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Comparisons of digestive enzyme activities and the effect of different protein sources on gut performance between 2 g- and 5 g-Pacific white shrimp, *Penaeus vannamei*

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Abstract - The objective of this study is to compare digestive enzyme activities between 2 g-shrimp and 5g-shrimp fed with similar diet. Also, it is extended to compare the gut performance including gut passage time (GPT), gut retention time (GRT), gut passage rate (GPR) after feeding with diets of different protein sources. The activities of three digestive enzymes in digestive organs including stomach, hepatopancreas and intestine in the 2 g-shrimp and 5 g-shrimp were compared. Overall, the activities of trypsin, lipase and amylase were found to be higher in the 5 g-shrimp than those of 2 g-shrimp in

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all three organs tested. In addition, there was no trypsin and lipase activities detected in the intestine of the 2 g-shrimp. The level of lipase activity in the stomach of 2 g-shrimp was 8-times lower than those of the 5 g-shrimp. The feeding experiment was performed to compare the efficiencies of the gut performance in the 2 g-shrimp and 5g-shrimp post feeding with the diets containing different protein sources. Three diet formulae that varied in proportion of fish meal (FM) and soybean meal (SBM) including F1 (30% FM), F2 (10% FM + 28% SBM), and F3 (42% SBM) to result in acceptable total crude protein contents for penaeid shrimp ranging 37% were prepared. The gut performance indicators include gut passage time (GPT), gut retention time (GRT), and gut passage rate (GPR). There were no significant differences among gut performance indicators of the 5 g-shrimp fed with 3 different diets. In contrast, the 2 g-shrimp fed with F3 demonstrated highest GPT, GRT and those fed with F1 revealed highest GPR. Taken together, the results suggest that the digestive functions of the 2 g-shrimp are underdeveloped and the SBM diet retained longer and moved with slow rate in the digestive tract. Further study to demonstrate the adaptability of the 2 g-shrimp to different feed if shrimp has been fed for a long time.

Keywords: Gut performance, fish meal, soybean meal, growth performance, digestive enzymes

1. Introduction

In an intensive shrimp production, feed is the main variable cost and represents up to 50% of the total expense for raising a crop. A better understanding of the mechanism of digestion and nutrition requirement at different developmental stages of shrimp is necessary for design overall diet quality to optimize the use of nutrients and to enhance animal growth. The digestive organs of shrimp include mouth, foregut (stomach), midgut and midgut gland (hepatopancreas), and hindgut (intestine). The midgut is the primary absorptive area of the digestive tract. The hepatopancreas is the shrimp's primary digestive gland and surrounds the posterior stomach and anterior midgut. Feed enters the mouth through the esophagus and stomach, where they are enzymatically digested into small particles with digestive enzymes. The stomach consists of anterior and posterior chambers and extends posteriorly to the midpoint of the hepatopancreas. The posterior chamber contains a gastric sieve. This sieve screens masticated food for delivery to the hepatopancreas. If the ingesta are small enough, it will pass the sieve into the hepatopancreatic primary ducts. The remainder of the ingesta passes into the midgut, where the absorption also occurs. The midgut extends to the sixth abdominal somite and fecal material is contained in a peritrophic membrane. The non-absorbed ingesta will further pass through the hindgut to be excreted as feces (Štrus et al., 2019). The study of digestion enzymatic activities in each digestive organ is important to improve mechanisms of digestion and design of nutritional needs.

Recently, extensive research on soybean meal (SBM) as an alternative protein source to fishmeal (FM) has been conducted to assess their potential impact on feed quality and shrimp growth. SBM

is the most extensively utilized plant-based protein in aquaculture due to its availability, affordability, excellent digestibility, and essential amino acid profile. In this study, the experiments were performed into 2 phases; firstly is the comparisons of the enzyme activities including those of trypsin, lipase and amylase in the hepatopancreas, stomach and intestine of 2 g-shrimp and 5g-shrimp, and secondly, to compare the gut performance including gut passage time (GPT), gut retention time (GRT) and gut passage rate (GPR) after feeding with diets with different protein sources (F1-F3) for one meal. The results from the study will help to design the feed formula suitable for each developmental stage of the whiteleg shrimp, *P. vannamei*.

2. Materials and methods

2.1 Feed formulation and production

Three isonitrogenous and isocaloric feeds (37% crude protein and 360 Kcal/100g feed) were formulated with different fish meal (FM) and soybean meal (SBM) proportions. The F2 feed was composed of 10% FM and 28% SBM, while F1 was FM feed (30% FM without SBM) and F3 was SM feed (42% SBM without FM). The composition of the 3 feed formulae and their proximate analysis were determined by Central Laboratory (Thailand) Co., Ltd. (Table 1). The protein content, fat content, moisture, crude fiber and ash were determined by AOAC (2019), carbohydrate and calories were determined by n-house method TE-CH-169 based on Method of Analysis for Nutrition Labeling (Sullivan & Carpenter, 1993).

Table 1. Composition (g/100 g feed) of three diets used in this study.

Ingredients	g/100g feed (as-is-basis)		
	F1	F2	F3
Fish meal (62% Protein)	30	10	0
Soybean meal (48% Protein)	0	28	42
Poultry meal (65% Protein)	13	13	13
Wheat gluten (82% Protein)	3	3	3
Wheat flour	20	20	20
Rice broken	25.15	16.05	9.57
Squid-liver meal (50% Protein)	3	3	3
Methionine	0.06	0.1	0.17
Lysine	0.23	0.17	0.25
Vitamins	1	1	1
Minerals	1	1	1
STAY C vitamin C 35%	0.1	0.1	0.1
Marine fish oil	0	2	3
Soybean oil	0.46	0.20	0.08
Lecithin	1	1	1
Monocalcium phosphate	0	1.38	2.84

2.2 Shrimp specimens and culture conditions

The experimental animals used in this study were handled according to the Thai national guidelines on the care and use of animals for scientific purposes under permits BT-Animal 05/2565 and MUSC64–035–584 from the Institutional Care and Use Committee, BIOTEC, NSTDA, and Faculty of Science, Mahidol University. Specific pathogen-free (SPF) shrimp were reared in the hatchery of the Faculty of Agriculture and Natural Resources, Rajamangala University of Technology Tawan-ok (RMUTTO), Chonburi, Thailand until the sizes reached fresh weights of approximately 2 g (approximately 4 weeks old) or 5 g (approximately 8 weeks old). A total number of 600 shrimp of 2 g and number of 100 shrimp of 5 g were acclimatized in 1,000 L tanks containing 800 L of 20 ppt saline water with sufficient aeration and fed at 5% body weight daily with the commercial feed available in the market. During the culture period, water quality was maintained at pH 7.8 -8.0, dissolved oxygen >4 mg/L, alkalinity >100 mg/L, total ammonia <1 mg/L, nitrite <0.4 mg/L, water temperature at 28- 30°C. The overall experimental design using SPF shrimp was shown in Figure 1.

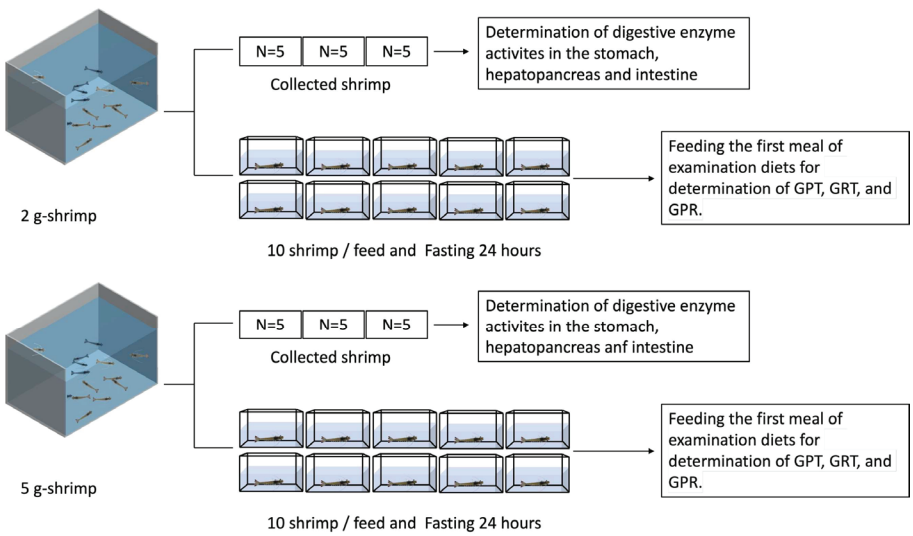


Figure 1. Diagram of the overall experiments and measurement in this study.

2.3 Comparisons of digestive enzyme activities between 2g- and 5g-shrimp

Before experimental feeding, three tissues; stomach, hepatopancreas, and intestine were separately collected from 15 individuals each of 2 g-shrimp and 5 g-shrimp. For each group of shrimp weight, the 3 samples (n=3) with 5 shrimp each were prepared. The crude enzyme extracts were prepared by individually grinding the samples on ice with 50 mM Tris-HCl buffer containing 200 mM NaCl (pH 8) at a ratio of 1:1 (w/v) (Rungruangsak-Torrissen, 2007). The homogenate was centrifuged at 15,000 ×g, 4°C for 60 minutes. The collected supernatant was referred to as the crude enzyme extract (CEE) and kept at

-80°C until used to determine the protein concentration using Bradford's reagent (Bio-rad, USA) and to measure enzyme activities.

The amylase activity assay was modified from the method by Areekijseree et al. (2006). Briefly, 4 µg protein of CEE was mixed with 25 µl of 1% starch dissolved in 100 mM Tris-HCl containing 6 mM NaCl (pH 8). The mixture was incubated at 37 °C for 15 minutes. Amylase activity was determined using 3,5 Dinitrosalicylic acid (DNS) as a substrate and measured absorbance at a wavelength of 540 nm. For the construction of the standard curve, the set of maltose was prepared with different concentrations at 5, 10, 15, 20, and 25 mM and used as the standard for amylase-digested products.

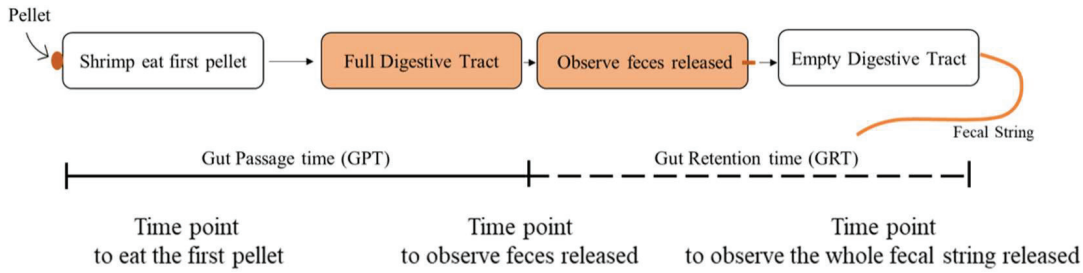
Lipase activity was measured following Versaw et al. (1989) with a modification for microplate (96 well plate) assay. The assay mixture comprised 5 µl of 100mM sodium taurocholate, 100 µl of 50mM Tris-HCl (pH 8.0), 4 µg protein of CEE (1 µl), and 1 µl of 200mM β-naphthyl caprylate. The reaction mixture was incubated at 37 °C for 30 min, then 1 µl of 100 mM fast blue BB was added, and the reaction was stopped by adding 10 µl 0.72N trichloroacetic acid and 136 µl of 1:1 (v/v) ethyl acetate/ethanol solution. The reaction mixtures were then measured for absorbance at 540 nm using a microplate reader (VERSA max tunable, Molecular device, USA). A set of 1, 2, 4, 6, 8, and 10 mM β-naphthol was assayed in parallel to construct a standard curve.

Trypsin-specific activity measurement was modified from the method described by Rungruangsak-Torrissen (2007). Briefly, 200 µl of 1.25 mM Bensoyl-L-arginine-p-nitroanilide (BAPNA), a specific substrate for trypsin, was added into each well of 96 well plates containing 4 µg protein of CEE. The solutions were incubated at 37 °C and absorbance (A_{410}) measured every minute for 10 minutes. For construction of a standard curve, the set of p-nitroaniline was prepared at different concentrations of 1, 2, 4, 6, 8, and 10 mM.

2.4 Determination of gut passage time (GPT), gut passage rate (GPR) and gut retention time (GRT)

The effect of three diets (F1-F3) on gut performance (GPT, GPR, and GRT) was investigated in 2 g-shrimp and 5 g-shrimp. Determination of gut performance employed shrimp only at the intermolt stage. Shrimp were reared in individual acrylic tanks and were starved for one day to clear their gastrointestinal contents. The experimental shrimp were then separately fed with F1, F2, and F3 at 1.5% BW (10 shrimp/ feeding group) for one meal. The GPT, GPR, and GRT values were subsequently determined.

The GPT and GRT were recorded according to the duration of post feeding shown in Figure 2. GPT is defined as the elapsed time between the first ingestion of a feed pellet and its earliest or first defecation. GRT is defined as the time elapsed between the first defecation and the occurrence of an empty intestine with release of the fecal string into the water. The gut length (GL) of each individual shrimp was measured to calculate the gut passage rate (GPR), which indicated the rate of gut content movement. Determination of GPT, GPR and GRT were modified from Beseres et al. (2006).



Gut passage rate (GPR) = Gut length (millimeter) / GPT (minute)

Figure 2. Schematic diagram to measure gut passage time (GPT, min), gut retention time (GRT, min), and gut retention time (GRT, min) in this study.

2.5 Calculations and statistical analysis

The value of GPT, GRT, and GPR as well as digestive enzyme activities in this study were analyzed by IBM SPSS Statistics version 22. One-way ANOVA and Tukey HSD method (Tukey, 1977) was used to compare the data among groups. The digestive enzyme activity were analyzed using independent sampled t-test of 2 g-shrimp and 5 g-shrimp. Differences were statistically significant when $p < 0.05$.

3. Results

3.1 Proximate composition of the diets

Proximate analysis

Although most parameters measured in the feed diets were similar among the three feeds, the feed diets with the component of SBM (F2 and F3) showed higher fiber contents than those of F1 (Table 2).

Table 2. Proximate analysis of the test feeds

Components	g/100g feed (as-is-basis)		
	F1	F2	F3
Protein	37.8	37.34	37.09
Fat	6.07	6.44	6.60
Carbohydrate	39.71	39.77	38.11
Fiber	0.39	1.26	1.80
Ash	7.76	7.24	7.63
Moisture	8.27	8.01	8.77
Calories	364.67	366.4	360.20

3.2 Comparisons of digestive enzymes in 2g- and 5g- shrimp

Before feeding with F1-F3 diet, three enzymatic activities were determined in the 2 g-shrimp and 5 g-shrimp and the results are shown in Table 3 and Figure 3. The digestive enzyme activity were analyzed using independent sampled t-test of 2 g-shrimp and 5 g-shrimp. Overall results

revealed that the 5 g-shrimp exhibited higher activities of the three enzymes (amylase, lipase and trypsin) in the stomach and intestine than did the 2 g-shrimp (P<0.05). There were no significant differences in the hepatopancreatic amylase or lipase activities among the 5 g-shrimp and 2 g-shrimp (Figure 3A-B). The hepatopancreas of the 5 g-shrimp showed higher trypsin activity than that of the 2 g-shrimp (Figure 3C).

Table 3. Determination of amylase, lipase and trypsin activities in 2g- and 5g-shrimp

	Stomach	**HP**	**Intestine**
Amylase (*mM Maltose/min/mg protein*)			
2g	343.06±98.36[a]	270.57±11.40[a]	108.61±0.43[a]
5g	556.71±39.65[b]	249.76±29.48[a]	353.43±15.51[b]
p-value	0.025	0.318	0.002
Lipase (*mM β-Naphthol/min/mg protein*)			
2g	3.70±3.98 [a]	33.39±2.90[a]	0.00±0.00[a]
5g	27.63±5.29[b]	34.48±3.62[a]	21.58±3.53[b]
p-value	0.003	0.705	0.009
Trypsin (*mM p*-Nitroaniline/min/mg protein)			
2g	133.70±24.65[a]	562.62±20.74 [a]	0.00±0.00[a]
5g	410.98±35.07[b]	979.30±48.42[b]	90.48±23.84[b]
p-value	0.000	0.000	0.003

Note: The values were shown as mean ± standard deviation (SD). The different superscript letters in the same column represent statistically significant differences (p < 0.05).

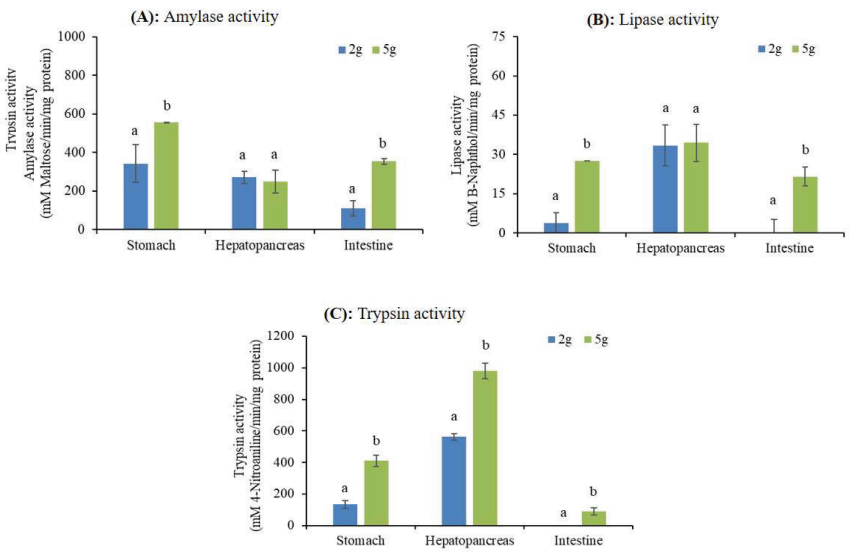


Figure 3. Determination of amylase, lipase, and trypsin in the digestive tissues of 2g- and 5g- shrimp. The protein lysates were derived from the stomach, hepatopancreas, and intestine. The enzymatic activity was expressed in mM of substrate/min/mg protein.

3.3 Comparisons of gut performance among 2g- and 5g-shrimp after feeding one meal with three different diets

To compare gut performance between 2 g-shrimp and 5 g-shrimp, the experimental shrimp were starved for one day before feeding with three different diets for one meal. Shrimp fed with F1 finished all pellets faster than those fed with F2 and F3. The GPT, GRT, and GPR of 2 g-shrimp and 5 g-shrimp are shown in Table 4 and Figure 4. After feeding, there were no significant differences in GPT, GRT, and GPR among the 3 diet groups (p = 0.130, p = 0.667, and p = 0.229) in the 5 g-shrimp, as shown in Figure 4A. In contrast, there were significant differences in GPT, GRT, and GPR of 2 g-shrimp among the groups fed with three different diets, as shown in Figure 4B. The 2 g-shrimp fed with the F3 diet revealed the longest GPT and GRT followed by those fed with F2 and F1, respectively (p = 0.001). For GPR, the group fed with the F1 diet showed the highest rate of gut content movement, compared to those fed with F2 and F3 diets (p = 0.000).

Table 4. Determination of GPT, GRT and GPR in the 2g- and 5g-shrimp after fed with 3 different diets.

Diets	Weight (g)	GPT (min)	GRT (min)	GPR (mm/min)
2g-shrimp				
F1	2.41±0.40^{a}	26.30±4.83^{a}	74.70±11.89^{a}	1.89±0.32^{b}
F2	2.22±0.32^{a}	32.70±3.74^{a}	78.50±13.53^{a}	1.47±0.20^{a}
F3	2.64±0.62^{a}	43.50±14.42^{b}	103.00±22.36^{b}	1.20±0.36^{a}
p-value	0.127	0.001	0.001	0.000
5g-shrimp				
F1	5.41±0.37^{a}	36.40±10.30^{a}	101.10±25.54^{a}	1.81±0.41^{a}
F2	5.59±0.29^{a}	48.30±21.55^{a}	96.60±21.55^{a}	1.49±0.43^{a}
F3	5.44±0.39^{a}	42.50±10.80^{a}	91.20±25.98^{a}	1.62±0.38^{a}
p-value	0.487	0.130	0.667	0.229

Note: The values were shown as mean ± standard deviation (SD). The different superscript letters in the same column represent statistically significant differences ($p < 0.05$).

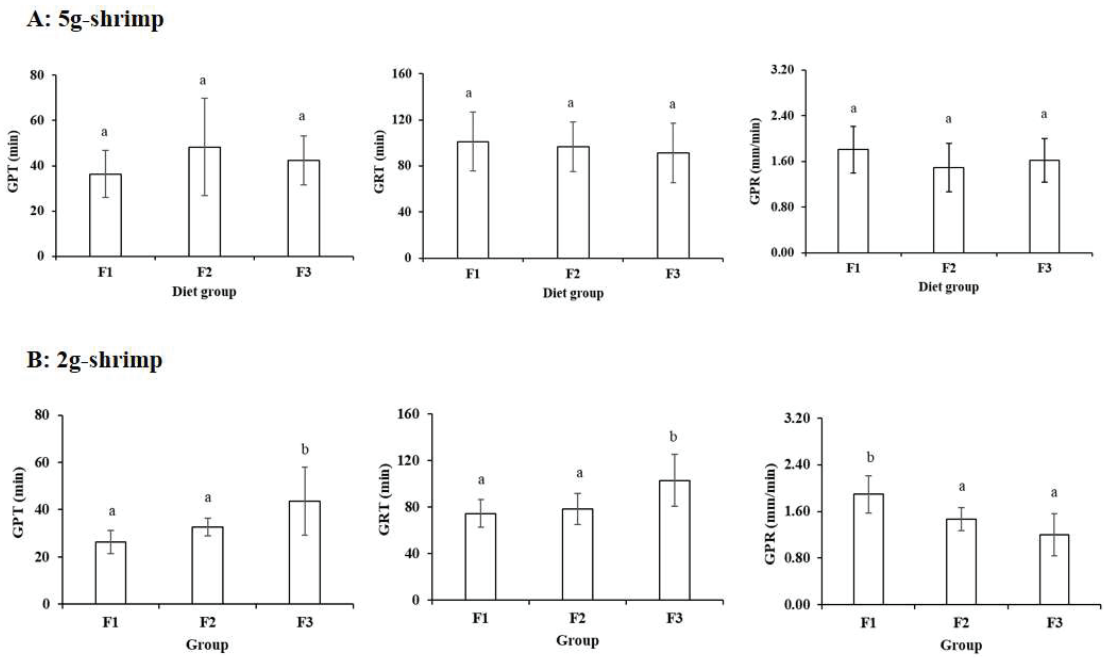


Figure 4. Gut passage time (GPT), gut retention time (GRT), and gut passage rate (GPR) of shrimp fed three different diets. A: 5 g-shrimp and B: 2 g-shrimp. The different superscript letters in each study indicate statistically significant differences ($p < 0.05$).

4. Discussion

The feed formulae were adjusted to meet a requirement of shrimp commercial feed, as shown in the proximate analysis in Table 2. These ingredients were added at different concentrations to balance essential amino acids, essential fatty acids and phosphorus according to the requirements of shrimp to ensure each diet contained similar nutritional values. Thus, we could focus on the effects of quantity of fishmeal and/or soybean meal in the diet on gut performance without other factors that might interfere the results. Generally, balancing the nutritional values of animal diet has been done by feed mill, especially when fishmeal and/or fish oil was replaced by another ingredient.

Digestive enzyme activities including those of amylase, lipase, and trypsin were detected in the digestive tissues including the stomach, hepatopancreas, and intestine of the 5 g-shrimp. In contrast with the 2 g-shrimp, very low levels of amylase activity were found in the intestine together with low lipase and trypsin activities in both the stomach and intestine. These results suggest that the digestion process of the 2 g shrimp was not fully functional when compared to the 5 g-shrimp. A study conducted by Gamboa-Delgado et al., (2003) agreed with our results in that significant increases in activities of lipase and chymotrypsin were observed as shrimp grew (2-12 g). Trypsin activity showed a peak at 5 g-stage and amylase activity increased two-fold after 2 g-stage. Protein is a major essential macromolecule highly required for juvenile shrimp growth (Aaqillah-Amr, et al., 2021). Trypsin, a major digestive protease found in the penaeids shrimp, has been emphasized as contributing to the process of protein digestion (Galgani

et al., 1985). A study performed by Shao et al. (2018) proposed that trypsin was the key regulator to determining growth performances in 2 g-whiteleg shrimp fed with different diets. Thus, the low trypsin activity in the stomach and hepatopancreas in our study probably contributed to lower digestibility in the 2 g-shrimp when compared to 5 g-shrimp.

The gut passage (transit) time (GPT) and rate (GPR) refer to the timing and velocity of ingesta transportation from feeding to defecation along the digestive tract that reflects the ability of digestion and absorption efficiencies (McGaw & Curtis, 2013). While gut retention time (GRT) could be considered by the duration of all crude ingesta remaining in the tract. All these parameters would be expected indicators of gut performance. Besides enzymatic levels, the under-development of the digestion process of the 2 g-shrimp was also demonstrated by the detection of the changes in the gut performance after feeding with the three diets with different protein sources (F1 = 30% FM, F2 = 10% FM + 28% SBM and F3 = 42% SBM) for one meal. There was no effect of protein sources on gut performance (GPT, GRT, and GPR) of the 5 g-shrimp (Figure 4). In contrast, longer GPT, GRT, and shorter GPR were found in the 2 g-shrimp fed with F3 than those fed with F1and F2. The highest GPT and GRT were found in the 2 g-shrimp fed with F3 diet which contained highest fiber content. The next step will be to determine the effect of fiber in the diet contributes to the high passage time and retention time of the digesta in the digestive tract.

5. Conclusion

The results from this study suggest that the functions of 3 important digestive enzymes were not fully developed in the 2 g-shrimp. To increase the effectiveness of digestion and absorption, shrimp must prolong their GPT and GRT with shortened GPR. The next experiment to study if the adaptability of shrimp to varying dietary components has an impact on animal growth performance.

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