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COMPARISON OF NUTRITIONAL AND PROTEASE ACTIVITY PROFILES OF TWO LIVE FEED CANDIDATES OF *Pseudodiaptomus* SPECIES

Rina Puji Astuti, Alim Isnansetyo#, Rarastoeti Pratiwi, and Suwarno Hadisusanto

Universitas Gadjah Mada, Indonesia
Bulaksumur, Caturtunggal, Kec. Depok, Kabupaten Sleman, Daerah Istimewa Yogyakarta 55281, Indonesia

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ABSTRACT

Pseudodiaptomus species are one of the copepods species as a superior live feed to date due to their nutrition and digestive enzyme contents. Some of them have been used as natural food for rearing marine fish larvae. The purposes of this study were to compare the nutritional and protease activity between two species of *Pseudodiaptomus* originated from Indonesian waters, and to determine more superior species to cultivate. Two different feeds i.e. *Thalassiosira* sp. and milk powder were used to grow the *Pseudodiaptomus* species. Analysis of amino acids (AAs) and fatty acids (FAs) profiles were carried out for both the *Pseudodiaptomus* species samples and the feeds, while the protease activity assay was carried out only for the *Pseudodiaptomus* species samples. Results indicated that the nutritional and protease activity profiles of *Pseudodiaptomus* were affected by the types of feed. *Pseudodiaptomus* code P61 was more superior to *Pseudodiaptomus* code P71. This code P61 species contained a wide variety of essential fatty acids and exhibited stable protease activity under the different feeding treatments. However, P61 contained a lower total AA content than P71. Both of them could be cultivated because they were complementary in nutrients to each other.

KEYWORDS: copepods; fatty acid; amino acid; enzyme activity; microcrustacea

INTRODUCTION

Copepods are microcrustaceans that have an important role in maintaining the fishery resources and for fish larvae rearing in hatcheries (Drillet *et al*., 2011) because their nutritional contents are best suited to the nutrient requirement of marine fish larvae (Rayner *et al*., 2015; Rasdi & Qin, 2016). Barroso *et al*. (2013) have reported that *Centropomus parallelus* larvae which are fed with copepods as initial feed contain a better fatty acid composition than those are fed with rotifer. Exogenous enzymes produced by the copepods are other advantages of copepods use, which support the growth of fish larvae.The enzymes play a noteworthy role in the digestion of fish larvae (Zaleha *et al*., 2012). Exogenous enzymes, including protease, lipase, carbohydrate enzymes, and phytase are widely used as fish feed additives worldwide because of their ability to improve nutrient absorption, especially in the early larval phase (Ji *et al*., 2008; Zheng *et al*., 2020). Therefore, the availability of copepods is very crucial for the early life stage of fish larvae, especially marine fish.

Pseudodiaptomus species is a potential copepod to be cultivated. *P. hessei* (Noyon & Froneman, 2014), *P. annandalei* (Rayner *et al*., 2015), *P. inopinus* (Matsui *et al*., 2021) have been profiled their fatty acid, while *Pseudodiaptomus* species code 61 (P61) and code 71 (P71) from Indonesian waters are being characterized and profiled. In our prior study, P61 found in Kulonprogo, Yogyakarta Province, Indonesia; might be a new species based on its morphology and molecular characteristics, while P71 was identified as *P. trihamatus*. *P. trihamatus*is abundant in estuary waters in Pejarakan, Buleleng, Bali Province. Till this moment, nutritional profiles of *P. trihamatus* have not been investigated yet. Therefore, the purposes of this study are to know the most appropriate *Pseudodiaptomus* species for a live feed of marine fish larvae by comparing the amino acid, fatty acid, and protease activity profiles of two *Pseudodiaptomus* species under two different feeds treatment.

Correspondence: Department of Fisheries, Faculty of Agriculture, Universitas Gadjah Mada.
Jl. Flora, Bulaksumur, Yogyakarta, Indonesia
Email: *isnansetyo@ugm.ac.id*

MATERIALS AND METHODS

Sample Preparation for Amino Acids and Prpotease Activity Profiles

Pseudodiaptomus species codes P61 and P71 were collected from shrimp and grouper ponds in Kulonprogo, Yogyakarta and Pejarakan, Bali, respectively in August 2018 and September 2020. The samples of adult *Pseudodiaptomus* for analysis were developed from eggs hatched by 200 gravid females. The rearing was carried out in an 8 L volume of filtered seawater using a 14 L plastic container, at 28-30 ppt salinity and 28°C with a 12:12 hour photoperiod. They were maintained for 14 and 10 days feeding with either 4,500 cells/mL of *Thallassiosira* sp. or 1 mg/L of milk powder (MP). *Pseudodiaptomus* were fasted for 24 hours in seawater and rinsed with fresh water before being harvested. Furthermore, 500-1,000 individuals were freeze-dried for 15 hours to be used in amino acids and fatty acids analyses. In addition, 100 individuals were also fasted and then harvested for protease activity analyses. These samples were stored at -80°C before being analyzed. Amino acids and fatty acids analyses were also conducted for *Thallassiosira* sp. and milk powder as feed.

Analysis of Amino Acids

A total individual number of 500 freeze-dried adult *Pseudodiaptomus* species were placed into a screw tube and added with 2 mL of 6 N HCl. Nitrogen gas was added into the tube for 0.5-1 minute and the tube was closed immediately. The tube was heated at 110°C for 24 hours, then kept at room temperature until it reaches room temperature level. The obtained solutions were transferred into a rotary evaporator flask. The tube was rinsed with distilled water 2-3 times, and combined in the rotary evaporator flask. These samples were dried using a rotary evaporator at 80°C. HCl0.01 N was added until reached 10 mL in volume and then filtered with 0.45 μm millipore paper (Sartorius™). Kalium borate buffer pH 10.4 was added to the sample in a ratio of 1:1 (v/v). Each 5 μL sample was transferred into an empty vial, then 25 μL of OPA solution was added, and left for one minute at room temperature. A sample of 5 μL was injected into the HPLC column (Thermo Scientific ODS-2 Hyersil). Buffer A and buffer B were used as mobile phase with 1 mL/minute of mobile phase flow rate, with a fluorescence detector. Buffer A was composed of 2 g Na-Asetat (pH 6.5), 0.5 g Na-EDTA, 90 mL methanol, and 15 mL tetrahidrofuran dilute in 1,000 mL high purity water which was filtered through 0.45 μm millipore paper. Buffer B was composed of 95% methanol in high purity water which was filtered through 0.45 μm millipore paper. The HPLC was conditioned by adding a gradient mobile phase, namely buffer B in 5% at 0.01 minute, 5% at two minute, 35% at 13 minute, 35% at 15 minute, 70% at 20 minute, 90% at 22 minute, 100% at 25 minute, 0% at 28 minute, and 0% at 35 minute.The concentration of amino acids was measured in mole while the result of the calculation was expressed in % (w/w). The injection was carried out in triple.

Analysis of Fatty Acids

Fatty acids methyl ester (FAME) synthesis was carried out according to the one-step method adopted from Indarti *et al*. (2005) using 1,000 adults *Pseudodiaptomus* and 0.1 mg of the feed samples. The Analysis of fatty acid compounds was carried out by gas chromatography/mass spectrometry (GC/MS) (Agilent), by injecting 1 μL of FAME suspension into HP-5MS 5% Phenyl methyl siloxane columns (Agilent™) at 280°C with a mobile phase of helium gas at a flow rate of 1 mL/minute for 39.667 minutes. The fatty acids were identified based on the Willey 09TH.L database.

Analysis of Protease Activity

Protease activity analysis were carried out using the Pierce Protease Assay Kit number 23263 (Thermo Scientific, USA). A total of 100 adult individuals *Psedodiaptomus* were crushed, dissolved in 200 μL assay buffer (BupH Borate Buffer), and centrifuged at 12,000 g for 10 minutes. The microplate well was added with 50 μL supernatant of sample and 100 μL of succinylated casein solution. The blank well was added with 100 μL of assay buffer. Trinitrobenzene sulfonic acid 50 μL was added to each well. The plate was incubated at 37°C for 20 minutes. The absorbance was measured at 450 nm with a microplate reader (BioTek ELX800). Protease activity was measured by subtracting the blank absorbance from the sample absorbance ("A450). This ("A450) was plotting to the linear regression equation of delta protease standard curve.

Data Analysis

The data were presented in mean ± standard deviation (SD). The statistical analysis was carried out with SPSS version 14.0. Independent-samples T-test was used to compare amino acid contents between species as well as between feed types. The fatty acid data were carried out with a descriptive analysis. The data of protease activity was analyzed with one-way ANOVA.

RESULTS AND DISCUSSION

The feeding treatments using the two types of feed with good nutrient contents (Table 1) were applied to cultivate two species of *Pseudodiaptomus* species code P61 and P71. When the two species were cultivated under *Thalassiosira* sp. feeding treatment, P61 showed a lower concentration of amino acid (AA) than P71. Milk powder (MP) feeding treatment increased AA concentration in P61 as well as P71 but a higher increase was found in P61 than in P71 (Table 2). Each *Pseudodiaptomus* species in this study showed different responses toward the feeding treatments. When P71 was fed with *Thalassiosira* sp., it exhibited a more noticeable increase in AA content than feeding with MP, in contrast, P61 fed with MP produced a higher AA content.

Within the metabolic pathways, AAs act as a regulatory substance. Essential and non-essential AAs play an important role in increasing the growth and survival of the larvae. Glycine is one of the non-essential AAs that plays a role in gluconeogenesis, increases the efficiency of nutrient absorption, and plays an essential role in osmoregulation (Li *et al*., 2009). Glycine supplementation in feed enhances the growth and immune response of *Litopenaeus vannamei* shrimp (Xie *et al*., 2014). This study indicated that the glycine concentrations in P71 and P61 were significantly different. Under *Thalassiosira* sp. feeding treatment, the P71 (2.97 ± 0.00%) showed a higher glycine content than P61 (1.22 ± 0.00%). On the other hand, glycine content in P61 was higher (1.88 ± 0.00%) than in P71 (1.75 ± 0.00%) under MP feeding treatment. Amino acids content in P61 and P71 were significantly different between the two *Pseudodiaptomus* species either under *Thalassiosira* sp. or MP feeding treatments, except for the lysine. The AA analysis results also indicated the interaction between the species of *Pseudodiaptomus* and the type of feed toward the total AA content.

Pseudodiaptomus species are able to synthesize fatty acids (FAs). This study found that P61 contained more diverse FAs than P71 under *Thalassiosira* sp. feeding treatment, which were 18 and 5 FAs, respectively. The P61 is likely able to synthesize FAs, including

Table 1. Detectable fatty acids of *Thallassiosira* sp. and milk powder

***Thalassiosira* sp.**	**Milk powder**
Methyl tetradecanoate	Methyl tetradecanoate
Oleic acid	Ethyl Oleate
Pentadecanoic acid	Pentadecanoic acid
Methyl palmitoleate	Methyl palmitoleate
Methyl palmitate	Methyl palmitate
Hexadecanoic acid	Hexadecanoic acid
Linoleic acid	Linoleic acid
cis-5,8,11,14,17-Eicosapentaenoic acid	Methyl myristoleate
4,7,10,13,16,19-Docosahexaenoic acid	Methyl pentadecanoate
Butyl 6,9,12,15-octadecatetraenoat	Methyl caproate
Methyl 8,11,14,17-eicosatetraenoat	Tetradecanoic acid
Methyl oleate	Methyl n-pentadecanoate
Methyl stearate	Methyl caprylate
Hexadecatrienoic acid	Decanoic acid
Ethyl 9-hexadecenoate	Methyl 15-methylhexadecanoate
Methyl lignocerate	Ethyl palmitate
	Trimethyl citrate
	cis-10-Heptadecenoic acid
	Heptadecanoic acid
	Methyl laurate
	Methyl elaidate
	Stearic acid
	Methyl 10-trans,12-cis-octadecadienoat
	Pentanoic acid
	Methyl linolelaid
	Methyl eicosanoate
	c-2,c-3-epoxy-t-6-methylcyclohept-4-en-r-1-ol

Table 2. Amino acids concentrations (% w/w) in *Pseudodiaptomus* sp. (P61) and *Pseudodiaptomus* sp. (P71) fed with *Thallassiosira* sp. and milk powder

Amino acid		*Thalassiosira* sp.	Milk powder	*Thalassiosira* sp.		Milk powder	
				P61	P71	P61	P71
Essential	Histidine	0.49 ± 0.00^{b}	0.54 ± 0.01^{a}	0.59 ± 0.00^{b}	1.52 ± 0.01^{a}	0.84 ± 0.00^{a}	0.75 ± 0.01^{b}
	Threonine	0.94 ± 0.01^{a}	0.7 ± 0.00^{b}	0.85 ±0.00^{b}	1.76 ± 0.00^{a}	0.99 ± 0.00^{b}	1.14 ± 0.01^{a}
	Arginine	1.96 ± 0.00^{a}	1.69 ± 0.01^{b}	4.86 ± 0.00^{b}	11.26 ± 0.01^{a}	12.41 ± 0.01^{a}	7.32 ± 0.09^{b}
	Tyrosine	0.32 ± 0.01^{b}	0.68 ± 0.00^{a}	0.82 ± 0.01^{b}	1.64 ± 0.01^{a}	0.84 ± 0.00^{b}	1.06 ± 0.01^{a}
	Valine	0.99 ± 0.01^{a}	0.97 ± 0.00^{b}	0.96 ± 0.00^{b}	2.07 ± 0.01^{a}	1.14 ± 0.00^{b}	1.33 ± 0.01^{a}
	Methionine	0.41 ± 0.00^{a}	0.30 ± 0.01^{b}	0.31 ± 0.00^{b}	0.63 ± 0.01^{a}	0.36 ± 0.00^{a}	0.26 ± 0.01^{b}
	Ileucine	0.67 ± 0.01^{b}	0.84 ± 0.00^{a}	0.76 ± 0.01^{b}	1.46 ± 0.00^{a}	0.83 ± 0.00^{b}	0.91 ± 0.01^{a}
	Leucine	1.38 ± 0.01^{b}	1.56 ± 0.00^{a}	1.37 ± 0.01^{b}	2.66 ± 0.01^{a}	1.48 ± 0.00^{b}	1.65 ± 0.00^{a}
	Lysine	1.23 ± 0.01^{b}	1.45 ± 0.01^{a}	1.59 ± 0.01^{b}	3.65 ± 0.00^{a}	2.21 ± 0.00^{a}	2.20 ± 0.06^{ab}
	Phenylalanine	1.02 ± 0.01^{a}	0.81 ± 0.00^{b}	0.79 ± 0.00^{b}	1.46 ± 0.01^{a}	0.82 ± 0.00^{b}	0.99 ± 0.02^{a}
Non-essential	Aspartic acid	1.87 ± 0.00^{a}	1.01 ± 0.00^{b}	1.59 ± 0.01^{b}	3.27 ± 0.01^{a}	1.88 ± 0.00^{b}	2.10 ± 0.00^{a}
	Serine	0.96 ± 0.01^{a}	0.86 ± 0.00^{b}	0.83 ± 0.00^{b}	1.66 ± 0.00^{a}	0.97 ± 0.00^{b}	1.14 ± 0.01^{a}
	Glutamate	2.65 ± 0.00^{b}	3.87 ± 0.01^{a}	2.85 ± 0.02^{b}	5.69 ± 0.01^{a}	3.32 ± 0.00^{b}	3.58 ± 0.00^{a}
	Glycine	1.01 ± 0.00^{a}	0.28 ± 0.00^{b}	1.22 ± 0.00^{b}	2.97 ± 0.00^{a}	1.88 ± 0.00^{a}	1.75 ± 0.00^{b}
	Alanine	1.23 ± 0.00^{a}	0.5 ± 0.00^{b}	1.31 ± 0.01^{b}	2.59 ± 0.00^{a}	1.50 ± 0.01^{b}	1.77 ± 0.00^{a}
Total		**17.14 ± 0.01^{a}**	**16.06 ± 0.01^{b}**	**20.69 ± 0.01^{b}**	**44.27 ± 0.01^{a}**	**31.47 ± 0.01^{a}**	**27.96 ± 0.01^{b}**

Description: The AA value represents the means ± standard deviation (n = 3). The different superscript letters in the same row indicate significant differences between *Thallassiosira* sp. and milk powder and between P61 and P71 species (T-test, $P < 0.05$) in each pair of the column

linoleic acid (ALA), arachidonic acid (ARA), and eicosapentaenoic acid (EPA), which are highly unsaturated fatty acids (HUFA). The three fatty acids play the important role in immunity, structures of cell membranes, eye migration, pigmentation, ion balance regulating, growth, and survival of marine fish larvae (Barroso *et al*., 2017; Jardine *et al*., 2020; Mejri *et al*., 2021). The DHA was the only HUFA detected in the MP treatment of P61. However, DHA and EPA were detected in the MP treatment of P71. *Pseudodiaptomus* code P71 which was fed with MP synthesized FAs in a wide diversity than P71 which was fed with *Thalassiosira* sp. Although in this study EPA and DHA were not detected in MP, these two FAs were detected in P61 and P71. This result indicated that both P61 and P71 are likely able to synthesize DHA and/or EPA from linoleic acid. This finding resolves that P61 and P71 are the superior species in DHA and EPA synthesis as limited copepods species are able to extend the linolenic acid chain into EPA and DHA (Bell *et al*., 2007).

The two *Pseudodiaptomus* species showed different abilities in synthesizing FAs under both *Thalassiosira* sp. or MP feeding treatment (Table 3). Based on the diversity of synthesized FAs, P61 fed with *Thalassiosira* sp. appeared with higher FAs synthesis ability than P71. Under the MP feeding treatment, both *Pseudodiaptomus* species showed asimilar synthesize ability of FAs. However, P71 synthesized two essential fatty acids of DHA and EPA, while P61 synthesized DHA only. Milk powder contained more variations of FAs (27), but EPA and DHA were not detected in this feed, except linoleic acid. Although there were only 16 types of FAs in *Thalassiosira* sp., this phytoplankton contained more complete essential FAs such as linoleic acid, EPA, and DHA.

The feed types affect the synthesis of FAs. This study revealed that P61 and P71 synthesized less essential FAs under MP feeding treatment than under *Thalassiosira* sp. feeding treatment. This is in line with the research reported by Rasdi *et al*. (2015) finding that the content of both unsaturated, monosaturated, and polyunsaturated FAs depends on the feeding treatment. Regarding FAs metabolism, Matsui *et al*. (2021) have found that *P. inopinus* contains higher DHA (16.18 ± 5.02%) than in the algal mixture (6.05 ± 0.10%). However, the EPA concentration in *P. inopinus* (15.28 ± 2.53%) is almost the same as in the feed (17.43 ±

Table 3. Detectable fatty acids in P61 and P71 under *Thallassiosira* sp. and milk powder feeding treatments

Feeding treatments	P61	P71
Thalassiosira sp.	Cyclohexamine Tetradecanoic acid Pentadecanoic acid Hexadecatrienoic acid 9-Hexadecenoic acid Hexadecanoic acid Methyl gamma.-linolenoate Linoleic acid Oleic acid 11-Octadecenoic acid Methyl stearate Methyl Arachidonate cis-5,8,11,14,17 Eicosapentaenoic Dehydroabietic acid Methyl 6,9,12,15,18-heneicosapenta Methyl lignocerate Cholesta-3,5-diene Cholest-5-ene	Hexanoic acid Methyl tetradecanoate Niobe oil Methyl palmitate Octadecanoic acid 5, 8, 11, 14, 17-Eicosapentaenoic acid
Milk powder	Methyl palmitate Methyl oleate Methyl myristate Methyl stearate 4, 7, 10, 13, 16, 19-Docosahexanoic acid cis-4, 7, 10, 13, 16, 19- Docosahexanoic acid Methyl capronate Methyl 3-acetylpropanoate	Methyl palmitate Methyl oleate Methyl myristate Methyl stearate 4, 7, 10, 13, 16, 19-Docosahexaenoic acid cis-5, 8, 11, 14, 17-Eicosapentaenoic Methyl capronate Methyl palmitoleate

0.61%). Meanwhile, Blanda *et al*. (2017) reported that *P. annandalei* in outdoor Taiwanese aquaculture ponds was consistently able to synthesize PUFA which were higher than the PUFA contained in sestons. Moreover, Nielsen *et al*. (2020) stated that *P. annandalei* and *Apocycloproyi* are able to produce high concentrations of DHA when these zooplanktons are fed with *Dunaliella tertiolecta*, *Rhodomonas salina*, and *Saccharomyces cerevisiae*, although the feeds contain only low or even undetectable DHA. However, this present study found that the concentration of EPA synthesized by the two species of P61 and P71 was proportional to the concentration of EPA from the consumed feed. Similarly, *P. hassei* is known to change its fatty acid composition along with the changes of feed types as copepods are able to accumulate FAs, especially EPA and DHA from the feed consumed (Siqwepu *et al*., 2017). In general, feed sources containing higher EPA are better suited for copepods feed. This study indicated that both P61 and P71 are able to synthesize DHA and EPA. It seems with *Cyclopina kasignete* and *Attheyellaris pinosa* can synthesize EPA and DHA although fed with a feed that is low or even undetectable in EPA and DHA contents (Rasdi *et al*., 2015) due to EPA and DHA are synthesizable from ALA (Kabeya *et al*., 2021).

Besides FAs and AAs, under the *Thalassiosira* sp. feeding treatment, the protease activity of P61 was 1.024 units/mL, it was 1.5 times lower than that of P71 which was 1.518 units/mL. Meanwhile, under the MP feeding treatment, the protease activity of P61 was higher eight times (0.823 units/mL) than that of P71 (0.106 units/mL) (Figure 1). These results reveal their varied abilities to digest feed between each species.

Information about the protease activity profile was the other criteria to determine the superior copepod species. The P71 tended to have higher protease activity under the *Thalassiosira* sp. feeding treatment than in P61, but it showed lower protease activity under the MP treatment. While P61 showed high protease activity on both types of feeding treatment. In addition, P61 showed more complete FAs under both feeding treatments. This indicated a wider ability of P61 to consume various types of feed than P71.

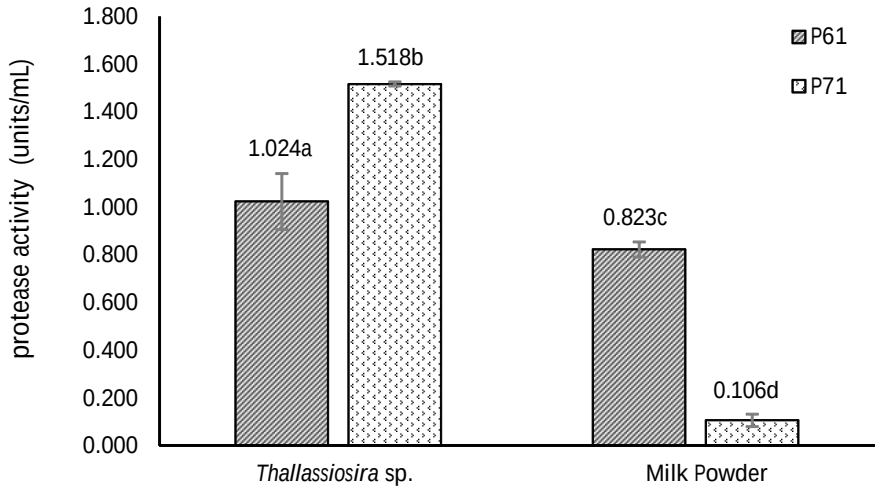

Figure 1. The protease activity of P61 and P71 under the *Thallassiosira* sp. and milk powder feeding treatments (Different letters on the bar indicate significant differences between feeding groups ($P < 0.05$)).

In addition, P61 ability to synthesize methyl arachidonate, which was a group of ARA, was advantageous and required to consider for determining superior *Pseudodiaptomus* species for live feed candidates. Thus, ARA plays an important role in supporting the pigmentation process and eye migration, as well as the growth and survival of either tropical and also cold water fish larvae (Mejri *et al*., 2021). Moreover, the content of ARA and DHA in larval tissue is closely related to eicosanoid metabolism, response to stress, and genes related to skeleton development (El Kertaoui *et al*., 2021), although to achieve those, it was necessary DHA, EPA, and ARA in the correct ratio (Boglino *et al*., 2014; El Kertaoui *et al*., 2021). Even though these three essential FAs are not required at high levels by marine fish larvae, the FAs are essentially present in a live feed to support the metamorphosis and growth of fish larvae. *Pseudodiaptomus* species containing a complete long-chain PUFA is an ideal live feed for the marine fish larvae.

CONCLUSION

It can be concluded that *Pseudodiaptomus* code 61 (P61) was superior to *Pseudodiaptomus* code 71 (P71) in terms of essential fatty acid content for larval feeds. However, both species were feasibly cultivated. This was due to their amino acid content and protease activity that complement each other. Thus, they were simultaneously applicable as feed for fish larvae.

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