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Supplementation Effect of Dietary Carvacrol and Thymol Polyphenols from Oregano *Origanum vulgare* on Growth Performance and Health Condition of Pacific White Shrimp *Litopenaeus vannamei*

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Authors' contributions

This work was carried out in collaboration among all authors. All authors read and approved the final manuscript.

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ABSTRACT

Two growth trials were conducted for 60-days using aquaria tanks (growth trial 1) and 50-days using commercial culture tank (growth trial 2) to assess the potential effect on using phytogenic feed additives (Regavit Aqua^{TM}, Ecopharm Hellas, Greece) containing thymol and carvacrol extracted from Oregano plant *Origanum Vulgare* (OP) by incorporating the OP powder into the diet formulation for trial 1 and top-dressing process of OP liquid for trial 2. The antimicrobial properties of carvacrol and thymol polyphenols were also determined by using minimum inhibitory concentration (MIC) analysis against *Vibrio harveyi* and *Vibrio parahaemolyticus.* From the aquaria tank (trial 1), the growth performance, including final average body weight (ABW), percentage weight gain (PWG) and thermal growth coefficient (TGC) of group of shrimp fed with OP were compared to the control treatment. Using top-dressing process (trial 2), the top coating process to the commercial diet enhance the growth performances of shrimp treated with OP compared to the control treatment. From, trial 1, there was clear evidence for elevated hemocyte activity and increased lysozyme in shrimp fed diets supplemented with OP compared to the control treatments. MIC analysis performed in the present study also revealed the ability of OP to inhibit the growth of bacteria at the concentration of 2×10^{1} µg mL^{-1}. The results indicate that the inclusion of OP up to 0.6% both in direct inclusion within the diet or using top-dressing process significantly increases the growth and non-specific immune in white shrimps *L. vannamei*.

Keywords Litopenaeus vannamei; growth performance; health; polyphenols; Origanum Vulgare.

1. INTRODUCTION

The current formulation of aquafeed that has a tendency to increase the inclusion levels of plant-protein sources and minimize the use of fish meal (FM) need to assure the fulfillment of specific nutrient requirement as well as the bioavailability of those nutrients to support the optimum growth and health condition of fish and shrimp [1-3]. Several alternative protein sources that has been used to replace fish meal and other animal proteins in shrimp feed, including soybean meal, cotton seed meal, and corn products [4-6]. However, wider use of plant-protein sources in shrimp diet may be hindered by the negative aspects associated with the presence of anti-nutritional factors such as plant lectins, phytic acid, saponins, phytosterols, and allergens, as well as toxin materials [7,8], which responsible to the low efficiency on nutrient use, sub-optimal growth, histomorphological change in the digestive system; and become more susceptible to the pathogen and diseases infection [9,10]. To mitigate the challenges on replacing FM with plant-protein sources, feed supplementation with phytogenic additives has been suggested [11]. Plant-derived products or phytogenic additives may improve nutrient retention and promote the growth and health condition of fish and shrimp [11-14].

According to Kesselring et al. [11] and Sutili et al. [15], classification of phytogenic additives in aquafeed can be divided based on botanical origin, processing methods and composition, and usually known as herbal products, spices, aromatic oleoresins and essential oils. Concerning to essential oil, their main characteristics as the volatile, natural, and complex compounds has been known as an antiseptic, including bactericidal, fungicidal, virucidal and medicinal properties; anti-inflammatory, anti-microbial, spasmolytic and locally anesthetic remedies [16]. Other than volatile compounds, the presence of non-volatile compounds extracted from various plants, mainly polyphenols, also play significant role in health through the regulation of metabolism, weight, chronic disease, and cell proliferation [17,18]. In crustaceans, the increase in the number of studies with polyphenols has been mainly due to the ability of this phenolic compounds to serve as melanosis inhibitor, antioxidant, anti-inflammatory, and antimicrobial activities as well as the promising alternatives to sulfiting agents [19]. Polyphenols can be found in vegetables, nuts, seeds, fruits, flowers and tree barks [20]. Among the sources, Oregano (*Origanum vulgare*) belonging to *Lamiaceae* family were considered as a rich source of polyphenols and frequently used as feed additives in aquaculture [21,22]. Recent studies have indicated the ability of polyphenols from Oregano *O. vulgare* improved the antioxidant and immune response of fish, including Zebrafish, *Danio rerio* [23] and rainbow trout, *Oncorhynchus mykiss* [24]. Such beneficial effects are attributable to the bioactive molecules, especially carvacrol and thymol that

show strong antioxidant activity due to their ability to neutralize the oxygen free radicals (ROS) in tissues and cells [15]. Since the nutrition research using carvacrol and thymol as significant polyphenols is limited in shrimp, the aimed of this study is to evaluate the efficacy of carvacrol and thymol polyphenols to enhance the growth and health condition of shrimp *Litopenaeus vannamei* by using two application method, namely direct inclusion of oregano polyphenols (OP) in the diet and top-dressing of commercial diet method. In addition to that, the ability of carvacrol and thymol to inhibit the growth of *Vibrio harveyi* and *Vibrio parahaemolyticus* as the significant pathogen in shrimp culture system was also determined.

2. MATERIALS AND METHODS

2.1 Preparation of Oregano Polyphenols (OP)

Oregano plant material (*Origanum Vulgare*; Hirtum or also known as greek oregano) were harvested and subjected to commercial steam distillation-condensation processes to extract the natural oregano essential oil (OEO). Natural oregano essential oil (OEO, Regavit Aqua^{TM}, Ecopharm Hellas, Greece) were polyphenols contained with carvacrol (78-82%) and thymol (1.4-2.4%) as determined by gas-chromatography. The formulated OEO products (5% wt/wt) are available as powder with attapulgite as carrier and as liquid-emulsion.

2.2 Experimental Diets

2.2.1 Experimental diets for controlled condition

Four isonitrogenous (36% crude protein) and iso-lipidic diet (6% crude fat) were formulated to contain 0.2; 0.4 and 0.6 % oregano polyphenols (OP) provided by Ecopharm Hellas, Greece (Table 1). Fish meal (FM), soybean meal (SBM) and Corn gluten meal (CGM) act as the protein source. On preparing the control and experimental diet, all dietary ingredients were first grounded into small particles using hammer mill and then passed to a 250 µm mesh sieve. All dry ingredients were mixed in a food mixer (Hobart, Troy, OH, USA). To enhance the distribution, OP powder were mixed with other micro-ingredients, including calcium phosphate-dibasic, vitamin and mineral premix and then added to the mash. Wheat products were then added into the mixture of micro and dry ingredients prior to the final addition of soy-lecithin and fish oil. The mixed ingredients were conditioned in a steam injection conditioner for 10 – 15 s prior to pelleting. The pellet temperature just after pelleting process was in the range of 80 - 82°C. After drying under the sun and in a cooler, the pellets (2-mm diameter) were stored in a sealed bag at 4°C until further use. The experimental diets were analyzed at PT Saraswanti Indo Genetech (Bogor, Indonesia).

2.2.2 Experimental diets for commercial application

The liquid form of OP with amount of 2; 4 and 6 mL (labeled as 0.2; 0.4 and 0.6% OP) were applied uniformly in 1 Kg of commercial feed (CJ, Code SA, Indonesia) with 35% crude protein, 6% crude fat, 11% moisture content, and 13% of ash content. The control feed were not prepared with OP. The OP-coated feeds were air dried under shade for 1 – 1.5 h before feeding.

2.3 Feeding Trial

For the trial using aquaria tank (growth trial-1), Pacific white shrimp (PWS) *Litopenaeus vannamei* were obtained from private commercial shrimp hatchery PT. Maju Tambak Sumur (Kalianda, Lampung, Indonesia) and nursed in a semi-indoor recirculating system. Dissolved oxygen was maintained within acceptable range for Vannamei using air stones in each culture tank and the sump tank using a common airline connected to a regenerative blower. Shrimp were hand-fed with a commercial feed (CJ feed, Code SA, Lampung, Indonesia) for three weeks until reached the suitable size. Shrimp (1.05±0.03 g initial mean weight) were stocked into 70 x 35 x 40 cm (98 L) tanks with 15 shrimp per aquarium per tank. Six replicate groups of shrimp were offered with experimental diets four times per day based on the historical shrimp research data assuming the normal growth of shrimp and employing a standard feeding ratio as much as 1.6. Daily amount of feed were then adjusted based on the daily observation for mortality for each tank during the observation period.

For the growth trial using commercial application method (growth-trial 2), A 6000 healthy PWS on the stage of post larvae (PL) 7 with average weight ~ 0.03 g were distributed into 12 plastic tanks (diameter 2.2 m with volume of 3000 L per tank). The tank were connected and operated as a recirculation system. Dissolved oxygen was maintained within acceptable range for shrimp

using blower and air stones in each culture tank. The PL's were acclimatized for 3 h and immediately fed using experimental diets for four times per day. The feed amounts were prepared similar with the growth trial using aquaria tank. The daily amounts of feed were adjusted based on the observed feed consumption and daily mortality.

2.4 Water Quality and Growth Performance Analysis

Water quality parameters including dissolved oxygen (DO), water temperature (T), pH, and salinity of the water were measured four times daily (5 AM; 10 AM, 2 PM and 6 PM) using a real-time measurement system (Aqua TROLL 500 Multiparameter Sonde instrument). Meanwhile, the Total ammonia-nitrogen (TAN), alkalinity, nitrate, nitrite, and ammonia were measured once a week by using photometer (Water link®, Spin Touch®, LaMotte). All data obtained from both feeding trial were stored to an application (AquaEasy apps, Bosch, Singapore) for data traceability and recording system purposes. At the termination of the feeding period, the shrimp in each aquaria tank were group counted and individually weighed, and from the commercial tanks were group weighed to calculate the final biomass, final weight, percentage weight gain (PWG), feed conversion ratio (FCR), percentage survival (SR) and thermal unit growth coefficient (TGC), as follows:

PWG = (average individual final weight-average individual initial weight) / (average individual initial weight) × 100

$$\text{FCR} = \frac{feed\ given\ (g)}{alive\ weigh\ gain\ of\ shrimp\ (g)}$$

$$\text{SR} = \frac{final\ number\ of\ shrimp}{initial\ number\ of\ shrimp} \times 100$$

$$\text{TGC} = \frac{FBW^{1/3} - IBW^{1/3}}{\sum TD} \times 100,$$

where FBW is the final body weight, IBW is the initial body weight, T is the temperature (^{0}C) and D is the number of feeding days.

2.5 Total Haemocyte Count

At the termination of the feeding trial using aquaria tank, hemolymph was sampled from three individual shrimp per aquaria tank, or eighteen shrimp per dietary treatment, to determine the total haemocyte count (THC). Hemolymph (100 µL) of individual shrimp was withdrawn from the pleopod base of the second abdominal segment with a sterile 1-mL syringe (25 G × 13 mm needle). Before the hemolymph extraction, the syringe was loaded with a precooled (4°C) solution (10%-EDTA, Na2) used as an anticoagulant. The hemolymph with anti-coagulant solution was diluted in 150 µL of formaldehyde (4%) and then 20 µL were placed on a hemocytometer (Neubauer) for the THC measurement using an optical microscope (Olympus, DP72).

2.6 Lysozyme Activity Analysis

At the termination of the feeding trial using aquaria tank, the lysozyme activity of shrimp was measured by using a commercial kit (Sigma-Aldrich, Cat. No. LY0100). Six shrimp per dietary treatment were used for the lysozyme activity analysis. The results were defined by the lysis of *Micrococcus lysodeikticus* cells. The reactions were conducted at room temperature and the absorbance of the samples was measured at 450 nm using an ultraviolet/visible spectrophotometer (Perkin Elmer, Lambda XLS, USA):

$$\text{Lysozyme activity}\ \left(\frac{\text{Units}}{\text{mL}}\right) = \frac{(\Delta \text{A}450/\min Test - \Delta \text{A}450/\min Blank)\ (df)}{(0.001)(0.03)}$$

df = dilution factor
0.001 = ΔA_{450} as per the unit definition
0.03 = Volume (in mL) of enzyme solution

2.7 Minimum Inhibitory Concentration (MIC) Analysis

Minimum inhibitory concentration (MIC) determinations were carried out by using agar diffusion assay to evaluate the efficacy of OP against *Vibrio harveyi* and *Vibrio parahaemolyticus*. As the positive control, oxytetracycline (OTC) was used against similar bacteria. For the test, standard 8-mm paper discs were placed on the surface of the agar and concentrations ranging from $2 \times 10^{-6} - 2 \times 10^{1}$ µg mL^{-1} of both OP and OTC were added. Plates were analyzed individually after 24 h of incubation period to determine the MIC and the average values from three repeats were taken in determination of the final MIC.

2.8 Statistical Analysis

Data on the growth parameters, total haemocyte counts, lysozyme activity and minimum inhibitory

concentration were analyzed using regression and one-way analysis of variance (ANOVA) to determine significant differences among treatments, followed by Tukey's multiple comparison tests to determine the difference between the means among the treatments. All statistical analyses were conducted using SAS system (V9.4. SAS Institute, Cary, NC, USA).

3. RESULTS

3.1 Water Quality Analysis

The results for physical water quality analysis in both trials almost similar and within the acceptable range for PWS (Table 2). Parameters such as pH, dissolved oxygen (DO), temperature, and salinity for the growth trial using aquaria tank in the morning are in the range of 7.82 ± 0.35; 5.68 ± 1.14 mgL^{-1}; 28.51 ± 1.13 °C; and 27.55 ±1.68 ‰, respectively. Meanwhile in the afternoon in the range of 7.95 ± 0.77; 6.04 ± 1.09 mgL^{-1}; 29.22 ± 0.68 °C; and 27.86 ± 1.55 ‰, respectively. For the growth trial using commercial tanks, the results for pH, dissolved oxygen (DO), temperature, and salinity in the morning were in the range of 7.41 ± 0.16; 5.02 ± 0.37 mgL^{-1}; 28.41 ± 0.42 °C; and 22.14 ± 1.29 ‰, respectively. The results for in the afternoon were in the range of 7.52 ± 0.96; 5.04 ± 0.52; 29.62 ± 0.47; and 23.25 ± 1.44 ‰, respectively. The results for chemical parameters focusing on Nitrite-nitrogen (NO_2-N), Nitrate-nitrogen (NO_3-N), and Ammonia (NH_3) for growth trial using aquaria tanks in the range of 0.023 ± 0.009 mgL^{-1}; 12.891 ± 2.328 mgL^{-1}; and 0.019 ± 0.011 mgL^{-1}. Meanwhile for the growth trials using commercial tanks were in the range of 2.821 ± 0.468 mgL^{-1}; 74.251 ± 5.543 mgL^{-1}; and 0.011 ± 0.017 mgL^{-1}.

3.2 Growth Trial

3.2.1 Growth trial using aquaria tanks

Growth results from the trial 1 (aquaria tank) are presented in Table 3. A continued positive growth performance of PWS was obtained with the increase of dietary OP from 0.2 to 0.6%. Among the dietary treatment, the highest FBW, PWG, and TGC were obtained in group of shrimp fed with 0.6% OP. In terms of FCR, the group of shrimp fed with 0.6% OP also showed the highest efficiency among the dietary treatment. There is no significant difference on survival between the group of shrimp fed with 0.4 and 0.6% OP, but still better compared to the control group.

3.2.2 Growth trial using commercial tanks

From the commercial trial or second growth trial conducted for 50 d, there is still clear-dose response, where the highest FBW, TGC and PWG were found in 0.4% OP before starting to plateau and have a decreasing trend in the group of shrimp treated with 0.6% OP, but still significantly higher than control treatment (P<0.05). The lowest FCR were found in the group of shrimp fed with 0.4% OP and then displayed an increasing trend with the inclusion of 0.6% OP. There were no significant differences in terms of survival among the dietary treatment (P>0.05).

3.3 Total Haemocyte Counts and Lysozyme Activity from Aquaria Tank Trials

The total haemocyte counts and the lysozyme activity were both significantly increased after fed the shrimp with dietary OP for 60 days. The total haemocyte counts (10^6 CFU mL-1) were higher in the group of shrimp fed with 0.6% OP compared to other dietary treatment (Fig. 1). For the lysozyme activity, a significant increase were showed in the group of shrimp fed with 0.2% OP and then decrease as the inclusion level of dietary OP increase.

3.4 Minimum Inhibitory Analysis

Table 5 showed good inhibition of *Vibrio parahaemolyticus* (VP) and *Vibrio harveyi* (VH) by using OP in disc diffusion assays. The diameter of zone inhibition on the use of OP was compared with the use of oxytetracycline (OTC) to evaluate the effectiveness of polyphenols extracted from oregano plant. The zone inhibition measured 13.2 mm using OP with concentration of 2×10^1 µg mL^{-1} in comparison with OTC 12.4 mm against VP. Meanwhile, the zone inhibition using OP with similar concentration level was 17.0 mm in comparison with OTC 17.3 mm against VP.

Table 1. Composition (%, *as is*) of diets consisting several inclusion levels of oregano polyphenols (OP, Regavit Aqua™, Ecopharm Hellas, Greece) powder and fed to *L. vannamei* over 60 days of culture period

Ingredients (% *as is*)	Diet code			
	Control	**0.2% OP**	**0.4% OP**	**0.6% OP**
Menhaden Fishmeal[1]	10.00	10.00	10.00	10.00
Soybean meal[1]	43.00	43.00	43.00	43.00
Corn Gluten Meal[1]	10.00	10.00	10.00	10.00
Menhaden fish oil[1]	5.64	5.64	5.64	5.64
Soy-Lecithin[2]	1.00	1.00	1.00	1.00
Powder of oregano polyphenols[3]	0.00	0.20	0.40	0.60
Corn starch[2]	8.06	7.86	7.66	7.46
Wheat products[4]	17.00	17.00	17.00	17.00
Mineral premix[5]	0.70	0.70	0.70	0.70
Vitamin premix[6]	1.90	1.90	1.90	1.90
Calcium phosphate-dibasic[2]	2.50	2.50	2.50	2.50
Choline chloride[5]	0.20	0.20	0.20	0.20
Proximate analysis				
Ash Content (%)	9.18	9.25	9.17	9.15
Total Fat (%)	58.77	59.14	59.18	59.33
Moisture Content (%)	9.55	9.49	9.68	9.71
Protein Content (%)	36.14	36.11	36.25	36.01

1 PT FKS Multi Agro, Tbk. Jakarta, Indonesia

2 PT Rajawali Mitra Pakanindo, Banten, Indonesia

3 Regavit aqua™, RA, Ecopharm Hellas, Greece

4 PT Pundi Kencana, Cilegon, Banten, Indonesia

5 Trace mineral premix (g/100 g premix): cobalt chloride, 0.004; cupric sulfate pentahydrate, 0.550; ferrous sulfate, 2.000; magnesium sulfate anhydrous, 13.862; manganese sulfate monohydrate, 0.650; potassium iodide, 0.067; sodium selenite, 0.010; zinc sulfate heptahydrate, 13.193; alpha-cellulose, 69.664.

6 Vitamin premix (g/kg premix): thiamin·HCL, 4.95; riboflavin, 3.83; pyridoxine·HCL, 4.00; Ca-pantothenate, 10.00; nicotinic acid, 10.00; biotin, 0.50; folic acid, 4.00; cyanocobalamin, 0.05; inositol, 25.00; vitamin A acetate (500,000 IU/g), 0.32; vitamin D3 (1,000,000 IU/g), 80.00; menadione, 0.50; alpha-cellulose, 856.81

Table 2. Water quality analysis for physical and chemical parameters for both growth trials

No	Parameters	Unit	Growth trial 1		Growth trial 2	
			AM	**PM**	**AM**	**PM**
Physical parameters						
1	pH		7.82 ± 0.35	7.95 ± 0.77	7.41 ± 0.16	7.52 ± 0.96
2	Dissolved oxygen	mgL^{-1}	5.68 ± 1.14	6.04 ± 1.09	5.02 ± 0.37	5.04 ± 0.52
3	Temperature	°C	28.51 ± 1.13	29.22 ± 0.68	28.41 ± 0.42	29.62 ± 0.47
4	Salinity	‰	27.55 ±1.68	27.86 ± 1.55	22.14 ± 1.29	23.25 ± 1.44
Biological parameters						
1	Nitrite-nitrogen (NO_2-N)	mgL^{-1}	0.023 ± 0.009		2.821 ± 0.468	
2	Nitrate-nitrogen (NO_3-N)	mgL^{-1}	12.891 ± 2.328		74.251 ± 5.543	
3	Ammonia (NH_3)	mgL^{-1}	0.019 ± 0.011		0.011 ± 0.017	

Table 3. Growth performance of Pacific white shrimp *Litopenaeus vannamei* (1.05±0.03 g as the initial mean weight) fed experimental diets for 60 d with several inclusion levels of oregano polyphenols powder (OP, Ecopharm Hellas, Greece). Values represent the mean of six replicates. Results in the same columns with different superscript letter are significantly different (*P<0.05*) based on analysis of variance followed by Tukey's multiple comparison test.

Treatment	FBW[1] (g)	FCR[2]	TGC[3]	PWG (%)[4]	SR (%)[5]
Control	9,62[a]	1.97[b]	0,0746[a]	822,8523[a]	83,33
0.2% OP[7]	10,67[b]	1.76[a]	0,0797[b]	930,9534[b]	84,58
0.4% OP	10,67[b]	1.80[ab]	0,0800[b]	940,5838[b]	94,44
0.6% OP	11,30[b]	1.65[a]	0,0825[b]	986,9305[b]	94,58
P-value	**0.0343**	**0.0086**	**0.0351**	**0.0464**	**0.0224**
PSE[6]	**0.0844**	**0.0161**	**0.0011**	**84.6361**	**0.7633**

Note: [1]FBW = Final body weight; [2]FCR = Feed conversion ratio; [3]TGC = Thermal growth coefficient; [4]PWG= Percentage weight gain; [5]SR = survival rate; [6]PSE = Pooled standard error, and [7]OP = Oregano polyphenols.

Table 4. Growth performance of Pacific white shrimp *Litopenaeus vannamei* (Mean initial post larva- 7: weight ~ 0.03 g) fed with top-dressing diets with several inclusion levels of oregano essential oils in the liquid form for 50 d. Values represent the mean of three replicates. Results in the same columns with different superscript letter are significantly different (*P<0.05*) based on analysis of variance followed by Tukey's multiple comparison test

Treatment	FBW[1] (g)	FCR[2]	TGC[3]	PWG (g)[4]	SR (%)[5]
Control	4.73[a]	1.44[a]	0.1060[a]	15668.00[a]	91.27
0.2% OP	5.21[b]	1.24[b]	0.1102[b]	17265.24[b]	96.13
0.4% OP	5.44[b]	1.18[b]	0.1121[b]	18018.00[b]	97.33
0.6% OP	5.06[b]	1.34[ab]	0.1089[b]	16751.60[b]	92.20
P-value	**0.0066**	**0.0080**	**0.0058**	**0.0067**	**0.2006**
PSE[6]	**0.1004**	**0.0402**	**0.0008**	**334.6117**	**0.1158**

Note: [1]FBW = Final body weight; [2]FCR = Feed conversion ratio; [3]TGC = Thermal growth coefficient; [4]PWG= Percentage weight gain; [5]SR = survival rate; and [6]PSE = Pooled standard error

Table 5. Results of the antimicrobial activity test (diameter of the inhibition zone in mm) of the oregano polyphenols (OP) and Oxytetracycline (OTC) by the paper disk method

No	Concentration (µg mL^{-1})	Inhibition Zone (mm)			
		OTC VP[1] (mm)	OP VP[2] (mm)	OTC VH[3] (mm)	OP VH[4] (mm)
1.	2×10^{-6}	-	-	-	-
2.	2×10^{-5}	-	-	-	-
3.	2×10^{-4}	-	-	-	-
4.	2×10^{-3}	-	-	-	-
5.	2×10^{-2}	-	-	-	-
6.	2×10^{-1}	-	-	-	-
7.	2×10^{0}	-	-	-	-
8.	2×10^{1}	12.4	13.2	17.3	17.0

[1] Oxytetracycline against Vibrio parahaemolyticus (OTCVP)
[2] Oxytetracycline against Vibrio harveyi (OTCVH)
[3] Oregano polyphenols against Vibrio parahaemolyticus (OP VP)
[4] Oregano polyphenols against Vibrio harveyi (OP VH)
(-) absence of inhibition zone detected
The data represents mean values of triplicate determinations. Means with different letters within a treatment indicate significant differences at P<0.05 by ANOVA nad Tukey's multiple comparison test

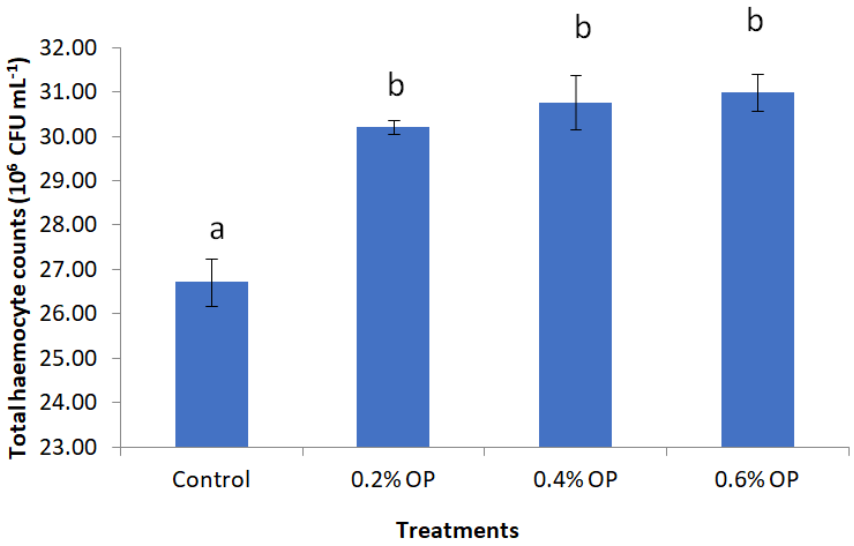

Fig. 1. Value of total haemocyte count (10^6 cells mL^{-1}) of Pacific white shrimp *L. vannamei* after being fed with several inclusion levels of oregano polyphenols (OP, Regavit Aqua™, Ecopharm Hellas, Greece) for 60 days from growth trial 1. Values represent the mean of eighteen replicates (P< 0.05)

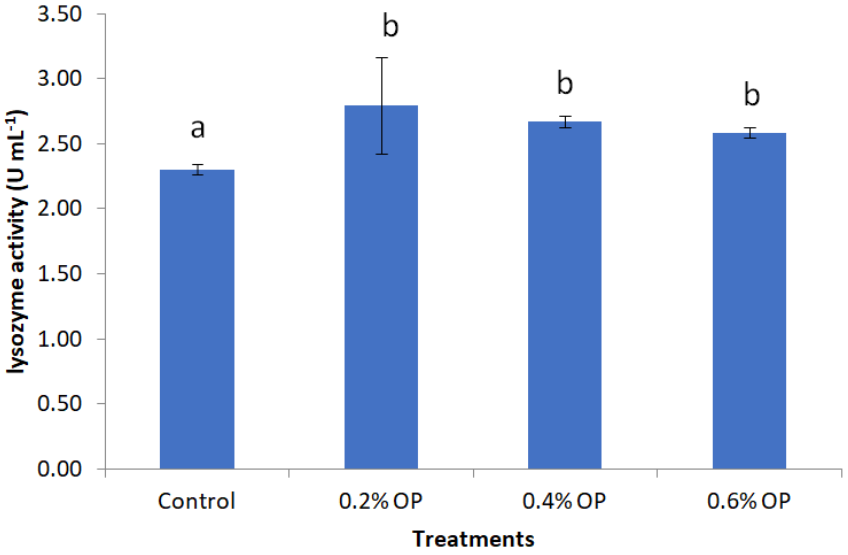

Fig. 2. Value of lysozyme activity (U mL^{-1}) of Pacific white shrimp *L. vannamei* after being after being fed with several inclusion levels of oregano polyphenols (OP, Regavit Aqua™, Ecopharm Hellas, Greece) for 60 days from growth trial 1. Values represent the mean of five replicates (P< 0.05)

4. DISCUSSION

Herbal-derived feed additives and supplements derived from plants and their extracts, which are used as supplements in fish and shrimp diets can provide various benefits such as improved growth performance, disease resistance, gut health, and stress reduction [25-27]. In this research using aquaria tank, group of shrimp fed with 0.2; 0.4 and 0.6% of polyphenols with carvacrol and thymol (OP) obtained from oregano plant (OP) have higher final body weight (FBW), thermal growth coefficient (TGC) and percentage weight gain (PWG) compared to the group of shrimp fed without OP. In addition, from the second growth trial using out-door tanks and

top-dressing methods to the commercial diet, the growth of shrimp fed with 0.2; 0.4 and 0.6 % of OP also have a significant higher FBW, TGC and PWG compared to the control treatment, despite numerically, the growth of shrimp fed with 0.6% OP has a decreasing trend compared to the group of shrimp fed with 0.4% OP, but still significantly better compared to the control treatment. In broiler, the supplementation of phytogenic product containing with equal mixtures of thymol and carvacrol significantly enhance the weight gain and feed efficiency [28]. In aquaculture, positive effect of thymol-carvacrol powder to significantly enhance the feed efficiency and growth performance has been observed in rainbow trout, *Oncorhynchus mykiss* [29], juveniles of great sturgeon, *Huso huso* Linnaeus, 1758 [30], channel catfish, *Ictalurus punctatus* [31], and Yellowtail Tetra, *Astyanax altiparanae* [32]. According to Ahmadifar et al. [29], the increased growth performance in fish could be due to the synergistic interactions between OP and the gastro-intestinal microflora that can enhance the metabolic process, digestibility and absorption of nutrients.

Thymol and carvacrol are two natural compounds that may have another mode of action than stimulating the growth, including as the health-promoting ingredients in the food industry, antimicrobial activity, antiviral activity, anthelminthic activity, immune stimulation, anti-inflammatory and antioxidative activity [28,33]. However, the mechanisms of herbal-derived feed additives in improving the animal health have yet to be firmly established as many complex interactions between the plant-active substances and host factors [34]. Current hypothesis suggesting that the effect may be related to the ability of the active substances to inhibit the growth of bacteria [34]. In this research, we report the ability of mixture of thymol and carvacrol to significantly enhance the total haemocyte counts (THC in 10^6 CFU mL^{-1}) and lysozyme activity of shrimp *L. vannamei* in trial 1. The results showed the antimicrobial activity of OP also works in shrimp that only rely on the non-specific immune system.

In shrimp, haemocytes play significant role not only in coagulation but also in the production of melanin via the Prophenoloxidase (ProPO) systems as an essential part of the defense mechanisms [35,36]. Thus, in most shrimp studies, THC are commonly used as a non-specific indicators for immune status and overall health condition (37]. In the present study, thymol and carvacrol were able to induce higher number of THC in shrimp compared to the control treatment. Recently, da Rosa COELHO et al. [38] reported that the THC number of *L. vannamei* fed with carvacrol at concentrations of 3 and 4 mg L^{-1} were increases compared to the control and the lowest inclusion level (1mg L^{-1}) of carvacrol. In addition, total numbers of THC present in the hemolymph of *Galleria mellonella* were also increases after exposure with oregano oil for 4 h and 72 h [39]. The THC in *Litopenaeus vannamei* can be influenced by various factors, including: (1) stress condition [40,41]; (2) environmental factors [42,43]; (3) disease and infections [44,45]; (4) feeding and nutrition [2,4]; (5) chemical exposures [46,47] and (6) medication and treatments [48,49]. The overall enhanced of THC number in group of shrimp fed with thymol and carvacrol polyphenols may show a strongest effect on improving the health condition of shrimp.

Our study also demonstrated that the inclusion of OP could enhance the lysozyme activity under controlled environment compared to the control treatment. Moreover, through the MIC analysis we found that the *Vibrio harveyi* and *Vibrio parahaemolyticus* showed sensitivity to OP at the concentration of 2×10^1 µg mL^{-1}. The efficiency of the therapeutic products has been investigated in *L. vannamei* and showed that the combination of thymol and carvacrol and extracts of oregano oil are effective for inhibiting the bacterial growth with MIC values equal or lower than 3 mg L^{-1} [50]. The antioxidants and antimicrobial effects of carvacrol and thymol have been confirmed in several studies, suggesting their essential role to improve the functional foods [51]. In the context of Vannamei, lysozymes, also known as muramidases, is considered an important component of the innate immune system, providing a first line of defense against bacterial pathogens in their aquatic environment [52,53]. Lysozyme are mainly synthesized in a variety of exocrine glands and then secreted into body fluids to protect shrimp against bacterial infection by catalyzing their cell wall, which subsequently lyses the cell [54-57]. Considering the impact of polyphenols to enhance the lysozyme activities and inhibit the growth of *V. harveyi* and *V. parahaemolyticus* at the concentration of 2×10^1 µg mL^{-1}, application of polyphenols with carvacrol and thymol could become an alternative strategy to completely replace the use of antibiotics in shrimp industry.

5. CONCLUSION

In conclusion, polyphenols with carvacrol and thymol obtained from oregano plant (OP) enhanced the growth performance of shrimp, reduced the feed intakes, increase the number of total haemocyte counts and lysozyme activity as well as inhibit the growth of *Vibrio harveyi* and *Vibrio parahaemolyticus* as the significant pathogen in shrimp industry. Integrating the application of polyphenols with carvacrol and thymol with good management practices into a comprehensive strategy can significantly contribute to optimizing the productivity and profitability of the industry. Thus, it may be assumed that dietary OP up to 0.6% both directly include in the diet formulation or using top-dressing methods can promote growth and health of Vannamei in controlled and stressful conditions.

ETHICAL STATEMENT

All procedures were conducted in accordance with the Indonesian Animal (Scientific Procedures) SNI 01-7252-2006 and SNI 8008:2014, approved by institutional ethical review committees (Politeknik Ahli Usaha Perikanan) and conducted under supervision of Dr. Romi Novriadi.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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