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

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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. trihamatusis 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.

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MATERIALS AND METHODS

Sample Preparation for Amino Acids and Protease 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. The rearing was carried out in a 14 L plastic container, at 28-30 ppt salinity and 28°C with a 12:12 hour photoperiod. The rearing was carried out according to the one-step method adopted from 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 Fatty Acids

Fatty acids methyl ester (FAME) synthesis was carried out according to the one-step method adopted from 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 analyses were carried out using the Pierce Protease Kit number 23263 (Thermo Scientific, USA). A total of 100 adult individuals Pseudodiaptomus 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.

Table 1. Detectable fatty acids of Thalassiosira 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                      |
| c-2,c-3-epoxy-t-6-methylcyclohept-4-en-r-1-ol          | Trimethyl citrate                    |

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...
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 milk powder 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 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 ± 5.02%).

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 synthesis 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.

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

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

Total: 17.14 ± 0.01<sup>a</sup> 16.06 ± 0.01<sup>b</sup> 20.69 ± 0.01<sup>b</sup> 44.27 ± 0.01<sup>b</sup> 31.47 ± 0.01<sup>a</sup> 27.96 ± 0.01<sup>b</sup>

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

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

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.
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). 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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