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Particulated microalgae composite (PMC) as substitute for live microalgae in culture of juvenile Manila clam *Ruditapes philippinarum* (A. Adams and Reeve, 1850): A feasibility analysis

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ABSTRACT

To evaluate the feasibility of particulated microalgae composite (PMC) as live microalgae substitute in artificial breeding of Manila clam *Ruditapes philippinarum*, effects of replacing *Dicrateria inornata* with partial or total PMC on the growth, digestive enzyme activities, nutritional ingredient composition and microbial composition in faeces of juvenile clams were investigated. The results showed that 50 and 100% PMC replacement did not affect the mean shell length, mean body weight and survival rate of juvenile clams ($p>0.05$). Juvenile clams fed with 100% PMC replacement had higher lipase and pepsin activities than those fed with 100% *D. inornata* ($p<0.05$), while 50% PMC replacement only increased lipase activity ($p<0.05$). Essential amino acid (EAA) and polyunsaturated fatty acid (PUFA) contents exhibited no obvious differences between clams fed with and without PMC replacement ($p>0.05$). Significant increase in delicious amino acid contents ($p<0.05$) was observed with 100% PMC replacement. PMC replacement did not affect the diversity of dominant phyla in faeces, but the abundance of each dominant phylum. Similar relative abundance of faecal bacteria was observed when *D. inornata* was replaced with 50 and 100% PMC. PMC appeared to be a potential substitute for microalgae in artificial Manila clam breeding.

Keywords: *Dicrateria inornata*, Microalgae substitute, Particulated microalgae composite, *Ruditapes philippinarum*

Introduction

The Manila clam *Ruditapes philippinarum* (A. Adams and Reeve, 1850), is one of the major aquaculture species in Asian, American and European countries (Liang *et al.,* 2019). In China, aquaculture of *R. philippinarum* developed rapidly in coastal areas of the Yellow Sea, Bohai Sea and East Sea since the 1990s (Fang and Lin, 2016). The annual production of this species in China is as high as 3.97 million t, accounting for about 27.57% of the national shellfish production in 2019 (Yu *et al.,* 2020). In the traditional culture methods of Manila clam, the seeds needed for aquaculture were mainly collected from intertidal zones. However, the harvesting of wild seeds is always uncontrollable, seasonally restricted and labour intensive. Developing artificial breeding technology can increase the stability of seed supply and shorten the production cycle, leading to increased production and profit of Manila clam farming. In China, mass breeding technology with short production cycle has been developed in the hatchery (Zhang *et al.,* 2006).

Manila clam is a filter-feeding bivalve and mainly feeds on live microalgae in seawater. Seed production of filter-feeding bivalves in the hatcheries relies heavily on live microalgae production. Therefore, sufficient microalgae culture is an important prerequisite in artificial breeding of Manila clam. However, the culture of microalgae is costly and often unpredictable (Pettersen *et al.,* 2010) and the production in large quantities is often accompanied by various technical difficulties. Culturing adequate microalgae to meet daily feeding requirement of clam has become a heavy burden to many hatcheries. In some hatcheries of China, juvenile Manila clams have to be transferred to the mudflat to grow-out until they reach market-size seed, although juvenile clam is especially vulnerable to environmental challenge in the entire breeding cycle (Huo *et al.,* 2018). Therefore, in Manila clam seed production, alternative food sources are needed. The partial or total replacement of microalgal foods with a cost-effective and easy to handle substitute, such as dried algae diet (Laing *et al.,* 1990), microalga detritus (Yin *et al.,* 2019), concentrated algal pastes (Ponis *et al.,* 2008), dietary lipid emulsion (Ehteshami *et al.,* 2016), baker's yeast (Tanyaros *et al.,* 2016) and microencapsulated diets (Willer and Aldridge, 2017), have been attempted for the artificial breeding of clam. In addition to being cost-effective and easy to handle, there are many other requirements associated with the development of microalgae substitute. These diets must have the equivalent nutrient value and need to be easily digested and assimilated. Moreover, substitute diets need to remain in suspension because deposition easily cause bacterial contamination.

In this study, we developed particulated microalgae composite (PMC) with dried microalgae and fermented soybean meal as the main ingredients. To assess the feasibility of PMC as substitute for live microalgae in the culture of juvenile manila clam, we investigated the effects of PMC replacement on the growth, survival rate, digestive enzyme activity and nutritional ingredient composition as well as bacterial diversity in the faeces of juvenile clams.

Materials and methods

PMC preparation

The main ingredients of PMC comprised spray-dried *Chlorella vulgaris*, *Spirulina platensis* powder and fermented soybean meal which were smashed to particle size below 20 μm with HMB-20-S water-cooled pulveriser and then mixed with a mass ratio of 4.5:4.5:1 to prepare composite algal powder. Composite algal powder, granular sodium alginate and 2% calcium chloride solution were mixed at a mass ratio of 25:5:1.25 and stirred well, then injected into a hydraulic press to obtain block-shaped compound feed (4 x 4 x 2.5 cm) at a pressure of 87.5 kg cm^2. Before feeding, feed block was weighed, introduced into a 500-mesh bag and scrubbed to particulates in sand-filtered seawater.

Organisms and cultivation

R. philippinarum juveniles were provided by Haisheng Aquaculture Co. Ltd. of Tianjin, China. After being sieved through 4 and 6 mm mesh sieves sequentially, juvenile clams were unfed for 7 days in constantly aerated sand-filtered seawater. The rearing seawater was refreshed daily and the dissolved oxygen was maintained above 5 mg l^{-1}, salinity fluctuated from 29 to 31 ppt and temperature ranged from 24 to 26°C.

Dicrateria inornate, the most common algal species used in Manila clam culture, was obtained from Institute of Oceanology, Chinese Academy of Sciences. *D. inornata* was cultured in F2 medium (Guillard, 1974) at 25±2°C under natural illumination. When *D. inornata* cells reached the late logarithmic phase (approximately 5×10^6 nos. ml^{-1}), culture medium was harvested and used to feed juvenile clams directly.

Experimental design

About 9000 juvenile clams (mean shell length 5.02±0.37 mm, mean body weight 0.0261±0.0152 g, n=40) were randomly divided into three groups with three replicate tanks. Each tank (34.5 × 24 × 10.5 cm) was filled with 6 l sand-filtered seawater or *D. inornata* culture medium. Clams were fed with different diets (Table 1) twice daily at 6:00 and 18:00 hrs for 28 days. In hatcheries, *D. inornata* culture medium in the late logarithmic phase were directly used to culture juvenile Manila clam. Clams cultured in *D. inornata* culture medium (approximately 5×10^6 cells ml^{-1}) were treated as control. Before feeding, the cell density of *D. inornata* was measured under a microscope and the dry weight of *D. inornata* in 6 l culture medium was estimated. PMC replaced the same dry weight of *D. inornata* at 50 and 100% ration in the other two treatments. Before each feeding, 100% seawater was exchanged to remove the remaining diet. During the experimental period, salinity ranged from 28 to 32 ppt and water temperature ranged from 23.8 to 28.2°C. Growth, survival rate, digestive enzyme activity and nutritional ingredients of clams were measured at the end of the experiment. The deposited faecal matter were siphoned out before water exchange every 7 days. The faecal samples were filtered with 0.45 μm filter and stored at -20°C before the bacterial diversity was analysed.

Growth performance

At day 28, forty juveniles from each replicate tank were randomly sampled to record shell length (antero-posterior axis parallel to the hinge) with vernier caliper to the nearest 0.01 mm and to measure body weight using an electronic scale with accuracy of 0.0001 g. Water on the surface of clams was removed using filter paper to minimise measurement errors. Dead clams were picked out and counted before feeding. Clams were considered dead when the shell opened and did not respond to touch.

Collection of tissue samples

The whole soft tissues of calms were sampled after being starved for 12 h. All tissue samples were frozen in liquid nitrogen for more than 15 min and then stored at -80°C for subsequent analysis.

Table 1. The proportion of *D. inornate* and PMC in the experiment diets

Treatment	Composition of different diets per day	
	D. inornata	PMC
D. inornata	100%	-
D. inornata+PMC	50%	50%
PMC	-	100%

Digestive enzyme activity analysis

For enzyme activity determination, approximately 0.2 g soft tissues were homogenised in 1.8 ml ice-cold physiological saline, then centrifuged (4°C, 2500 rpm, 10 min). Lipase, amylase and pepsin activities of the supernatant were evaluated using commercial assay kits (Jiancheng Bioengineering Institute, Nanjing, China) following the manufacturer's instructions. Total protein content of supernatant was assayed according to Bradford (1976) using bovine serum albumin as a standard.

Nutritional ingredient analysis

After acid hydrolysis according to the method of Dai *et al*. (2014), the total amino acids (AA) were determined by a liquid chromatograph (Waters ACQUITY UPLC I-CLASS, Waters) and mass spectrometer (Xevo TQ-XS, Waters) integrated with a column (BEH Amide 1.7 µM 100 x 2.1 mm). Total lipid was extracted by sonication in dichloromethane-methanol (2:1, v/v) according to the method of Yin *et al*. (2019) and the extracted lipids were esterified with 0.5 M KOH methanol. Fatty acid methyl esters (FAME) were analysed using a Phillips PU 4410 gas chromatograph equipped with a fused silica capillary column (BPX-70; 25 m × 0.32 mm × 0.25 µm film) and FID detector.

Faecal bacterial diversity analysis

The genomic DNA of faecal bacteria was extracted using CTAB protocol and examined by agarose gel electrophoresis. PCR amplification and sequencing of the V4 region of bacterial 16S rRNA gene were conducted according to the method of Bi *et al*. (2019). PCR amplifications were performed with Phusion® High-Fidelity PCR Master Mix (New England Biolabs) and the primers used were 515F (5′-GTGCCAGCMG CCGCGGTAA-3′) and 806R (5′-GGACTACHVGGG TWTCTAAT-3′) (Caporaso *et al*., 2011). The same volume of 1 × loading buffer (containing SYB green) was mixed with PCR products and detected by 2% agarose gel electrophoresis. PCR products of 400-450 bp were selected and purified using Qiagen Gel Extraction Kit (Germany). TruSeq® DNA PCR-Free Sample Preparation Kit (Illumina, USA) was used to generate sequencing libraries and index codes were added. The library quality was evaluated using the Qubit® 2.0 Fluorometer (Thermo Scientific) and Agilent Bioanalyzer 2100 system. Finally, 250 bp paired-end reads were obtained after using the NovaSeQ6000 platform to sequence the library.

Paired-end reads were allocated based on the unique barcode of the sample and truncated by cutting off the barcode and primer sequence. Paired-end reads were merged using FLASH (V1.2.7) (Magoc and Salzberg, 2011). According to the Qiime (V1.9.1) quality controlled procedure (Caporaso *et al*., 2010), the raw tags were quality-filtered under specific filtering conditions to obtain high-quality clean tags (Bokulich *et al.,* 2013). To detect chimera sequences, the tags were compared with Gold database using UCHIME Algorithm (Edgar *et al*., 2011) and then removed the chimera sequences (Haas *et al*., 2011) and obtained the effective tags. Sequence analysis was performed using Uparse v7.0.1001 software (Edgar, 2013). Sequences with similarity ≥97% were allocated to the same OTUs. A representative sequence of each OTU was screened for further annotation. For each representative sequence, the GreenGene Database (Desantis *et al*., 2006) based on the RDP classifier (Version 2.2) (Wang *et al*., 2007) algorithm was used to annotate taxonomic information. To investigate the phylogenetic relationship of different OTUs and the difference of the dominant species in different groups, we performed multiple sequence alignment using the MUSCLE software (ver.) (Edgar, 2004). OTU abundance information was normalised using a standard of sequence number corresponding to the sample with the least sequences. All sequences were submitted to NCBI-GenBank (Acc. No. SRP321001).

Statistical analysis

Data are expressed as means±SD and were subjected to one-way analysis of variance (ANOVA, SPSS ver. 21.0) to determine significant differences between treatments. The significance of variation ($p<0.05$) was determined by least significant difference multiple-range test.

Results

Growth performance

As shown in Table 2, the mean shell length of clams fed with 50 and 100% PMC replacement did not significantly differ from those fed with 100% *D. inornata* ($p>0.05$). There was no significant difference in the mean body weight of clams fed with 50 and 100% PMC replacement and those fed with 100% *D. inornata* ($p<0.05$). Replacing 50 and 100% of *D. inornata* with PMC did not affect the survival rate significantly ($p>0.05$).

Digestive enzyme activity

Effects of PMC replacement on the digestive enzyme activities of *R. philippinarum* juveniles are shown in Table 3. Clams fed with 50 and 100% PMC replacement had higher lipase activity than those fed with 100% *D. inornata* ($p<0.05$), while PMC replacement did not affect amylase activity significantly ($p>0.05$). Pepsin activity of clams fed with 100% *D. inornata* did not differ from those fed with 50% PMC replacement ($p>0.05$), but was significantly lower than those fed with 100% PMC replacement ($p<0.05$).

Table 2. Effects of PMC replacement on the growth performance and survival rate of *R. philippinarum* juveniles

Parameter	*D. inornata*	*D. inornata*+PMC	PMC
Shell length (mm)	6.04±0.39	6.32±0.36	6.26±0.50
Body weight (g)	0.0388±0.009	0.0473±0.017	0.0399±0.005
Survival (%)	78.53±0.83	77.33±3.30	74.33±4.41

Values are represented as means±SD. values sharing no letters are not significantly different ($p>0.05$).

Table 3. Effects of PMC replacement on the digestive enzyme activities (U mg of protein^{-1}) of *R. philippinarum* juveniles

Parameter	*D. inornata*	*D. inornata*+PMC	PMC
Lipase	1.07±0.42^{b}	3.06±1.49^{a}	4.71±1.08^{a}
Amylase	0.04±0.01	0.04±0.001	0.08±0.06
Pepsin	0.26±0.13^{b}	0.83±0.53^{ab}	1.01±0.38^{a}

Values are represented as means±SD. Values sharing the same letters or no letters are not significantly different ($p>0.05$), whereas those with different letters are significantly different ($p<0.05$).

Amino acid composition

The amino acid composition of juvenile clams fed with different diets are shown in Table 4. Replacing 50 and 100% *D. inornata* with PMC could significantly increase glutamine contents ($p<0.05$). Proline, serine, tyrosine and glycine contents of clams fed with 100% PMC replacement were higher than those fed with 100% *D. inornata* ($p<0.05$) while no difference in these amino acid contents was observed between clams fed with 50% PMC replacement and 100% *D. inornata*. Clams fed with 50% PMC replacement had higher γ-aminobutyric acid contents than those fed with 100% *D. inornata* ($p<0.05$) and there was no difference between clams fed with 100% *D. inornata* and 100% PMC replacement. Replacing 100% *D. inornata* with PMC could significantly increase delicious amino acid (DAA) contents ($p<0.05$). Free amino acid (FAA), essential amino acid (EAA) and non-essential amino acid (NEAA) contents exhibited no obvious differences between clams fed with and without PMC replacement ($p>0.05$).

Fatty acid composition

The fatty acid profiles of *R. philippinarum* in three groups are presented in Table 5. The primary components

Table 4. Effects of PMC replacement on free amino acid composition of *R. philippinarum* juveniles (µg g wet weight^{-1})

Amino acid	*D. inornata*	*D. inornata*+PMC	PMC
Alanine#	322.21±100.53	397.13±30.89	408.27±104.75
Arginine	253.42±56.09	264.75±64.04	324.43±170.37
Asparagine	43.32±15.74	47.30±6.61	57.08±16.11
Aspartic acid#	272.39±134.58	339.49±64.11	413.43±19.78
Glutamine	49.23±19.02^{b}	90.93±29.24^{a}	93.27±25.59^{a}
Glutamate#	240.94±40.81	341.02±97.05	299.08±48.02
Histidine*	43.40±22.01	36.74±0.06	45.96±10.08
Isoleucine*	84.84±44.84	85.94±38.64	98.06±20.50
Leucine*	108.20±33.77	133.13±37.62	187.52±93.10
Lysine*	200.83±53.66	197.35±118.84	228.83±71.20
Methionine*	55.26±28.80	63.59±22.26	62.57±9.90
Phenylalanine*	71.41±43.70	94.37±24.03	86.43±5.78
Proline	47.75±28.55^{b}	72.41±8.15^{b}	116.94±32.88^{a}
Serine	112.33±20.21^{b}	157.92±41.75^{b}	242.32±69.77^{a}
Threonine*	90.53±50.80	142.24±48.38	101.00±27.14
Tryptophan*	17.73±6.71	15.90±7.06	19.80±4.68
Tyrosine	63.58±6.28^{b}	57.20±27.04^{b}	99.72±17.49^{a}
Valine*	130.61±111.40	137.26±60.39	148.07±13.93
Taurine	1391.58±615.56	1636.17±493.24	937.12±419.98
Ornithine	42.21±15.93	48.31±26.84	39.34±6.06
Glycine#	315.12±215.89^{b}	649.65±161.86^{ab}	720.50±284.88^{a}
γ-aminobutyric acid	0.82±0.34^{b}	1.73±0.74^{a}	0.91±0.51^{ab}
Protein (%)	9.27±0.24^{a}	8.79±0.53^{ab}	7.89±0.45^{b}
FAA	3966.96±1393.70	5019.39±718.71	4738.56±883.21
EAA	802.80±328.68	906.51±222.13	978.25±145.55
NEAA	3154.90±1065.14	4103.99±682.06	3752.43±770.78
DAA	1150.67±466.46^{b}	1727.28±320.35^{ab}	1841.28±422.00^{a}
EAA/FAA (%)	19.98±1.18	18.20±4.76	20.79±2.08
EAA/NEAA (%)	25.06±1.83	22.57±7.18	26.37±3.32

Values are represented as means±SD. Values sharing the same letters are not significantly different ($p>0.05$), whereas those with different letters are significantly different ($p<0.05$). FAA: Free amino acids; EAA: Essential amino acids (*); NEAA: Nonessential amino acids; DAA: Delicious or flavour amino acids (#).

of fatty acids in groups fed with 50 and 100% PMC replacement were C22:6n-3(DHA), C20:2, C16:0 and C18:2n-6c. In group fed with 100% *D. inornata*, C22:6n-3 (DHA), C20:4n-6(ARA), C20:5n-3(EPA) and C16:0 were the primary components of fatty acids. The contents of C11:0, C16:0, C23:0, C18:1n-9c, C18:2n-6c, C18:3n-6 and C20:2 in clams fed with 50 and 100% PMC replacement were higher than those fed with 100% *D. inornata* ($p<0.05$), while higher C14:0 content was found in clams fed with 100% *D. inornata* ($p<0.05$). There was no significant difference in C22:6n-3(DHA) content between groups fed with 100% *D. inornata* and 50% PMC replacement ($p>0.05$), while clams fed with 100% PMC replacement showed lower C22:6n-3(DHA) content than those fed with 100% *D. inornata* ($p<0.05$). No difference was observed in ∑PUFA and ∑SFA contents between three groups and clams fed with 100% PMC replacement showed higher ∑MUFA content than those fed with 100% *D. inornata* ($p<0.05$). ∑n-3/∑n-6 value decreased with increased PMC replacement.

Bacterial diversity in the faeces

The bacterial profiles of the 36 samples analysed generated 48 distinctive bacterial phyla. Taxonomic composition of the faecal microbiota at phylum level among 3 diet groups was compared based on the mean of the relative abundance (Fig. 1). *R. philippinarum* in 3 diet groups had similar top 10 phyla, including Proteobacteria, Bacteroidetes, Actinobacteria, Verrucomicrobia, Firmicutes, Planctomycetes, Acidobacteria and Chloroflexi besides unidentified bacteria and unidentified archaea. However, the relative abundance of top 10 phyla varied greatly among 3 diet groups. Proteobacteria was the first dominant phylum in all the 3 diet groups. The relative abundance of Proteobacteria fed with 100% *D. inornate*, 50% *D. inornate*+50% PMC and 100% PMC varied from 59.43 to 67.65%, 65.18 to 79.04% and 63.39 to 84.69%, respectively. Bacteroidetes, the second dominant phylum in all the 3 diet groups, represented 13.18-25.01% (100% *D. inornate*), 15.85-19.96% (50% *D. inornate*+50% PMC) and 10.23-25.83% (100% PMC) of all sequences. Function of faecal bacteria was predicted and the relative abundance of functional annotations in 3 diet groups is presented in Fig. 2. Similar relative abundant functions were observed in 3 diet groups. The relative abundance of metabolism, including amino acid, carbohydrate, energy, lipid and metabolism of cofactors and vitamins was nearly 50% in all 3 diet groups.

Table 5. Effects of PMC replacement on fatty acid composition of *R. philippinarum* juveniles (µg g wet weight^{-1})

Fatty acid	*D. inornata*	*D. inornata*+PMC	PMC
C11:0	-	0.04±0.07	0.02±0.03
C12:0	0.31±0.03	0.37±0.23	0.33±0.11
C14:0	12.67±1.65^{a}	5.60±1.78^{b}	3.12±0.44^{b}
C15:0	3.84±0.60	3.01±1.72	3.42±0.87
C16:0	85.74±20.23^{b}	126.80±72.26^{a}	162.08±29.84^{a}
C17:0	25.94±1.60	26.47±11.41	27.81±5.80
C18:0	68.94±7.87	71.37±40.60	83.60±17.14
C20:0	33.31±1.95	33.19±1.40	33.85±2.36
C23:0	0.66±0.22^{b}	1.81±0.52^{a}	2.27±0.69^{a}
C24:0	0.73±0.06	0.73±0.36	0.64±0.06
C16:1	26.00±8.30	17.45±4.11	15.17±2.70
C18:1n-9c	56.88±14.05^{b}	105.20±23.93^{a}	117.91±18.17^{a}
C18:2n-6c	21.37±4.37^{c}	119.58±42.53^{b}	190.93±28.59^{a}
C18:3n-6	-	19.33±13.18^{b}	46.57±5.56^{a}
C18:3n-3(LNA)	21.95±3.62	26.31±3.16	28.19±2.57
C20:2	56.37±11.38^{b}	162.88±29.35^{a}	183.56±26.01^{a}
C20:3n-6	15.24±13.20^{b}	26.22±22.86^{b}	56.47±2.01^{a}
C20:4n-6(ARA)	131.98±7.88	118.25±42.73	108.36±18.62
C20:5n-3(EPA)	104.67±19.20	97.27±45.61	68.35±6.63
C22:6n-3(DHA)	310.80±19.11^{a}	290.36±71.26^{a}	208.59±17.63^{b}
∑SFA	232.13±30.26	269.39±128.81	317.11±56.24
∑MUFA	82.88±22.09^{b}	122.65±28.02^{ab}	133.08±20.12^{a}
∑PUFA	662.38±73.80	860.19±249.80	891.02±101.92
∑n-3	126.63±22.71	123.59±48.76	96.55±9.19
∑n-6	168.59±22.51^{b}	283.37±119.75^{ab}	402.33±52.00^{a}
∑n-3/∑n-6	0.75±0.04^{a}	0.44±0.03^{b}	0.24±0.01^{c}

Values are represented as means±SD. Values sharing the same letters are not significantly different ($p>0.05$), whereas those with different letters are significantly different ($p<0.05$). LNA: Alpha-linolenic acid; ARA: Arachidonic acid; EPA: Eicosapentaenoic acid; DHA: Docosahexaenoic acid; ∑SFA: Total saturated fatty acids; ∑MUFA: Total monounsaturated fatty acids; ∑PUFA: Total polyunsaturated fatty acids; - represents not detected.

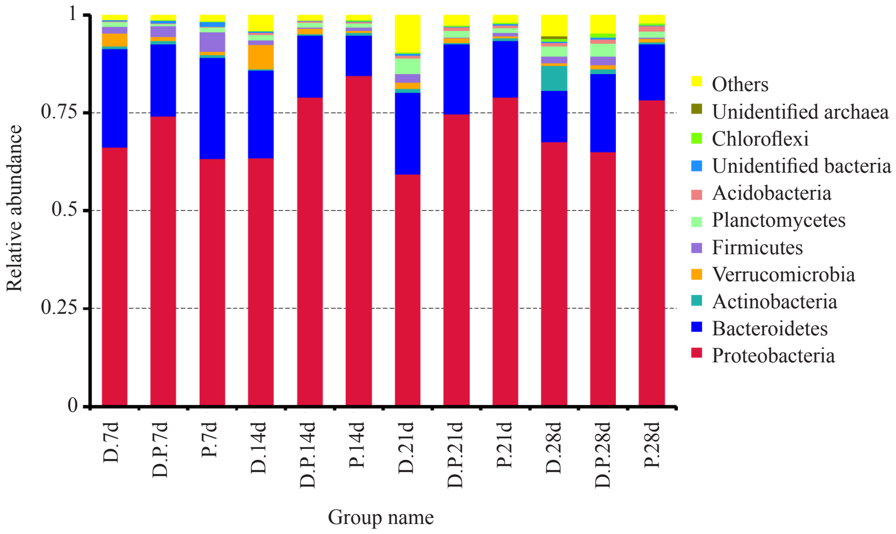

Fig. 1. Composition of the faecal microbiota at phylum level in 3 diet groups. D: Group fed with 100% *D. inornate*, D. P: Group fed with 50% *D. inornate* and 50% PMC replacement, P: Group fed with 100% PMC replacement

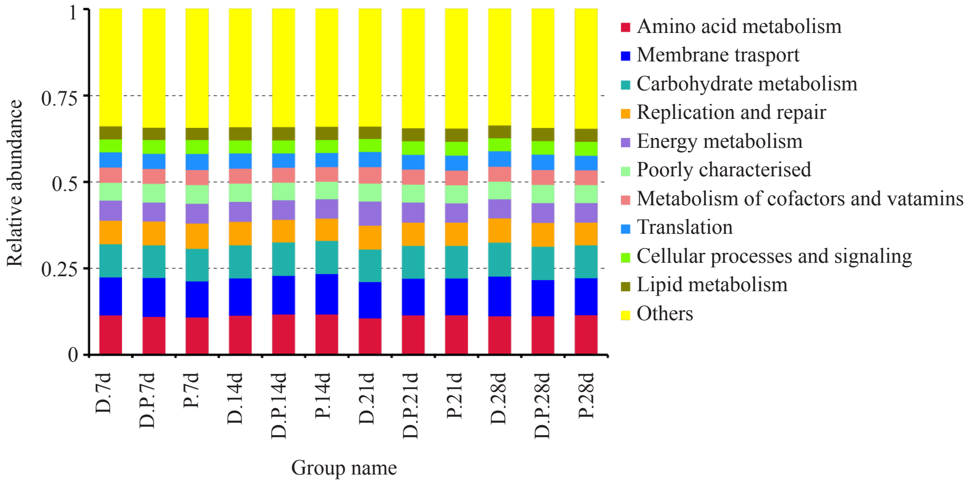

Fig. 2. Functional prediction of the faecal microbiota in 3 diet groups. D: Group fed with 100% *D. inornate*, D. P: Group fed with 50% *D. inornate* and 50% PMC replacement, P: Group fed with 100% PMC replacement

Discussion

Microalgae are the primary food source of bivalves in the hatcheries and considering the nutritional properties, concentrated and dried microalgae appear to be the best alternative products for bivalve juveniles particularly (Robert and Trintignac, 1997). Flagellates and diatoms with high nutritional value for some bivalve larvae or juveniles, such as *Isochrysis* (Liu *et al.,* 2009), *Pavlova luther* (Delaunay *et al.,* 1993) and *Chaetoceros* (Brown and Robert, 2002; Rivero-Rodríguez *et al*., 2007) are commercially reliable microalgae used in hatcheries. As compared to the monospecific microalgae, mixed microalgae diets always showed better stimulatory effects on bivalve productivity because they could complement the nutritional profile and provide balanced essential nutrients (Webb and Chu, 1983; Ragg *et al.,* 2010). In this study, we prepared PMC added with both dried *C. vulgaris* and *S. platensis* powder. *Cholrella* powder has been widely used in fish feed for its advantages in aquaculture, including growth promotion, improving feeding rate and regulating innate immune defense of fish (Kim *et al.,* 2002; Spolaore *et al.,* 2006; Xu *et al.,* 2014). *Spirulina* powder can be used as a protein substitute or functional additive in aquaculture (Ibrahem *et al.,* 2013; Kim *et al.,* 2013). Unlike some species of diatoms and flagellates cultured in sizeable quantities with difficulty, both *Cholrella* and *Spirulina* belong to the most utilised microalgae, which subsequently profit their commercial applications in the substitute diets. In aquaculture, soybean meal has the potential to be used as sustainable, low-cost protein source for its favourable amino acid profile, palatability and digestibility (Watanabe, 2002; Barnes *et al.,* 2014). However, numerous anti-nutritional factors of soybean meal limits its use in fish diets (Iwashita *et al.,* 2008). Heating and fermentation can reduce the amount of thermolabile antinutritional factors (Barrows *et al.,* 2007; Teng *et al.,* 2012). Fermented soybean meal has been suggested to be the primary protein source for some cultured fish (Yamamoto *et al.,* 2010). In this work, fermented soybean meal was supplemented to mixed *Cholrella* and *Spirulina* powder to prepare composite algal powder.

Filter-feeding bivalves have high retaining rates for particles with diameter ranging from 1.4 to 4 μm, which increases with growth (Shumway *et al.,* 1985; Tezuka *et al.,* 2009). The larvae of *R. philippinarum* and *R. decussatus* could ingest single-cell detritus less than 20 μm (Uchida and Numaguchi, 1996; Camacho *et al.,* 2004). To facilitate intake of *R. philippinarum* juveniles, we smashed *Cholrella, Spirulina* powder and fermented soybean meal to particle size below 20 *μ*m. Smashed microalgae powder and fermented soybean meal were added with granular sodium alginate and 2% calcium chloride solution and then compressed to feed blocks. Sodium alginate could cross-link with calcium ions to form a gel, which helped the feed blocks slowly release nutrient substances. Our previous study has proved that the daily release of feed block was about 2 g dry weight under simulated natural flow conditions of seawater. In the cold season, slow-release feed blocks were suitable to be used to deal with the shortage of natural diet in the natural culture conditions. Under indoor culture conditions, without flow velocity, we scrubbed feed blocks to suitable particulate composite before feeding. When PMC replaced 50 or 100% live *D. inornata*, both mean body weight and mean shell length of juvenile clams were similar to those fed with 100% live *D. inornata*. In addition, replacement of 50 or 100% *D. inornata* with PMC did not affect survival rate of juvenile clams. From the growth performance and survival rate of the clams, it is evident that PMC could not only be ingested, digested and assimilated by juvenile clams, but also meet their nutritional requirements.

Aquatic animal growth relies heavily on the nutrient utilisation and digestive enzymes play important roles in nutrient digestion. Their activities directly reflect the digestive capacity and the nutritional status of aquatic animals (Furne *et al.,* 2005). Not only diet size but also diet composition could affect digestive enzyme activities (Baragi and Lowell, 1986; Pedersen and Andersen, 1992). In the current study, partial or total replacement of *D. inornata* with PMC did not affect amylase activity, but increased lipase and pepsin activity, suggesting there was greater difference in dietary protein and lipid than carbohydrate between PMC and *D. inornata*. Both lipase and pepsin activity increased with increase of PMC replacement. This further demonstrated that juvenile Manila clams have the capacity to adapt their digestive physiology in response to the changes in dietary compositions.

There were nine essential amino acids (EAA) in Manila clams, including methionine, leucine, isoleucine, lysine, phenylalanine, tyrosine, threonine, histidine and valine and the nutritional value of protein was largely determined by the content and composition of these EAA. There was no significant difference in these nine EAA contents between clams fed with *D. inornata* and PMC, indicating that total or partial replacement of *D. inornata* with PMC did not affect EAA content and composition of clams. The flavour quality of aquatic organisms mainly depends on the content of delicious amino acids (DAA) contained in their edible parts. In this study, replacing 100% *D. inornata* with PMC led to significant increase in DAA contents. This indicates that PMC could improve the taste of clams and in perspective of protein nutrition, PMC was suitable to replace micrògalgae as a substitute. The growth and development of bivalve larvae and post-larvae necessitates three types of long-chained polyunsaturated fatty acids (LC-PUFA); docosahexaenoic acid (DHA, 22:6n-3), eicosapentaenoic acid (EPA, 20:5n-3) and arachidonic acid (ARA, 20:4n-6) (Langdon and Waldock, 1981; Pernet *et al.,* 2005). However, bivalves lack the ability to biosynthesize these LC-PUFA and have to depend on dietary source (Laing *et al.,* 1990; Freites *et al.,* 2002). In the current study, these LC-PUFA accounted for a significant proportion of PUFA in juvenile clam tissue. Such findings are consistent with previous studies (Freites *et al.,* 2002; Pettersen *et al.,* 2010). Replacement of 50 or 100% *D. inornata* with PMC did not affect ARA and EPA contents of juvenile clams, but decreased DHA contents. The results suggest that PMC could provide ARA and EPA similar to *D. inornata*, but with fewer DHA. With the increased replacement of PMC, DHA content of clam juveniles decreased, however, survival rate of clam juveniles decreased insignificantly. In other bivalve species, inconsistent results were found. Pettersen *et al.* (2010) found a distinct correlation between body DHA content and survival which showed the importance of DHA on larval health of blue mussel and Pernet and Tremblay (2004) got similar results in the sea scallop.

The gut microbiota plays an essential role in the maintenance of host health, including regulating host metabolism (Karasov and Carey, 2009), protecting the host from colonisation by pathogens (Quigley, 2006), enhancing gut development (Castillo *et al.,* 2007) and stimulating development of immunity (Hrncir *et al.,* 2008). Diet is considered a key factor that influence the gut microbiota composition (Tremaroli and Backhed, 2012). It was difficult to sample the gut of juvenile *R. philippinarum* with mean shell length of 5-6 mm and bacterial diversity and abundance in faeces was used to evaluate gut bacterial composition according to the previous studies (Kim *et al.,* 2015; Han *et al.,* 2017). *R. philippinarum* in 3 diet groups had similar top 10 phyla. Proteobacteria and Bacteroidetes were the first and second dominant phyla in faeces of *R. philippinarum* in the 3 diet groups. Consistent with our results, Liu *et al*. (2020) found Proteobacteria (39.3 -84.1%) and Bacteroidetes (7.1-32.6%) as the top two phyla in the gut of scallop *Chlamys nobilis*. Besides Proteobacteria and Bacteroidetes, *R. philippinarum* and *C. nobilis* shared the other two dominant phyla of Actinobacteria and Firmicutes. Products of intestinal bacterial metabolism acted as signaling molecules and influence the host's metabolism (Tremaroli and Backhed, 2012). In humans and other vertebrates, it has been revealed that gut microbiota produces many metabolites, including short-chain fatty acids (Immerseel *et al*., 2010), essential vitamins (Hill, 1997), amino acids (Mardinoglu *et al.,* 2015) and digestive enzymes that act in conjunction with the host (Tremaroli and Backhed, 2012; Carmody and Turnbaugh, 2014). Similar relative abundance of faecal bacterial functions, especially metabolism, were observed in clams fed with or without PMC replacement. Replacement of 50 or 100% *D. nornate* with PMC diet for 28 days had no significant effect on the metabolic functions of gut bacteria and would not affect the host's metabolism obviously.

In conclusion, PMC is cost-effective and easy to handle in perspective of production technology and the price of ingredients. Results of the feeding experiments clearly demonstrated that partial (50%) and total (100%) replacement of *D. inornata* with PMC did not affect growth performance and survival rate of juvenile Manila clams. Results of evaluation of the digestive enzyme activities, amino acid and fatty acid composition and the abundance of faecal bacterial functions, clearly indicates PMC is an appropriate substitute to replace live microalgae as feed in the culture of juvenile clams.

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