rotifer stock cultures were disinfected using 10 mg l^{-1} of oxytetracycline for 24 h and the treatment was repeated after 24 h to obtain a pure stock of *B. plicatilis* (Hagiwara *et al*., 1994). Scaling up of *B. plicatilis* was carried out initially in 500 ml conical flasks containing 300 ml filtered sterile seawater. The flasks were inoculated with rotifers at 2 nos. ml^{-1} and 4 ml fresh algae were added to the flasks on the first day and subsequently doubled till the final harvest on day 7. Mass culture was carried out in cylindro-conical fibreglass tanks of 65 l capacity. A modified batch culture system was followed for rotifer culture (Lubzens and Zmora, 2003). The culture in each tank was started with one-fifth of its volume filled with dense algal cultures. Four culture trials were conducted during the study period. Rotifers were stocked at a density of 50 nos. ml^{-1}. Continuous aeration was provided in all the culture tanks. The culture temperature varied from 24 to 28 °C. Volume of the culture medium was increased daily with 30 ppt seawater and 10-30% of the water from the culture tanks was removed as and when needed. Whenever the culture water became free from algae, freshly harvested algae were introduced to the culture. Final volume of the tank (65 l) was reached after 4-5 days and the rotifers were concentrated by siphoning out and sieving through 20- 30 µ bolting cloth. Harvested rotifers were washed with distilled water to remove the salt content and residues of food material. Rotifer samples thus collected were used for wet weight determination and stored in refrigerator for further analysis.

Biochemical analysis of rotifer

Rotifers were washed with distilled water on glass fiber filter, dried to a constant weight at 60 °C, (Theilacker and Kimball, 1984) and weighed to a precision of ±2 µg. Samples were scraped off from the glass fibre filter for further analyses. For ash estimation, a pre-weighed amount of dry powdered sample in silica crucible was ignited in a muffle furnace (Barnstead, USA), at 550 °C for 4 h till all the organic matter was burnt out leaving no carbon residue. The ignited content was weighed after cooling to room temperature and the difference in weight was taken as the ash content of the tissue (Ben-Amotz *et al*., 1987).

The ash content values obtained in percentage were subtracted from the dry weight to find out the ash free dry weight and expressed as percentage dry matter. Protein content in the rotifer samples was determined by Lowry method (Lowry *et al*., 1951). The phenol-sulphuric acid method of Dubois *et al.* (1956) was followed to estimate the carbohydrate content in the rotifer samples.

Amino acid analysis was performed by reverse-phase high-performance liquid chromatography after pre-column derivatization by phenyl isothiocyanate (PITC) by a modified method adapted from Fierabracci *et al*. (1991). PITC derivatives were prepared by adding PITC reagent (ethanol-TEA-water-PITC at a ratio of 7:1:1:1), mixed well and incubated at room temperature for 30 minutes, and the samples allowed to dry under vacuum. Diluent was added in each sample, which was then processed for filtration (0.45 µm syringe filter) and then 20 µl was injected. HPLC was performed using a Waters 1525 Binary HPLC pump and Waters 2487 Dual Absorbance Detector. Data was processed and analyzed using Waters Breeze software. Operating conditions were: column temperature - 38 °C, column - picotag (Waters, pico tag system); absorbance - 254 µm; pump pressure - 1500-1700 psi.

Bligh and Dyer (1959) method was followed for the estimation of total lipids in the sample (Christie, 1982). The caloric content was measured with Parr 6725 semi-micro bomb calorimeter (Theilacker and McMaster, 1971).

Benzoic acid was added to the samples weighing less than 40 mg as an aid for the combustion. Energy content per gram benzoic acid was 26.45 KJ g^{-1}. From the gross heat value of the total sample, caloric content of benzoic acid was reduced to get the value for dry rotifer sample. The average of four determinations was expressed as cal. g^{-1} dry rotifer.

Statistical analysis

All data are presented as mean ± S.D. and were subjected to a one-way analysis of variance (ANOVA) (Zar, 1984). Significant difference among means ($p< 0.05$) were tested with Duncan's multiple range tests. All statistical analyses were conducted using SPSS for Windows (Statistical Package for Social Sciences, Windows Version, Chicago, IL, USA) and Microsoft Excel.

Results and discussion

The rotifer, *B. plicatilis* for the present study was cultured by using four different feeds and biochemical studies were conducted. Results of the present study showed that the nutritional value of rotifer was influenced by the type of feed given. Scott and Baynes (1978) observed that the type of algae used as food for *B. plicatilis* had significant effect on the biochemical composition. The biochemical composition of rotifer, *B. plicatilis* cultured using selected diets are given in Table 1. Algal diets were found to be better than the bacterial supplemented (*B. licheniformis* along with *Nannochloropsis* sp.) diet in terms of biochemical composition. Watanabe *et al*. (1983) reported that the feed organism used for culture affected the proximate composition of both the rotifer and the brine shrimp. It is well established that in rotifers, the fatty acid profile is chiefly determined by the diet (Lubzens *et al*., 1989; Rainuzzo *et al*., 1994; Isik *et al.,* 1999) and most of the studies shows that the nutritional quality of *B. plicatilis* depend on the transfer of dietary components from its diet (Watanabe *et al*., 1979; Gatesoupe and Luquet, 1981; Ben-Amotz *et al*., 1987; Frolov *et al*., 1991; Lubzens *et al*., 1995; Lie *et al*. 1997). It is generally known that different algal foods can yield substantially different reproductive rates (Hirayama *et al*., 1979; Snell *et al*., 1983; James and Rezeq, 1988). Navarro and Yufera (1998) reported that food type had a significant effect on the maximal population density of *B. plicatilis*. Ash content obtained for the rotifer samples during the present study showed variation among treatments. Ash content was high for *B. plicatilis* fed with a combination of *B. licheniformis* and *Nannochloropsis* sp. Micro algae as individual feeds and in combination did not show significant difference ($p>0.05$) in ash values. Fernandez-Reiriz *et al*. (1993) have reported ash values between 13.3% and 16.7% which is almost similar to that obtained in the present study. Caric *et al*. (1993) have found out that the ash content varies with the growth phase and type of feed for rotifer.

The mean protein level in rotifers fed with different types of feeds varied between 29.19 ± 0.23% and 46.02 ± 0.23% with the highest protein percentages in rotifers cultured with *I. galbana* (46.02 ± 0.23%) and the lowest protein level in rotifers fed *Nannochloropsis* sp. along with *B. licheniformis* (29.19 ± 0.23%). Caric *et al*. (1993) showed that protein content could vary with the type of food and growth phase and the values ranged from 34% to 52%. Ben-Amotz *et al*. (1987) have reported a much wider range of crude protein from 28-51%. The reason for varying protein levels in the present study may be attributed to the influence of feed, growth phases or feeding rates of different feeds. The influence of the biochemical composition of food on that of the rotifer *B. plicatilis* has been studied and found that there is a positive correlation between the protein content of the food and that of the rotifers and the percentage protein values ranged between 28.8% and 61.3% with different feed types (Millamena *et al*., 1990; Frolov *et al*., 1991; Lie *et al*., 1997; Nhu, 2004; Srivastava *et al*., 2006).

Marked differences in the lipid content of rotifers in various treatments were observed (Table 1). Relatively higher lipid content (36.1 ± 0.07%) was obtained for the

Table 1. Nutritional composition (% dry matter) of the rotifers fed on different feeds and dry weight (% of wet wt) [1, 2]

Parameter	Diets			
	Nannochloropsis sp.	*I. galbana*	*Nannochloropsis* sp. + *I. galbana* (1:1)	*B. licheniformis* + *Nannochloropsis* sp. (1:1)
Ash (%)	5.2 ± 0.06[a]	5.3 ± 0.27[a]	6.4 ± 0.31[a]	7.8 ±0.13[b]
Ash free dry weight (%)	94.8 ± 0.29[a]	94.7 ± 0.33[a]	93.6 ± 0.29a	92.2±0.46[b]
Moisture (%)	95.4 ± 0.20[a]	96.2 ± 0.20[b]	96.06 ± 0.06[b]	94.6 ± 0.1[c]
Protein (%)	32.1 ± 0.83[b]	46.02±0.23[c]	38.3 ± 0.29[d]	29.19 ± 0.23[e]
Carbohydrate (%)	14.62 ± 0.10[ab]	14 ± 0.03[c]	11.31 ± 0.21[d]	14.44 ± 0.24[b]
Total Lipid (%)	19.85 ± 0.19[b]	36.1 ± 0.07[c]	35.14 ± 0.29[d]	15.91±0.11[e]
Dry matter (%)	4.6 ±0.20[a]	3.8 ± 0.20[b]	3.94 ± 0.56[b]	5.4 ±0.10[c]
Calorific value (Kcal g^{-1})	17.61	16.52	17.15	14.01

[1]Mean± s.d. of three replicate groups; [2] Means within the same rows with different superscript letters are significantly different ($p<0.05$).

rotifer cultured with *I. galbana* as compared to those using a combination of *Nannochloropsis* sp. and *I. galbana* (35.14 ± 0.29%). The lipid content (19.85 ± 0.19%) of rotifers fed with *B. licheniformis* along with *Nannochloropsis* sp. was found to have a lipid content of 15.91 ± 0.11%. A significant difference ($p<0.05$) in lipid content was observed between the treatments. The total lipid content of *B. plicatilis* grown using different diets in the present study was found to be between 15.91% and 36.1%. James *et al*. (1987) reported a lower lipid level for rotifers fed with yeast alone and a lipid content of 13.1% along with *Chlorella* sp. for 24 h. Caric *et al*. (1993), who studied the chemical composition of rotifers fed on different food at three growth phases, found that the total lipid as percentage dry matter varied between 8.5% and 19.4%. The fat levels in the Rotifera vary according to their diet and age: from 5.4 to 17.1% on dry matter with an average of about 12% at the age of 2 days. Age seems to influence the fat level, in fact a decreasing trend with increasing age. Scott and Baynes (1978) studied the effect of algal diet and temperature on the biochemical composition of the rotifer, *B. plicatilis* and found that there was very little difference in lipid composition between cultures. In contrast, the studies conducted by James and Rezeq (1988) showed that the lipid synthesis in rotifers depends on the quality of the algal species or diets used in the culture system. The lipid values obtained from his study using different feed types varied between 4.5% and 20.1%. In the present investigation, the highest lipid content of 36.1 ± 0.07% was obtained from the rotifer fed with *I. galbana*. Dendrinos and Thorpe (1987) reported that lipids usually range from 14.0 to 28.5% in rotifer. Millmena *et al*. (1990) reported that *I. galbana* had the highest fat content of 16% among the algal species. Frolov *et al*. (1991) found that the lipid content of rotifers correlates very well with the lipid content of their food. A strong degree of correlation between the lipid content of rotifers and their food was also noted by Watanabe *et al*. (1978a). Total lipid levels in rotifers ranging between 8.1 and 20.8% have been reported (Ben-Amotz *et al*., 1987; Millamena *et al*., 1990; Frolov *et al*., 1991; Isik *et al*., 1999; Nhu, 2004). King *et al.* (2002) observed high level of DHA in rotifers fed 24 h on *I. galbana*. This might be the reason for high lipid level in rotifers fed with *I. galbana* in the present study. Fernandez-Reiriz and Labarta (1996) found that there was a higher total lipid content for rotifers fed with a mixture of *I. galbana* and *Nannochloropsis gaditana*. In the present study also, rotifers fed with a combination of *Nannochloropsis* and *I. galbana* gave higher lipid levels.

The amino acid analysis of rotifers fed on *Nannochloropsis* sp. alone was determined and the results given in Table 2. The total amino acid content excluding tryptophan was found to be ranging between 24.14 and 41.18 g 100 rotifer^{-1}. Compared to levels reported by Aragão *et al.* (2004b), the rotifers from the present experiments were low in methionine. Methionine has previously been discussed as a potential limiting AA for growth in fish larvae (Helland *et al*., 2003; Aragão *et al*., 2004b). Histidine was low in all the treatments and it was reported that histidine was low and suggested to be deficient in rotifers fed to gilthead sea bream larvae (Aragão *et al*., 2004b).

Table 2. Amino acid profile of rotifer cultured using different diets (expressed as g 100 g rotifer^{-1}).

Amino acids	(a)	(b)	(c)	(d)
Aspartic acid	3.67±0.18	4.58±0.14	3.96±0.05	2.53±0.06
Glutamic acid	4.34±0.26	5.19±0.18	4.55±0.01	3.75±0.09
Serine	2.86±0.40	4.02±0.15	3.01±0.14	2.45±0.14
Glycine	1.21±0.01	1.59±0.45	1.39±0.12	1.02±0.17
Histidine	0.21±0.01	0.85±0.41	0.42±0.11	.14±0.02
Arginine	2.13±0.40	3.01±0.12	2.59±0.13	1.95±0.14
Threonine	1.70±0.23	2.14±0.15	2.10±0.08	1.52±0.16
Alanine	1.57±0.25	2.48±0.17	2.01±0.04	1.41±0.09
Proline	1.63±0.45	2.54±0.25	1.85±0.11	1.35±0.12
Tyrosine	0.95±0.14	1.24±0.29	1.05±0.15	0.64±0.14
Valine	1.22±0.17	2.01±0.18	1.35±0.17	1.02±0.11
Methionine	0.28±0.16	0.78±0.19	0.52±0.13	0.15±0.13
Cystine	0.03±0.14	0.12±0.32	0.43±0.14	0.02±0.15
Isoleucine	1.15±0.23	2.10±0.34	1.75±0.12	1.00±0.21
Leucine	2.04±0.32	3.01±0.41	2.91±0.14	1.23±0.15
Phenylalanine	1.67±0.25	2.54±0.5	1.87±0.11	2.01±0.14
Lysine	2.27±0.14	2.98±0.24	2.46±0.11	1.95±0.17
TOTAL	28.93±0.35	41.18±0.13	34.22±0.12	24.14±0.14

Values are expressed as mean of AA ± SD, n = 3;
(a) *Nannochloropsis* sp. (b) *I. galbana;* (c) *Nannochloropsis* sp. + *I. galbana;* (d) *B. licheniformis* + *Nannochloropsis* sp.

Energy content was determined by using a Parr 6725 semi micro bomb calorimeter and expressed as $Kcal^{-1}$ g dry rotifer sample. The energy content obtained per gram dry rotifer sample was 14.01 - 17.61 Kcal (Table 1).

The carbohydrate (CHO) content of rotifer samples in the present study using different diets ranged between 11.3% and 14.6% (Table 1). There was no significant difference ($p>0.05$) for the CHO values obtained for *Nannochloropsis* sp. and a combination of *Nannochloropsis* sp. and *B. licheniformis*. For other feed types the CHO content varied significantly ($p<0.05$). Reported value for two days old *B. plicatilis* was around 13.8% on dry matter (Scott and Baynes, 1978; Yoshida and Hoshii, 1978) and with increasing age this level decreased. Caric *et al*. (1993) observed that the CHO values vary with growth phase and the type of feed and the values obtained were between 2 % and 6.5%. Ben-Amotz *et al*. (1987) found out the proximate CHO values of *B. plicatilis* cultured on different diets and the values ranged between 7 and 44.9%. Studies by Frolov *et al*. (1991) revealed that the carbohydrate values can range between 17.4% and 26.8%. Frolov and Pankov (1992) studied the effect of starvation on the biochemical composition of the rotifer *B. plicatilis* and found that the carbohydrate proportion decreased from 21.2% to 12.7% and then increased to 14.1%. Scott and Baynes (1978) also found out the CHO content for rotifers, effect of algal diet and temperature on the biochemical composition of the rotifer, *B. plicatilis*.

From the present results, it has been concluded that *I. galbana* is the best diet among the tested ones for *B. plicatilis* in terms of protein and lipid content. Bacterial supplementation should be aimed only in terms of probiotic effect and not for nutritional enhancement.

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Date of Receipt : 15.10.2009
Date of Acceptance : 29.04.2011