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IN VITRO ANTIMICROBIAL ACTIVITY OF CARVACROL AGAINST SHRIMP PATHOGENS AND ITS USE AS FEED ADDITIVE FOR THE PACIFIC WHITE SHRIMP*

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ABSTRACT

This study aimed to evaluate the *in vitro* effect of the phenolic compound carvacrol on different microorganisms of importance in shrimp farming, and it's *in vivo* effect on zootechnical, immunological and microbiological performance, and resistance of *Litopenaeus vannamei* challenged with *Vibrio parahaemolyticus*. In particular, the antimicrobial activity of carvacrol was evaluated *in vitro* by analysis of the minimum inhibitory concentration (MIC) and by agar diffusion with Gram-negative and Gram-positive bacteria. The *in vivo* experiment was conducted using different concentrations of carvacrol (1, 3, 4 and 6 mg mL^{-1}) added to shrimp feed, together with a control diet without carvacrol. After four weeks, zootechnical, immunological and microbiological parameters and resistance of animals challenged with *V. parahaemolyticus* were evaluated. The MIC of carvacrol was 0.078 mg mL^{-1} for *Vibrio alginolyticus* and *Vibrio harveyi*, while for the other bacteria, it was 0.156 mg mL^{-1} of carvacrol. The greatest halos of inhibition were observed in *V. parahaemolyticus* and *V. harveyi* with significant differences demonstrated for the other microorganisms, except *Escherichia coli*. The *in vivo* results showed no significant differences among treatments. In conclusion, the antimicrobial activity of carvacrol was confirmed with Gram-negative and Gram-positive bacteria, and it is suggested that its antimicrobial potential is more effective against *Vibrio* spp. However, the concentrations of carvacrol used *in vivo* did not affect the parameters evaluated.

Keywords: aquaculture; *Litopenaeus vannamei*, essential oil; food additive; *Vibrio*.

ATIVIDADE ANTIMICROBIANA *IN VITRO* DO CARVACROL CONTRA PATÓGENOS DE CAMARÕES E SEU USO COMO ADITIVO ALIMENTAR NA DIETA DO CAMARÃO-BRANCO-DO-PACÍFICO

RESUMO

O objetivo do presente estudo foi avaliar o efeito *in vitro* do carvacrol contra diferentes microrganismos de importância na carcinicultura e o seu efeito *in vivo* no desempenho zootécnico, imunológico, microbiológico e na resistência de *Litopenaeus vannamei* desafiados com *Vibrio parahaemolyticus.* A atividade antimicrobiana do carvacrol foi realizada *in vitro* pela análise da concentração inibitória mínima (CIM) e por difusão em ágar, com bactérias Gram-negativas e Gram-positivas. Para o experimento *in vivo* foram adicionadas diferentes concentrações do carvacrol (1, 3, 4 e 6 mg mL^{-1}) na alimentação dos camarões e uma dieta controle. Após quatro semanas, foram avaliados parâmetros zootécnicos, imunológicos, microbiológicos e a resistência dos animais desafiados com *V. parahaemolyticus.* A CIM do carvacrol para *Vibrio alginolyticus* e *Vibrio harveyi* foi de 0,078 mg mL^{-1}, enquanto nas demais bactérias foi de 0,156 mg mL^{-1} de carvacrol. Os maiores halos de inibição foram observados em *V. parahaemolyticus* e *V. harveyi* e demonstraram diferenças significativas em relação aos demais microrganismos, exceto *Escherichia coli*. Os resultados *in vivo* não demonstraram diferenças significativas entre os tratamentos. Em conclusão, a atividade antimicrobiana do carvacrol foi confirmada com bactérias Gram-negativas e Gram-positivas e sugere-se que seu potencial antimicrobiano seja mais eficaz contra *Vibrio* spp. No entanto, as concentrações de carvacrol utilizadas *in vivo* não afetaram os parâmetros avaliados.

Palavras-chave: aquicultura; *Litopenaeus vannamei;* óleo essencial; aditivo alimentar; *Vibrio*.

INTRODUCTION

The Pacific white shrimp *Litopenaeus vannamei* is the most profitable and commercially important species in shrimp farming, with a global production of approximately 5 million tonnes in 2018 (ABCC, 2017; FAO, 2020). However, with the intensification of production, these animals are affected by viral and bacterial infectious diseases that cause serious economic losses owing to decreased production (Chakraborty and Hancz, 2011). Among these diseases are those caused by Gram-negative bacteria of the genus *Vibrio.* These microorganisms are part of the natural microbiota of the peneid shrimps and under stressful conditions can cause infections. As an example, stand out the infections by *Vibrio parahaemolyticus*, responsible for acute hepatopancreatic necrosis disease (AHPND), which severely affects the development of aquatic shrimp worldwide (Santos et al., 2020).

Many producers in aquaculture use chemotherapeutic agents, such as antibiotics, to prevent diseases. However, prolonged use of chemical substances can accumulate in aquatic animals and thus select antibiotic-resistant microbiota (Alderman and Hastings, 1998). Moreover, the chemical residues could affect the environment and human health developing resistant bacteria against drugs for human medicine (Chakraborty and Hancz, 2011).

In the search for alternatives to the use of antibiotics, studies have tested the effectiveness of various herbal medicines for aquaculture. Such medicines contain natural organic compounds that do not harm the environment, animal health or human health (Talpur et al., 2013). Among these phytotherapics is carvacrol, a phenolic monoterpene abundant in plants belonging to the Lamiaceae family, such as oregano (*Origanum vulgare*) and thyme (*Thymus vulgaris*) (Rattanachaikunsopon and Phumkhachorn, 2010). This phenolic compound has attracted significant attention because of its biological properties, e.g., antibacterial, antifungal, antiviral and antioxidant activities, among others (Nostro et al., 2004; Guimarães et al., 2010; Chavan and Tupe, 2014; Sánchez et al., 2015). In addition, carvacrol has been approved by the U.S. Food and Drug Administration (FDA, 2017) as a compound with no toxic effects on humans.

In vitro studies have demonstrated the strong antimicrobial activity of carvacrol, which has already been investigated against such pathogenic microorganisms as *Escherichia coli*, *Salmonella typhimurium*, and *Edwardsiella tarda*, among others (Cosentino et al., 1999; Rattanachaikunsopon and Phumkhachorn, 2010; Carvalho et al., 2018; Stratakos et al., 2018). *In vivo* studies in fish showed that carvacrol could improve zootechnical performance and hematological indices, and reduce mortality caused by *Vibrio anguillarum* and *E. tarda* (Rattanachaikunsopon and Phumkhachorn, 2010; Ahmadifar et al., 2011; Volpatti et al., 2013).

In crustaceans, carvacrol has been tested in previous studies. For example, the shrimp *L. vannamei* was fed diets containing essential oils of oregano (*Lippia berlandieri* Schauer) with different levels of thymol and carvacrol, followed by challenge with *Vibrio vulnificus*, *V. parahaemolyticus* and *Vibrio cholerae*. The concentration of bacteria in the muscle and hepatopancreas was then evaluated. Substantial inhibition of Vibrionaceae growth was demonstrated, regardless of the essential oil used (Gracia-Valenzuela et al., 2014). In addition, Baruah et al. (2017) demonstrated that Artemia larvae treated by immersion with carvacrol exhibited significantly increased survival compared to the control.

Essential oils, such as oregano and thyme, are composed of terpenes which several studies have considered to be responsible for the antimicrobial activity of these oils (Samy and Gopalakrishnakone, 2010). Therefore, the study of these isolated compounds against microorganisms can lead to therapies that minimize the losses caused by diseases and represent an alternative to the use of antibiotics. Furthermore, compounds from plants are environmentally friendly, sustainable and safe. In this context, the use of carvacrol could prove to be a promising alternative to improve the immunocompetence of animals against pathogens, such as vibrios. Despite this evidence, the literature provides scant studies demonstrating the use of carvacrol in marine shrimp. Therefore, this study aimed to evaluate the *in vitro* effect of carvacrol against different microorganisms of importance in shrimp farming, and it's *in vivo* effect on zootechnical, immunological and microbiological performance, and resistance, of *L. vannamei* challenged with *V. parahaemolyticus*.

MATERIALS AND METHODS

The experiments were conducted at the Laboratory of Marine Shrimp (LCM). The diets were manufactured at the Aquaculture Species Nutrition Laboratory (LABNUTRI), and the experimental challenge with *V. parahaemolyticus* was carried out at the Aquaculture Pathology Study Center (NEPAQ). All laboratories form part of the Federal University of Santa Catarina (UFSC) in Florianópolis (SC).

Carvacrol

The phytotherapeutic carvacrol, at a concentration of 10 mg mL^{-1}, was provided in nanoemulsion form by the Laboratory of Plant Morphogenesis and Biochemistry at UFSC. The compound was prepared by emulsification with Tween® 80 (1:1, w/w), packed in an amber bottle, and stored at room temperature.

Bacterial Strains

The strains of vibrionaceae used in the *in vitro* tests were as follows: *V. harveyi* ATCC 14126, *V. alginolyticus* BCCM 2068 and *V. parahaemolyticus* ATCC 17802. In addition, *E. coli* ATCC 25102 and *Staphylococcus aureus* ATCC 25923 were used, serving as Gram-negative and Gram-positive standard microorganisms, respectively. Also, *Lactobacillus plantarum* (CPQBA 007 07 DRM01), considered a probiotic, was used to test its potential to boost the potency of carvacrol in future experiments. *V. parahaemolyticus* was chosen for the *in vivo* tests because of its impact on shrimp farming.

Preparation of Bacterial Suspensions used *in vitro*

The bacteria were initially sown in a broth culture medium. For *L. plantarum*, the medium used was MRS (Man Rogosa Sharpe Agar) with 3% NaCl and incubated at 35°C for 24 h. For the vibrios, the medium used was BHI (Brain Heart Infusion) with 3% NaCl, and for the other bacteria, BHI 0% was used, all incubated at 30°C for 24 h.

For the methodology of minimum inhibitory concentration (MIC), the bacterial suspensions were centrifuged (15 min at 1,800 x *g*) and diluted in saline solution (0 and 3%) until a concentration of 0.5 on the McFarland scale was obtained. This corresponds to approximately 1.5 x 10^8 CFU mL^{-1}. For the agar diffusion inhibition test, the bacterial suspensions were diluted in saline and used at a concentration 1 x 10^6 CFU mL^{-1}. All *in vitro* tests were conducted according to the National Committee for Clinical Laboratory Standards (NCCLS, 2006) with some modifications.

In vitro Effect of Carvacrol on Different Microorganisms

Analysis of minimum inhibitory concentration (MIC)

The *in vitro* inhibitory effect of carvacrol was evaluated against the microorganisms described above. The minimum inhibitory concentration (MIC) methodology was employed, using a 96-well microplate (U-shaped bottom). 100 µL of culture medium were added to all wells, being: 1) SPW (Salt peptone water- 1% peptone, 3% NaCl, pH 7.4) for use with vibrios; 2) PB (Poor Broth - 1% peptone, 0.5% NaCl, pH 7.4) for use with *E. coli* and *S. aureus;* and 3) MRS plus 3% NaCl for use with *L. plantarum*. Together with the culture media, 100 µL of carvacrol (concentration of 10 mg mL^{-1}) were added to the first well, and serial dilution (5; 2.5; 1.25; 0.625; 0.312; 0.156; 0.078; 0.039; 0.019; 0.0098; 0.0049; 0.0024 mg mL^{-1} of carvacrol) was performed until the 12th well. Subsequently, 20 µL of the bacteria at a concentration of 0.5 on the McFarland scale were added to all wells. For the controls, only the culture media were used, with or without the bacteria (positive and negative control, respectively). The microplates were incubated at 30°C or 35°C (*L. plantarum*) for 24 h, and bacterial growth was analyzed using the colorimetric method. For this, 30 µL of Sodium Resazurin solution (0.01%, Sigma-Aldrich, MO – USA) were added, and the results were read after 1 hour, considering the immediate concentration when the color appears (Araujo and Longo, 2016). The tests for each bacterium were performed in triplicate.

Analysis by agar diffusion

The inhibition of carvacrol in agar diffusion was evaluated against the microorganisms described previously in Bacterial Strains. After the bacterial suspensions were diluted, according to the methodology described previously in Preparation of Bacterial Suspensions used *in vitro*, they were sown in plates containing agar culture medium with MRS plus 3% NaCl for *L. plantarum* and TSA (Tryptic Soy Agar) for the other bacteria. Next, 50 µL of carvacrol were added to three 0.8 mm diameter wells, and each sample was performed in triplicate. The plates were incubated for 24 h, and the diameters of the inhibition halos around each well were measured in mm.

Biological Material

The research was conducted with the marine shrimp *L. vannamei* acquired from Aquatec Aquacultura Ltda. (Rio Grande do Norte, Brazil). The animals were grown at the LCM in a biofloc system until they reached the ideal weight for the beginning of the experimental tests.

Preparation of Experimental Diets

The diets were formulated with the aid of Optimal Formula 2000® software, version 19102009, based on the recommendations and nutritional requirements for *L. vannamei* (Gong et al., 2000; NRC, 2011; Zhou et al., 2012). The dry ingredients were crushed and sieved at 600 µm. Next, the micro-ingredients were homogenized and added to the macro-ingredients. Then, the oils, soy lecithin and water (200 mL kg^{-1} of the diet) were added. The resulting mixture was pelleted in a micro-extruder (Inbramaq MX-40), dried in an oven at 40°C, and the finished feed was refrigerated at 4°C until use. For the *in vivo* experiment, a control diet (without carvacrol) was tested, and to the other diets, containing the same ingredients were added different concentrations of carvacrol (1, 3, 4 and, 6 mg mL^{-1}). As it is a highly volatile compound, the corresponding volume of each concentration of carvacrol was pipetted directly into the feed 20 min before feeding. These concentrations were defined according to the results obtained in the *in vitro* analyses of the present study. The percentage composition of the feed was analyzed according to AOAC (2005) by the laboratory analysis company CBO Ltda. (Valinhos, São Paulo, Brazil), and the ingredients are listed in Table 1.

Experimental Design

After a fifteen-day acclimatization period, the four-week experiment started. This experiment consisted of evaluating the *in vivo* effect of carvacrol on *L. vannamei* shrimp kept in clear water. The design was entirely random, with three replications, totaling fifteen tanks where the diets described above were tested. The polyethylene units contained 400 L of water which was renewed every other day. Food scraps, feces and molts were removed.

The experimental units were constantly aerated and heated with a thermostat to maintain the temperature at 29 ± 0.5°C. These units were populated with twenty-five shrimp with an average weight of 4.96 ± 0,14 g at the start of the experiment. Feeding occurred four times a day and was adjusted according to biweekly biometrics and survival, following an estimated scheduled conversion (Ray et al., 2010). In addition, throughout the experiment, water quality parameters, such as dissolved oxygen and temperature, were monitored once a day (YSI 55, YSI Incorporated, Yellow Springs, OH, USA). Analyses of pH (pHmetro Tecnal®), salinity (Eco-Sense YSI EC30), alkalinity by the APHA method (APHA, 2005), nitrite, and total ammonia performed according to Strickland and Parsons (1972) were monitored once a week.

Table 1. Formulation and percentage composition of the feed.

Ingredients	g kg^{-1}
Wheat flour	150.00
Soybean meal	324.63
Carboxymethylcellulose	5.00
Soy lecithin	25.00
Monocalcium phosphate	25.00
Soy oil	10.00
Vitamin C	0.70
Vitamin premix[1]	5.00
Mineral premix[2]	17.00
Magnesium sulphate	15.00
Kaolin	100.00
Sodium chloride	12.00
Potassium chloride	10.00
Methionine	5.00
Fish residue	150.00
Offal flour	125.67
Fish oil	20.00
Carvacrol[3]	
Composition (%)	
Moisture	9.04
Crude protein	39.60
Ethereal extract	8.71
Crude fibre	1.87
Ash	17.65

[1]Vitamin premix: vit. A - 900 mg kg^{-1}; vit. D3 - 25 mg kg^{-1}; vit. E – 46,900 mg kg^{-1}; vit. K3 – 1,400 mg kg^{-1}; cobalamin (B12) – 50 mg kg^{-1}; pyridoxine (B6) – 33,000 mg kg^{-1}; riboflavin – 20,000 mg kg^{-1}; nicotinic acid – 70,000 mg kg^{-1}; pantothenic acid – 40,000 mg kg^{-1}; biotin – 750 mg kg^{-1}; and folic acid – 3,000 mg kg-^{1}. [2]Mineral premix: copper – 2,330 mg kg^{-1}; zinc – 10,000 mg kg^{-1}; manganese – 6,500 mg kg^{-1}; selenium - 125 mg kg^{-1}; iodine – 1,000 mg kg^{-1}; cobalt – 50 mg kg^{-1}; magnesium – 20 g kg^{-1}; and potassium – 6.1 g kg^{-1}. [3]Carvacrol: Carvacrol was pipetted directly into the feed at each feeding, using the concentrations defined for the treatments (1, 3, 4 and 6 mg mL^{-1}).

Analysis of Immunological Parameters

At the end of the experiment, the hemolymph was collected from the ventral sinus of ten prawns per tank (three pools per treatment). For this purpose, sterile 1 mL syringes cooled to 4°C were used. From the collected hemolymph, 50 µL were fixed in modified Alsever anticoagulant solution (MAS, Modified Alsever Solution - 27 mM sodium citrate, 9 mM EDTA, 115 mM glucose, 336 mM NaCl, pH 7.2) with 4% formaldehyde for total hemocyte count (THC). The remainder was coagulated at 4°C, macerated and centrifuged at 6,000 x *g* for 10 min to obtain the serum, which was then aliquoted and stored at -20°C. The number of hemocytes per milliliter of hemolymph was estimated by direct counting in a Neubauer chamber. The calculation of total serum protein concentration was performed in a 96-well microplate (flat-bottomed) and estimated using the Bradford method (Bradford, 1976) with bovine serum albumin as a standard. All tests were carried out in triplicate. The activity of the phenoloxidase (PO) enzyme was determined by spectrophotometry (DO 490 nm) based on the formation of DOPA-chromium pigment after the oxidation of the substrate L-dihydroxyphenylalanine (L-DOPA). The serum samples were diluted (1:15) in TBS-1 (1 mM Tris, 336 mM NaCl, 5 mM $CaCl_2$, 10 mM $MgCl_2$, pH 7.6). Of this solution, 50 µL were incubated in an equal volume of trypsin (Sigma-Aldrich, 1 mg mL^{-1}) enzyme inducer in a 96-well microplate (flat-bottomed) for 5 min at room temperature. After incubation, 50 µL of L-DOPA (Sigma-Aldrich, 3 mg mL^{-1}) were added to all wells. In the controls, trypsin was replaced by TBS, and the formation of DOPA-chromium was monitored after 5, 10 and 15 min. PO activity was expressed in units of enzymatic activity (U) by varying 0.001 in absorbance/minute/milligram of protein (Söderhäll and Häll, 1984). For the analysis of serum agglutination titer, 50 µL of TBS-2 solution (50 mM Tris, 5 mM $MgCl_2$, 10 mM $CaCl_2$, 150 mM NaCl, pH 7.4) were initially deposited in all wells of the microplate (U-shaped bottom). Then, 50 µL of the serum diluted (1:15) in TBS-2 was added to the first well, and serial dilutions were performed until the 12th well. 50 µL of 2% canine erythrocyte solution were added to each well, mixed and incubated for 2 h in a humid chamber at room temperature. In the control wells, the serum from the shrimp was replaced by TBS-2. Serum agglutination titer was defined as the reciprocal of the last dilution capable of agglutinating erythrocytes (Maggioni et al., 2004).

Analysis of Intestinal Tract Microbiota

For this analysis, the intestines of ten shrimp per tank (three pools per treatment) were sampled, totaling thirty shrimp per treatment. The intestinal tracts were aseptically extracted, homogenized in a grinder and diluted serially (1/10) in sterile 3% saline. Then, they were sown in TSA (Tryptic Soy Agar) and TCBS (Thiosulfate Citrate Bile Sucrose agar) to count total heterotrophic bacteria and *Vibrio* spp., respectively. The intestines sown in the plates were incubated in an oven at 30°C for 24 h, and then total colony forming units (CFU) counts were performed.

Experimental Challenge with *Vibrio parahaemolyticus*

After cultivation, the animals were transferred to NEPAQ where ten shrimp were distributed in 15 experimental units of 100 L, totaling 30 animals per treatment. Through the dorsal part of the first abdominal segment, the prawns were injected with 100 µL of *V. parahaemolyticus* solution at a concentration of 1 x 10^7 UFC mL^{-1} according to the LD_{50} test previously performed. The animals were monitored for 48 h, and after that, mortality was assessed.

Assessment of Zootechnical Parameters

At the end of the experiment, the final biometrics of all shrimp cultivated in each experimental unit was performed to obtain the zootechnical parameters, as shown below:

- Weekly Weight Gain (g/s) = {[final average weight (g) - initial average weight (g)]/days of cultivation} x 7;
- Total weight (g) of the animals at the end of the experiment;
- Feed Conversion Ratio (FCR) = feed consumed (kg) / shrimp biomass produced (kg);

- Survival (%) = (final number of shrimp / initial number of shrimp) x 100.

Statistical Analysis

Bacterial count data from the intestinal tract were transformed to $\log^{10}(x + 1)$, and those with agglutinating titer were transformed to $\log^{2}(x + 1)$ before being subjected to statistical analysis. The homoscedasticity and normality of all data were assessed using the Bartlett and Shapiro-Wilk tests, respectively. When considered parametric, data were submitted to one-way analysis of variance (One-way ANOVA), followed by the Tukey test, and non-parametric data were submitted to the Kruskal-Wallis test. All statistical tests were performed using the Statistica 13.5 program (TIBCO Software, Inc.), using a 5% significance level. Mortality data after the challenge were analyzed by Kaplan-Meier using GraphPad Prism 5.0 software.

RESULTS

In vitro Effects of Carvacrol against different Microorganisms

Analysis of the minimum inhibitory concentration (MIC)

The results of the minimum inhibitory concentration of carvacrol against different microorganisms are described in Table 2. Carvacrol inhibited *V. alginolyticus* and *V. harveyi* at a lower concentration than the other microorganisms. For the vibrios, the inhibition concentrations were 0.078 mg mL^{-1} of carvacrol, while a concentration of at least of 0.156 mg mL^{-1} of carvacrol was required to inhibit other bacteria.

Analysis by agar diffusion

The agar diffusion tested carvacrol against different microorganisms, and the results are in Table 3. As noted, carvacrol showed the highest inhibition halos against *V. parahaemolyticus* and *V. harveyi* with 13.75 mm and 12.92 mm, respectively. In addition, these vibrios showed significant differences relative to the other microorganisms tested, except E. *coli*, which was the same. The lowest inhibition halos were observed in *S. aureus*, followed by *V. alginolyticus* and *E. coli* with 9.17 mm, 9.63 mm and 9.92 mm, respectively. However, it was not possible to observe bacterial growth or the formation of inhibition halos for *L. plantarum*.

Water Quality Parameters

Temperature and dissolved oxygen were maintained at 28.73 ± 0.75 °C and 5.64 ± 0.40 mg L^{-1}, respectively, and remained constant throughout the experiment. In addition, the mean for salinity was 34.30 ± 0.49 g L^{-1}, pH 8.15 ± 0.04, alkalinity 121.12 ± 15.17 mg $CaCO_3$ L^{-1}, total ammonia 0.91 ± 0.26 mg L^{-1} and nitrite 0.39 ± 0.26 mg L^{-1}. The parameters remained within the appropriate standards established by Van Wyk and Scarpa (1999) for the species, and none of the parameters showed significant differences (p >0.05) among treatments.

Table 2. Minimum inhibitory concentration (MIC; mean ± standard deviation) of carvacrol against different microorganisms (n = 3).

Microorganisms	MIC (mg mL^{-1})
Vibrio alginolyticus	0.0781±0.00
Vibrio harveyi	0.0781±0.02
Vibrio parahaemolyticus	0.1563±0.04
Staphylococcus aureus	0.1563±0.00
Escherichia coli	0.1563±0.00
Lactobacillus plantarum	0.1563±0.04

Table 3. Inhibition halos (mm; mean ± standard deviation) using the agar diffusion method with carvacrol against different microorganisms (n = 3).

Microorganisms (1 x 10^6 UFC mL^{-1})	Inhibition halos (mm)
Vibrio alginolyticus	9.63±0.18[a]
Vibrio harveyi	12.92±0.82[b]
Vibrio parahaemolyticus	13.75±0.12[b]
Staphylococcus aureus	9.17±0.47[a]
Escherichia coli	9.92±2.0[ab]
Lactobacillus plantarum	No bacterial growth

Different letters indicate a significant difference obtained by One-way ANOVA followed by the Tukey test (p = 0.0028).

Immunological Parameters

As described in Table 4, there were no significant differences (p >0.05) in any of the immunological parameters analyzed, such as Total Hemocyte Count (THC), total serum protein concentration, Phenoloxidase Activity (PO) and serum agglutination titer among treatments.

Intestinal Tract Microbiota

The bacterial count of the intestinal tract of the shrimp did not present a significant difference between treatments (p >0.05), as shown in Figure 1.

Experimental Challenge with *Vibrio parahaemolyticus*

After 48 hours, no significant difference was demonstrated among treatments in animals challenged with *V. parahaemolyticus* (p >0.05) (Figure 2). The animals treated with 3 mg mL^{-1} of carvacrol reached mortalities of 20%, followed by treatments with 4 and 6 mg mL^{-1} with mortalities of 16.67%, 1 mg mL^{-1} with 15% and the control group with 13.3%.

Zootechnical Parameters

After the four-week, no significant differences (p >0.05) were observed in any of the zootechnical parameters analyzed, such as weekly weight gain (WWG), final weight, feed conversion ratio (FCR) and survival, as shown in Table 5.

Table 4. Immunological parameters (mean ± standard deviation) of *Litopenaeus vannamei* fed with carvacrol at concentrations of 1, 3, 4 and 6 mg mL^{-1} and a control diet without the addition of carvacrol (n = 3).

Treatments	THC ($x10^6$ Cells mL^{-1})	Protein Concentration (mg mL^{-1})	PO Activity (unit min^{-1} mg^{-1} Protein)	Agglutination Titer (log^2 x + 1)
Control	51.28±0.63	212.12±0.42	11.06±1.76	9.33±0.57
1 mg mL^{-1}	24.95±0.80	213.75±3.54	5.19±2.84	10.05±0.83
3 mg mL^{-1}	56.03±0.20	212.07±0.45	8.14±4.96	9.80±0.33
4 mg mL^{-1}	77.96±0.16	214.25±4.57	7.13±2.56	9.24± 0.66
6 mg mL^{-1}	41.44±0.66	213.71±1.49	7.26±2.02	10±0.0
***p* value**	0.391	0.751	0.281	0.354

THC: Total Hemocyte Count; PO: Phenoloxidase Activity.

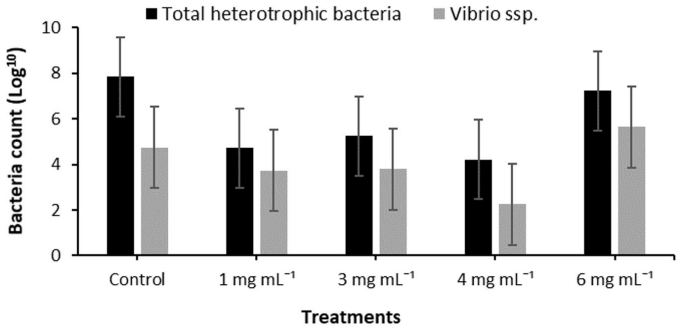

Figure 1. Bacterial count from the intestinal tract of *Litopenaeus vannamei* fed with carvacrol at concentrations of 1, 3, 4 and 6 mg mL^{-1} and a control diet without the addition of carvacrol. Results are presented as mean ± standard deviation of the triplicates, and the bars indicate the standard deviation of the mean. Total heterotrophic bacteria, $p = 0.655$; *Vibrio* spp., $p = 0.785$.

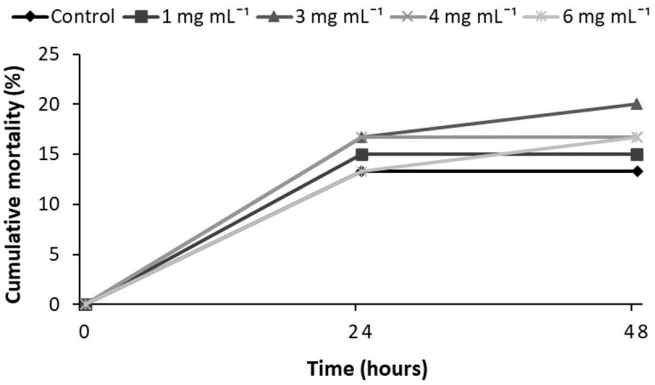

Figure 2. Cumulative mortality of *Litopenaeus vannamei* fed with carvacrol at concentrations of 1, 3, 4 and 6 mg mL^{-1} and a control diet without the addition of carvacrol. Results are presented as mean of the triplicates. $p = 0.978$.

Table 5. Zootechnical parameters (mean ± standard deviation) of *Litopenaeus vannamei* fed with carvacrol at concentrations of 1, 3, 4 and 6 mg mL^{-1} and a control diet without the addition of carvacrol (n = 3).

Treatment	WWG (g)	Final weight (g)	FCR	Survival (%)
Control	1.74±0.08	11.87±0.02	1.4±0.02	100±0.0
1 mg mL^{-1}	1.69±0.06	11.83±0.32	1.33±0.07	100±0.0
3 mg mL^{-1}	1.63±0.10	12.12±0.59	1.37±0.08	98.7±2.31
4 mg mL^{-1}	1.78±0.17	11.83±0.38	1.45±0.06	98.6±2.41
6 mg mL^{-1}	1.67±0.03	11.25±0.28	1.43±0.02	100±0.0
***p* value**	0.458	0.126	0.231	0.518

WWG: Weekly Weight Gain; FCR: Feed Conversion Ratio.

DISCUSSION

In recent years, the use of antimicrobial compounds derived from plants has attracted growing interest in aquaculture, as they may represent a promising alternative to the use of antibiotics (Abutbul et al., 2004; Rattanachaikunsopon and Phumkhachorn, 2009). Carvacrol has emerged as a bioactive compound for therapeutic purposes and has been extensively tested due to its high antimicrobial activity against a wide range of bacteria (Marinelli et al., 2018).

In vitro studies have demonstrated the antimicrobial action of carvacrol against *V. parahaemolyticus*, *S. aureus* and *Pseudomonas fluorescens* which presented a MIC of 0.5, 0.125 and 0.5 mg mL^{-1}, respectively (Fang et al., 2019). In another study, carvacrol was evaluated against other microorganisms, such as strains of *E. coli*, showing a MIC of 0.064 mg mL^{-1}, and *S. typhimurium*, showing a MIC of carvacrol of 0.12 mg mL^{-1} (Lemos et al., 2017; Lima et al., 2017). In addition, thyme essential oil (*T. vulgaris*), containing 60% carvacrol, was evaluated against *V. vulnificus*, *Vibrio fluvialis* and *V. parahaemolyticus* with a MIC of carvacrol of 0.156 mg mL^{-1},

and for *V. alginolyticus*, it was 0.078 mg mL^{-1} (Snoussi et al., 2008; Hajlaoui et al., 2010). The latter concentrations are consistent with the findings in the present study, in which *V. alginolyticus* and *V. harveyi* demonstrated growth inhibition up to the concentration of 0.078 mg mL^{-1}, while the other bacteria obtained inhibition only at the concentration of 0.156 mg mL^{-1} of carvacrol. Thus, it is possible to confirm the potential antimicrobial activity of this phenolic compound against all the bacteria tested in our study, including probiotic bacteria.

However, in the agar diffusion test, it was not possible to observe bacterial growth of *L. plantarum* or the formation of inhibition halos, even using diluted carvacrol. This could be explained by the slow growth rate of probiotic bacteria compared to the other tested bacteria, or carvacrol may have been incorporated into the agar, inhibiting the growth of the probiotic. Studies report that bacterial type can influence the effectiveness of essential oils. For example, gram-positive bacteria are more susceptible to essential oils than gram-negative bacteria, which can be explained by the difference in the structure of their membranes (Nevas et al., 2004; Gilles et al., 2010), although this was not assessed or confirmed in the present study.

For the other microorganisms in the agar diffusion test, *V. parahaemolyticus* and *V. harveyi* presented the greatest inhibition halos and were statistically different relative to the other tested microorganisms, except for *E. coli*, as they were significantly the same. Our results corroborate the studies by Snoussi et al. (2008) who used essential oil of thyme (*T. vulgaris*) that contained 60% of carvacrol. Their results showed that the inhibition halo obtained for *V. alginolyticus* was 13.3 mm, *V. parahaemolyticus* 14.66 mm, *V. vulnificus* 12.66 mm and *V. fluvialis* 13 mm. In addition, Guarda et al. (2011) obtained inhibition halos of 9.0 mm for *E. coli* and 11 mm for *S. aureus*. These data are consistent with the results obtained in the present study.

From the promising results obtained by the *in vitro* tests in our study, we defined different concentrations of carvacrol to be added to the diets of the Pacific white shrimp. The better performance of aquatic animals has already been demonstrated in a study by Ahmadifar et al. (2011) using carvacrol and thymol as an additive for *Oncorhynchus mykiss*. They observed that animals fed diets containing 2 and 3 g kg^{-1} of the compounds exhibited higher weight gain, length and final weight and immunological parameters influenced. However, studies using different concentrations of carvacrol (0.025% and 0.05%) as a food additive for *Dicentrarchus labrax* reported that the inclusion of this compound did not affect the growth performance or immune response of the animals (Volpatti et al., 2013). The same was observed in our study, where different concentrations of carvacrol (1, 3, 4 and 6 mg mL^{-1}) did not affect the performance or immune response of *L. vannamei* in terms of the parameters evaluated.

It is known that the intestines of animals play a vital role in the absorption and metabolism of nutrients and act as one of the first lines of defense against pathogens and environmental stress (Garrett et al., 2010; Rombout et al., 2011). In this context, we evaluated the intestinal microbiota of *L. vannamei* fed different concentrations of carvacrol and despite the apparent abundance of bacteria in the treatment of 6 mg mL^{-1} compared with the other treatments, no significant differences were detected between the groups.

Regardless of the excellent results obtained in the *in vitro* tests of the present study, the different concentrations of carvacrol affected neither the zootechnical, immunological and intestinal microbiota performance, nor the resistance of animals challenged with *V. parahaemolyticus*. To explain this outcome, the use of essential oil may be more effective than its pure and isolated compounds, such as carvacrol, considering the synergism among all the components present in essential oil, as already demonstrated in the literature (Zheng et al., 2009; Lemos et al., 2017). Furthermore, Nostro et al. (2012) showed that carvacrol could be more effective when the pH of the medium is acidic, a likely consequence of the greater hydrophobicity that results in better fragmentation of the membrane lipids. Marinelli et al. (2018) evidence this mechanism of action where the outer membrane and the cytoplasmic bacteria are considered the main sites of carvacrol activity resulting in functional and structural changes to the membranes, interference in the synthesis and function of nucleic acids, coagulation of the cytoplasm and leakage of its constituents, metabolic imbalance and inhibition of Quorum sensing (QS) (Knobloch et al., 1989; Sikkema et al., 1995). In addition, according to Astashkina et al. (2012), *in vitro* models do not fully represent *in vivo* physiology and, therefore, may not provide enough information to understand with accuracy the biological properties of the studied compound.

CONCLUSION

Carvacrol presents antimicrobial activity with Gram-negative and Gram-positive bacteria, suggesting that this action is more effective against *Vibrio* spp. The use of carvacrol in the diet did not affect the zootechnical, immunological, intestinal microbiota performance and the resistance of the shrimp challenged with *V. parahaemolyticus*. These results can help for future histological and metagenomic studies that target the mechanisms of action of carvacrol in the intestinal tract.

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