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Composting fishwastes as low-cost and stable diet for culturing *Brachionus rotundiformis* Tschugunoff (Rotifera): Influence on water quality and microbiota

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ABSTRACT

The demand for the rotifers *Brachionus* spp., which is considered indispensable for larviculture, has increased considerably in the tropical countries. Although freshly cultured microalga is the common rotifer diet, the culture protocols are laborious and costly, thus limit sufficient production of rotifers, and consequently disrupt fish seedling production programs in the microalgae-based hatcheries. This study aimed at developing an alternative low-cost and stable fishwaste diet (FWD), for the rotifers. The diets i.e. FWD_1 (fishwastes only), FWD_2 (FWD_1 + carbon source) and control (*Nannochloropsis oculata* + *Chlorella vulgaris* only) were used to determine the population density, specific growth rate and dietary value of the rotifer *Brachionus rotundiformis* (SS-type) in 30 l tanks of sea water (22 ppt), in which 20 rotifers ml^{-1} were stocked and semi-continuously cultured for 18 days at 28 ± 1 °C without aeration. The coefficient of variation (CV) of the mean specific growth rate was calculated to determine stability of the cultures. The culture medium and rotifer gut were aseptically screened for bacteria, which were counted and reported as colony-forming units (CFU ml^{-1}). The rotifers and microflora were harvested and analyzed for total lipids. FWD_2 produced significantly higher rotifer density than FWD_1 and control diet ($p < 0.05$), but no significant difference was detected between FWD_1 and control cultures ($p > 0.05$). Up to 1188 ± 70 rotifers ml^{-1} were obtained on day 13 day with FWD_2. There was no significant effect of FWD on the CV of the cultures which were 0.11 ± 0.05, 0.07 ± 0.02 and 0.08 ± 0.04 for FWD_1, FWD_2 and control cultures, respectively ($p > 0.05$). There was significantly higher DO in the control- than in the FWD-tanks, and higher NH_3-N in the FWD- than in the control-tanks ($p < 0.05$). There was no significant effect of FWD on the pH in the diets ($p > 0.05$). However, the water quality values were within the acceptable limits for aquaculture. The FWD-rotifers ingested significantly higher CFU ml^{-1} of bacteria ($1.02 \pm 0.12 \times 10^7$) than the control-rotifers ($8.25 \pm 2.19 \times 10^6$ CFU ml^{-1}). About 0.35 and 0.39 mg/g of DHA and EPA, respectively was obtained in the FWD_2-fed rotifers, and both were under detectable limit in the control-rotifers. The FWD may have contained essential nutrients and probiotics, which explains the higher rotifer population density in the FWD cultures than in the control cultures. This study offers an opportunity to reduce or eliminate the need for the expensive on-site microalgal production, and rotifer enrichment emulsions, toward a more cost-effective aquaculture, especially in the tropical countries.

1. Introduction

Successful marine fish larviculture depends on the availability of high quality live food resources (Sorgeloos et al., 2001). Among the live food resources, rotifers, especially of the genus *Brachionus* are essentially preferred as first exogenous food for most marine fish larvae and, the demand for *Brachionus rotundiformis* (SS-type) for small-mouth fish larvae is overwhelming (Lubzens et al., 1995). For example, in Japan alone, an average hatchery requires about 20 billion rotifers day^{-1}, where 20,000 to 100,000 rotifers per fish larvae are needed to raise marine fish during its first 20–30 days (Fushimi, 1989). Regular supply of such a high rotifer demand is indeed stressful to many hatcheries, primarily due to the laborious nature and high cost of microalgal production. Other shortcomings of the fresh microalgae include susceptibility to contaminations, seasonal quality variations and short shelf life (about 2–3 weeks under normal refrigeration) (Laing, 1991).

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Alternatively, cheaper diets such as baker's yeast have been used but culture instabilities due to imbalances of vitamin B_{12} and bacterial flora are common (Hirayama and Funamoto, 1983; Hino, 1993). Other studies have also reported the use of waste-grown bacteria and/or synthetic medium grown-bacteria as rotifer diet. For example, Probiotic culture systems (PCS) have been developed for a variety of domestic animals and fish (Hirata, 1980) and even rotifers species (Hirata et al., 1998). Loo et al. (2016) employed an easily and cheaply cultured phototrophic bacteria (PB) from industrial wastes of palm oil mill effluent (POME) to culture *Brachionus rotundiformis*. Other products e.g. condensed microalgae and artificial diets such as Selco (Inve-Co. Ltd.) are also commercially available but costly for most fish farmers, especially in the developing countries, which are potential future leaders in marine larviculture production. In addition, the micro-particulate feeds have problems of settling, clumping, nutrient leach and low digestibility, thus limit production of fish juveniles (Lavens and Sorgeloos, 1996). In the recent past, high-density and ultra-high-density rotifer culture systems (about 20,000–30,000 ind ml^{-1}) have been developed in Japan using algal pastes (reviewed in Yoshimatsu and Hossain, 2014). However, such rotifer production systems require sophisticated and costly technologies. These limitations have constrained their adoption by aquaculture industry in many developing countries, where aquaculture is important for food production. Therefore, studies to develop simple, low-cost, and stable methods of seeding, growing and complete harvest of live food diets are relevant priorities for aquaculture especially in the less developed countries, where cases of nutritional insecurity are common.

Due to the increasing global demand for fish and processed fish products, about 64 million tons of fishwastes e.g. heads, viscera, skin, bones, fins, air bladder, scales, blood, liver, gonads and guts etc. are generated annually (Rai et al., 2010). These wastes cause environmental pollution if poorly disposed. The fishwastes are substrate for microbial growth, some of which have probiotic properties such as lactic acid bacteria (LAB) (Balcazar et al., 2008). The probiotics enhance growth and immunity of rotifers and fish larvae either individually or in combination (Gatesoupe, 1999). In addition, the fishwastes contain appreciable amounts of recoverable essential bio-molecules including the *n*-3 series of the long chain polyunsaturated fatty acids (PUFAs) e.g. eicosapentaenoic acid (EPA, 20:5*n*-3) and docosahexaenoic acid (DHA, 22:6*n*-3) (Cho et al., 2014). These elements are critical for growth and survival of fish larvae (Harel et al., 2002). Being filter feeders and top predators in the microbial web, it is hypothesized that rotifers can transfer the essential bio-molecules from the fishwastes to fish larvae through bacteria-rotifer-fish trophic pathway and/or through direct ingestion of micro-fishwaste particles present in the culture media.

Composing organic matter yields huge densities of bacterial cells under optimum carbon/nitrogen (C/N) ratio (Avnimelech, 2003). Usually, optimum C/N ratio ensures immobilization of inorganic nitrogen into huge bacterial proteins (biomass) and restores good water quality by removing toxic ammonia (Azim and Little, 2008). The bacterial proteins are known to improve population growth and nutrition content of rotifers (Yasuda and Taga, 1980; Hagiwara et al., 1994). However, the use of bacteria for live food culture has been confined at experimental scales, and is mainly added as supplements to microalgae or baker's yeast. This study explored the feasibility of promoting simultaneous growth of high dense bacterial biomass and rotifers in the same culture facilities, where the rotifers feed directly on the microbial flora and/or micro-fishwaste particles as the sole source of nutrition. So far, protocols have been developed for converting specific fishwastes into other useful products such as fertilizers (López-Mosquera et al., 2011), silage (Ferraz de Arruda et al., 2007), collagen (Nagai and Suzuki, 2000) and essential enzymes and minerals (Rebah and Miled, 2013). Previously, we developed a protocol for making fishwaste diet (FWD) for rotifers, but the efficacy of the FWD for mass culture of rotifers is not yet documented. Hence the objectives of this study are, 1) to determine the population growth and mictic reproduction of the rotifer *B. rotundiformis* fed with the FWD and 2), to analyze the total fatty acid content of the rotifers and bacterial flora in the FWD cultures. This study offers an opportunity to reduce or eliminate the need for the expensive on-site microalgal production, and rotifer enrichment emulsions, toward a more cost-effective aquaculture, especially in the tropical countries. In addition, conversion of disposable wastes such as fishwastes into products of economic benefit would also limit environmental pollution sources.

2. Materials and methods

2.1. Fishwaste diet (FWD)

Fishwastes (heads) of the Chub mackerel; *Scomber japonicus* Houttuyn 1782 were obtained from Nagasaki fish market and frozen at − 80 °C in the laboratory for further use. *S. japonicus* is a pelagic marine fish with appreciable amounts of PUFAs (Cho et al., 2014). The fish heads were preferred because they are mostly inedible and therefore, are considered absolute wastes. The fish heads were crushed using mortar and pestle (to increase surface area for microbial growth) before weighing 0.5 g of the fishwaste per litre of culture medium using a digital scale (Mettler Toledo – AG204, Japan). Other details of the FWD preparation protocol have been extensively described in our patent registered under number: P00201609066 in Indonesia. This study considered addition of carbon source to the FWD protocol as an improvement to the diet. For that reason, carbon source (wheat flour) was calculated according to Ebeling et al., 2006 and added to maintain an optimum C/N ratio of 16. The diets i.e. FWD_1 (15 g fishwastes), FWD_2 (FWD_1 + 6 g of wheat flour) and control (7.0 × 10^6 cells ml^{-1} of *Chlorella vulgaris* (Araujo and Hagiwara, 2005)) were investigated as food source for the euryhaline rotifer, *B. rotundiformis*. *C. vulgaris* diet was regularly supplied by Chlorella Industry Co. Ltd., Fukuoka, Japan.

Calculation of optimum C/N ratio for the experiment

Diet used in 30 l tank = 0.015 kg, crude protein (CP) of fishwaste = 15% (Cho et al., 2014), Carbohydrate in fishwaste = 14% (Cho et al., 2014). CP of wheat = 10%, carbohydrate in wheat = 76%.

Total Ammonium Nitrogen (TAN) produced: = Diet used (kg) × CP × 0.144[a] = 0.015 kg × 0.15 × 0.144 = **3.24 × 10^{-4} kg**

Carbon requirement = [TAN × 15.17[b]] − [Diet used (kg) × % carbon in feed] = [0.000324 kg × 15.17] − [0.015 × 0.14] = **2.81 × 10^{-3} kg**

Carbon constant = Carbon in wheat − [CP of wheat × 0.16[c] × 15.17] = **0.52**

Carbohydrate needed = carbon requirement / carbon constant = **5.44 × 10^{-3} kg**

C/N ratio = carbohydrate needed / TAN = **16**

Note:

[a]Ebeling constant (Ebeling et al., 2006)

[b]15.17 = the constant representing the required carbohydrate needed to eliminate 1 unit of nitrogen (Ebeling et al., 2006)

[c]0.16 = the constant in the ammonia generation equation assumes that protein is 16% nitrogen (Ebeling et al., 2006)

2.2. Experimental design

Stock culture of the rotifer, *B. rotundiformis* (SS-type, Perth strain; 90–100 μm) was used. Every diet was triplicated in polycarbonate tanks

(blue in color) each containing 30 l of artificial seawater (22 ppt.), in which, 20 rotifers ml^{-1} were inoculated on the same day as FWD application (day 0) and, semi-continuously cultured without aeration for 18 days. Artificial seawater was made by dissolving salt (Marine Art Hi Co. Ltd., Japan) in tap water then subjecting to aeration overnight to expel chlorine. The temperature of the culture media was maintained at 28 ± 1 °C in a water bath with heater. Three-1 ml of culture water was sampled at top 10 cm from each tank, fixed with lugol and then rotifer numbers were counted under stereo microscope at × 25 magnification to estimate rotifer population density. At every exponential growth phase, partial harvesting was done by replacing 50% of the culture medium with new artificial seawater and fresh diet applied. The specific growth rate (SGR) was calculated as: $r = [\ln N_t - \ln N_o] / t$, where, N_o = initial population density, N_t = population density after the time (t) and t = time in days (i.e. at day 4, 9 and 13). The coefficient of variation (CV) of the mean SGR was computed to determine the stability of the cultures on day 4, 9 and 13 in each diet.

2.3. Water analysis

Dissolved oxygen (DO) ($mg\ l^{-1}$) was measured in the culture tanks using a multi-parameter water meter (YSI-model 85/10 FT, Yellow Springs, Ohio USA). The pH was measured by pH meter (HM-30G DKK TOA, Japan) while unionized ammonia (NH_3-N) ($mg\ l^{-1}$) was determined by a Photometer system for water analysis (Palintest® 8000 Ltd., USA) according to the company's manual.

2.4. Mictic reproduction

The mictic reproduction of the rotifer was monitored using batch cultures in glass jars containing 4 l of seawater (22 ppt) with initial density of 20 rotifers ml^{-1}. The rotifers were incubated at 28 ± 1 °C under total darkness with each diet (i.e. FWD_1, FWD_2 and control) for 10 days without aeration or water exchange. The diet quantities were similar to the previous experiments. Rotifer population density was daily counted in three-1 ml from each diet group. In the same sample, number of fertilized and unfertilized mictic females were counted based on the type of egg they carried (Hagiwara et al., 1988). Then the rotifer mixis rate was calculated as follows:

$$\text{Mixis}\ (\%) = \left[\frac{mictic\ females}{amictic\ females + mictic\ females}\right] \times 100\ \%$$

2.5. Microbiology

The rotifer gut and culture medium were separately screened for bacteria under sterile conditions. For gut microbial analysis, rotifers were sampled from FWD_2 and control tank and separately rinsed thoroughly with distilled water to remove external flora. The rotifers were collected using filter paper from beneath plankton sieve (45 μm) and transferred into a sterile 1.5 ml Eppendorf tube and homogenized using sterile pellet pestle (Sigma-Aldrich Z35997-1EA) to expose the internal gut microflora. The homogenized samples were re-suspended in 1 ml of sterile (Milli-Q) water, vortexed for 10 s and filtered through a 10 μm net to remove rotifer tissues. One-ml of the homogenate was serially diluted up to 10^{-6}. Similar dilutions were done for the culture medium sampled from FWD_2 and control tank (after filtering out rotifers and residues using 10 μm plankton net). For platting, the Zobell marine agar (Difco™ 2216, Becton, Dickinson & Co. France) was used. Then, 0.1 ml of the respective diluted samples was inoculated over the surface of the solidified agar in triplicates. The plates were incubated upside down (to avoid vapor condensation on the agar) at 32 °C for 48 h. The bacterial colony forming units (CFU) were calculated as CFU ml^{-1} = (No. of colonies × dilution factor) / inoculated volume (ml).

2.5.1. Fatty acid analysis

The rotifers were harvested using 50 μm plankton net, washed and dried as mentioned previously, and were kept frozen at − 80 °C until chemical analysis. Visible dense microbial suspensions (i.e. bioflocs) were collected from the FWD tanks and centrifuged at 8000 × g for 5 min. The biofloc pellet was aseptically collected and frozen at − 80 °C until total lipid analysis. Some bacterial colonies isolated from FWD culture medium as described previously were grown in rotating tubes each containing 5 ml of marine agar broth (Pearcore Trypto-soy, Eiken, Japan) for 24 h at 37 °C. The bacteria were also centrifuged and preserved at − 80 °C for lipid analysis. Total lipid and fatty acid composition analysis were conducted at Chlorella Industry CO., LTD. Fukuoka, Japan. The sample methanolysates were prepared at 100 °C for 2 h after the addition of 2 M hydrogen chloride methanol. Fatty acid methyl esters (FAME) were extracted by n-hexane. Gas chromatography analysis was performed using a GC-2010 (Shimadzu Scientific Instruments, Inc.) equipped with a HR-SS-10 column (Shinwa Chemical Industries, Ltd.). The column temperature was regulated at 150 to 220 °C. Individual fatty acids were quantified by means of the response factor to 15:0 fatty acids as the internal standard (Folch et al., 1957).

2.6. Microparticles distribution

The FWD_1, which was not blended with wheat flour, was employed to determine the microparticles size and distribution in the culture media to avoid interference from wheat flour particles. Here, two treatments i.e. FWD_1 only, and FWD_1 + rotifers (20 rotifers ml^{-1} initial density) were each triplicated in tanks containing 30 l of artificial seawater (22 ppt) and cultured for 18 days without water exchange in experimental design similar to 1.2 above. From day 8 to 17, about 10 ml of culture water was sampled daily from each tank and filtered through 10 μm net to remove residues and/or rotifers. The microparticle concentration and their mean sizes in each sample were determined using the particle distribution analyzer (Sysmex PDA-500, USA).

2.7. Data analysis

The data was analyzed using R statistical software (version 3.2.1 of the R Foundation for Statistical Computing Platform © 2015). The Bartlett test was used to test for the homogeneity of variances. Two-way analysis of variance (ANOVA) was used to test the effects of FWD and culture days on water quality, rotifer population densities, SGR and mixis rate. Wilcoxon rank sum test and student t-test were used to compare the mean particle concentration and sizes in each treatment, respectively. Where significant differences were detected, the Tukey's HSD Post hoc test was performed to locate them ($p < 0.05$).

3. Results

3.1. Rotifer population growth and mictic response

There was significant effect of FWD ($F = 18.81$, $p < 0.05$), culture days ($F = 102.59$, $p < 0.05$) and their interaction ($F = 5.94$, $p < 0.05$) on the rotifer population density. The rotifers fed with FWD_2 had significantly higher density than those fed with FWD_1 and control diet on day 9, 12 and 13 (Tukey HSD test, $p < 0.05$), but declined significantly on day 17 and 18 (Tukey HSD test, $p = 0.00$) (Fig. 1). There was no significant difference between population density of the FWD_1-rotifers and control-rotifers except on day 17 and 18, where density of the control-rotifers significantly exceeded those in given FWD_1 (Tukey HSD test, $p = 0.00$) (Fig. 1). Highest population densities were 883.6 ± 84.6, 1188 ± 69.7 and 830.3 ± 109.7 ind ml^{-1} on day 13 for the rotifers fed with FWD_1, FWD_2 and control diet, respectively (Fig. 1). The SGR was significantly influenced by the FWD ($F = 13.63$, $p = 0.00$) and culture days ($F = 28.16$, $p = 0.00$) where the FWD_2-fed rotifers had significantly higher SGR than the control-rotifers on day 9 (Tukey HSD test, $p = 0.00$),

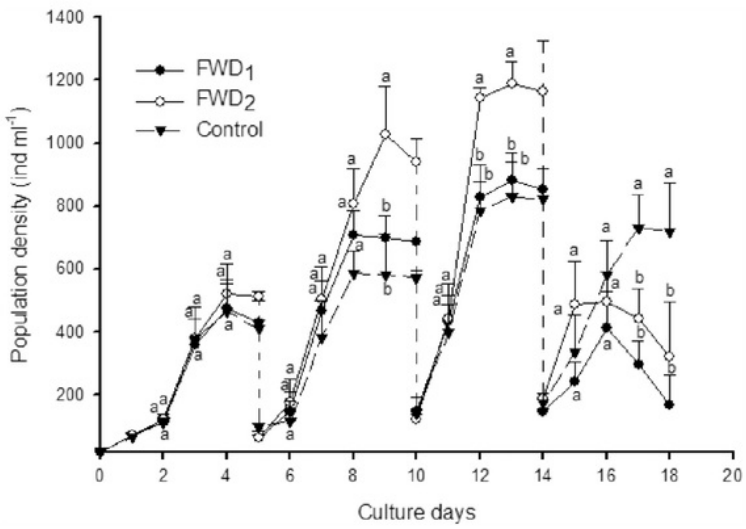

Fig. 1. Population density curves of the rotifers cultured with FWD and control diets. Half of culture medium was replaced with new media on day 5, 10 and 14 as shown by the dotted lines. The values represent mean ± SD, $n = 3$. Different letters each day denote significant differences at $p < 0.05$. Two-way ANOVA, Tukey HSD test, a > b.

Table 1
The specific growth rate (SGR) at day 4, 9 and 13 of the rotifers cultured in the FWD and control tanks; Two-way ANOVA, Tukey HSD test, different superscripts each day indicate significant differences at $p < 0.05$; $n = 3$, a > b.

Day	Treatments		
	FWD_1	FWD_2	Control
4	0.78 ± 0.04^{a}	0.81 ± 0.04^{a}	0.78 ± 0.04^{a}
9	0.60 ± 0.09^{ab}	0.69 ± 0.06^{a}	0.44 ± 0.02^{b}
13	0.61 ± 0.07^{a}	0.76 ± 0.05^{a}	0.58 ± 0.07^{a}

but no significant difference occurred between the FWD_1-rotifers and the control-rotifers ($p = 0.11$) (Table). There was no significant effect of FWD on the CV of the cultures (One-way ANOVA, $F = 0.94$, $p = 0.43$). The CV values were 0.11 ± 0.05, 0.07 ± 0.02 and 0.08 ± 0.04 for FWD_1, FWD_2 and control tanks, respectively. The rotifer mictic induction was significantly affected by FWD ($F = 114.89$, $p = 0.00$), culture days ($F = 15.13$, $p = 0.00$) and their interaction ($F = 4.53$, $p = 0.00$). The control-rotifers had significantly ($p = 0.00$) higher mixis rate than those given FWD. About 20% mixis rate was realized for the control-rotifers compared to about 5% of the FWD-rotifers within 10 days (Fig. 2).

3.2. Water analysis and microbiology

The FWD significantly affected DO, NH_3-N but not pH (Table 2). The range and mean values of water quality parameters are presented in Table 3, while their daily fluctuation curves in the culture tanks are shown in Fig. 3. The bacterial colonies were counted at $\times 10^{-4}$ dilutions. The FWD significantly affected the CFU (ml^{-1}) (One-way ANOVA, $F = 15.11$, $p = 0.01$), where the FWD-rotifers ingested significantly higher CFU ($1.02 \pm 0.12 \times 10^{7}$ CFU ml^{-1}) of bacteria than control-rotifers ($8.25 \pm 2.19 \times 10^{6}$ CFU ml^{-1}) (Tukey HSD test, $p = 0.03$). However, there was no significant difference of CFU in the culture medium of FWD and that of control, and between the rotifer gut and the culture medium in each diet (Tukey HSD test, $p > 0.05$) (Fig. 4).

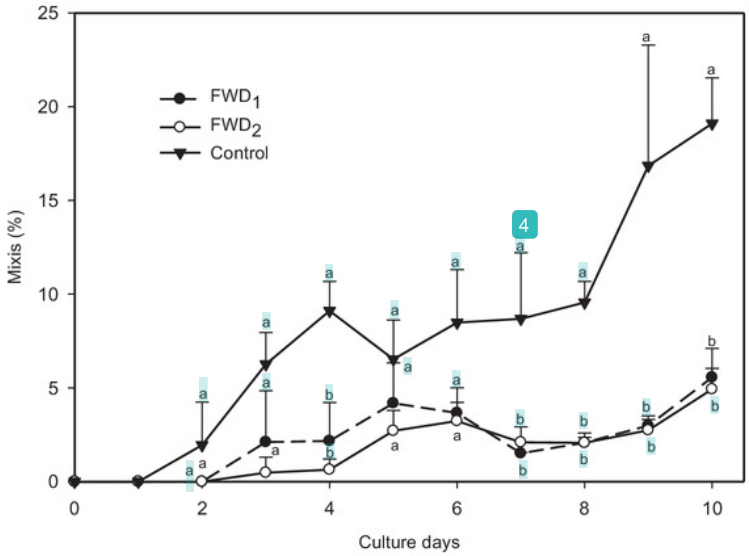

Fig. 2. The percent mixis rate of rotifers batch cultured in 400 ml glass jars. The values represent means ± SD. Different letters each day denote significant difference at $p < 0.05$; Two-way ANOVA, Tukey HSD, $n = 3$, a > b.

Table 2
Two-way ANOVA output of the effects of FWD and culture period on the mean of water quality parameters in all the treatments. df: degrees of freedom, SS: sum of squares, MS: mean square, *F*: F-ratio, *P*: level of significance (*denote significant difference at $p < 0.05$, $n = 3$).

	df	SS	MS	*F*	*P*
Dissolved oxygen (DO) mg l^{-1}					
Day	18	258.88	14.38	1288.25	0.00*
FWD	2	43.50	21.75	1948.24	0.00*
Day × FWD	36	12.96	0.36	32.26	0.00*
Residuals	114	1.27	0.01		
Ammonia (NH_3-N) mg l^{-1}					
Day	18	18.38	1.02	267.26	0.00*
FWD	2	2.35	1.17	308.15	0.00*
Day × FWD	36	2.43	0.06	17.68	0.00*
Residuals	114	0.43	0.00		
pH					
Day	18	0.44	0.02	7.53	0.00*
FWD	2	0.02	0.01	4.08	0.01*
Day × FWD	36	0.05	0.00	0.45	0.95
Residuals	114	0.37	0.00		

3.3. Fatty acids analysis

About 0.35 and 0.39 mg/g of DHA and EPA, respectively was obtained in the rotifers fed with FWD_2 and both were under detectable limit in the control-rotifers. The total lipid analysis was not done for the rotifers fed with FWD_1, bioflocs and broth-cultured bacteria due to insufficient sample quantities (i.e. < 10 g). The DHA/EPA ratios were as follows: bioflocs, 2.7; broth-cultured bacteria, 0.0; FWD_1-rotifers, 0.9; FWD_2-rotifers, 0.9 and control-rotifers, 0.0. The DHA/ARA (20:4 n-6) ratio was 15.5 and 7.5 for the broth-cultured bacteria and bioflocs, respectively, but was under detectable limit in the FWD- and control-rotifers (Table 4).

3.4. Microparticles distribution

Only particle sizes between 0.61 and 6.0 μm were recognized by the particle counter machine. There was significantly higher concentration of particles in the FWD tanks without rotifers ($2.56 \pm 0.19 \times 10^7$ ml^{-1}) than in the FWD tanks with rotifers ($3.46 \pm 1.75 \times 10^5$ ml^{-1}) (Wilcoxon rank sum test, $W = 886$, $p = 0.00$). Meanwhile, the mean particle sizes were significantly larger (2.71 ± 0.27 μm) in the FWD tanks with rotifers than in the FWD tanks without rotifers (2.01 ± 0.33 μm) (Student *t*-test, $t = -8.58$, df = 29, $p = 0.00$). Visually, the FWD tanks without rotifers were cloudier than the FWD tanks with rotifers.

4. Discussion

In the recent past, biosynthesis and utilization of microbial proteins has attracted the attention of aquaculture nutritionists (Avnimelech, 2003; De Schryver et al., 2008). The microbial proteins are generated through heterotrophic microbial growth that is stimulated when nutrients from organic materials are recycled at optimal C/N ratios (Ebeling et al., 2006; Azim and Little, 2008). To date, scientific evidence of converting fishwastes into microbial proteins as food source for rotifers are scanty. This study has developed a cheap and stable FWD and, determined its usefulness as a food source for the rotifer, *B. rotundiformis*. Higher SGR and population density of the rotifers cultured with FWD_2 compared to the control-rotifers corresponded to a high microbial biomass in the rotifer gut and, a low mixis rate. FWD_2 produced up to 1200 rotifers ml^{-1} bi-weekly, beyond which, the system became exhausted and required a complete restart. In this regard, the FWD seems to have caused proliferation of high bacterial flocs as food source for the rotifers. The ingestion of the bacterial proteins by rotifers favored parthenogenetic reproduction at the expense of mixis induction. Our study corroborates the findings of Ooms-Wilms (1997) that rotifer densities and/or egg/female ratio dynamics in natural eutrophic waters are positively correlated with bacterial load. A generally lower coefficient of variation in all the treatments indicated the stability of FWD cultures just as those fed with normal *C. vulgaris* diet.

Water quality aspects also influenced rotifer growth. For example, lower NH_3-N level recorded in FWD_2 than in FWD_1 (Fig. 2) probably favored faster rotifer growth rate, thus depicting carbon source addition (i.e. maintaining high C/N ratio) as an improvement of the FWD technology. Literature reports show that optimal C/N ratio facilitates biochemical transformation of excess NH_3-N into microbial biomass and restores good water quality in efficiently functioning biofloc systems (Avnimelech, 2003; Azim and Little, 2008). Whereas the pH and DO values in this study are comparable to those of standard rotifer cultures (Yu and Hirayama, 1986), NH_3-N values in FWD tanks appeared to be slightly higher than in normal cultures, perhaps due to the deamination process of FWD. However, the NH_3-N values are still below the lethal concentration level (LC_{50}) for normal rotifer mass cultures (Yu and Hirayama, 1986). In addition, the pH was relatively stable (7.51–7.77) in all the cultures. Unstable pH is detrimental to rotifers as higher pH (above 8.0) favors production of toxic ammonia in the cultures (Yu and Hirayama, 1986). The FWD-fed rotifers appeared to have crashed after 2 weeks of culture, during which the NH_3-N level was about 2.0 mg l^{-1}. This observation reflects those of previous studies that accumulation of NH_3-N in culture tanks significantly impedes mass production of rotifers (Yu and Hirayama, 1986; Dhert et al., 2001). Further technologies should be investigated to stabilize FWD-rotifer cultures beyond 2 weeks. It may have been possible to prolong the life of the FWD cultures through frequent harvests or large quantity harvests (i.e. > 50%).

Meanwhile, the FWD caused proliferation of microflora, which formed rotifer diet as shown by ingestion of higher CFU ml^{-1} of bacterial by the FWD-rotifers than the control-rotifers (Fig. 3). Indeed, lower rotifer reproduction has been reported in bacteria-free cultures than those in the cultures supplemented with microbial flora (Tinh et al., 2006). Under optimal conditions, bacteria form flocs attach to detritus to reach a size large enough to be eaten by rotifers (Hino and Hirano, 1980; Hino, 1993). Some bacterial species that are commonly found in rotifer cultures e.g. *Bacillus* sp. are known to have probiotic properties to the rotifers (Duy et al., 2017). Probiotic bacteria produce vital chemicals e.g. protease, lipase and vitamins that promote growth, reduce the stress, aid in the digestive processes and increase the reproduction of cultured animals (Yu et al., 1988, 1989; Gatesoupe, 1991;

Table 3
The ranges and means ± SD of water quality parameters for 18 days in the FWD and control tanks; Two-way ANOVA, Tukey HSD test, different superscripts in a row indicate significant differences at $p < 0.05$; $n = 54$, a > b > c.

Water parameters	Treatments					
	FWD_1		FWD_2		Control	
	Range	Mean	Range	Mean	Range	Mean
DO (mg l^{-1})	0.61–5.22	2.09 ± 1.32^b	0.49–5.23	1.97 ± 1.41^c	1.51–5.31	3.09 ± 1.05^a
NH_3-N (mg l^{-1})	0.00–2.31	0.88 ± 0.45^a	0.00–1.69	0.81 ± 0.34^b	0.00–0.89	0.60 ± 0.24^c
pH	7.51–7.77	7.63 ± 0.07^a	7.44–7.73	7.61 ± 0.07^a	7.52–7.79	7.64 ± 0.07^a

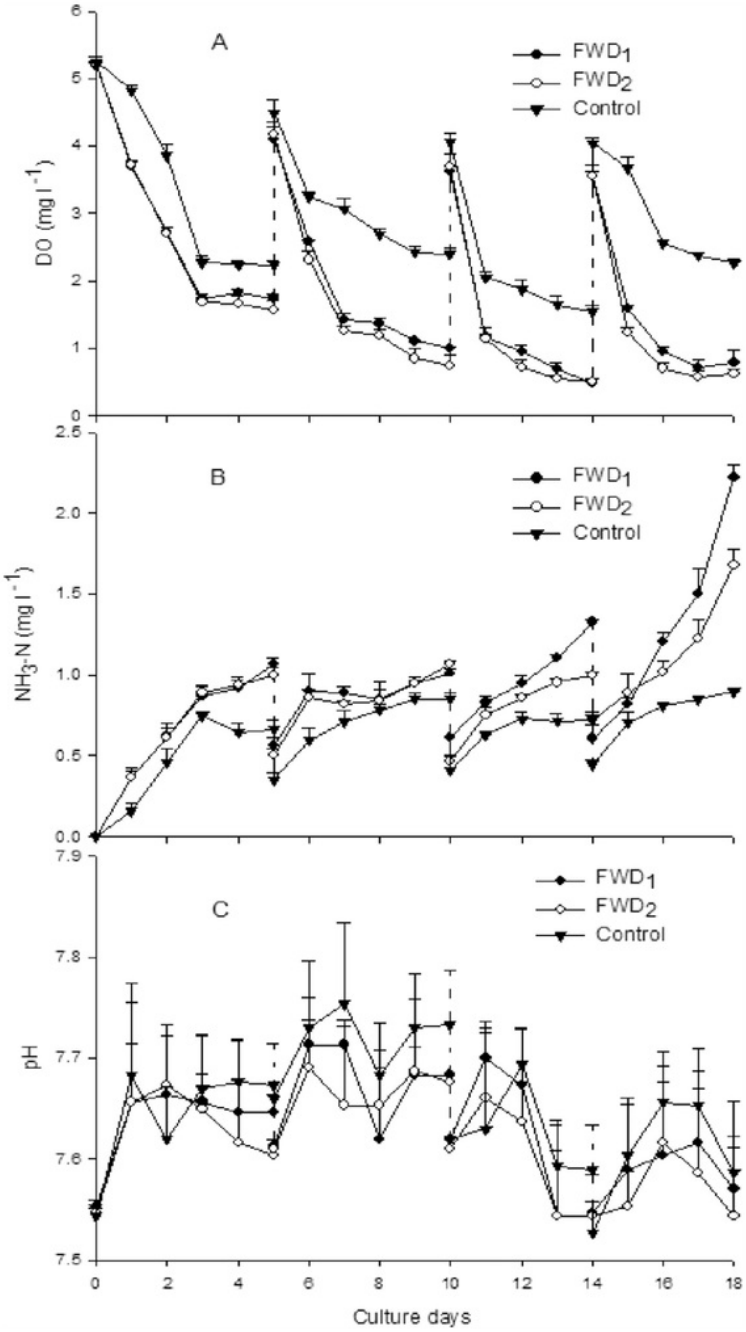

Fig. 3. Daily fluctuations of the water quality parameters in the culture tanks. The values are mean ± SD, $n = 3$. Two-way ANOVA, Tukey HSD test, $p < 0.05$; half of culture water was replaced with new media on day 5, 10 and 14 as shown by the dotted lines.

Douillet, 2000; Planas et al., 2004). Probiotics are also known to reduce occurrence of pathogenic bacteria and quorum sensing (Vazquez et al., 2005). In addition, some probiotics strains of *Bacillus* sp. produce chemical metabolites e.g. gamma aminobutyric acid (GABA) (Li and Cao, 2010), an organic acid that stabilizes rotifer cultures and enhances parthenogenetic reproduction of rotifer progenies during sub-optimal conditions (Gallardo et al., 2000; Assavaaree and Hagiwara, 2011; Ogello et al., 2017). Despite the positive effects of bacteria in rotifer cultures (Douillet, 2000), certain bacteria e.g. *Vibrio anguillarum*, that also commonly found in rotifer cultures could be problematic for successful culture of marine fish larvae (Yu et al., 1988; Frans et al., 2011). Therefore, further studies should focus on comprehensive molecular characterization of the microflora including their succession patterns over time in the FWD cultures.

The suitability of marine fish larval diet is determined by the content and dietary balance of the essential fatty acids e.g. DHA, EPA and ARA (Sargent et al., 1997, 1999; Boglino et al., 2014). The presence these essential fatty acids at optimal amount is indeed critical for proper growth, development and survival of fish larvae (Watanabe et al., 1983). In this study, FWD enhanced the DHA/EPA ratio of the

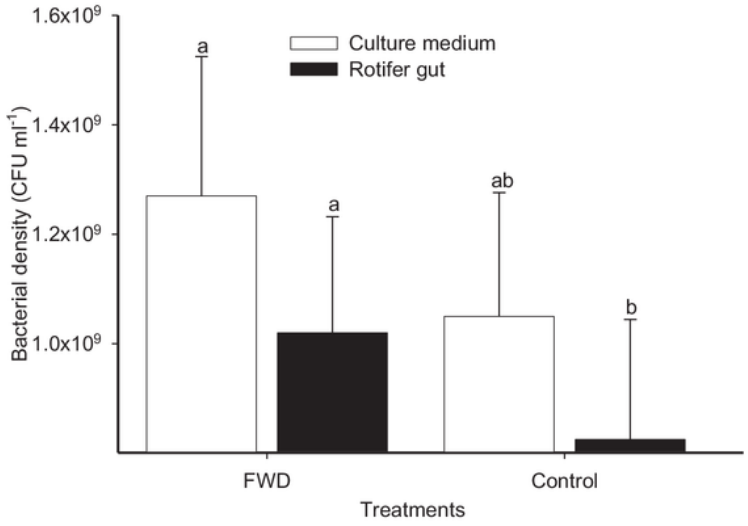

Fig. 4. The bacterial colony forming units (CFU/ml) in the culture medium and rotifer gut in each treatment at $\times 10^4$ dilution rate. Different letters denote significant differences at $p < 0.05$: Tukey HSD test, $n = 3$, a > b.

Table 4
Total lipids (mg/g dry weight) and fatty acid composition (%) in total lipids of raw fishwaste, broth-cultured bacteria, bioflocs, FWD-cultured rotifers and control-rotifers; Note: DHA, docosahexaenoic acid (22:6 *n*-3); EPA, eicosapentaenoic acid (20:5 *n*-3). -*Means total lipids not measured due to limited sample quantity; [a]other unidentified biomolecules.

	Raw fishwaste	Bacteria (cultured)	Bioflocs (in medium)	Rotifer samples from		
				FWD_1	FWD_2	Control
Total lipids (mg/g dry weight)	28.7	-*	-*	-*	20.5	11.1
Fatty acids (%)						
C14:0	3.7	4.5	3.5	4.5	5.4	1.8
C14:1	0.2	0.5	0.2	1.5	2.3	1.0
C16:0	18.7	21.6	18.9	17.7	15.2	15.1
C16:1	2.1	3.6	2.2	2.1	3.0	0.2
C16:2	1.3	1.0	1.3	0.1	0.6	0.3
C18:0	6.9	7.0	7.0	3.0	4.3	4.8
C18:1	14.5	12.8	14.9	6.3	4.8	2.0
C18:2 n-6	1.4	0.8	1.4	4.3	4.1	30.8
C18:3 n-3	0.8	0.4	0.8	0.3	0.5	5.7
C20:0	0.8	0.7	0.8	0.2	0.3	0.2
C20:1	0.3	1.5	0.3	0.6	0.6	1.4
C20:4 n-6	2.4	0.2	2.4	0.0	0.0	0.4
C20:5 n-3	6.5	0.0	6.6	1.6	1.9	0.0
C22:0	0.3	0.0	0.3	0.0	0.0	0.0
C22:1	0.4	1.5	0.4	14.2	15.1	0.6
C24:0	1.3	0.3	1.3	0.6	1.4	0.0
C24:1	0.4	0.9	0.4	1.2	0.8	0.4
C22:5 n-3	2.3	0.3	2.2	0.4	0.5	0.0
C22:6 n-3	18.7	3.1	18.0	1.5	1.7	0.0
Others[a]	16.8	39.2	17.1	40.6	37.4	35.0
DHA/EPA	2.9	0.0	2.7	0.9	0.9	0.0
DHA/ARA	7.8	1.3	90.0	0.0	0.0	0.0
Total	100	100.0	100.0	100.0	100.0	100.0

rotifers by 0.9 while the DHA/EPA and DHA/ARA of the bioflocs were 2.7 and 7.5, respectively (Table 4). It is speculated that the rotifers may have obtained the EPA and DHA via FWD-bacteria trophic pathway, thus portraying bacteria as conduits for transporting the bio-molecules. On the other hand, the rotifers may have also obtained the EPA and DHA through direct ingestion of microparticles as shown by the higher concentration of microparticles in the FWD only tanks than in the FWD + rotifer tanks. This observation reaffirms the finding of Rothhaupt (1990) that rotifers can ingest bacteria-sized particles in the absence of microalgae or at low microalgae density. However, these particles may have also included bacteria and rotifer feces. Hino et al. (1997) reported that rotifers can ingest their own feces, thus increasing their food conversion ratio.

The DHA/EPA ratio of 0.9 reported for the FWD-fed rotifers in this study still falls below the recommended threshold ratio of 2.0 required for effective marine fish larval nutrition (Sargent et al., 1997). More studies are needed to boost the nutrition level of the FWD. The optimum HUFAs ratios are species specific, and are about 10:5:1, for DHA/EPA/ARA, respectively for most marine fishes (Sargent et al., 1999). However, the FWD developed in this study promises as a major leap toward developing a cheap and nutritionally rich diet for the rotifers. Based on the food value of the bioflocs, the FWD culture fluid can be considered a cheaper enrichment medium for low quality larval fish foods e.g. bakers' yeast and, may reduce the need for the expensive enrichment emulsions. However, further studies are needed for this suggestion. Lewis et al. (1998) reported that certain species of bacteria e.g. *Shewanella* sp. can produce sufficient DHA and EPA for enriching rotifer cultures. Other home-made emulsion products have been produced using fish oil and egg yolk as cheap ways of enriching rotifer cultures (Hirayama and Funamoto, 1983), but these products are prone to oxidation and have short shelf life that limits their application in aquaculture. The bacteria-held PUFAs are more protected against oxidation, and provide a variety of other natural nutrients that meet the species-specific nutritional requirements of the rotifers, and their predators (Harel et al., 2002). The FWD technology is a universal innovation that can be applied for both marine and freshwater live food production. The estimated cost of rotifer mass production using large scale batches is USD 4.5/million rotifers where feeds, live algae and yeast account for 72, 50 and 22% of the production costs, respectively (Treece, 1995). Comparatively, the FWD in the current study is significantly cheaper because fishwaste, which is the main ingredient in the diet, can be obtained for free. In addition, the efficacy of the FWD can be improved by addition of cheap carbon source. In general, the estimated cost of rotifer production in culture tanks using FWD is approximately USD 0.01/million rotifers. The special features of this FWD technology are that probiotic medium is produced from fish wastes that have little or no commercial value and, that the final product is fed to the rotifer culture in the form of a liquid. FWD may provide the means to develop more economic ways of producing rotifers as food for commercially important species 8n small-scale tropical hatcheries.

5. Conclusion

The FWD is a cheap and stable product that can produce up to 1200 rotifers ml^{-1} bi-weekly, thus presents a self sustaining biotechnology for production of high density of DHA- and EPA-rich rotifers without microalgae, for aquaculture. FWD causes proliferation of probiotic bacterial flora, suppresses rotifer mictic cycle and favors parthenogenetic reproduction of rotifers. The FWD offers an opportunity to 1) reduce environmental pollution sources by reusing poorly discarded fishwastes; 2) reduce or eliminate direct dependence on the immediately cultured or the expensive on-site microalgae production and, 3) to lower the cost of rotifer enrichment, thus making it convenient for aquaculture production, especially in the less developed countries, where malnutrition is prevalent. Further studies are recommended to investigate the mass culture potential of other planktonic live food resources e.g. copepods, cladocerans and *Artemia* using the FWD and, the dietary value of the FWD-fed live foods for larviculture.

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