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Growth and health performance of pacific white shrimp fed diets with varying protein levels and citral supplementation

Kinerja pertumbuhan dan kesehatan udang vaname yang diberi pakan dengan kadar protein dan suplementasi citral yang berbeda

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ABSTRACT

Citral is known for its antimicrobial, antioxidant, and anti-diabetic properties. This study evaluates the effects of dietary citral supplementation on glucose absorption, growth, feed utilization, and health of Pacific white leg shrimp (*Litopenaeus vannamei*) fed different protein levels. A 2×3 factorial experiment was conducted with two variables: protein content (30% and 35%) and citral concentrations (0, 50, and 75 mg/kg). Shrimp (3.22 ± 0.01 g) were reared in 180 L tanks (30 shrimp/tank) for 60 days. Citral supplementation increased blood glucose levels in the first two hours post-feeding, with a faster return to basal levels. Growth and feed efficiency also improved with citral diets. After stress testing, citral and dietary protein showed a synergistic effect on superoxide dismutase (SOD), with the highest level in the 75 mg/kg citral, 35% protein diet, correlating with higher post-challenge survival. Total haemocyte count (THC), phenoloxidase (PO) activity, respiratory burst (RB), and blood clotting time improved with citral supplementation, while protein level only affected RB. After Vibrio challenge, higher protein increased THC and reduced clotting time, while citral enhanced THC, PO, and RB. The highest post-challenge survival was observed in shrimp fed 75 mg/kg citral with 35% protein ($p<0.05$). These findings suggest dietary citral supplementation may enhance shrimp health and resilience against stress and Vibrio infection.

Keywords: Citral, *Cymbopogon citratus*, post-prandial glucose, *Litopenaeus vannamei*, *V. parahaemolyticus*

ABSTRAK

Citral dikenal memiliki sifat antimikroba, antioksidan, dan anti-diabetes. Studi ini mengevaluasi efek suplementasi citral dalam pakan terhadap absorpsi glukosa, pertumbuhan, pemanfaatan pakan, dan kesehatan udang vaname (*Litopenaeus vannamei*) yang diberi pakan dengan kadar protein berbeda. Percobaan faktorial 2×3 dilakukan dengan dua variabel: kadar protein (30% dan 35%) serta konsentrasi citral (0, 50, dan 75 mg/kg). Udang (3,22 ± 0,01 g) dipelihara dalam tangki 180 L (30 ekor/tangki) selama 60 hari. Suplementasi citral meningkatkan kadar glukosa darah dalam dua jam pertama setelah makan, dengan penurunan lebih cepat ke tingkat basal. Pertumbuhan dan efisiensi pakan juga meningkat dengan pakan yang mengandung citral. Setelah uji stres, citral dan protein menunjukkan efek sinergis terhadap kadar superoksida dismutase (SOD), dengan kadar tertinggi pada perlakuan 75 mg/kg citral dalam diet protein 35%, yang berkorelasi dengan tingkat kelangsungan hidup pasca tantangan yang lebih tinggi. Jumlah hemosit total (THC), aktivitas fenoloksidase (PO), respiratory burst (RB), dan waktu pembekuan darah meningkat dengan suplementasi citral, sementara kadar protein hanya berpengaruh pada RB. Setelah tantangan Vibrio, kadar protein yang lebih tinggi meningkatkan THC dan mempercepat pembekuan darah, sedangkan citral meningkatkan THC, PO dan RB. Kelangsungan hidup pasca tantangan tertinggi ditemukan pada udang yang diberi 75 mg/kg citral dengan protein 35% ($p<0,05$). Hasil ini menunjukkan bahwa suplementasi citral dalam pakan dapat meningkatkan kesehatan dan ketahanan udang terhadap stres dan infeksi Vibrio.

Kata kunci: Citral, *Cymbopogon citratus*, glukosa pasca makan, *Litopenaeus vannamei*, *V. parahaemolyticus*

INTRODUCTION

The cultivation of Pacific white shrimp, *Litopenaeus vannamei* continues to face a number of challenges, including disease, environmental stress, and feed utilization. Bacterial diseases such as vibriosis are currently reported as one of the main causes of shrimp culture failure (Kumar *et al.,* 2021). Meanwhile, climate change and the environment increase the dynamics of the aquatic environment's quality, which can potentially stress shrimp or other aquaculture organisms. Furthermore, shrimp farming is dealing with rising feed costs as a result of rising feed raw material prices, one of which is the price of raw material used as a protein source. Sufficient energy and protein in feed support tissue maintenance, metabolism, and the moulting process, which are essential for shrimp growth.

Low protein feed can lead to non-optimal growth, weight loss, and even death due to unmet metabolic requirements. Excessively high protein levels can increase production costs and potentially pollute the environment. Studies have shown that shrimp experience decreased protein efficiency at very high protein levels, as excess protein is used for metabolic energy rather than tissue growth. The higher the protein digestibility, the more protein can be effectively used by the shrimp's body. Protein digestibility tends to decrease with protein content above 35% in the feed (Prakoso *et al.,* 2020).

Protein requirements for *vannamei* shrimp range from 32% to 48% depending on shrimp size, water conditions, and feed characteristics such as protein quality, energy content, and palatability (Yun *et al.,* 2016). Because protein is the most expensive nutrient in aquaculture feed formulation, a high protein content in feed can increase feed costs. A protein diet can also lead to an increase in nitrogen excretion into the water in the form of ammonia (Baki & Yucel, 2017). Thus, the utilization of protein must be pursued as optimally and efficiently as possible. The utilization of dietary protein will be related to the availability of non-protein energy sources (lipids and carbohydrates). One way to improve protein utilization efficiency is by increasing the carbohydrate content in feed formulations as an energy source (Zhang *et al.,* 2022).

Carbohydrates are becoming more popular in aquaculture feed because they are less expensive than fat and have high availability. However, most aquatic organisms, including prawns, have carbohydrate utilization limitations (Zhang *et al.,* 2022). Because shrimp growth is influenced by the rate of molting, which involves the synthesis of chitin and requires glucose as a precursor for shell formation and hormone control, the shrimp carbohydrate requirement is quite high (Gao *et al.,* 2021). Pacific white prawns have a good ability to digest carbohydrates, due to bacteria isolated from the digestive tract of *L. vannamei* have the capacity for extracellular enzyme production, including amylases, which are relevant in the digestive processes of this species. *L. vannamei* uses carbohydrates as a direct source of metabolic energy, for chitin synthesis, and for nucleic acid synthesis (Tzuc *et al.,* 2014). Shrimp tend to have less ability to digest and regulate plasma glucose concentrations.

The utilization of carbohydrates by aquatic animals is relatively low relative that of terrestrial animals. The ability of shrimp to utilize digested carbohydrates is limited due to low regulation of plasma glucose and low capacity in the absorption of plasma glucose (Zhang *et al.,* 2022; Wang *et al.,* 2016). One of the strategies to improve carbohydrate digestion capacity and blood glucose absorption activity is the addition of phyto-additives derived from natural ingredients such as citral. Citral, a mixture of two monoterpene aldehyde isomers: geranial (trans-citral, citral A) and neural (cis-citral, citral B), is an active ingredient found in some plants such as the citronella plant Cymbopogon citratus (Mori *et al.,* 2019; Liakos *et al.,* 2016) that may have beneficial effects on carbohydrate utilization. Monoterpenes are the primary constituents of essential oils, which are isoprenoid, lipophilic, and volatile.

By exposing adipocyte 3T3-L1, monoterpenes may facilitate glucose uptake. Citral (R-(+)-limonene and (R) - (+) b-citronellol have also been shown to increase human glucose absorption by 17.4% (Tan *et al.,* 2016). Citral content in citronella oil has been shown to activate peroxisome proliferator receptor gene expression (PPARs), which is involved in fat and carbohydrate metabolism (Katsukawa *et al.,* 2010). Citral, according to Mishra *et al*. (2019), has an antidyslipidemic function by lowering blood cholesterol levels, and functions anti-diabetic through increasing plasma insulin and utilisation of glucose through regulation of activity of enzymes. The addition of citral

has been shown to increase tilapia growth, with an optimum concentration of 400 mg/kg feed (Al-Sagheer *et al.,* 2018). Citral also has anti-inflammatory, antibacterial, and antivirulence properties (Liu *et al.,* 2020), immunomodulator, antiseptic, and fungistatic properties (Li *et al.,* 2022). In this context, the present study aimed to evaluate the effect of citral supplementation on feed utilization, growth and health performance of Pacific white shrimp fed with different dietary protein levels.

MATERIALS AND METHODS

Experimental diets and design

Six isoenergetic experimental diets were formulated with two protein levels, 30% and 35%, and three citral levels, 0 mg/kg, 50 mg/kg, and 75 mg/kg (Table 1). The inclusion level of citral was chosen based on previous study by Pratama *et al*. (2023). Citral (Sigma Aldrich C83007) was weighed according to the concentration of each treatment and diluted with water as much as 160

Table 1. Experimental diet formulation and proximate composition.

Ingredient (%)	30FA0	35FA0	30FA50	30FA75	35FA50	35FA75
Fishmeal	17.50	22.00	17.50	17.50	22.00	22.00
Corn Gluten Meal	8.00	8.00	8.00	8.00	8.00	8.00
Meat Bone Meal	10.00	12.00	10.00	10.00	12.00	12.00
Wheat Pollard	14.00	16.00	14.00	14.00	16.00	16.00
Corn Meal	13.00	14.00	13.00	13.00	14.00	14.00
Soybean Meal	16.00	17.00	16.00	16.00	17.00	17.00
Tapioca	4.00	4.00	4.00	4.00	4.00	4.00
Squid Oil	2.00	2.00	2.00	2.00	2.00	2.00
Fish Oil	2.00	2.00	2.00	2.00	2.00	2.00
Lysine	0.30	0.30	0.30	0.30	0.30	0.30
Lecithine	0.80	0.80	0.80	0.80	0.80	0.80
DL-Methionine	0.30	0.30	0.30	0.30	0.30	0.30
Choline Chloride	0.30	0.30	0.30	0.30	0.30	0.30
Mono-calcium phosphate	1.00	1.00	1.00	1.00	1.00	1.00
Cholesterol	0.30	0.30	0.30	0.30	0.30	0.30
Citral	0.00	0.00	0.05	0.075	0.05	0.075
Vitamin Mix	1.00	1.00	1.00	1.00	1.00	1.00
Mineral Mix	1.00	1.00	1.00	1.00	1.00	1.00
Carboxymethy cellulose (CMC)	3.00	3.00	3.00	3.00	3.00	3.00

Table 2. Proximate analysis of experimental diets.

Composition	Protein content and concentration of fitoadditive citral mg/kg dry feed					
	P30FA0	P30FA50	P30FA75	P35FA0	P35FA50	P35FA75
Moisture (%)	8.85	8.43	8.61	8.35	8.83	8.85
Crude Protein (%)	30.35	30.88	30.76	35.23	35	35.85
Crude Lipid (%)	7.14	7.73	7.47	7.72	7.62	7.14
Crude Fiber (%)	2.25	2.86	2.26	2.58	2.84	2.25
NFE (%)	39.88	35.98	38.51	34.91	33.35	34.38
Ash (%)	11.52	12.06	11.64	11.48	12.36	11.52
GE[2] (kcal GE/kg)	4006	4015	4034	4118	4043	4088

mL/kg of feed and subsequently mixed with other raw materials until homogeneous. The dough was later pelletized and oven dried at 100°C for four hours. The experimental diet was subsequently kept in airtight plastic until further use.

Experimental set-up

Specific pathogen-free shrimp post larvae (PL8) were obtained from a local hatchery, PT. Suri Tani Pemuka, Anyer, Indonesia and maintained in a fiber tank for 30 days before until they reached the experimental size. Eighteen fiber tanks previously filled with 180 L of seawater located semi outdoor at the Vocational Campus Fisheries Pond, IPB University, Indonesia were used as the experimental units. Shrimp juveniles with an initial weight of 3.22 ± 0.01 g was distributed randomly into each tank at a density of 30 shrimp/tank and maintained for 60 days. Feeding was offered four times daily at an initial feeding level of 8% biomass, and later adjusted according to the biomass estimated after sampling. Sampling was done every two weeks by weighing about 10 shrimp from each tank. Daily siphoning and weekly water exchange (30%) was performed to discharge fecal material and to maintain the water quality. Seawater was added occasionally to replace water loss due to siphoning and evaporation. Water quality parameters such as salinity, dissolved oxygen, pH and temperature were measured daily, whereas total ammoniacal nitrogen (TAN), nitrite and nitrate were measured every week.

Research parameter

Post prandial glucose

Post prandial plasma glucose was determined following the method described in Watanabe (1988) with modification. Before the test, the shrimp was fasted for about 24 hours and the water was completely changed to ensure no additional food available in the water. Feeding was given at about 8% of the shrimp biomass. Post prandial glucose was done by measuring blood glucose concentration before feeding (0), and 1, 3, 5, 12, 24 h after feeding with each experimental diet. Three shrimp was taken for each sampling point, anesthetized in ice-cold water, and blood was collected using 1 mL syringe which had been rinsed with anticoagulant (EDTA). Blood was centrifuged at 6,000 rpm for about five minutes to obtain plasma. Subsequently, glucose concentration in blood plasma was analyzed by using ortho-toluidine reaction.

Growth performance

Growth performance parameters observed were survival rate (SR) and specific growth rate (SGR), all determined on day 60 after the administration of citral supplementation. Growth performance was calculated using Zonneveld *et al*. (1991) formula:

$$SR\ (\%) = \frac{N_t}{N_0} \times 100$$

$$SGR\ (\%/\ day) = \frac{\ln W_t - \ln W_0}{t} \times 100$$

Note:

Nt = Amount of fish at the end of the treatment (fish)

N0 = Amount of fish at the beginning of the treatment (fish)

Wt = Final body weight (g)

W0 = Initial body weight (g)

t = Experimental period (days)

Feed utilization

Analysis of white shrimp feed utilization was observed was total feed efficiency, protein retention, fat retention and post-prandial glucose. Feed efficiency given to white leg shrimp is calculated using the formula proposed by NRC (2011). Feed efficiency (FE) was calculated using the formula:

$$FE = \frac{Wt - W0}{F} \times 100$$

Note:

Wt = Final body weight (g)

W0 = Initial body weight (g)

Protein retention was calculated by proximate analysis of the body protein of the test shrimp at the start and end of culture. The difference between the amount of protein in the shrimp body at the end and the beginning of rearing compared to the amount of feed protein consumed by shrimp during the rearing period (Takeuchi, 1988). Fat retention was calculated by proximate analysis of the test shrimp's body fat at the study's beginning and end.

Furthermore, a comparison is made with the amount of feed fat consumed (g) during the rearing period (Takeuchi, 1988). The measurement of blood plasma glucose levels followed the method of Watanabe (1988), which was modified at 0, 1, 3, 5, 12, and 24 hours of each control treatment

and the addition of citral to feed. The test shrimp used for each treatment were three replicates. Blood collection was carried out at the 5th leg of the white shrimp using a syringe that had been rinsed with EDTA solution to prevent blood clots. Blood serum was centrifuged at 6,000 rpm for five minutes to obtain blood plasma. Next, blood plasma glucose was analyzed using an ortho-toluidine reagent and in a spectrophotometer at λ = 530 nm.

Immune responses

Immune response parameters were observed on day 60 (before the challenge test) and day 67 (after the challenge test). The immune responses were determined by a sample of 0.5 mL shrimp hemolymph taken from the base of the first swimming leg using a 1 mL syringe filled with 0.5 mL of anticoagulant (30 mM trisodium citrate, 0.34 M sodium chloride, 10 mM EDTA, 0.12 M glucose, 4°C, pH 7.55). The total hemocyte count was calculated according study conducted by Huynh *et al*. (2018). PO activity was measured spectrophotometrically by recording dopachrome formation produced from L-di hydroxyphenyl alanine (L-DOPA) (Liu & Chen, 2004). Meanwhile, respiratory burst (RB) activity is often measured by assessing the reduction of nitroblue tetrazolium (NBT) to formazan, which indicates superoxide anion production (Arthikala *et al.,* 2017). Hepatopancreas from two prawns were collected from each tank for superoxide dismutase activity measurement, which was carried out using SOD colorimetric test kit (Sigma Aldrich).

The hypoxia stress test was performed during the final rearing period by transferring 10 intermolt prawns from each tank into a plastic bag filled with 5 L of seawater without any oxygen addition or aeration (Liu *et al.,* 2015). After that, the plastic bag was tightly tied up, and prawn survival was observed for 24 hours. The dissolved oxygen was measured at the initial and after 24 hours observation. Hemolymph clotting time refers to the methodology of Jussila *et al*. (2001) hemolymph samples (20 µL) from each shrimp (n = 20 organisms) stored in capillary microtubes for 1.55 mm diameter hematocrit (Brand SD); The coagulation time for each sample was calculated using repeated inversions. Time was counted from when the needle was inserted into the shrimp's ventral sinus until the microtube's hemolymph flow stopped.

Challenge test

The challenge test of white shrimp against *V. parahaemolyticus* was carried out for seven days after 60 days of rearing with test feed. It was done by injecting *V. parahaemolyticus* 10^6 CFU mL (LD_{50}) intramuscularly on the back between the second and third segments with 100 µl per shrimp. The survival rate was calculated by:

$$SR\ (\%) = \frac{\text{Number of live shrimps after challenge test}}{\text{number of injected shrimp}} \times 100$$

Data analysis

Data in this study were analyzed using Microsoft Excel 2010 and by variance (TWO WAY ANOVA) using SPSS ver. 25 software. If the obtained results were significantly different ($p<0.05$), further tests were carried out using Tukey's test.

RESULT AND DISCUSSION

Post prandial glucose

Postprandial glucose levels showed significant differences between treatments ($P<0.05$) starting at the first hour of observation after feeding. Shrimp blood glucose values one hour to three hours after feeding experienced a significant increase in the citral addition treatment compared to those of the control ($p<0.05$) (Figure 1). Meanwhile, the decrease in blood glucose levels in all citral treatments showed a faster time and was significantly different than the control ($p<0.05$).

Two-way ANOVA analysis on postprandial glucose levels (Table 3) showed that the addition of citral to feed consistently had a significant effect on this parameter, except at 0 and 24 hours where blood glucose levels had returned to basal values. Meanwhile, the protein content of the feed appeared to have a significant effect only at the 3rd hour of observation. A significant interaction was also shown in the parameter post prandial glucose levels, especially at the first and fifth hours of observation, which indicated that during these hours, the effect of citral on this parameter was influenced by the protein content of the feed.

Growth performance

The results of supplementation of citral on a diet compared to controls, the administration of dietary citral P35FA75 increased the value of the specific growth rate and final body weight.

The survival rates in citral treatments were not significantly different from controls (p>0.05). Adding citral to the feed treatment with lower protein content also resulted in higher shrimp growth than shrimp-fed feed with higher protein content without adding citral (Table 4).

Feed utilization

Overall, compared to controls, the administration of dietary citral was able to increase the value of the feed efficiency. The highest value was shown by the addition of citral with a concentration of 75 mg/kg in both treatments with 30% (P30FA75) and 35% (P35FA75) protein content and was significantly different compared to P30FA0 and P35FA0 as a control (P<0.05) (Table 4). The results of the two-way ANOVA analysis showed that the citral addition factor had more influence on feed efficiency than protein content. The parameters of protein retention and fat retention show the same thing. The highest protein retention was seen in the P35FA75 treatment, which showed a 16% increase compared to the control with the same protein content (P<0.05).

The fat retention value increased by 11% with the addition of 75 mg/kg citral to 35% protein feed but was not significantly different from P30FA75 (P>0.05). Post-prandial glucose levels showed significant differences between treatments (P<0.05) starting at the first hour of observation after feeding. Shrimp blood glucose values one hour to three hours after feeding experienced a significant increase in the citral

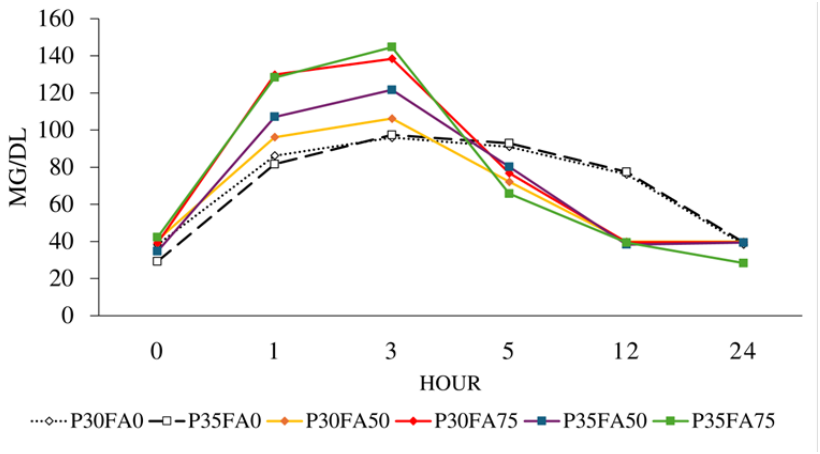

Figure 1. Postprandial glucose levels (mg/dL) of white shrimp that fed with different protein levels with the addition of citral for 24 hours of observation.

Table 3. Post prandial glucose levels (mg/dL) of penaeid shrimp fed with different protein levels with the addition of citral for 24 hours of observation.

Treatment	Hour-					
	0	1	3	5	12	24
P30FA0	38.19 ± 1.02^{ab}	86.18 ± 2.50^{cd}	95.88 ± 1.71^{c}	91.03 ± 3.06^{a}	76.18 ± 1.39^{a}	38.3 ± 1.09^{a}
P35FA0	29.21 ± 1.14^{ab}	81.64 ± 0.91^{bc}	97.39 ± 2.95^{c}	92.85 ±0.66^{ab}	77.39 ± 3.97^{b}	39.36 ± 2.53^{a}
P30FA50	40.88 ± 0.92^{ab}	96.18 ± 3.4^{a}	106.18 ± 2.78^{a}	72.09 ± 2.50^{b}	39.82 ± 1.89^{b}	39.82 ± 1.89^{a}
P30FA75	39.21 ± 1.09^{ab}	129.82 ± 2.78^{b}	138.45 ± 2.50^{b}	76.79 ± 1.32^{b}	39.52 ± 1.32^{b}	39.52 ± 1.32^{a}
P35FA50	34.67 ± 1.58^{a}	107.09 ± 3.53^{a}	121.64 ± 3.55^{a}	80.12 ± 2.48^{c}	38.3 ± 1.09^{b}	39.36 ± 2.53^{a}
P35FA75	42.24 ± 0.8^{b}	128.3 ± 0.6^{d}	144.67 ± 2.23^{c}	65.73 ± 0.95^{a}	39.36 ± 2.53^{a}	28.3 ± 4.08^{a}
P Value						
P	0.38	0.455	0.004	0.812	0.936	0.102
FA	0.21	0.001	0.001	0.001	0.001	0.078
PxFA	0.122	0.025	0.064	0.002	0.836	0.055

Note: Mean values ± standard deviations followed by different uppercase letters in the same row indicate significant differences (p<0.05).

addiction treatment compared to the control (P<0.05). Meanwhile, the decrease in blood glucose levels in all citral treatments showed a faster time and was significantly different than the control (P<0.05).

Immune responses and stress resistance

The results of measurements of the stress resistance (Table 5) in the hypoxic condition showed that P35FA75 treatment has the highest value (63.33 ± 0.06a) compared to that of other treatments, but was only significantly different from the control treatment at 30% protein content (40.00 ± 0.10b) (p<0.05). While the SOD value seemed to be affected by both treatment factors protein content and citral addition level, where the increase in protein content and addition rate resulted in a higher SOD value, with the highest value seen in the P35FA75 treatment (p<0.05). The immune responses of white shrimp, covering THC, RB activity, and PO activity after 60 days of rearing treatment and a challenge test with *V. parahaemolyticus* on days 67. Total hemocyte count after treatment diet supplementation of citral (day 60) and after a challenge test day 67) with *V. parahaemolyticus* are displayed in Table 6 and 7.

The results showed that the THC value in the citral treatment groups was higher than the control (p<0.05). The same result was found in the THC value after the challenge test, which tended to be higher in all citral treatment groups compared to the positive control (p<0.05). After the challenge test, the P35FA75 treatment group obtained a higher THC value of 13.25 ± 0.66^{a} cell/mm^{-3} compared to the P35FAO positive

Table 4. Growth performance and feed utilization.

Treatment	Parameter					
	SR (%)	Wt (g)	SGR (%)	FE (%)	RP (%)	RL (%)
P30FA0	96.67 ± 7.07^{a}	9.0 ± 0.69^{a}	2.82 ± 0.05^{c}	39.46 ± 3.42^{b}	14.61 ± 0.52^{c}	6.67 ± 4.25^{b}
P30FA50	91.11 ± 2.36^{a}	12.00 ± 1.30^{a}	3.61 ± 0.25^{ab}	61.28 ± 0.86^{ab}	24.53 ± 0.68^{b}	13.45 ± 6.03^{ab}
P30FA75	97.78 ± 3.85^{a}	11.65 ± 1.50^{a}	3.53 ± 0.26^{ab}	73.23 ± 1.30^{a}	26.47 ± 2.15^{ab}	14.68 ± 5.31^{a}
P35FA0	88.88 ± 7.07^{a}	9.89 ± 0.59^{a}	3.16 ± 0.10^{bc}	43.28 ± 5.86^{b}	16.36 ± 1.47^{c}	9.51 ± 7.38^{ab}
P35FA50	96.67 ± 7.07^{a}	11.56 ± 1.22^{a}	3.62 ± 0.32^{ab}	64.55 ± 2.65^{ab}	30.37 ± 3.73^{ab}	16.40 ± 6.80^{ab}
P35FA75	96.67 ± 5.77^{a}	12.16 ± 0.21^{a}	3.76 ± 0.03^{a}	79.07 ± 0.22^{a}	31.56 ± 2.45^{a}	17.92 ± 7.43^{a}
P value						
P	0.817	0.650	0.069	0.342	0.133	0.582
FA	0.334	0.021	0.001	0.001	0.000	0.001
P×FA	0.088	0.722	0.388	0.966	0.952	0.001

Note: Mean values ± standard deviations followed by different uppercase letters in the same row indicate significant differences (p<0.05).

Table 5. Stress resistance of white shrimp.

Treatment	Stress Resistance	
	Survival rate after hypoxia stress challenge (%)	SOD (% inhibition)
P30FA0	40.00 ± 0.10^{b}	11.90 ± 1.19^{c}
P30FA50	50.00 ± 0.00^{ab}	19.45 ± 3.00^{b}
P30FA75	56.67 ± 0.06^{a}	24.21 ± 0.69^{b}
P35FA0	50.00 ±0.10^{ab}	12.30 ± 2.48^{c}
P35FA50	53.33 ± 0.06^{ab}	19.84 ± 3.44^{b}
P35FA75	63.33 ± 0.06^{a}	31.35 ± 1.37^{a}
P value		
P	0.069	0.029
FA	0.011	0.001
PxFA	0.723	0.036

Note: Mean values ± standard deviations followed by different uppercase letters in the same row indicate significant differences (p<0.05).

treatment group, which had a THC value of 5.50 ± 0.10^{de} cell/mm^{-3}. The results of the respiratory burst (RB) measurement signified that after the treatment, the addition of 50 mg/kg and 75 mg/kg of citral treatment groups were significantly different ($p<0.05$) from the control.

However, the RB value after the challenge test in the citral treatment groups was quite different from the positive control ($p<0.05$). After treatment with citral, the PO value of white shrimp in the P35FA75 treatment group had significantly different results ($p<0.05$) from the control. After a challenge test on day 67, had a significant difference ($p<0.05$) in all treatment groups (Table 7). Meanwhile, the blood clotting time measurement results showed that after the citral administration, the values showed a faster time of clotting compared to the control (Table 7). After a challenge test on day 67, the blood clotting time treatment groups significantly differed from the positive control ($p<0.05$).

Table 6. Immune response of white shrimp that fed with different levels of protein and citral addition for 60 days of rearing period.

Treatment	Time of Sampling	Parameter			
		THC (10^6 cell/mm^{-3})	PO (OD 490n M/100 µl)	RB (O.D 630n M/10 µl)	Blood Clotting Time (s)
P30FA0 (-)	H60	6.25 ± 0.31^{b}	0.128 ± 0.0006^{b}	0.152 ± 0.009 ^{c}	70.33 ± 9.02 ^{a}
P30FA50	H60	7.05 ± 0.47^{b}	0.189 ± 0.003^{a}	0.183 ± 0.006 ^{b}	44.67 ± 5.30 ^{b}
P30FA75	H60	8.50 ± 0.58^{a}	0.175 ± 0.011^{a}	0.273 ± 0.021^{a}	36.67 ± 2.03 ^{b}
P35FA0 (-)	H60	6.45 ± 0.31^{b}	0.130 ± 0.006^{b}	0.164 ± 0.009 ^{c}	47.67 ± 3.71 ^{b}
P35FA50	H60	7.10 ± 0.47^{b}	0.168 ± 0.018^{a}	0.280 ± 0.014 ^{a}	43.00 ± 3.51 ^{b}
P35FA75	H60	9.20 ± 0.84^{a}	0.188 ± 0.004^{a}	0.281 ± 0.021^{a}	28.00 ± 2.52 ^{b}
P value					
P		0.236	0.600	0.001	0.329
FA		0.001	0.001	0.001	0.001
P×FA		0.565	0.033	0.001	0.020

Note: Mean values ± standard deviations followed by different uppercase letters in the same row indicate significant differences ($p<0.05$).

Table 7. Immune response of white shrimp after challenge test of seven days.

Treatment	Time of Sampling	Parameters			
		THC (10^6 cell/mm^{-3})	PO (OD 490n M/100 µl)	RB (O.D 630n M/10 µl)	Blood Clotting Time (s)
P30FA0 (-)	H62	7.75 ± 0.13	0.165 ± 0.008	0.274 ± 0.013	63.67 ± 3.71
P35FA0 (-)	H62	7.15 ± 0.36	0.173 ± 0.010	0.311 ± 0.008	66.33 ± 4.48
P30FA0 (+)	H62	5.25 ± 0.30^{b}	0.126 ± 0.009^{b}	0.259 ± 0.011^{b}	102.67 ± 10.14^{ab}
P30FA50	H62	8.10 ± 0.65^{c}	0.255 ± 0.011^{ab}	0.355 ± 0.008^{a}	123.67 ± 6.06^{b}
P30FA75	H62	11.25 ± 0.46^{ab}	0.279 ± 0.007^{ab}	0.374 ± 0.005^{a}	114.67 ± 4.18^{ab}
P35FA0 (+)	H62	5.50 ± 0.10^{de}	0.167 ± 0.020^{d}	0.246 ± 0.0016^{b}	118.67 ± 8.69^{ab}
P35FA50	H62	9.45 ± 0.83^{bc}	0.233 ± 0.027^{b}	0.342 ± 0.015^{a}	90.67 ± 1.45^{ab}
P35FA75	H62	13.25 ± 0.66^{a}	0.288±0.009^{a}	0.383 ± 0.007^{a}	86.00 ± 3.21^{a}
P value					
P		0.021	0.295	0.529	0.003
FA		0.001	0.001	0.001	0.601
PxFA		0.316	0.034	0.528	0.062

Note: Mean values ± standard deviations followed by different uppercase letters in the same row indicate significant differences ($p<0.05$).

Resistance to Vibrio parahaemolyticus

The value of survival rate (SR) of the white shrimp after undergoing a challenge test with *V. parahaemolyticus* in P35FA75 treatment groups was higher ($p<0.05$) than the positive control group P35FA0, with P30FA75 treatment group not significantly different ($p>0.05$). The highest survival rate was found in the P35FA75 treatment, which obtained a survival rate of 90.00% (Table 8).

Discussion

Increasing non-protein energy sources like carbohydrates in feed can minimize the use of dietary protein as an energy source in crustaceans (Singha *et al.,* 2023). For this reason, another approach is needed to increase the capacity to utilize carbohydrates, one of which is by adding phyto-additives such as citral. The results of this study indicated that citral could play a role in regulating blood sugar levels after eating (post-prandial glucose). From the 1st to 3rd hour observation after feeding, it was seen that all treatments showed an increase in blood glucose levels which indicated that the glucose absorption process was the result of digestion of the feed consumed. At this time of observation, blood glucose levels were higher in all citral treatments those in control at 30% and 35% protein levels.

This may indicates that citral treatment can increase the activity of carbohydrate digestion and absorption of glucose from the intestine into the blood, with an increase in line with the increase in citral concentration in the feed. The results of previous studies indicate that increased glucose absorption may occur due to the increased activity of carbohydrase enzymes in the shrimp digestive tract which facilitated the digestion of carbohydrates (Akter *et al.,* 2016). Pratama *et al*. (2023) shows that adding citral at a dose of 100 mg/kg of feed could increase the activity of the amylase enzyme by up to 69%. In the observation five hours after feeding, it was seen that all citral treatments had shown a decrease in blood glucose levels of 32 to 55% of blood glucose levels in the third hour. Whereas, in the control treatment, the reduction was only around 4%.

This indicated that citral could play a role in transporting glucose from the blood into cells in various tissues that require glucose as an energy source and liver and muscle cells to store it in the form of glycogen. This indication was confirmed by observational data at the 12th hour, which showed that glucose levels in all citral treatments had returned to the basal value. In contrast, in the control treatment, the basal blood glucose value was only seen at the 24th hour of observation. Citral was found to lower blood glucose levels and raise insulin plasma concentrations in diabetic rats in a prior study (Mishra *et al.,* 2019). This was thought to be related to the role of citral in increasing insulin secretion, which facilitated blood glucose absorption in various tissues (Djahi *et al.,* 2021).

In addition to regulating the increased absorption of glucose into tissue cells, insulin also acts on the liver to increase glycogenesis. Besides increasing insulin secretion, the role of citral in carbohydrate metabolism was also associated with increased glycolytic activity

Table 8. Value of survival rate regarding resistance to *Vibrio parahaemolyticus*.

Treatments	Survival Rate (%)
P30FA0	36.67 ± 0.06^{c}
P30FA50	60.00 ± 0.13^{abc}
P30FA75	70.00 ± 0.14^{a}
P35FA0	40.00 ± 0.11^{bc}
P35FA50	80.00 ± 0.20^{ab}
P35FA75	90.00 ± 0.17^{a}
P value	
P	0.208
FA	0.001
PxFA	0.864

Note: Mean values ± standard deviations followed by different uppercase letters in the same row indicate significant differences ($p<0.05$).

(Duan *et al.,* 2020). Xu *et al*. (2018) reported that administering citral could restore the decreased glucokinase (GCK) gene expression level due to feeding with high-fat content in rats. OuYang *et al*. (2018) also stated that citral could change mitochondrial morphology and suppress the citrate cycle (TCA) and the glycolysis process in *Penicillium* dictated.

The positive role of citral on carbohydrate metabolism goes hand in hand with increasing feed utilization and growth performance. The results of this study showed that the LPS, EP, RP and RL values increased significantly in the citral treatment compared to those of the control regardless of the protein content of the feed used ($p<0.05$). Giving citral can increase the retention of protein and fat in shrimp fed with either 30% or 35% protein content, which indicates that citral could play a role in increasing the efficiency of feed protein and fat utilization. The increase in protein retention was thought to be related to the increase in the availability of carbohydrates as an energy source so that the protein that can be used for growth becomes higher (protein-sparing effect). At the same time, the increase in fat retention was thought to be caused by two things, namely the use of carbohydrates which substitute for fat as an energy source or an increase in body fat levels.

The conversion of excess glucose in shrimp into fat was also supported by the rise in the body fat content of the shrimp from around 4-5% in the control treatment to 6.5-7.6% in the treatment with citral addition. Research on mud crabs shows that high levels of feed carbohydrates increase body fat levels due to increased expression of the fatty acid synthase (fas) gene (Zhan *et al.,* 2020). Citral in feed can increase enzyme activity to accelerate the breakdown of glucose into pyruvate acid in the glycolysis process. Li *et al*. (2015) explained the enzymes that play a role in glycolysis: hexokinase, phosphofructokinase, and pyruvate kinase. In addition to these three key enzymes, an important enzyme, glucokinase, catalyzed the conversion of glucose to glucose-6-phosphate.

Mishra *et al*. (2019) explained that hexokinase, glucokinase and pyruvate kinase activities decreased in the liver of rats with diabetes. Citral increased the activity of this enzyme because glycolysis can be activated, and glucose utilization can be improved. Optimal protein utilization impacts feed utilization efficiency. Fast glucose absorption can produce energy which can prevent protein catabolism into energy used for growth. Better feed utilization in citral treatment is reflected in the value of feed efficiency and shrimp growth rate, which is also better than control.

The addition of citral in feed can play a role in increasing shrimp resistance to hypoxic stress, and this was indicated by the post-stress test shrimp survival rate, which was higher than the control in both the 30% and 35% protein feed groups, with the highest post-stress test survival rate indicated by P35FA75 ($p<0.05$). This increase in resistance to stress is closely related to the increase in antioxidant capacity in shrimp-fed citral, as noted in the SOD value. In this study, it was seen that both protein content and citral addition had a positive effect on the SOD value. Hypoxic conditions modify the activity of cytochrome chains which are responsible for mitochondrial oxidative phosphorylation, resulting in decreased synthesis of adenosine triphosphate (ATP) and increased reactive oxygen species (ROS) together with reduced activity of cellular antioxidant systems, which can cause oxidative stress (Coimbra-Costa *et al.,* 2017). Citral can protect IEC-6 cells against aspirin-induced oxidative stress, which can recognize triggers of natural antioxidant substances (Bouzenna *et al.,* 2017). The SOD value can describe the ability of shrimp to overcome free radicals such as ROS, which are produced by metabolic processes.

Pratama *et al*. (2023) also stated that white leg shrimp fed citral at a dose of 75 mg/kg of feed could increase the SOD value and decrease the MDA value compared to the control. Citral also increased the SOD activity of common snook at a dose of 1.76 mg/kg of feed (Mori *et al.,* 2019); this happens because citral has phytochemical compounds in the form of terpenoids which function as a free radical barrier and can reduce ROS levels and function to improve the activity of SOD, catalase enzymes, GPx, and GR. Antioxidant and cytoprotective effects on citral can play a role in overcoming oxidative damage caused by hydrogen peroxide (H_2O_2) in endothelial cells (Safaeian *et al.,* 2020). Excess ROS causes oxidative stress, which induces changes in aquatic organisms' lipids, proteins, and nucleic acids (Xu *et al.,* 2018).

The results of this study indicated that the addition of citral has a positive effect on the immune response of shrimp both after 60 days of rearing and after the challenge test of *V. parahaemolyticus* bacteria. White leg shrimp do not have an immune system generally found in

vertebrate animals (a specific immune system) but rely on non-specific immune system mechanisms in dealing with oxidative or environmental stress (Bao *et al.,* 2019). Adding citral to the feed can improve the white leg shrimp's cellular and humoral immune systems. Chastain *et al*. (2022) explained that citral can increase the THC value of *Galleria mellonella* (Lepidoptera: Pyralidae) larvae.

Dietary citral supplementation in shrimp feed can enhance various aspects of shrimp health and growth performance. One notable effect is its impact on the immune system, particularly humoral immune responses. Citral supplementation has been shown to increase phenoloxidase (PO) activity, a crucial component of the shrimp immune system. PO plays a key role in melanin production, which helps in pathogen inactivation. A study found that the highest PO activity was observed at a citral concentration of 75 mg/kg in feed (Zheng *et al.,* 2025; Pratama *et al.,* 2023; Muahiddah *et al.,* 2022).

The active proPO system, together with several other molecules, carries out self-defense responses, namely melanin formation, recognition of disease agents, adhesion, and communication between cells (Liu & Chen, 2004). Tassanakajon *et al.* (2011) particularly at high densities (of which intensive farming represents an extreme example stated that if a pathogen enters the shrimp's body, phagocytosis occurs in the hemolymph by hyaline and semiregular cells, destroys the pathogen by PO activity and also activates antibacterial action by antimicrobial peptides (AMPs) such as paladins, crusting and anti-lipopolysaccharide factors (ALFs). It is reported that PO activity in shrimp can be increased by adding various essential oils such as thymol, vanillin and thyme (Tomazelli *et al.,* 2017), but the mechanism of action of essential oils in affecting the shrimp immune system is still unknown. RB activity is a series of processes that destroy phagocytized microbial particles that involve the release of degradative enzymes into the phagosome and the production of reactive oxygen intermediate (ROI) (Thomas, 2017).

This study's results indicate that adding citral to feed can enhance the van name shrimp's immune system to fight pathogenic bacterial infections and increase its ability as an antimicrobial compound. Adding citral can accelerate the rate of blood clotting in white leg shrimp. As blood coagulation is an important defense response for crustaceans, an increase in normal clotting time should indicate an impaired (slower) response to the presence of a stress agent (Wang *et al.,* 2013). The tendency for shorter clotting times in the citral treatment means progressive acclimatization to the stressor. Positive interactions were shown at the protein level treatment in synergy with citral; this occurred due to specific interactions between compounds in essential oils and proteins in the immune system and counteracting toxins that can reduce health levels.

The immune system in crustaceans is primarily innate and relies on various protein-based processes for defense against pathogens. This system is characterized by the activation of specific proteins in response to pathogen-associated molecular patterns (PAMPs), which include components from bacteria, fungi, and viruses. Upon recognition of PAMPs, PRPs activate several immune pathways leading to the release of effector molecules that help eliminate pathogens.

This activation triggers processes like phagocytosis, encapsulation, and the production of reactive oxygen species (Tran *et al.,* 2022; Huang *et al.,* 2020). The increase in the immune response of shrimp after being given feed containing citral was seen in the challenge test results. Citral is known to have antimicrobial and antivirulent properties against V. *parahaemolyticus* bacteria (Cao *et al.,* 2021). The results of this study indicated that the survival after the challenge test of *V. parahaemolyticus* bacteria in the citral-treated shrimp was higher than the control ($p<0.05$), which confirms the research of Sun *et al*. (2019) that citral has an inhibitory effect on the virulence factor of V. *parahaemolyticus*.

CONCLUSION

The addition of citral can increase the utilization of carbohydrates by increasing the amount and speed of absorption of glucose, increasing the efficiency of feed protein utilization, growth performance and shrimp health as well as resistance to stress and vibriosis. The best growth and health performance was shown by the addition of 75 mg/kg citral with 35% protein content.

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