A THESIS
FOR THE DEGREE OF MASTER OF SCIENCE

Dietary Riboflavin Requirement of Pacific White Shrimp (*Litopenaeus vannamei*)

Meepavita Gamage Kokila Sanjeewani Malkumari

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(Supervised by Professor Kyeong-Jun Lee)

A thesis submitted in partial fulfilment of the requirement for the degree
of
MASTER OF SCIENCE
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한글 요약문

본 연구는 흰다리새우 (*Litopenaeus vannamei*)를 대상으로 riboflavin 의 요구량을 규명하고 성장, 사료효율, 선천적 면역력 및 사료 소화율에 미치는 영향을 조사하고자 수행되었다. 총 7 개의 실험사료는 riboflavin 의 함량을 각각 0, 10, 20, 30, 40, 50, 60 mg/kg 농도로 기초사료에 첨가하였다 (R0, R10, R20, R30, R40, R50, R60). 새우 (평균무게, 0.17 ± 0.00 g)는 실험사료구 당 4 반복으로 배치했으며, 실험사료는 1 일 6 회에 나누어 8 주에 걸쳐 공급하였다. 성장률, 일간 성장률, 단백질이용효율은 riboflavin 첨가구가 대조구에 비해 유의적으로 높게 나타났다 ($p < 0.05$). 실험새우의 phenoloxidase, nitroblue tetrazolium, superoxide dismutase, glutathione peroxidase 활성은 R40 구가 가장 높았다. Lysozyme 활성은 R30, R40 구가 R60 구에 비해 유의적으로 높게 나타났다 ($p < 0.05$). 장 융모는 R50, R60 구가 다른 실험구에 비해 유의적으로 길었으며, R0 구가 가장 짧게 나타났습니다 ($p < 0.05$). 사료의 건조물 및 단백질의 명시적 소화율 계수는 리보플라빈 함량에 영향을 받지 않았습니다 ($p < 0.05$). 건물 및 단백질소화율은 모든 실험구 사이에 유의적인 차이를 보이지 않았다. 전어체 내 일반성분은 모든 실험구간에 유의적인 차이가 없었다. 따라서, 사료 내 riboflavin 의 첨가는 흰다리새우의 성장, 사료효율, 비특이적 면역력, 장 융모의 길이를 증진 시킬 수 있을 것으로 사료된다. 흰다리새우 사료 내 riboflavin 의 적정 첨가 함량은 40.9 mg/kg 로 판단된다.

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ABSTRACT

This study was conducted to determine the dietary riboflavin requirement and its effects on growth performance, feed utilization, innate immunity and diet digestibility of *Litopenaeus vannamei*. A riboflavin-free basal diet (R0) was formulated as a control and six other diets were prepared by adding riboflavin of 10, 20, 30, 40, 50 and 60 mg/kg to the basal diet (designated as R10, R20, R30, R40, R50 and R60, respectively). Quadruplicate groups of shrimp (initial average weight 0.17 ± 0.00 g) were fed the diets six times a day for 8 weeks. Weight gain, specific growth rate and protein efficiency ratio were significantly increased by riboflavin ($p < 0.05$). The maximum values were observed in shrimp fed R40 diet. The highest activities of phenoloxidase, nitro blue tetrazolium, superoxide dismutase and glutathione peroxidase were observed in shrimp fed R40 diet. Lysozyme activity was significantly higher in shrimp fed R30 and R40 diets than that of shrimp fed R60 diet ($p < 0.05$). Intestinal villi were significantly longer in shrimp fed R50 and R60 diets compared to those of all other groups while the shortest villi were observed in R0 group ($p < 0.05$). Intestinal villi were clearly distinguished in shrimp fed higher levels of riboflavin compared to those of shrimp fed R0 and R10 diets. Apparent digestibility coefficients of dry matter and protein in diets were not significantly affected by riboflavin levels ($p < 0.05$). Whole-body proximate composition and hemolymph biochemical parameters were not significantly altered by dietary riboflavin ($p < 0.05$). Therefore, the results of this study indicate that riboflavin is essential to enhance growth performance, feed utilization, non-specific immunity and intestine morphology of shrimp. An optimal riboflavin requirement for the maximum growth of *L. vannamei* seems to be approximately 40.9 mg/kg diet.

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1.Introduction

1.1 Riboflavin

Riboflavin (vitamin B2) is a water-soluble and heat-stable vitamin synthesized by plants and most microorganisms. It is essential for biochemical reactions in all living cells (Revuelta et al., 2017). Riboflavin was first documented in 1879 by Alexander Wynter Blyth, as a yellow, fluorescent pigment found in milk (Cardoso et al., 2012); however, the vitamin property of riboflavin was not established until the early 1930s (Northrop-Clewes, & Thurnham 2012). Riboflavin can be produced in large quantities through chemical synthesis and fermentation of sugar sources by genetically modified bacteria strains (Averianova et al., 2020; Revuelta et al., 2016). Chemical formulation of riboflavin is 7, 8-dimethyl-10-ribityl-isoalloxazine which consists of a flavin isoalloxazine ring bound to a ribitol side chain. It functions as a precursor of two coenzyme forms, flavin adenine dinucleotide (FAD) and flavin mononucleotide (FMN) after phosphorylation by riboflavin flavokinase and FAD pyrophosphorylase (Figure 1). Both coenzymes are primarily required for redox reactions and energy production in animals through the metabolism of carbohydrates, lipids, proteins and ketone bodies (Revuelta et al., 2017; Beztsinna et al., 2016).

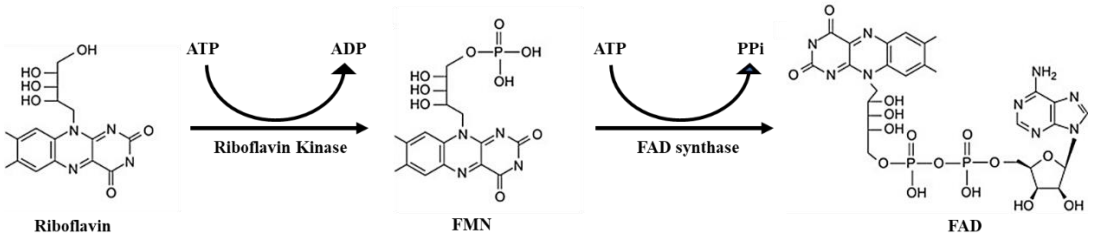

Figure 1: Flavin mononucleotide (FMN) is formed first by the action of riboflavin kinase on riboflavin. FMN is then converted to flavin adenine dinucleotide (FAD) by the action of FAD pyrophosphorylase with ATP.

1.2 Role of riboflavin.

Riboflavin protects human and animal tissues from oxidative stress by preventing lipid peroxidation. All aerobic cells produce free radicals, which cause tissue damage, known as reperfusion oxidative injury, leading to ageing, degenerative diseases, damage to cellular macromolecules (DNA, proteins, and lipids) and cell death (Venkataraman et al., 2013; Suwannasom et al., 2020). Free radicals are neutralized by antioxidant enzymes to prevent lipid peroxidation. Riboflavin directly regulates the activity of glutathione peroxidase which is one of main antioxidant enzymes neutralize H_2O_2 by oxidizing glutathione (GSH) to glutathione disulfide. The FAD form of riboflavin is required to activate glutathione reductase which induces the reversible reaction of converting glutathione disulfide to GSH (Figure 2) (Ashoori & Saedisomeolia, 2014).

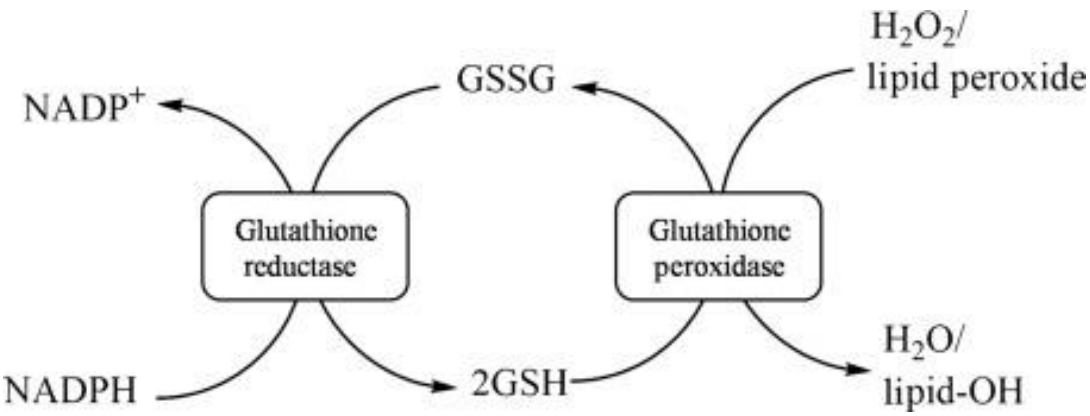

Figure 2: Conversion of oxidized glutathione (GSSG) to the reduced form (GSH) by glutathione reductase. Glutathione reductase requires riboflavin in the FAD coenzyme form for its activity. G-6P-D, glucose-6-phosphate dehydrogenase (Saedisomeolia & Ashoori 2018).

Riboflavin promotes the conversion of tryptophan into niacin (vitamin B3) together with pyridoxine (vitamin B6) (Gasco et al., 2018) and activates vitamin K, pyridoxine and folic acid (Vitamin B9) to their physiologically active forms (Pinto & Rivlin, 2013; Preusch &

Suttie, 1981). Furthermore, different studies of rats revealed that riboflavin can improve the early postnatal development of the gastrointestinal tract (Yates at el., 2003), iron absorption and mobilization (Powers, 2021; Powers, 1987).

1.3 Riboflavin in aquaculture

Riboflavin and other vitamins are often produced by gut microbiota in animals (Wong et al., 2014; LeBlanc et al., 2013). The quantity of vitamins produced via microbial synthesis is generally low in shrimp and other decapods because of their simple digestive tracts compared with mammals and birds (Conklin, 1989). Therefore, the overall riboflavin levels in practical feeds are prone to be insufficient to meet the dietary requirements of decapods despite of the presence of riboflavin in many feed materials (Written & Aulrich, 2019). The effects of dietary riboflavin supplementation on aquatic animals have been examined in numerous studies. The optimum level of dietary riboflavin improves growth, gill immunity, flesh quality, antioxidant system, intestinal health and mRNA expression of tight junction proteins in grass carp, *Ctenopharyngodon idella* (Jiang et al., 2019; Chen et al., 2015a; Chen et al., 2015b). Riboflavin can enhance growth and prevent deficiency signs such as anorexia, short-body dwarfism and cataracts in sunshine bass, *Morone chrysops* × *M. saxatilis* (Deng & Wilson, 2003) red hybrid tilapia, *Oreochromis mossambicus* x *O. niloticus* grown in seawater (Lim et al., 1993) and channel catfish, *Ictalurus punctatus* (Serrini et al., 1996). Kumar (2021) reported that dietary riboflavin improved the growth performance, antioxidant enzyme activities and immune responses of striped catfish, *Pangasianodon hypophthalmus* under stresses given by arsenic pollution (2.68 mg/L) and high temperature (34°C). Riboflavin increases the intestinal enzyme activities, protein efficiency and lipid utilization in addition to promoting growth performance in Jian carp, *Cyprinus carpio* var. Jian (Li et al., 2010). Dietary riboflavin positively affected RNA/DNA ratio of spotted snakehead, *Channa punctatus* (Zehra & Khan, 2018). Riboflavin alone or in combination with folic acid was reported to enhance growth, hemolymph biochemical, and immunological parameters of rainbow trout, *Oncorhynchus*

mykiss (Lameeihassankiadeh et al., 2019a; Lameeihassankiadeh et al., 2019b). In shrimp, optimum riboflavin levels in diets for *Penaeus monodon* and *Penaeus japonicus* were estimated as 22.3 and 80 mg/kg, respectively (Chen & Hwang, 1992; NRC, 1983). Whole-body riboflavin level of *P. monodon* was increased with the increase of dietary riboflavin levels although the growth, feed utilization and survival were not significantly affected (Chen & Hwang, 1992). However, *P. monodon* fed a riboflavin-deficient diet (0.48 mg/kg) showed deficiency signs including irritability, short-head dwarfism, protuberant cuticle and light coloration (Chen & Hwang, 1992). However, Reddy et al. (1999) did not observe any effect by deletion of riboflavin from juvenile *P. monodon* diets compared to shrimp fed diet containing 50 mg/kg riboflavin for 8 weeks. The deletion of riboflavin from dietary vitamin mixture led to increased growth rates of *P. monodon and* juvenile freshwater prawn, *Macrobrachium rosenbergii* (Heinen, 1984; Catacutan & Cruz, 1989).

1.4 Pacific white shrimp

Pacific white shrimp, *Litopenaeus vannamei*, is the most economically important shrimp species in the world aquaculture industry due to its fast growth rate and high nutrition value. *L. vannamei* production was about 5812.2 thousand tons in 2020 which was more than half of the total crustacean production in the world (FAO 2022). It is necessary to provide nutritionally balanced feed which meets their nutrient requirements for optimum growth performance, feed utilization efficiency and health condition. Nutritionally balanced feeds are also important to reduce production cost and water pollution in the aquaculture industry. According to previous studies, low or high dietary vitamin levels led to reduced shrimp growth and health status (Xia et al., 2015; Cui et al., 2016). The dietary requirements of other vitamins were previously determined for *L. vannamei*, including thiamin (Huang, et al., 2015), niacin (Xia et al., 2015; Hasanthi and Lee, 2023), pyridoxine (Cui et al., 2016; Li et al., 2010) and biotin (Xia et al., 2014). However, the optimum riboflavin requirement for *L. vannamei* remains unknown although it is available for *P. japonicus* and *P. monodon*. Therefore, this study was designed to

determine the dietary riboflavin requirement of *L. vannamei* through its effects on growth performance, feed utilization, innate immunity, antioxidant capacity, intestinal histology and nutrient digestibility in diet.

2.Materials and Methods

2.1. Experimental diets

Riboflavin-free basal diet (R0) was formulated as the control to contain 100 g/kg fish meal (tuna meal and sardine meal), squid liver meal 50 g/kg and 400 g/kg soybean meal as major protein sources. Fish oil was used as a major lipid source. The six experimental diets were prepared by adding riboflavin (99% purity) to the control diet at concentrations of 10, 20, 30, 40, 50, and 60 mg/kg (designated as R10, R20, R30, R40, R50 and R60 respectively). Ingredients were thoroughly mixed and a wet dough was prepared by adding fish oil and distilled water (10%). Then the homogenous mixture was pelleted by using a pelletizer (SP-50, Gum Gang Engineering, Korea) with a 2 mm die. The pellets were dried using an electrical drier (SI-2400, Shinil General Dryer Co., Ltd) at 24-25°C for 8 h to reduce the moisture content below 10%. Then stored at –20°C in air-tight polyethylene bags until used. The ingredients and proximate composition of the seven diets are shown in Table 1. The riboflavin content of diets was determined by high-performance liquid chromatography (HPLC) method of Hasselmann et al. (1989) and found to contain 1.41 (R0), 6.38 (R10), 17.87 (R20), 25.26 (R30), 37.11 (R40), 46.01 (R50), and 55.11 (R60) mg/kg riboflavin in the dry diet.

Figure 3: Experimental diet preparation for Pacific white shrimp (*Litopenaeus vannamei*).

Table 1: Formulation and proximate composition of the experimental diets for Pacific white shrimp (*Litopenaeus vannamei*) (g/kg, dry matter basis).

Ingredients	Experimental Diets						
	R0	R10	R20	R30	R40	R50	R60
Fish meal (sardine)	50.0	50.0	50.0	50.0	50.0	50.0	50.0
Fish meal (tuna)	50.0	50.0	50.0	50.0	50.0	50.0	50.0
Soybean meal	400	400	400	400	400	400	400
Squid liver meal	50.0	50.0	50.0	50.0	50.0	50.0	50.0
Wheat flour	180	180	180	180	180	180	180
Starch	140	140	140	140	140	140	140
Fish oil	50.0	50.0	50.0	50.0	50.0	50.0	50.0
Mineral Mix[1]	20.0	20.0	20.0	20.0	20.0	20.0	20.0
Vitamin Mix[2]	10.0	10.0	10.0	10.0	10.0	10.0	10.0
Cholesterol	2.00	2.00	2.00	2.00	2.00	2.00	2.00
Lecithin	10.0	10.0	10.0	10.0	10.0	10.0	10.0
Monocalcium phosphate	30.0	30.0	30.0	30.0	30.0	30.0	30.0
Riboflavin[3]	0.00	0.01	0.02	0.03	0.04	0.05	0.06
Cellulose	8.00	7.99	7.98	7.97	7.96	7.95	7.94
Proximate composition (g/kg)							
Dry matter	927	926	923	928	926	923	926
Crude protein	347	347	343	346	345	344	343
Crude lipid	92.6	85.4	86.1	89.1	86.3	92.1	92.1
Crude ash	91.2	91.2	89.4	89.2	89.2	91.2	92.1
Riboflavin content (mg/kg)[4]	1.41	6.38	17.87	25.26	37.11	46.01	55.11

[1]Mineral mix (g/kg): $MgSO_4.7H_2O$, 80.0; $NaH_2PO_4.2H_2O$, 370.0; KCl, 130.0; Ferric citrate, 40.0; $ZnSO_4.7H_2O$, 20.0; Ca-lactate, 356.5; $CuCl_2$, 0.2; $AlCl_3.6H_2O$, 0.15; Na_2SeO_3, 0.01; $MnSO_4.H_2O$, 2.0; $CoCl_2.6H_2O$, 1.0.

[2]Vitamin mix (g/kg): β-carotene, 0.57; thiamin hydrochloride, 8.91; niacin 18.0; pantothenate, 18.0; pyridoxine hydrochloride, 7.2; cyanocobalamin, 0.09; ascorbic acid, 100.0; cholecalciferol, 144.0; α-tocopherol acetate, 20.0; menadione, 0.9; biotin, 0.9; folic acid, 7.2; inositol, 45.0.

[3]Xi'an Julong Bio-Tech Co., Ltd, Xi'an, China.

[4]Actual riboflavin content in diets

2.2. Experiment setting and feeding trial.

The experimental shrimp were acclimatized for two weeks before the feeding trial. During the acclimatization period, commercial feed (40% protein, 6% lipid, Woosung premium aqua feed, Korea) was supplied. Healthy and equal size shrimp (0.17 ± 0.00 g) were chosen at random and transferred to 215 L capacity 28 acrylic tanks. A total of 25 shrimp were stocked in each tank to be one of four replicates of diets. Tanks were filled with filtered and preheated seawater and aerated continuously. Feeds were delivered manually six times a day (08:00, 10:00, 12:00, 14:00, 16:00 and 18:00 h) for 8 weeks. Daily feeding rates were 12% of shrimp body weight at the beginning of the study and then gradually decreased to 3% at the end. The bulk weight and total shrimp count of each tank were measured fortnightly to adjust the feed amount according to the predicted specific growth rate (SGR). Approximately 70% of culture water was exchanged by preheated seawater every third day before the first feeding. Water quality was manipulated and recorded daily. During the feeding trial, different physiochemical parameters such as temperature, pH (Suntex TS-1), dissolved oxygen (Pro20 Dissolved Oxygen Meter, YSI, Yellow springs, OH, USA) and ammonia (Strickland and Parsons, 1972) were recorded as 28.0 - 31.0°C, 7.47 - 7.81, 5.70 - 6.77 mg/L and 0.04 - 0.10 mg/L respectively. The criteria of the Institutional Animal Care and Use Committee of Jeju National University were followed in the experimental protocols.

Figure 4: Experimental setting and feeding trial of Pacific white shrimp (*Litopenaeus vannamei*).

2.3. Sample collection

Shrimp were starved for a day before being sampled. Individuals from each tank were counted and weighed. The collected data was used to calculate weight gain (WG), SGR, feed conversion ratio (FCR), protein efficiency ratio (PER) and survival rate (Table 2). Three shrimp were randomly chosen from each tank (12 shrimp per treatment) and anesthetized by dipping them into an ice water bath. Then hemolymph was sampled from the base of the third walking leg puncture with 1 mL syringes with a 25-gauge needle and immediately mixed with the same volume of anticoagulant, Alsever's solution (A3551, Sigma-Aldrich, St. Louis, MO, USA). Hemolymph was centrifuged (800 × *g* for 20 min at 4°C) to separate the liquid part. The supernatant was removed and kept at –80°C for the analysis of the immune and blood parameters. The same individuals were dissected after collecting hemolymph and 1cm length intestine samples, just after hepatopancreas, were collected into Davidson's fixative solution for histological analysis. Another three additional shrimp per tank were kept at –20°C to analyze the whole-body proximate composition and muscle total antioxidant capacity.

Figure 5: Weight measurement and sample collection from Pacific white shrimp (*Litopenaeus vannamei*).

$$\text{Weight gain (\%)} = \frac{(\text{Final body weight} - \text{Initial body weight})}{\text{Initial body weight}} \times 100$$

$$\text{Specific Growth Rate (\%)} = \frac{(\ln \text{final body weight} - \ln \text{initial body weight})}{\text{Initial body weight}} \times 100$$

$$\text{Feed conversion ratio} = \frac{\text{Dry feed fed}}{\text{Wet weight gain}}$$

$$\text{Protein efficiency ratio} = \frac{\text{Wet weight gain}}{\text{Total protein given}}$$

$$\text{Survival (\%)} = \frac{\text{Final amount of shrimp}}{\text{Initial amount of shrimp}} \times 100$$

2.4. Sample analyses

2.4.1 Proximate analysis

Moisture and ash content of diets and whole-body were estimated by gravimetric analysis according to standard techniques AOAC (2005). Moisture content was measured by drying 2 g of samples in a heated oven at 125°C until obtained a constant weight. Ash content was determined by burning 2 g of samples in a muffle furnace at 550°C for four hours. According to Folch et al. (1957), crude lipid was quantified gravimetrically after extraction with chloroform-methanol mixture. Crude protein levels were analyzed by the Kjeldahl method in a Kjeltec 2300 semi-automated system (FOSS, Hilleroed, Denmark). First, samples were acid hydrolyzed by concentrated sulfuric acid. Then distillation process was conducted using Kjeltec system with Sodium hydroxide. Released ammonia gas was captured by 1% boric acid and titrate with 0.1N HCl.

2.4.2 NBT activity

Intracellular production of the super oxide anion in hemocytes was quantified using the NBT (Nitroblue Tetrazolium) reduction to formazan according to Song & Hsieh (1994) with slight modifications (Zhang et al., 2013). In brief, 200 µl Hanks' Balanced Salt Solution (HBSS) was added into a separate 2 mL eppendorf tube (for triplicate). 40 µl of hemolymph diluted in anticoagulant solution was transferred in eppendorf tube and incubated for 30 min at 25°C.

Next, 100 µl Zymosan (0.1% in Hank's solution) was added and incubated for 2 h at 37°C. NBT solution (0.3%) was added and incubated for 2 h at 37°C. Discarded supernatant after 600 µl methanol was added and centrifuged at 6500 rpm for 10 min. After fixed hemocytes were washed three times with 100 µl methanol (70%) and dried for 5 min. Formazan was dissolved with 700 µl of 2 M KOH and 800 µl DMSO (Dimethyl sulfoxide), and absorbance was read at 620 nm using a spectrophotometer.

2.4.3 Phenoloxidase activity

Phenoloxidase (PO) activity was measured spectrophotometrically by recording the formation of dopachrome produced from L-dihydroxyphenylalanine (L-DOPA, Sigma-Aldrich) using the method described by Hernández-López et al. (1996). In brief, 50 µL of sample was placed in a 96-well plate and pre-incubated with 50 µL of trypsin (1mg/mL in CAC buffer) for 30 min at 25°C. Then, 50 µL of L-DOPA (3 mg/mL in CAC buffer) was added. The CAC buffer includes 10 mM sodium cacodylate and 10 mM $CaCl_2$ at pH 7.0. After 10 min of incubation at 25°C, the absorbance was recorded at 490 nm using a microplate reader (UVM 340, Biochrom, Cambridge, UK). The optical density (OD) of the shrimp's phenoloxidase activity was expressed as dopachrome formation in 50 µl of cell-free hemolymph.

2.4.4 Anti-protease activity

The plasma anti-protease activity was measured according to the method described by Ellis (1990), with slight modifications (Magnadóttir et al., 1999). Briefly, 20µl of serum was incubated with 20 µl of standard trypsin solution (Type II-S, from porcine pancreas, 5 mg/mL, Sigma-Aldrich) for 10 min at 22°C. Then, 200 µl of phosphate buffer (0.1 M, pH 7.0) and 250 µl 2% azocasein (Sigma-Aldrich) were added and incubated for 1 h at 22°C. 500 µl of 10% trichloro acetic acid (TCA) was added and further incubated for 30 min at 22°C. The mixture was centrifuged at 6000 × g for 5 min and 100 µl of the supernatant was transferred to the wells of a 96 well flat-bottomed microplate containing 100 µl of 1N NaOH and OD was read at 430 nm. For a 100% positive control, buffer was replaced for serum, while for the negative control buffer replaced both serum and trypsin. The trypsin inhibition percentage was

calculated using the following equation:

$$\text{Trypsin inhibition (\%)} = \frac{A1 - A2}{A1} \times 100$$

Where A1 = Control trypsin activity (without serum); A2 = Activity of trypsin remained after serum addition.

2.4.5 Lysozyme activity

Lysozyme activity was observed through enzyme activity and bacterial target clearance during incubation according to Hultmark et al. (1980). *Micrococcus lysodeikticus* bacteria (0.75 mg/mL) was suspended in 0.1M phosphate buffer pH 6.4, 200 µl of bacteria suspension was placed in 96 well plate reader. Then, 20 µl of serum was added. Check the absorbance at 570 nm and incubate for 30 min at 37°C and checked the absorbance again at 570 nm. Lysozyme activity was measured by the reduction of bacteria (*Micrococcus lysodeikticus*) due to enzyme activity during incubation period.

2.4.6 GPx activity

GPx activity was assayed using kit (Biovision, Inc. California, USA). In this assay, cumene hydroperoxide was used as a peroxide substrate (ROOH), and glutathione reductase and NADPH (reduced nicotinamide adenine dinucleotide phosphate) were included in the reaction mixture. The change in 340 nm due to NADPH oxidation was monitored and was indicative of GPx activity. Briefly, 50 µl of serum was added to 40 µl reaction mixture and incubated for 15 min and then 10 µl cumene hydroperoxide was added and OD1 read at 340 nm. After 5 min of incubation OD2 read in 340 nm by a microplate reader. Activity of GPx was calculated as mU/mL.

2.4.7 Total antioxidant capacity

Total antioxidant capacity (TAC) was determined using a commercial kit (Antioxidant assay kit, Sigma-Aldrich; CS0790). Homogenize muscle samples (~ 100 mg) prepared using an assay buffer solution provided with the kit. Then, the samples were added with mixture of ABTS (2,2′-Azino-bis (3-ethylbenzthiazoline-6-sulfonic acid) and hydrogen peroxide (150 µl).

Hydrogen peroxide oxidize ABTS and produce ABTS+ (2,2′-Azino-bis (3-ethylbenzthiazoline-6-sulfonate). After 10 min of incubation, samples were added with metmyoglobin solution (100 µl). Metmyoglobin contains ferryl myoglobin which react with ABTS+ and produce a green color soluble chromogen. Antioxidants in the samples suppress the production of the ABTS+ in a concentration dependent manner and the color intensity decreases proportionally. The color intensity was determined using a plate reader at 405 nm. TAC values of samples were expressed as an equivalent of the mM concentration of a (±)-6-hydroxy-2, 5, 7, 8-tetramethylchromane-2-carboxylix acid (Trolox) solution. Trolox standard curve at the range of 0.00 – 0.42 mM was prepared for the assay.

$$\text{Antioxidant concentration (mM)} = \frac{\text{Absorbance of test sample at 405nm}}{\text{Slope of the standard curve}} \times \text{Dilution factor}$$

2.4.8 Superoxide dismutase (SOD)

Serum superoxide dismutase (SOD) activity was measured by the percentage reaction inhibition rate of enzyme with WST-1 (Water Soluble Tetrazolium dye) substrate and xanthine oxidase using a SOD Assay Kit (Sigma-Aldrich, 19160) according to the manufacturer's instructions. Each endpoint assay was monitored by absorbance at 450 nm (the absorbance wavelength for the colored product of WST-1 reaction with superoxide) after 20 min of reaction time at 37°C. The percentage inhibition was normalized by mg protein and presented as SOD activity units.

2.4.9 Biochemical analysis

Levels of cholesterol, glucose, triglyceride and total protein in hemolymph were analyzed using an automated blood analyzer (SLIM, SEAC Inc, Florence, Italy) following the instructions provided with reagents purchased from Stanbio Laboratories (Texas, USA).

$$\text{Total cholesterol (mg/dL)} = \frac{\text{Absorbance of sample}}{\text{Absorbance of standard}} \text{x200}$$

$$\text{Glucose (mg/dL)} = \frac{\text{Absorbance of sample}}{\text{Absorbance of standard}} \text{x100}$$

$$\text{Total protein (mg/dL)} = \frac{\text{Absorbance of sample}}{\text{Absorbance of standard}} \text{x10}$$

$$\text{Triglyceride (mg/dL)} = \frac{\text{Absorbance of sample}}{\text{Absorbance of standard}} \text{x200}$$

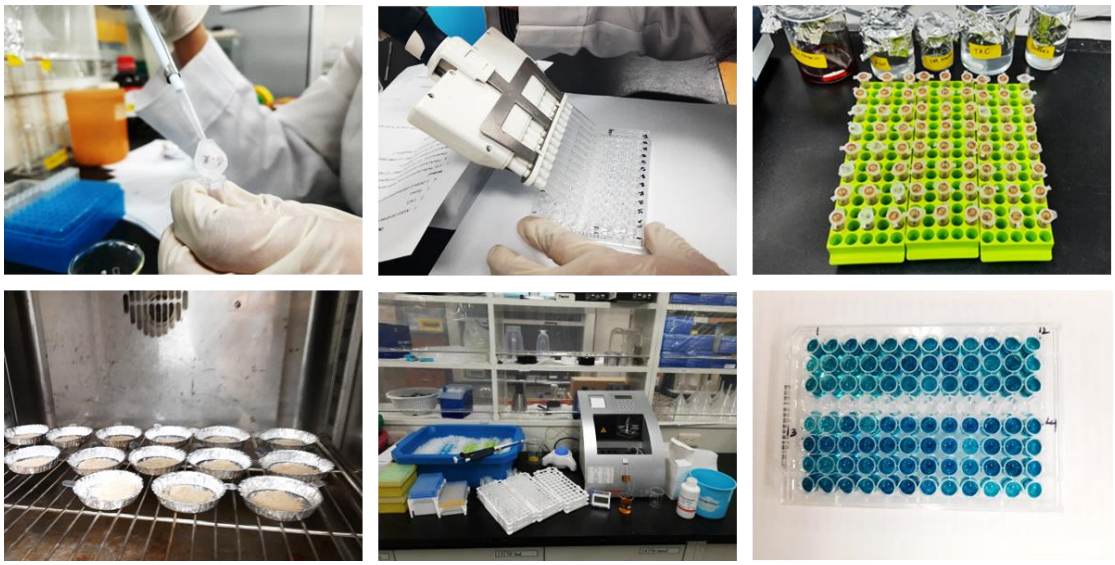

Figure 6: Whole-body and hemolymph analysis of Pacific white shrimp (*Litopenaeus vannamei*).

2.5. Intestinal histomorphology

Intestine samples (1 cm length midgut parts from three shrimp in each tank) were fixed in Davidson's solution for 24 h. Then it was passed through ethanol series to dehydrate and equilibrated in xylene. Then Samples were embedded with paraffin wax to make solid wax blocks. Paraffin blocks were sectioned (6 µm thick) with a rotary microtome (Leica RM2125RT), and multiple transverse sections were placed on a glass slide. Slides were dried at 35℃ for 24 h and stained after deparaffinization and rehydration, with Hematoxylin and Eosin (BBC biochemical, McKinney, TX, US). Intestine samples were observed using a microscope Leica DM 750 built with a camera (Leica ICC50E). The morphometric measurements of villi were measured using the image analyzing software (Leica Application Suite, version 4.13.0, Switzerland).

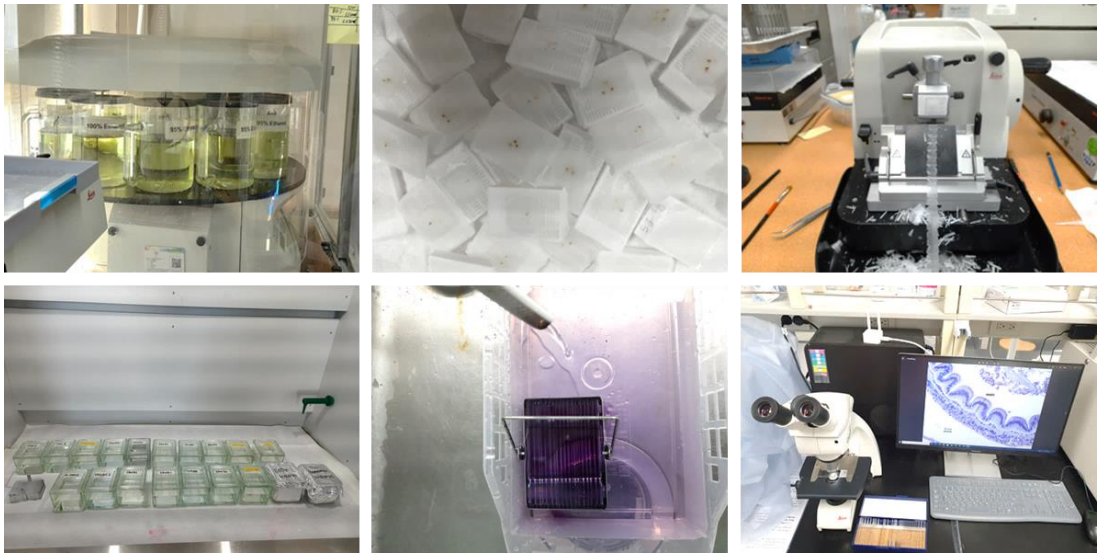

Figure 7: Histological sample dehydration, embedding, sectioning, H&E staining, and observation of Pacific white shrimp (*Litopenaeus vannamei*) intestine.

2.6. Digestibility trial

2.6.1 Digestibility diet

To estimate the apparent digestibility coefficients (ADC) of diets, Chromic oxide (Cr_2O_3) (Sigma-Aldrich, St. Louis, USA) was included in the diets as an inert indicator at a concentration of 1.0%. All dry ingredients were thoroughly mixed and extruded through a pelletizer machine (SP-50, Gum Gang Engineering, Korea) to ideal size after addition of fish oil and 10% distilled water. The pellets were dried with electric dryer at room temperature and stored at –20°C until used.

2.6.2 Fecal collection

Following the end of the feeding trial, the remaining shrimp in each treatment were used in a digestibility test with three replicates of 20 shrimps per tank (240 L). Shrimp were fed digestibility diet four times per day (08:00, 11:30, 15:00 and 18:30 h). Rearing water was exchanged after 30 min of feeding to remove leftover feed and feces on the bottom of tanks. Feces were collected from tanks' bottoms by siphoning each tank three times per day (10:30, 14:00 and 17:30 h) for one week. Collected feces were placed on a filter paper to drain water

and then frozen at –20℃ until analysis. Before freeze-drying, the daily fecal samples from each tank were pooled together.

2.6.3 Chromic oxide analyses

Chromic oxide content of diet and feces samples were analyzed by the method described by Divakaran et al. (2002). Ash samples of freeze-dried fecal samples and digestibility diet were prepared by burning 2 g in a muffle furnace at 550°C for four hours. Briefly, a known weight (5-10 mg) of ash samples containing chromic oxide was placed in glass test tubes. Then 4 mL of perchloric reagent was added along the sides of the test tube to wash down any adhering ash. Perchloric reagent was prepared as follows: 200 mL of concentrated nitric acid was added to 100 mL of distilled water, cooled down and then 200 mL perchloric acid (70%) was added. The test tubes were set in a heating block and heated at 300°C for 20 min, for oxidation of chromic oxide to monochromate (CrO_4^{2-}). Then the tubes were cooled down to room temperature and their contents were quantitatively transferred and made up to 25 mL in a volumetric flask by rinsing repeatedly with distilled water. The absorbance of samples was read at 350 nm using a spectrophotometer (Beckman DU-730, USA). A known weight (2 - 4 mg) of chromium oxide was similarly treated and used as standard.

2.6.4 Estimation of apparent digestibility coefficients

The ADCs for dry matter and protein of the experimental diets were calculated by the following formula (Cho et al., 1982):

$$\text{ADC of dry matter (\%)} = 100 - \frac{\%Cr_2O_3 \text{ in diet}}{\%Cr_2O_3 \text{ in feces}} \times 100$$

$$\text{ADC of protein (\%)} = 100 - 100 \times \frac{\%Cr_2O_3 \text{ in diet}}{\%Cr_2O_3 \text{ in feces}} \times \frac{\%Cr_2O_3 \text{ in diet}}{\%Cr_2O_3 \text{ in feces}}$$

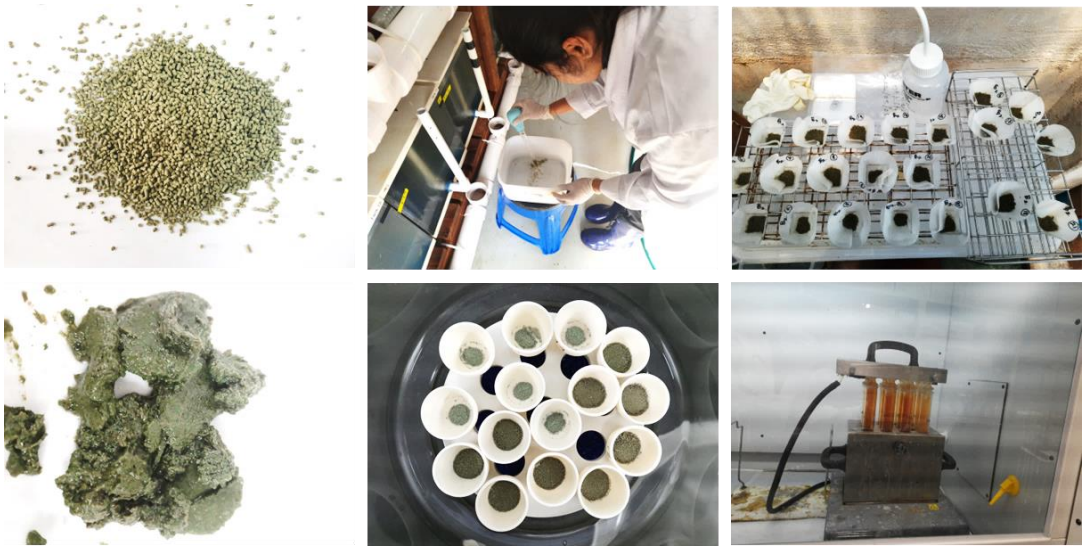

Figure 8: Digestibility diet, feces collection and analysis of feces.

2.7. Statistical analysis

Experimental diets were assigned using a completely randomized design. Data of all evaluated criteria were initially checked for normality. When normality assumptions were met, the means of all the parameters were compared by one-way analysis of variance (ANOVA) after arcsine transformation in SPSS version 20.0 (SPSS Inc., Chicago, IL, USA). The results were presented as mean ± standard deviation. When ANOVA of values was found to be significant, a post hoc comparison of means was performed using Tukey's multiple range test with a significance level of 5% significance level ($p < 0.05$). A follow-up trend analysis by orthogonal polynomial contrasts was run to determine whether the effect is linear and/or quadratic. A broken-line regression model was adopted for quantifying the riboflavin requirement based on WG (Robins 1986).

3. Results

3.1 Growth performance and feed utilization

The growth performance, feed utilization and survival of *L. vannamei* fed diets with increasing levels of riboflavin are provided in Table 2 and Figure 9. The growth performance of shrimp gradually increased with the increase of riboflavin up to R40 group. R0 group exhibited the lowest growth performance which was significantly lower than all other groups except for shrimp fed R10 diet. Final body weight (FBW), WG and SGR of shrimp fed R40 diet were significantly higher ($p < 0.05$) than those of shrimp fed R0, R10, R20 and R30 diets. However, results of the parameters of R50 and R60 groups were comparable with those of shrimp fed R20, R30 and R40 diets. A significantly higher PER value was observed in shrimp fed R40 diet compared to R0 group. However, FCR and survival were not significantly affected by dietary riboflavin. Moreover, FBW, WG and SGR exhibited significant linear and quadratic trends while FCR and PER exhibited significant quadratic trends with the range of dietary riboflavin tested in the feeding trial. The optimal dietary riboflavin level for higher WG was estimated as 40.9 mg/kg diet according to the two-slope broken-line model as shown in Figure 10.

Table 2: Growth performance, feed utilization and survival of Pacific white shrimp (*Litopenaeus vannamei*) fed the experimental diets for 8 weeks.

	FBW[1]	WG[2]	SGR (%)[3]	FCR[4]	PER[5]	Survival[6]
R0	7.90±0.22^{d}	4557±140^{d}	6.62±0.05^{d}	1.52±0.08	1.88±0.08^{b}	93.0±3.83
R10	8.10±0.12^{cd}	4674±76.0^{cd}	6.67±0.03^{cd}	1.51±0.05	1.90±0.06^{ab}	95.00±2.00
R20	8.27±0.12^{bc}	4781±73.1^{bc}	6.70±0.03^{bc}	1.49±0.07	1.97±0.08^{ab}	95.0±2.00
R30	8.39±0.17^{bc}	4873±69.0^{bc}	6.71±0.02^{bc}	1.46±0.07	2.05±0.10^{ab}	96.0±3.27
R40	8.83±0.10^{a}	5069±103^{a}	6.80±0.03^{a}	1.44±0.04	2.06±0.05^{a}	95.0±2.00
R50	8.50±0.10^{ab}	4950±14.5^{ab}	6.76±0.00^{ab}	1.51±0.06	1.92±0.07^{ab}	94.0±2.31
R60	8.57±0.16^{ab}	4928±109^{ab}	6.75±0.04^{ab}	1.50±0.06	1.94±0.07^{ab}	95.0±3.83
Pr >*F**						
ANOVA	0.00	0.00	0.00	0.27	0.02	0.84
Linear	0.00	0.00	0.00	0.16	0.12	0.60
Quadratic	0.00	0.01	0.01	0.01	0.03	0.29

Values are mean of quintuplicate groups and presented as mean ± *SD*. Values with different superscripts in the same column are significantly different ($p < 0.05$). The lack of superscript letters indicates no significant differences among treatments.

[1]Final body weight (g)

[2]Weight gain (%) = [(Final body weight – Initial body weight) / Initial body weight] x 100

[3]Specific Growth Rate (%) = [(ln final body weight – ln initial body weight) / days] x 100

[4]Feed conversion ratio = Dry feed fed / Wet weight gain

[5]Protein efficiency ratio = Wet weight gain / Total protein given

[6]Survival (%) = (Final amount of shrimp / Initial amount of shrimp) x 100

*Significance probability associate with the *F*-statistic

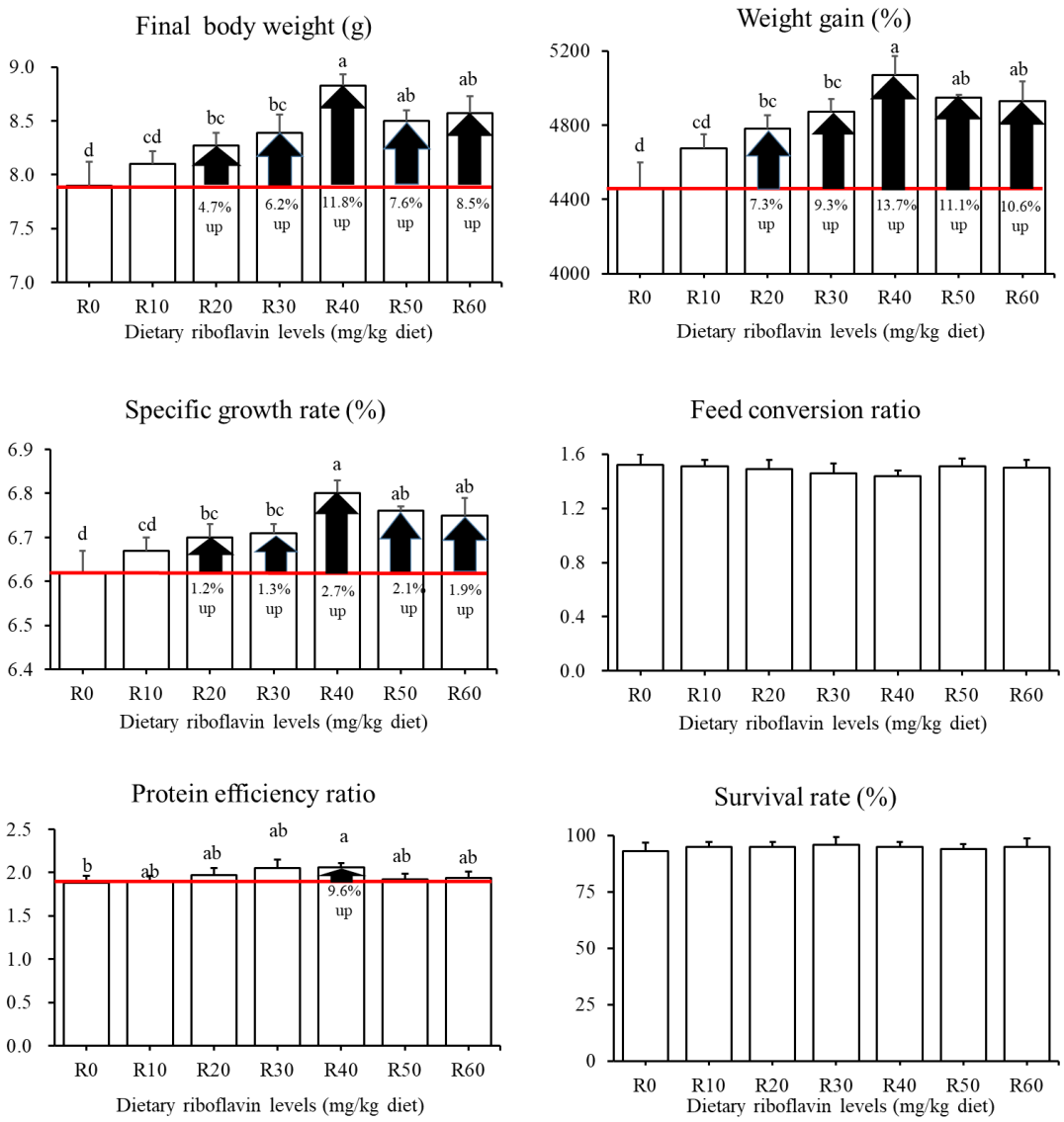

Figure 9: Growth performance, feed utilization and survival of Pacific white shrimp (*Litopenaeus vannamei*) fed the experimental diets for 8 weeks.

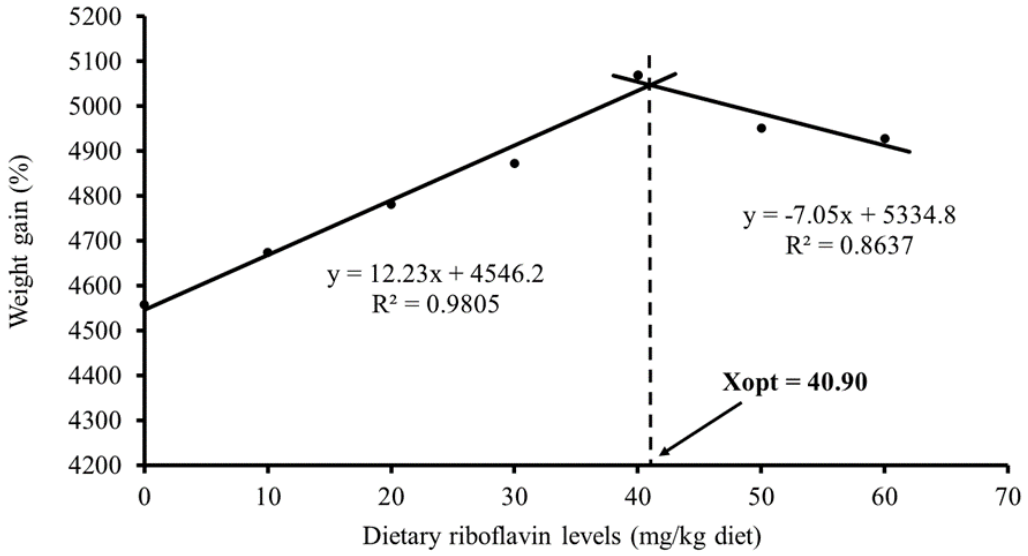

Figure 10: Relationship between the weight gain (%) and dietary riboflavin in levels by the broken-line regression analysis. X represents the optimum dietary riboflavin requirement for maximum weight gain (%) in Pacific white shrimp (*Litopenaeus vannamei*).

3.2 Non-specific immunity

Non-specific immune parameters are provided in Table 3 and Figure 11. PO activity was significantly higher in R40 group compared to that of R0 and R10 groups. The lysozyme activity was significantly higher in shrimp fed R30 and R40 diets than in the R60 diet. NBT activity was significantly higher in R40 group compared to R0, R10, R20, R50 and R60 groups. Antiprotease activity was not significantly affected by dietary riboflavin. Increased levels of dietary riboflavin linearly enhanced PO activity while lysozyme, NBT, antiprotease displayed a significant quadratic trend.

Table 3: Non-specific immune parameters of Pacific white shrimp (*Litopenaeus vannamei*) fed the experimental diets for 8 weeks.

	PO[1]	Lysozyme[2]	NBT[3]	Antiprotease[4]
R0	0.26±0.02^{b}	13.5±1.89^{ab}	3.38±0.01^{b}	10.4±0.59
R10	0.27±0.02^{b}	17.8±1.87^{ab}	3.29±0.11^{b}	13.0±0.40
R20	0.28±0.05^{ab}	17.8±2.42^{ab}	3.38±0.29^{b}	11.0±0.58
R30	0.36±0.06^{ab}	19.3±2.09^{a}	3.73±0.26^{ab}	12.5±0.76
R40	0.39±0.06^{a}	18.9±3.66^{a}	3.88±0.17^{a}	12.6±0.40
R50	0.35±0.03^{ab}	15.8±2.09^{ab}	3.30±0.25^{b}	10.6±0.74
R60	0.34±0.07^{ab}	12.3±0.96^{b}	3.30±0.24^{b}	10.1±2.99
Pr >*F**				
ANOVA	0.01	0.01	0.00	0.01
Linear	0.00	0.36	0.64	0.22
Quadratic	0.06	0.00	0.00	0.01

Values are mean of quintuplicate groups and presented as mean ± *SD*. Values with different superscripts in the same column are significantly different ($p < 0.05$).

[1]Phenoloxidase activity (absorbance).

[2]Lysozyme activity (μg/mL).

[3]Nitro blue tetrazolium activity (absorbance).

[4]Antiprotesase activity (% inhibition).

*Significance probability is associate with the *F*-statistic.

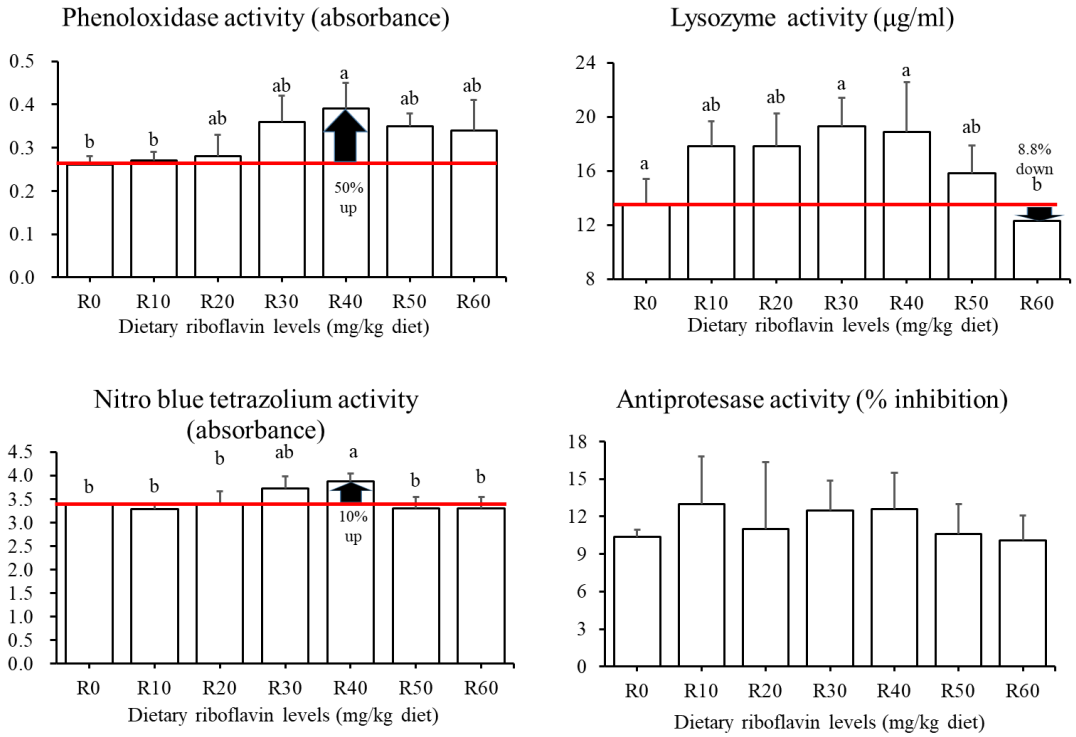

Figure 11: Non-specific immune parameters, antioxidant enzymes activity and muscle total antioxidant capacity of Pacific white shrimp (*Litopenaeus vannamei*) fed the experimental diets for 8 weeks.

3.3 Antioxidant enzymes activity

Antioxidant enzymes activity and muscle total antioxidant capacity are provided in Table 4 and Figure 12. The highest activities of SOD and GPx were observed in shrimp fed R40 diet. SOD activity was also significantly higher in R40 group compared with the R0 group. GPx activity was significantly higher in shrimp fed R40 diet compared to R0, R10 and R20 groups and comparable with R30, R50 and R60 groups. Interestingly, R0 group showed the lowest value, which was significantly lower than all other groups. The GPx activity of R20 group was comparable with R10, R30, R50 and R60 groups although the values are significantly lower than R40 group. Antiprotease and muscle TAC were not significantly affected by dietary riboflavin. Increased levels of dietary riboflavin quadratically enhanced SOD and muscle TAC while GPx activity displayed both linear and quadratic trends.

Table 4: Antioxidant enzymes activity and muscle total antioxidant capacity of Pacific white shrimp (*Litopenaeus vannamei*) fed the experimental diets for 8 weeks.

	SOD[1]	GPx[2]	TAC[3]
R0	56.1±0.52^{b}	175.2±5.85^{d}	0.29±0.03
R10	61.6±3.82^{ab}	188.9±5.16^{c}	0.30±0.05
R20	61.7±5.37^{ab}	196.2±5.02^{bc}	0.32±0.04
R30	61.6±2.36^{ab}	207.8±7.59^{ab}	0.33±0.02
R40	63.7±2.88^{a}	210.2±7.40^{a}	0.32±0.02
R50	63.2±2.41^{ab}	204.4±5.59^{ab}	0.29±0.04
R60	58.1±1.96^{ab}	200.3±5.59^{abc}	0.32±0.05
Pr >*F**			
ANOVA	0.02	0.00	0.38
Linear	0.18	0.00	0.80
Quadratic	0.00	0.00	0.03

Values are mean of quintuplicate groups and presented as mean ± *SD*. Values with different superscripts in the same column are significantly different ($p < 0.05$).

[1]Superoxide dismutase (% inhibition).

[2]Glutathione peroxidase (mU/mL).

[3]Total antioxidant capacity (μmol/mL).

*Significance probability is associate with the *F*-statistic.

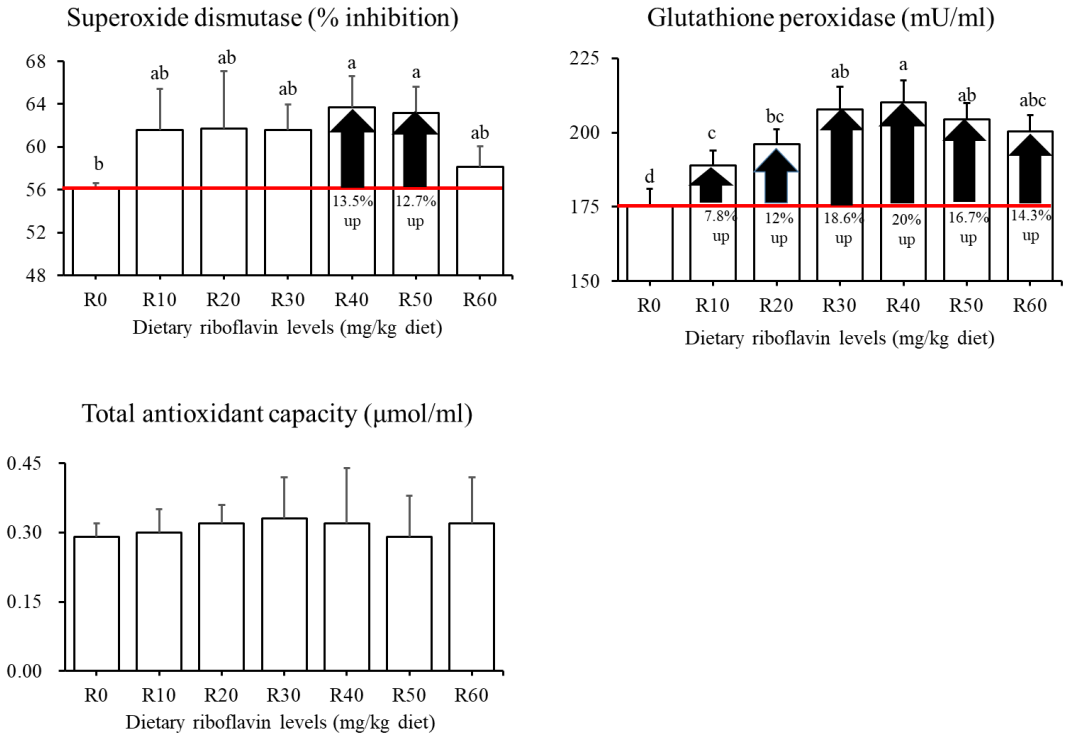

Figure 12: Antioxidant enzymes activity and muscle total antioxidant capacity of Pacific white shrimp (*Litopenaeus vannamei*) fed the experimental diets for 8 weeks.

3.4 Whole-body proximate composition

Whole-body protein, lipid, and ash levels of shrimp are presented in Table 4 and Figure 13. There was no significant difference in the whole-body crude lipid, crude protein and ash content among all the dietary groups. However, whole-body ash content showed a linearly decreasing trend with the increase of riboflavin levels in diets.

Table 5: Proximate composition (% wet weight basis) of Pacific white shrimp (*Litopenaeus vannamei*) fed the experimental diets for 8 weeks.

	Protein	Ash	Lipid
R0	18.20±1.98	3.40±0.47	1.46±0.01
R10	18.29±1.14	4.45±0.16	1.39±0.27
R20	18.89±0.73	3.96±0.86	1.34±0.15
R30	18.62±1.53	3.72±0.21	1.39±0.03
R40	19.38±0.62	3.45±0.46	1.14±0.07
R50	19.72±1.52	3.24±0.39	1.52±0.02
R60	18.01±1.80	2.92±0.11	1.51±0.22
Pr >*F**			
ANOVA	0.40	0.12	0.28
Linear	0.98	0.03	0.66
Quadratic	0.37	0.40	0.09

Values are mean of quintuplicate groups and are presented as mean ± *SD*. Values with different superscripts in the same column are significantly different ($p < 0.05$).

*Significance probability associated with the *F*-statistic

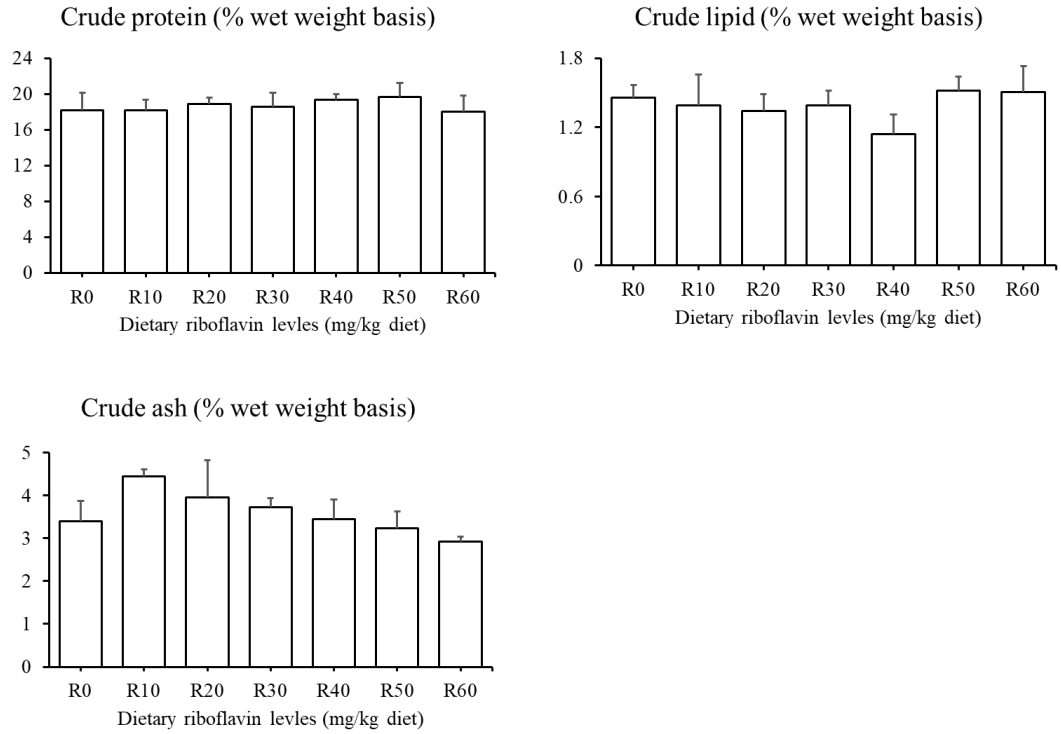

Figure 13: Proximate composition (% wet weight basis) of Pacific white shrimp (*Litopenaeus vannamei*) fed the experimental diets for 8 weeks.

3.5 Biochemical parameters

Hemolymph biochemical parameters are shown in Table 6 and Figure 14. Supplementation of riboflavin did not exhibit significant differences in hemolymph glucose, triglyceride, protein and cholesterol levels.

Table 6: Hemolymph biochemical parameters of Pacific white shrimp (*Litopenaeus vannamei*) fed the experimental diets for 8 weeks.

	Glucose[1]	Triglyceride[2]	T. Protein[3]	T. cholesterol[4]
R0	536±3.48	10.7±1.80	3.33±1.19	13.3±1.43
R10	533±7.97	9.32±1.12	3.40±1.01	13.4±1.53
R20	544±7.20	9.50±1.96	3.43±1.39	12.4±1.30
R30	547±6.52	10.9±1.48	3.84±0.93	13.4±1.54
R40	533±6.46	9.90±1.61	3.75±0.71	12.9±1.25
R50	535±10.3	10.1±1.28	3.73±0.78	12.9±2.65
R60	538±12.8	9.54±3.10	3. 60±1.47	12.4±1.72
Pr >*F**				
ANOVA	0.64	0.8	0.93	0.98
Linear	0.89	0.71	0.37	0.56
Quadratic	0.4	0.9	0.5	0.89

Values are mean of quintuplicate groups and presented as mean ± *SD*. Values with different superscripts in the same row are significantly different ($p < 0.05$)

[1]Glucose (mg/dL)

[2]Triglyceride (mg/dL)

[3]Total protein (g/dL)

[4]Total cholesterol (mg/dL)

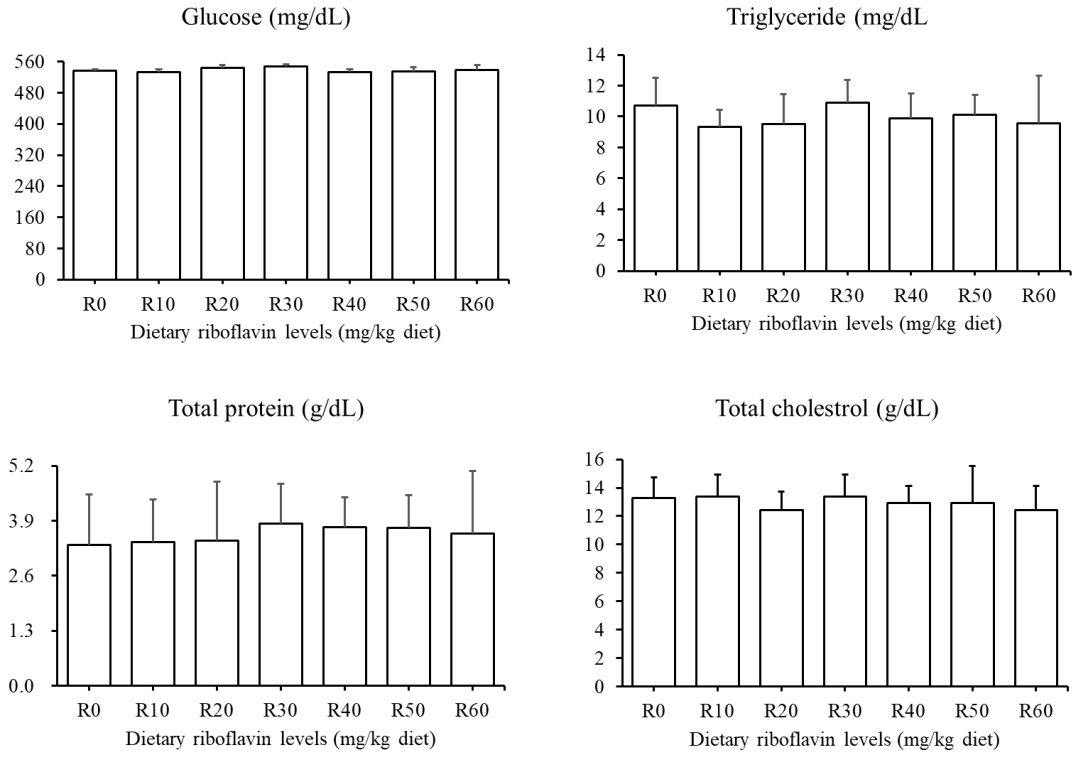

Figure 14: Hemolymph biochemical parameters of Pacific white shrimp (*Litopenaeus vannamei*) fed the experimental diets for 8 weeks.

3.6 Apparent digestibility coefficients

ADCp and ADCdm of the diets are presented in Table 7 and Figure 15. The ADCp and ADCdm were not significantly affected by the dietary supplementation of riboflavin.

Table 7: Apparent digestibility coefficients (%, ADC) for dry matter and protein of the experimental diets for Pacific white shrimp (*Litopenaeus vannamei*).

	ADCdm (%)[1]	ADCp (%)[2]
R0	93.1±0.62	86.1±0.24
R10	93.8±0.44	86.9±0.93
R20	94.1±1.02	86.6±2.31
R30	94.0±1.11	87.1±2.39
R40	94.3±0.15	87.9±0.91
R50	94.1±0.43	86.7±0.95
R60	93.6±0.85	86.2±1.84
Pr >*F**		
ANOVA	0.64	0.85
Linear	0.43	0.78
Quadratic	0.10	0.24

Values are mean of quintuplicate groups and presented as mean ±*SD*. Values with different superscripts in the same row are significantly different ($p < 0.05$).

[1]Apparent digestibility coefficient of dry matter (%)

[2]Apparent digestibility coefficient of a protein (%).

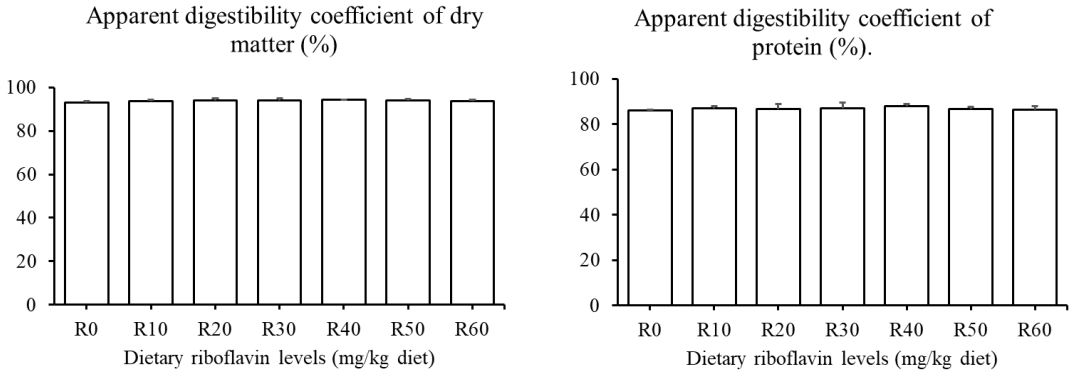

Figure 15: Apparent digestibility coefficients (%, ADC) for dry matter and protein of the experimental diets for Pacific white shrimp (*Litopenaeus vannamei*).

3.7 intestinal histomorphology

The villus height of shrimp intestine is presented in Figure 16. Villi were significantly longer in shrimp fed R50 and R60 diets compared to R0, R10 and R20 groups while values were comparable with R30 and R40 groups. Moreover, the intestinal villi of shrimp fed R0 diet were significantly shorter than that of shrimp fed other experimental diets. Photomicrographs of transverse sections of the intestine are provided in Figure 17. Villi were clearly distinguished in shrimp fed higher levels of riboflavin compared with R0 and R10 groups.

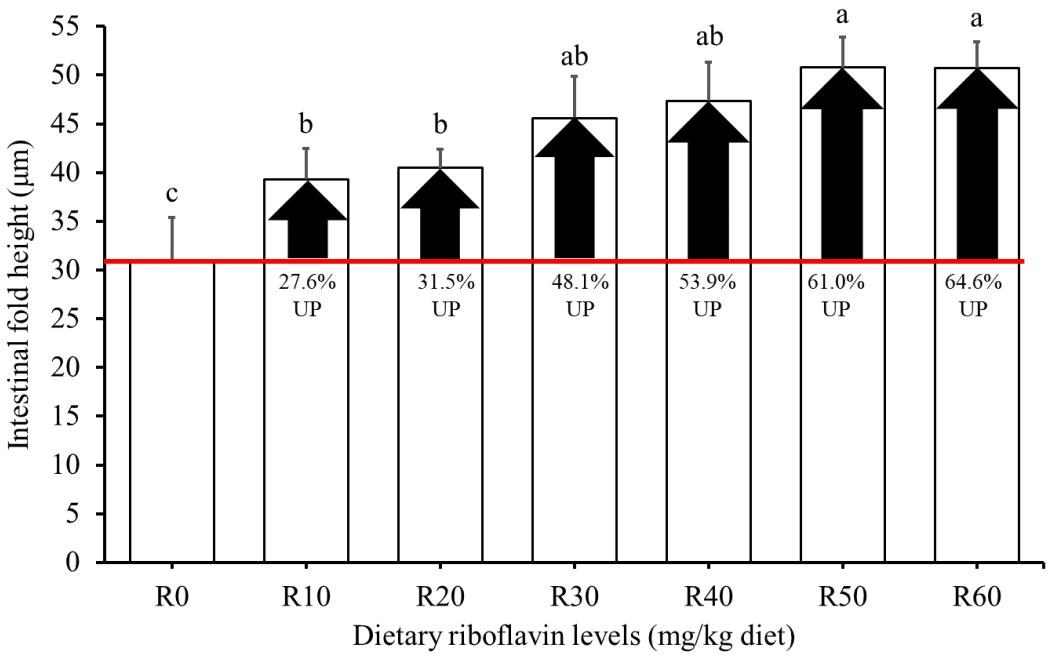

Figure 16: Intestinal villi height of Pacific white shrimp (*Litopenaeus vannamei*) fed the experimental diets for 8 weeks. Bars with different letters are significantly different ($p < 0.05$).

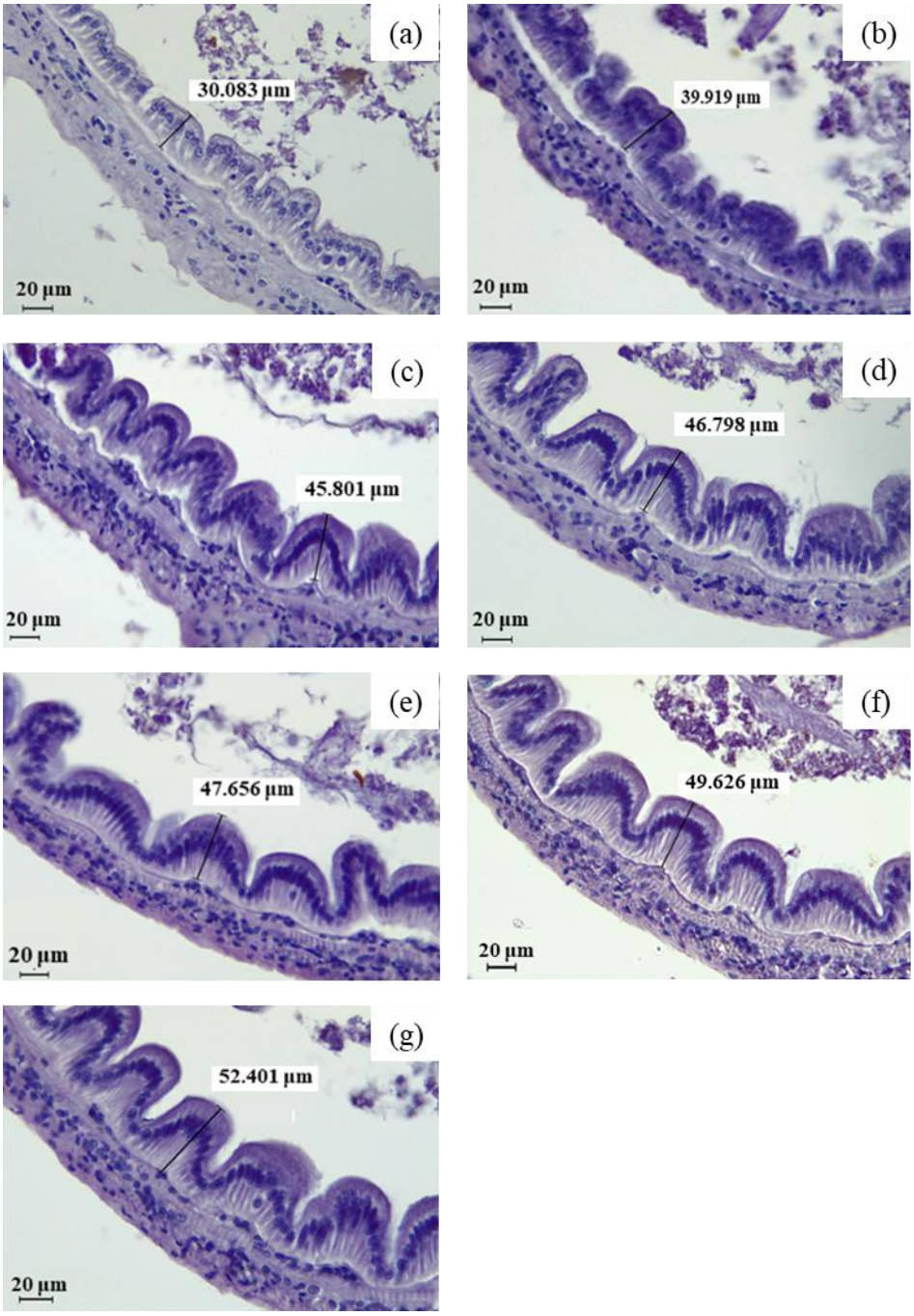

Figure 17: Representative photomicrographs of transverse HE-stained sections of the intestine of Pacific white shrimp (*Litopenaeus vannamei*) fed seven experimental diets for 8 weeks. (Villus height; magnification, × 40). (a) R0, (b) R10, (c) R20, (d) R30, (e) R40, (f) R50 and (g) R60 (The line segments show the changes in intestinal fold height in experimental groups).

4. Discussion

The results of this study demonstrated that optimal dietary riboflavin levels could enhance the growth performance and feed utilization efficiency in *L. vannamei.* The broken-line analysis on WG exhibited that the optimal riboflavin requirement to achieve maximum WG was 40.9 mg/kg. Nevertheless, this result is higher than the estimated levels for *P. monodon* 22.3 mg/kg (Chen & Hwang, 1992) and lower than the estimation for *P. japonicus* 80 mg/kg (NRC, 1983). According to Kanazawa (1985), larval stages of *P. japonicus* required apparently higher vitamin levels than juveniles. Shrimp gut is immature and they exhibit less vitamin storage capacity in juvenile stages (Hardy & Kaushik, 2021). Accordingly, riboflavin requirements can be different with the growth stage. The requirement of riboflavin for shrimp was considerably higher than that for many aquatic species, such as sunshine bass 3.45 mg/kg (Deng & Wilson, 2003), grass carp 6.65 mg/kg (Chen et al., 2015b), blue tilapia, *Oreochromis aureus* 6 mg/kg (Soliman & Wilson, 1992), Atlantic salmon, *Salmo salar* 10-12 mg/kg (Hemre et al., 2016) and sea cucumber (Selenka), *Apostichopus japonicus* 9.73-17.9 mg/kg (Okorie et al., 2011). Shrimp are robust, bottom-dwelling slow feeders (Aaqillah-Amr et al., 2021). Dry and sinking pellets are used for shrimp feeding (Obaldo et al., 2002). Derby et al. (2016) observed that the first pellets were consumed quickly by shrimp after feeding. Then they slowly consumed pellets with greater manipulation using their legs and mouthparts. Thus, nutrient leaching from the feeds always occurs while handling the pellets by mouthparts and legs, especially in the case of water-soluble nutrients including riboflavin can easily be leached into the water. Riboflavin and other B vitamins are synthesized by the gut microbiota of animals including fish (Uebanso et al., 2020). However, shrimp have a simple digestive tract compared with fish. The gut microbiota of shrimps might have synthesized little amount of riboflavin. Most animals' energy requirement is higher during their rapid development period; however, crustaceans need extra energy for their molting process (Conklin, 1989). Because of these reasons, the above difference in riboflavin requirement seems to occur between fishes and shrimp. Coenzyme forms (FAD and FMN) of riboflavin are essential for energy production through the metabolism of major nutrients (Revuelta

et al., 2017; Sepúlveda Cisternas et al., 2018). Therefore, riboflavin-supplemented groups might show higher growth than R0 group. Riboflavin activates other vitamins, such as vitamin B6, B9 and K. These vitamins are also important to improve shrimp and prawn growth (Asaikkutti et al.,2016; Cui et al., 2016; Shiau & Liu, 1994). Riboflavin may enhance shrimp growth because of these reasons.

PO system is one of the primary components of crustaceans' innate defense system. It is important for the melanization of pathogens (Charoensapsri et al., 2014; Cerenius & Söderhäll, 2004). Hemocytes release reactive oxygen species during the respiratory burst, which is important to protect the host animals from pathogens by phagocytosis (Chang et al., 2009). The present results showed that optimum dietary riboflavin supplementation can improve both PO and NBT activities of *L. vannamei*. Lysozyme is a glycolytic enzyme that functions as an antimicrobial agent in the innate immune system. Lysozyme can lead to cell death by cleaving the peptidoglycan component of bacterial cell walls (Oliver & Wells, 2015). Lysozyme activity was significantly higher in R30 and R40 groups while the lowest was observed in R60 group. The same trend was observed in lysozyme activity in intestine of grass carp with the highest riboflavin level (Chen et al., 2015b). However, Lysozyme activity was lowered by riboflavin deprivation in grass carp gills and intestine (Chen et al., 2015a; Chen et al., 2015b).

Riboflavin reacts with lysozymes under specific conditions, i.e., light-induced binding of riboflavin to lysozyme (Duran et al., 1983). The R60 group might induce that particular reaction due to high riboflavin availability in analyzed plasma samples in the present study resulting the lowest lysozyme level.

Oxidative stress occurs due to higher production of reactive oxygen species (ROS) and/or low ability to deactivate ROSs (Ashoori & Saedisomeolia, 2014). Glutathione (GSH) plays a key role in the cellular control of ROS (Jefferies et al., 2003). The activity of GSH is mediated by the action of GPx (Hayes & McLellan, 1999). In the present study, GPx activity was significantly higher in shrimp fed riboflavin containing diets compared to the control group. Jiang et al. (2019) observed that

supplementation of riboflavin could increase GSH content in grass carp muscle. GPx activity in the liver, gills and brain was increased with the riboflavin supplementation in striped catfish (Kumar, 2021). Chen et al. (2015) observed that riboflavin supplementation could reduce ROS and increase anti-superoxide anion (ASA) and anti-hydroxy radical (AHR) in gills of grass carp. ASA and AHR have ability to reduce ROS (Jiang et al., 2009). Riboflavin is a neglected antioxidative vitamin that acts as an antioxidant independently or together with the glutathione redox cycle (Averianova et al., 2020; Ashoori & Saedisomeolia, 2014). Riboflavin is likely to improve the antioxidant properties of glutathione which further improve the antioxidant potential in cells by deactivating ROSs (Huang et al., 2010). Therefore, improved GPx activity observed in the present study might be attributed to dietary riboflavin supplementation. However, Chen & Hwang (1992) mentioned that the glutathione reductase activity is an unreliable analysis to evaluate the riboflavin status of *P. monodon.* SOD is also an essential antioxidative defense system because it protects cells from free radical damages (Xie et al., 2012). In this study, hemolymph SOD activity was significantly influenced by the dietary riboflavin levels and showed a significant quadratic trend. Riboflavin deficient diets showed significantly lower SOD levels resulting in lipid peroxidation in grouper *Epinephelus coioides* (Huang et al., 2010) and Bloch (Zehra & Khan, 2018). Interestingly, relatively high levels of dietary riboflavin tend to reduce both GPx and SOD activities in the hemolymph as observed in R50 and R60 groups in the present study. Similar trends were observed in grass carp intestine when the dietary riboflavin level was increased (Chen et al., 2015b). Riboflavin is important to activation of other vitamins including niacin, pyridoxine and folic acid (Gasco et al 2018; Pinto & Rivlin, 2013). These three vitamins have been reported to improve the non-specific immune responses in shrimps and prawns (Asaikkutti et al., 2016; Cui et al., 2016; Xia et al., 2015). Hence, riboflavin might indirectly improve the non-specific immune response in shrimps. The activities of GPx and SOD is likely to be interfered in R50 and R60 groups due to the independent antioxidant action of riboflavin. Further investigations are needed to elucidate this phenomenon. TAC level in muscle represents the ability to inhibit lipid peroxidation and its relationship with enzymatic and non-enzymatic antioxidant

factors (Benzie & Strain, 1996). In the present study, muscle TAC results exhibited a significant quadratic trend in response to the increase in dietary riboflavin levels, although the levels were not significantly different between the groups. Therefore, optimum dietary riboflavin level for high antioxidant activity in *L. vannamei* might lie within this range.

Histological observations of villi illustrated significant effects of riboflavin on intestine morphology of *L. vannamei*. Surface area of the gut is expanded by increased villi height. In general, increased villi height indicates improved nutrient absorption (Wang et al., 2020). Riboflavin was reported to develop the digestive tract of rats by increasing villus growth and improving the functions of villi because of its function on the crypt sensing mechanism (Yates et al., 2003). The role of crypts is important for gut secretory and absorptive functions of animals (Carneiro-Filho et al., 2004). Qin et al. (2022) reported that riboflavin regulates renewal and cells differentiation in intestinal epithelial of piglets. They also suggested that riboflavin deficiency can induce inflammations in piglet intestine. Accordingly, villi height was significantly increased with the increase of dietary riboflavin in the present study. We assumed that an epithelium inflammation might occur in addition to malfunctions of gut crypts of shrimp fed riboflavin deficient diets because of their significantly lower villi heights. However, protein and dry matter ADCs of diets were not significantly affected by riboflavin in this study. In Jian carp, the activities of Na+, K+, ATPase and alkaline phosphate (AKP) were enhanced in the intestinal brush border by the dietary riboflavin supplementation (Li et al., 2010). An increase in activity of Na+, K+-ATPase in pig indirectly reflects an improved absorption of amino acids and glucose in the intestine (Rhoads et al.,1994). Activity of AKP was also important to improve nutrient absorption in addition to its ability to develop the gut epithelium (Cuvier-Péres & Kestemont, 2001). Digestibility of dry matter and protein were not significantly affected by riboflavin in the present study. FCR was also not significantly affected by riboflavin levels. However, feed intake was improved by riboflavin. In the present study, the digestibility trial was conducted using remaining experimental shrimp from each treatment. Therefore, we assumed that gut transition time might be lower in riboflavin deficient groups although nutrient digestibility was comparable in all diets in

shrimp fed experimental diets for eight weeks. The low gut transition time might also be one of responsible reasons for lower growth observed in the present study. In contrast, gut transition process might be accelerated in R50 and R60 groups with the increase of riboflavin level in diets. The well-developed villi in these groups support the assumption. However, it should be investigated in future studies.

Whole body proximate composition was not affected by different riboflavin levels in this study. However, whole-body ash content was reduced showing a significant linear trend with the riboflavin. Whole-body ash content was not significantly different in grass carp (Jiang et al., 2016) and Jian carp (Li et al., 2010) fed with different riboflavin levels. The Ash content of the carcass of Bloch was also not significantly changed with riboflavin supplementation (Zehra & Khan, 2016). However, significantly lower whole-body ash content was observed in sea cucumber (Selenka) with increasing riboflavin levels in diets (Okorie et al., 2011). Therefore, further experiments should be conducted to find the effect of riboflavin on the whole-body ash content of shrimp.

Riboflavin's effect on the biochemical parameters of most animals, especially riboflavin is important to maintain and balance cholesterol levels (Pinto & Cooper, 2014). In crustaceans, glucose often serves as the primary circulating carbohydrate, which might be linked to the body's overall carbohydrate storage. Wickson and Morgan, (1946) reported that supplementation of riboflavin can reduce the blood glucose level by stimulating glycogenolysis in rats, the same results were observed in Kumar, (2021) for stripped catfish. Total protein level was also increased with the supplementation of riboflavin for stripped catfish (Kumar, 2021). However, there was no clear evidence for the effect of riboflavin on crustacean hemolymph. In the present study hemolymph biochemical parameters were not affected by riboflavin levels. Shrimps may have the ability to maintain their biochemical parameters with little riboflavin amount or riboflavin sparing effect by another nutrient may occur in their body. This should be further investigated in future studies.

5. Conclusions

The optimal dietary riboflavin levels can improve the growth performance, feed utilization efficiency and non-specific immune response, and improve the intestinal villi height of shrimp. The optimal level of dietary riboflavin is predicted to be 40.9 mg/kg diet based on the two-slope regression study between weight gain and dietary riboflavin levels.

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