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Dietary inclusion of roselle improves body colouration and survival of the whiteleg shrimp (*Litopenaeus vannamei*) juveniles challenged against *Vibrio harveyi*

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Abstract

This study was conducted to investigate the use of fresh and heat-treated roselle meals on growth, body colouration, and survival against *Vibrio harveyi* infection of whiteleg shrimp, *Litopenaeus vannamei*. Fresh (FR) or heat-treated roselle (HR) meals were added at 10% in isoproteic (38%) and isolipidic (7%) diets. A control diet was prepared without any addition of roselle meal. These diets were fed to shrimp (initial body weight 1.26g±0.02g) in triplicate groups for 8 weeks then challenged with *V. harveyi*. The growth, feed efficiency, total carotenoid content, and survival of shrimp and total *Vibrio* spp. counts in the hepatopancreas post-challenge test were estimated. No significant differences were recorded in the growth, survival, and feed utilization of the shrimp fed the roselle-added diets (FR or HR) compared to the control group. A significantly higher total carotenoid concentration was detected in shrimp fed with the FR and HR diets compared to the control ($P<0.05$). The challenge test with *V. harveyi* showed that the shrimp specimens fed with FR and HR diets gained higher survival and a lower total number of *Vibrio* spp. count in the hepatopancreas compared to the control group. The present finding suggests that fresh or heat-treated roselle meals provide a good source of carotenoid and increase the resistance of shrimp against vibriosis without affecting the growth and feed utilization.

Keywords: Roselle, Heat-treated roselle, Body colouration, Whiteleg shrimp, *Vibro* sp.

Whiteleg shrimp (*Litopenaeus vannamei*) is among the most extensively cultured crustaceans in the world (FAO, 2022). In shrimp, the optimal appearance, specifically the body colour is a crucial factor in determining the purchasing decision of customers. The pink flesh colour in shrimp is derived from the carotenoid pigments (Ponce-Palafox et al.,2006) that is obtained through dietary accumulation as penaeid shrimps are unable to *de novo* synthesize carotenoids (Maoka, 2011). In their natural habitat, crustaceans obtain carotenoids from the consumption of microalgae or partially modified through metabolic pathways (Maoka, 2011). In intensive shrimp farming, dietary supplementation of carotenoids such as astaxanthin is widely used. However, the limited production and cost of astaxanthin have driven the need to find alternative sources for this pigment (Ponce-Palafox et al., 2006, Stachowiak and Szulc, 2021).

Previous studies showed that besides microalgae, other plant sources can be a good source of carotenoids for aquaculture animals (Supamattaya et al., 2005; Jomeh et al.,2021). Roselle, *Hibiscus sabdariffa*, is a flowering plant that grows in tropical and subtropical countries. The calyces are rich in anthocyanins which are water-soluble pigments responsible for colour in many plants. Besides their function as a pigment, anthocyanins are also a powerful antioxidant component (Tsai et al., 2002; Norhizan et al., 2010) and show anti-inflammatory (Xia et al.,2009; Mauray et al.,2010) and antimicrobial activities (Leito et al., 2005). In general, the roselle calyces are used to prepare herbal drinks, juices, and other delicacies (Amin et al.,2008). However, the usage of roselle was not limited to human consumption. Previously, roselle meal or extract has been tested in aquatic animals and showed improving the growth, hematological profile, antioxidant characteristics, and immunological parameters of the animals (Pérez-Escalante et al., 2012; Hoseini et al., 2021, Jomeh et al., 2021). In most practices, the roselle was discarded after the extraction. The production of roselle meals from the discarded or processed roselle could help in the complete utilization of the roselle.

In shrimp farming, disease outbreaks are a serious problem. The introduction of feed additives with antioxidant properties has been shown to enhance the immune response of the animal (Jomeh et al., 2021) and hence can minimize the severity of the disease outbreak. This study was conducted to investigate the potential use of fresh or heat-treated roselle meals in the diet of *L. vannamei.*

For this study the experimental shrimp were procured from a local shrimp farm and handled according to the Code of Practice for the Care and Use of Animals for Scientific Purposes of Universiti Malaysia Sabah.

Two types of roselle meals were prepared: fresh dried roselle (FR) and heat-treated roselle (HR) meals. For the FR meal, the roselles were dried in an oven at 40°C. The dried roselles were ground into homogenous powder and kept in -20°C freezer before use. The HR meal was prepared with the same batch of roselle after boiling with water in 1:1 ratio of roselle to water for about 20 minutes followed by roselle meal preparation as described in the FR meal preparation.

Isoproteic (crude protein, CP 37%) and isolipidic (crude lipid, CL 7%) diets were prepared using fishmeal and fish oil as the main protein and lipid source (Hu et al., 2008). The roselle meals were added at 10% into the experimental diets. Diet without roselle meals was served as control. The CP and CL of the diets were in the range of 37.0-37.1% and 6.9-7.5%, respectively (Table 1).

Table 1. Formulation of the experiment diets (g/100g dry weight basis)

Ingredients (g)	**Dietary Treatments**		
	Control	**FR**	**HR**
Fishmeal	40.9	38.8	38.8
Fresh roselle meal	-	10.0	-
By-product roselle meal	-	-	10.0
Shrimp meal	6.8	6.8	6.8
Fish oil	5.9	5.9	5.9
Vitamin Premix	4.0	4.0	4.0
Mineral Premix	3.0	3.0	3.0
Cholesterol	0.5	0.5	0.5
Tapioca starch	31.8	27.0	27.0
Carboxymethylcellulose	4.0	4.0	4.0
Proximate composition (%)			
Crude protein	37.1 ±0.10	37.1 ±0.26	37.0 ±0.17
Crude lipid	6.9 ±0.08	7.2 ±0.50	7.5 ±0.57
Dry matter	91.9 ±0.12	92.2 ±0.29	93.2 ±0.02
Ash	10.2 ±0.02	9.8 ±0.19	9.8 ±0.05

The juvenile shrimp of initial body weight (BW) of 1.2 ± 0.2g) were weighed individually and randomly stocked into triplicate tanks (50L tanks) at 20 shrimp/tank for each treatment. The shrimp specimens were fed four times daily for eight weeks at 10% of total biomass and then the biomass was adjusted based on the monitoring of feed intake (Mok et al., 2021). Water salinity 30±0.9ppt, temperature 28.20±0.56°C, dissolved oxygen 6.40±0.27mg L^{-1}, and pH at 7.40±0.15 were recorded throughout the rearing period. At the end of the feeding trial, the BW and body length (BL) were individually measured. Shrimp tail muscle (n=6) was sampled for body proximate analysis (AOAC, 1990) while the tail muscle with exoskeleton (n=6) was sampled for determination of total carotenoid concentration (TCC) (Arredondo-Figueroa et al.,2003). The body weight gain, specific growth rate, feed conversion ratio, and protein efficiency ratio were calculated at the end of the trial (Mok et al., 2021).

After sampling, the remaining shrimp specimens for each treatment were pooled for *V. harveyi* challenge test (Roque et al.,1998). For each treatment, 10 shrimp were exposed to 1.3 x 10^4 CFU ml^{-1} of *V. harveyi* VHJR7 suspension (Ransangan et al., 2012) in 50L tanks. Approximately 70% of the water in tanks was removed daily by the siphon method and replaced with sterilized seawater. The *V. harveyi* concentration in the water was maintained by daily inoculation into each tank. The inoculums used were monitored daily through thiosulphate citrate bile sucrose (TCBS) agar plate count (Austin, 1988). The shrimp were fed once daily at 5 to 10% of total biomass with the respective experimental diets, and mortalities were recorded. Due to the limited number of specimens, the test was done without replication. Therefore, the challenge test was terminated, and the survival of all treatments was recorded when any of the treatment tanks reached 50% mortality. Shrimp (n=3) were sampled to estimate the *Vibrio* spp. count in the hepatopancreas expressed as CFU g^{-1} tissue by spreading the hepatopancreas homogenate onto the TCBS plates and incubating for 24 hours at 37°C.

All the data were subjected to one-way ANOVA and Tukey's test (SPSS v20) at a significant level *of* $P<0.05$. Normality and homogeneity of variances were confirmed with Shapiro-Wilk and Levene tests.

The inclusion of 10% roselle meals in the diets (FR and HR) enhanced the final BW, BW gain, and specific growth rate of the shrimp although not significantly higher than the control group ($P>0.05$, Table 2). No significant difference was observed in the feed utilization of the shrimp between the treatment groups ($P>0.05$). There is limited study on the use of roselle in aquaculture particularly in shrimp (Hoseini et al., 2021). Roselle has been tested in extract or dried powder form and yielded varied results on aquatic animals (Perez-Escalante et al.,2012; Hoseini et al., 2021; Jomeh et al., 2021). Dietary inclusion of roselle powder in rainbow trout diet led to higher growth (Hoseini et al., 2021) while anthocyanin extracted from roselle showed no beneficial effect on growth (Jomeh et al., 2021). In contrast, a previous study showed that roselle extract enhanced the growth of Nile tilapia and goldfish, *Carassius auratus* (Perez-Escalante et al., 2012; El-Mesallamy et al., 2016). In the present study, the growth performance of shrimp fed HR was slightly higher than those fed FR. This could be due to the heat treatment of the roselle that reduced the anti-nutritional factors as shown in a study on rat feeding with boiled or dried roselle seed (Halimatul et al., 2007). The beneficial effect of the fresh or heat-treated roselle meals can be attributed to the anthocyanins in the roselle as a study reported that the anthocyanins were found to be thermostable up to 120 °C (Ursu et al., 2020).

Table 2. Growth performances and feed utilization of shrimp fed experimental diets

Parameters	Dietary Treatments		
	Control	**FR**	**HR**
Initial body weight (g)	1.24±0.03	1.24±0.04	1.26±0.02
Final body weight (g)	8.75±0.27	9.62± 0.12	10.06±0.02
Body weight gain (%)	605.65 ±72.62	675.81 ±23.86	689.41 ±22.79
Specific growth rate (% growth day^{-1})	3.48±0.19	3.65±0.05	3.71±0.05
Feed conversion ratio	1.27±0.80	1.25±0.83	1.25±0.87
Protein efficiency ratio	1.25±0.34	1.19±0.27	1.40±0.30

Values are mean ± standard deviation (n=3)

$$\text{Body weight gain (\%)} = \left[\frac{\text{Final BW (g)} - \text{initial BW (g)}}{\text{initial BW (g)}}\right] * 100$$

$$\text{Specific growth rate (SGR) (\% growth day}^{-1}) = \left[\frac{\text{In}\left(\text{Final BW (g)}\right) - \text{In (Initial BW (g))}}{\text{days of experiment}}\right] * 100$$

$$\text{Feed conversion ratio (FCR)} = \frac{\text{Feed consumed (g)}}{\text{Wet weight gained (g)}}$$

$$\text{Protein efficiency ratio (PER)} = \frac{\text{Wet weight gained of fish (g)}}{\text{protein intake (g)}}$$

The inclusion of dietary roselle did not affect the shrimp muscle moisture and protein contents (P>0.05, Table 3). However, a significant increase in muscle lipid was seen in shrimp fed the HR diet (p<0.05) compared to other treatments. The use of medicinal herbs and *Bacillus* sp. on whiteleg shrimp also significantly affected the body lipid content (Yu et al., 2009). The dietary inclusion of roselle seed meal did not alter the body lipid content of African catfish and tilapia (Fagbenro, 2005, El-Mesallamy et al., 2016). This could be due to species differences and the ingredients used. Despite the different results on the body proximate composition, the present study and investigations on goldfish showed that the dietary supplementation of roselle enhanced the body colouration (Perez-Esclante et al., 2012, Vanegas-Espinoza et al., 2019). In the present study, significantly higher TCC was demonstrated in the shrimp fed FR diet compared to the control (P<0.05, Table 3) while the TCC content was not significantly different between the FR and HR groups. In general, animals do not synthesize carotenoids *de novo*; the carotenoids found in animals are accumulated from the feed. This finding showed that the natural pigment anthocyanin from roselle can improve the body colouration of the shrimp. This is similar to other plant natural pigments that improve the body colouration of aquatic animals (Singh et al.,2021). The natural pigment derived from roselle can be an alternative carotenoid source for aquaculture animals.

Table 3. Proximate composition of the shrimp muscle (wet weight basis) and total carotenoid concentration of whole body of the shrimp.

Parameter	Dietary treatment		
	Control	**FR**	**HR**
Moisture (%)	73.21 ±0.29	74.99 ±0.56	74.08 ±0.70
Crude protein (%)	22.35 ±0.42	22.53 ±0.43	21.96 ±1.30
Crude lipid (%)	3.04 ±0.04^{a}	3.89 ±0.23^{b}	5.51 ±0.32^{c}
Total carotenoid concentration (μg g^{-1})	9.35 ±1.46^{a}	15.37 ±1.55^{b}	13.68 ±2.7^{ab}

Values are mean ± standard deviation (n=3). Mean value with different superscript within a row is significant different at P<0.05

In the post-challenge test with *V. harveyi*, the survival was higher in shrimp fed both roselle diets compared with the control (Table 4) and a significantly lower number of *Vibrio* spp. in the hepatopancreas was detected in shrimp fed the FR diet compared to the control (P<0.05). This finding was supported by previous studies on feed supplemented with roselle. The dietary inclusion of roselle is reported to enhance the antioxidant activities in rainbow trout (Hoseini et al., 2020), improve immune-related responses in rainbow trout (Jomeh et al., 2021), and increase the bactericidal activities against *Aeromonas hydrophila* infection in tilapia (El-Mesallamy et al., 2016). The protective effect against pathogens might be due to the anthocyanin in the roselle meals. Anthocyanin was previously reported to increase the innate immune response of Nile tilapia (Yilmaz, 2019).

Table 4. Survival of the shrimp and total *Vibrio* spp. count in the hepatopancreas on day 5 post challenges test

Parameter	Dietary treatment		
	Control	**FR**	**HR**
Survival (%)*	50	70	60
Vibrio spp. count (x10^{4} CFU ml^{-1})	25.67 ±4.51^{b}	12.00 ±2.65^{a}	17.67 ±2.52^{ab}

*In each treatment, 10 shrimp specimens were stocked in one tank without replicate tank (n=1)
Values are mean ± standard deviation, n=3. Mean value with different superscript within a row is significant different at P<0.05

The present study showed that roselle meal processed either from the fresh or heat-treated roselle can enhance the body colouration and the resistance of shrimp against vibriosis without affecting the growth and feed utilization of the shrimp *L. vannamei.*

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Conflict of interest

All authors declare that there is no conflict of interest.

Author contributions

Yong A.S.K. was involved in conception and design of the study. Mohamad N.M contributed to acquisition of data, sample analysis and drafting the manuscript. Yong A.S.K. and Mohamad Lal M.T. contributed to data analysis and revising the manuscript critically for important intellectual content. Yong A.S.K., Mohamad Lal M.T., and Mohamad N.M contributed to approval of the version of the manuscript to be published.

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