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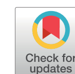
Essential oils mediated antivirulence therapy against vibriosis in *Penaeus vannamei*



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ABSTRACT

The emergence of new virulent *Vibrio* strains resistant to common antibiotics has caused significant economic losses to shrimp farming worldwide. It is mandatory to adopt new strategies to control shrimp farming related vibriosis. Essential oils (EOs) have several biological properties among of which the quorum sensing (QS) inhibitory activity is appealing for vibriosis control. In this work, we evaluated QS inhibitory activity of five EOs obtained from oregano (*Organum vulgare*), tea tree (*Melaleuca alternifolia*), lemongrass (*Cymbopogon citratus*), cinnamon (*Cinnamomum verum*) and thyme (*Thymus vulgaris*), at sublethal doses. EOs involvement in bioluminescence shutdown, biofilm formation and swarming motility was evaluated in four *Vibrio* strains of aquaculture interest including *V. harveyi*, *V. campbellii*, *V. vulnificus*, and *V. parahaemolyticus*. Oregano oil (EOOv) and tea tree oil (EOMa) were further tested in *in vivo* assays due to their significant effects ($P < 0.05$) on QS inhibition. EOOv was the most efficient one and exerted a comparable QS inhibitory effect to EOMa at a lower concentration *in vivo* ($2.5\,\mu g\,mL^{-1}$ of EOMa *versus* $1.0\,\mu g\,mL^{-1}$ of EOOv). The lowest active doses of EOOv and EOMa that inhibited QS had no toxic effects on hemocytes and larvae of *P. vannamei*. A challenge test was performed in *P. vannamei* postlarvae (PL8) with *V. campbellii*, grown previously in the presence of EOOv or EOMa at sublethal active doses. Our results indicated that both EOs affected the virulence of *V. campbellii* and were able to significantly ($P < 0.05$) reduce shrimp mortality (EOOv in a 40% while EOMa in a 32%). A field bioassay was also carried in earthen ponds to test two different concentrations of EOOv and EOMa for feed supplementation (2.5 and $5.0\,mg\,kg^{-1}$ respectively). EOOv increased significantly ($P < 0.05$) shrimp survival and yield at both doses, whereas EOMa increased shrimp survival and yield only at the highest dose. In conclusion, EOOv and EOMa constitute suitable alternatives to reduce vibrios virulence and to increase yield in shrimp culture systems.

1. Introduction

Shrimp farming is one of the most extensive aquaculture activities, with a production of 4.8 million tons in 2018 worldwide, four times higher than that produced two decades ago (FAO, 2018). Despite its significant growth, the shrimp farming industry has constantly been hit by viral and bacterial pathogens (Walker and Mohan, 2009; Flegel, 2012). Recently, the emergence of new strains of highly virulent vibrios, capable of causing severe mortalities have been reported within the shrimp farming industry (Tran et al., 2013; Phiwsaiya et al., 2017; Restrepo et al., 2018), It is estimated Thailand's shrimp farming industry lost 26 million USD in 2015 due to acute hepatopancreatic necrosis disease (AHPND) (Shinn et al., 2018) which is caused by pathogenic vibrios. Most strains of pathogenic vibrios have shown resistance to common antibiotics (Kitiyodom et al., 2010; Lai et al., 2015; Sotomayor et al., 2019), making them difficult to control in aquaculture production systems. To a large extent, the emergence of resistant strains is due to the misuse of antibiotics, commonly adopted by producers to treat vibriosis (Romero et al., 2012; Cabello et al., 2013; Chi et al., 2017; Thornber et al., 2019).

In this context, the adoption of new management strategies to control pathogenic vibrios strains in culture systems is essential. A promising low-risk and environmentally friendly strategy is antivirulence therapy (Hentzer et al., 2003; Rasko and Sperandio, 2010;

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Defoirdt et al., 2011). Antivirulence therapy is based on the interruption of bacterial communication, known as quorum sensing (QS) (Waters and Bassler, 2005; Clatworthy et al., 2007; Defoirdt, 2018). Antivirulence therapy minimizes the risk of microbial resistance (Lesic et al., 2007; Defoirdt, 2013; Totsika, 2016) as it inhibits virulence without affecting bacterial growth. Unlike antivirulence therapy, strategies based on antibiotics kill (bactericides) or inhibit (bacteriostatic) bacterial growth and therefore cause selective pressure within the pathogen community (Cabello, 2006; Watts et al., 2017). Bacteria communicate through QS using small chemical molecules called autoinducers (Nealson, 1977; Reading and Sperandio, 2006; Rutherford and Bassler, 2012), and acquire collective behaviors to regulate the expression of several virulence factors. QS is involved in factors such as: bioluminescence production (Dunlap, 1999; Defoirdt et al., 2008a), biofilm development (Nadell et al., 2008; Dickschat, 2010; Li and Tian, 2012), exopolysaccharide production (Marketon et al., 2003; Shrout and Nerenberg, 2012; Maunders and Welch, 2017), swarming motility (Daniels et al., 2004; Shrout et al., 2006), plasmid transfer (Piper and Farrand, 2000; Lang and Faure, 2014), secondary production of metabolites, and in interactions with the host and other microbes.

It is well known that bioluminescence, biofilm formation, swarming motility and toxin production in *Vibrio* species is mediated by the QS system (Henke and Bassler, 2004; Yildiz and Visick, 2009; Yang and Defoirdt, 2015; Liu et al., 2018; Noor et al., 2019), which allows vibrios to distinguish between high or low population density and coordinate the genetic expression of the entire community (Rutherford and Bassler, 2012; Defoirdt, 2018). Vibrios can launch a coordinated attack that facilitates the overcoming of the host's defense barriers thanks to QS mediated mechanisms (Defoirdt et al., 2005; Li and Nair, 2012). QS has been linked to the virulence of pathogenic vibrios important to aquaculture (Brackman et al., 2008; Natrah et al., 2011; Kiran et al., 2016; Defoirdt, 2019). Preventing vibrios communication or altering their QS mediated responses are appealing strategies to reduce or even abolish their virulence (Defoirdt et al., 2005; Defoirdt et al., 2008b; Zhao et al., 2018).

Recent studies have shown that natural products, specifically essential oils (EOs) at sublethal doses, can alter the QS system and thus the virulence of pathogenic bacteria (Ferro et al., 2016; Myszka et al., 2016; Camele et al., 2019). EOs have recently been proposed as an efficient and safe alternative for antibiotics replacement (Nazzaro et al., 2013; Yap et al., 2014; Omonijo et al., 2018), however, the potential use of EOs as anti-QS substances for the control of vibriosis in *P. vannamei* farming has not been evaluated yet. The purpose of this study was to identify EOs that can interfere with the QS of known pathogenic vibrios in *P. vannamei* farming. Five EOs were tested *in vitro* at sublethal doses for their ability to affect QS mediates processes, including bioluminescence expression, biofilm formation, and swarming motility in four pathogenic *Vibrio* strains. Our results indicate the feasibility of using EOs as virulence control agents for pathogenic vibrios in shrimp farming.

2. Materials and methods

2.1. Bacterial strains and growth conditions

Four vibrios were used in this study, *V. harveyi* (strain E22), *V. campbellii* (stain LM2013), *V. parahaemolyticus* (strain ATCC 27969) and *V. vulnificus* (strain S2). Vibrios strains were provided by the microbiology department of the National Center for Aquaculture and Marine Research (CENAIM, Ecuador). All strains were grown aerobically in Luria Bertani agar with 2% NaCl (LBa + 2% NaCl), by striae seeding and incubated for 18 h at 28 °C. Individual colonies were then transferred to Luria-Bertani broth supplemented with 2% NaCl (wt/vol) (LBb + 2% NaCl) and incubated at 28 °C with agitation (200 rpm) for 8 h growth. Vibrios cultures were diluted in LBb + 2% NaCl to obtain bacterial suspensions of 2×10^8 CFU mL^{-1} at an optical density (OD) of 0.4–0.6 at 600 nm. From this, serial dilutions were made in sterile saline solution (SS-2% NaCl) for downstream analyses.

Table 1
Minimum inhibitory concentration (MIC) and Maximum bactericidal concentration (MBC) for each of the essential oils evaluated. Values are expressed in (μg mL^{-1}). EOOv: essential oil of oregano; EOMa: essential oil of tea tree; EOCc: essential oil of lemongrass; EOCv: essential oil of cinnamon; EOTv: essential oil of and thyme.

Vibrio strains	(μg mL^{-1})	Essential oils (EO)				
		EOOv	EOMa	EOCc	EOCv	EOTv
V. campbellii	MIC	800	800	1500	1000	1900
	MBC	800	900	1500	1200	2000
V. harveyi	MIC	700	800	1000	900	2000
	MBC	800	800	1100	900	2100
V. vulnificus	MIC	900	1000	2000	1000	1800
	MBC	1100	1200	2200	1100	1800
V. parahaemolyticus	MIC	800	600	1400	1500	1500
	MBC	900	900	1500	1600	1500

2.2. Essential oils evaluation

Five essential oils (EOs) were evaluated, essential oil of *Organum vulgare* (EOOv), *Melaleuca alternifolia* (EOMa), *Cymbopogon citratus* (EOCc), *Cinnamomum verum* (EOCv) and *Thymus vulgaris* (EOTv). For the anti-QS assays, the EOs were emulsified in SS-2% NaCl + Tween-80 (1.0%). Tween-80 was used as emulsifying agent (Deng et al., 2016). For the *in vitro* toxicity tests, the EOs were emulsified in Hanks balanced salt solution (Gibco 14185-052) + Tween-80 (1.0%). It was previously determined that the dosage of the substance used as an emulsifier (Tw-80), does not influence the parameters evaluated.

2.3. Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of EOs

MIC and the MBC values were determined to establish sublethal doses, affecting only the QS indicators of bioluminescence, biofilm development and swarming motility, without affecting the viability of vibrios. MIC values were obtained using a microwell dilution assay technique previously described by Sokovicќ et al. (2010) with slight modification. Using 96-well microplates, 160 μL of LBb + 2% NaCl sterile were added to each well plus 20 μL of the EO previously adjusted at different final concentrations: 50 at 3000 μg mL^{-1}, and 20 μL bacterial suspension 2×10^8 CFU mL^{-1}. A positive control (containing inoculum but no EO) and negative control (containing EO but no inoculum) were included on each microplate, in addition to six replicates for each concentration of EOs and controls. Microplates were incubated at 28 °C for 24 h. This assay was conducted for each of the four strains. MIC values were determined as the lowest dose at which the wells showed no turbidity $OD_{600\ nm}$ (VarioskanLux_0315) from bacterial growth. For MBC determination, cultures of wells selected for the MIC and the wells containing the next three higher concentrations of EO were plated in TSA + 2% NaCl, incubated at 28° C for 24 h. The EO concentrations where no bacterial growth was observed after incubation were considered as MBC and expressed in mg L^{-1} EO.

2.4. Effect of EOs on the bioluminescence of V. harveyi and V. campbellii

Given that bioluminescence in *Vibrio* species is one of the phenotypes which is controlled by quorum sensing (Manefield et al., 2000), we examined the possibility that EO may affect bioluminescence in *V. harveyi* and *V. campbellii* wild type strains, following the methodology described by Nackerdien et al. (2008) with slight adaptations. Overnight cultures of vibrios were adjusted by optical density to $OD_{600} \sim$ (0.40 *V. harveyi*) and (0.46 *V. campbellii*), corresponding to

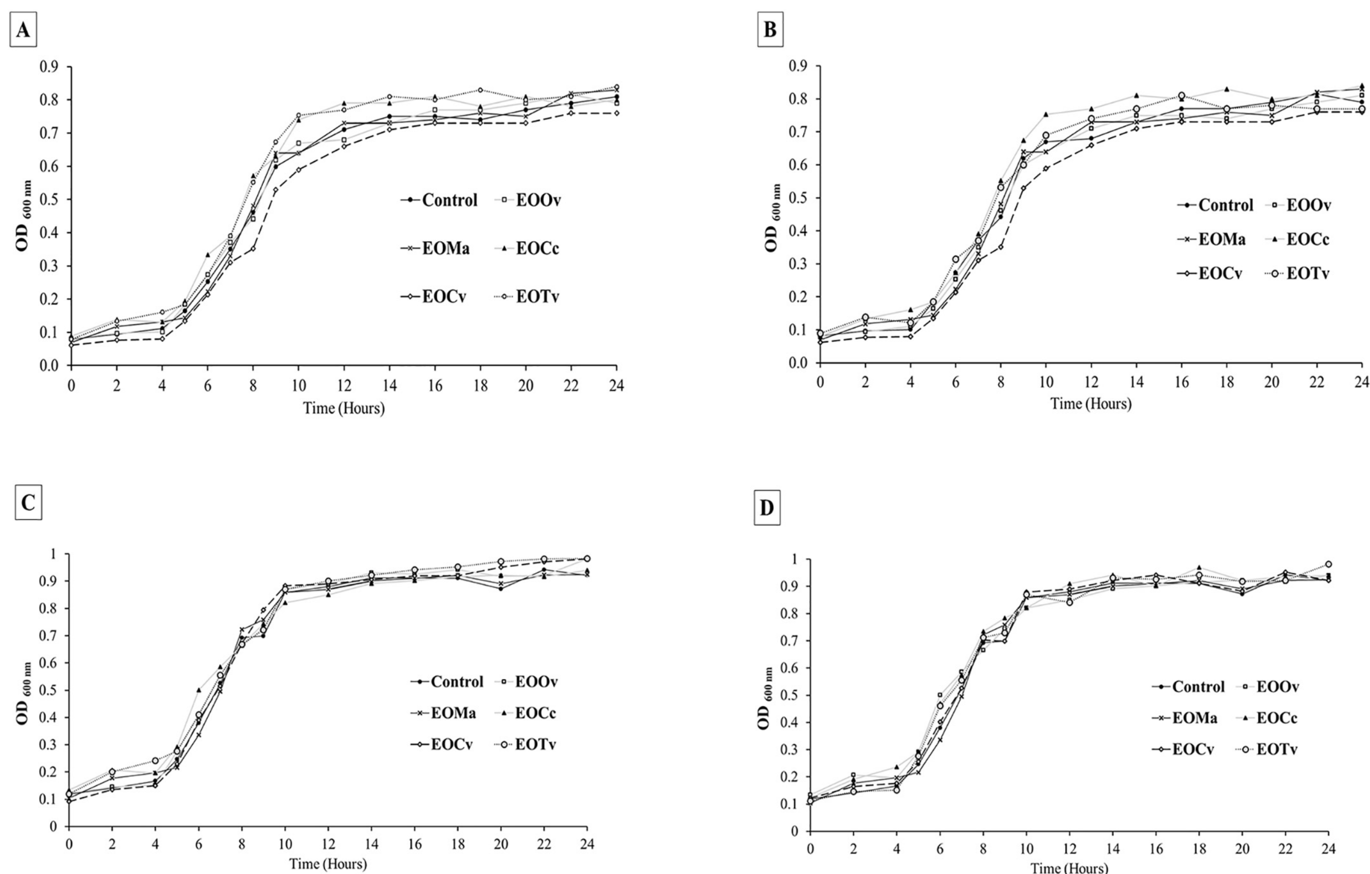

Fig. 1. Essential oil effect on the growth of *Vibrio* strains (A) *V. harveyi*, (B) *V. campbellii*, (C) *V. vulnificus* and (D) *V. parahaemolyticus*. All assayed concentrations were sublethal to the four *Vibrio* species.

10^8 CFU mL^{-1}. Bacterial suspensions were diluted 1:100 in fresh LBb + 2% NaCl sterile, which produced a bacterial concentration of approximately 10^6 CFU mL^{-1}. From this bacterial suspension, 20 µL was transferred to each well in a 96-well microplate, containing 180 µL of LB + 2% NaCl sterile at different sublethal concentrations (0.1, 0.25, 0.5, 1.0, 2.5, 5.0, 10.0 µg mL^{-1}) of each EO evaluated. A control was performed containing bacterial suspension + LBb + 2% NaCl without EO. Six replicates for each EO concentration and the control were included. Microplates were incubated at 28 °C for 12 h. The luminescent emission of vibrios was quantified by a luminescence detector (VarioskanLux_0315) every hour throughout the bioassay (12 h). Data were transformed to (%) bioluminescence considering 100% bioluminescence to the light emitted by the bacteria not treated with the EO.

2.5. Effect of EOs on the biofilm formation

The effect of EOs on biofilms of the four *Vibrio* strains, *V. harveyi*, *V. campbellii*, *V. parahaemolyticus* and *V. vulnificus*, was assessed. The bacterial biofilm biomass was stained with violet crystal (CV) and quantified spectrophotometrically following the methodology described by Djordjevic et al. (2002), with slight modifications. Briefly, bacterial suspensions (1×10^6 CFU mL^{-1}) for each *Vibrio* strain were prepared as previously described. Twenty µL of bacterial suspension was transferred to each well in a 96-well microplate containing 180 µL of LBb 2% NaCl sterile at different sublethal concentrations (0.1, 0.25, 0.5, 1.0, 2.5, 5.0, 10.0 µg mL^{-1}) of each EO evaluated. A control was performed containing bacterial suspension + LBb + 2% NaCl without EO with six replicates for each concentration. In addition, a commercial antibiotic (oxytetracycline) at a concentration of 50 µg mL^{-1} was included. The plates were incubated at 28 °C for 36 h. The planktonic cells were removed, and the generated biofilms were carefully washed twice using 200 µL of PBS (pH 7.2). The biofilms were dried at 50 °C for 30 min and stained with 220 µL of 0.1% CV (w/v) (Merck_ C0775) per well for 15 min. The plates were then rinsed to remove excess dye and dried at room temperature (26 °C). The impregnated CV was solubilized with 220 µL of ethanol. From the solubilized product, 150 µL of each well was transferred to a new 96-well plate and the optical density was read at 590 nm (OD_{590}). The data were transformed to (%) biofilm formation considering 100% biomass of untreated bacterial biofilms (negative control). The level of biofilm inhibition was calculated using the following formula:

Percent inhibition = [(OD Control OD Test)/OD Control] × 100.

2.6. Effect of EOs on swarming motility

The effect of the EOs on the swarming motility of the four vibrios mentioned above was also evaluated, following the method described by Fünfhaus et al. (2018) with slight adaptations. The LBb supplemented with 0.8% agar and 2% NaCl was autoclaved (SN510 Sterilizer). After the medium LB was cooled to 45 ± 3 °C, the EOs were added separately to each concentration to be evaluated (0.1, 0.5, 1.0, 2.5, 5.0, 10.0 µg mL^{-1}). The medium LB was dispensed in Petri dishes (100 × 15 mm). The plates were dried for 15 min and immediately following 5 µL of 2×10^6 CFU mL^{-1} vibrios inoculum was inoculated in the center of the plates. A control was included in a Petri dish with LB supplemented with 0.8% agar and 2% NaCl without EO. In addition, plates with the same amount of agar and ClNa + antibiotic oxytetracycline were included at a single dose (10 µg mL^{-1}). The plates were incubated at 26 °C for 72 h and the migration of the swarming motility was measured in mm. The swarming motility migration of the treated vibrios with EO was compared with the swarming motility migration of

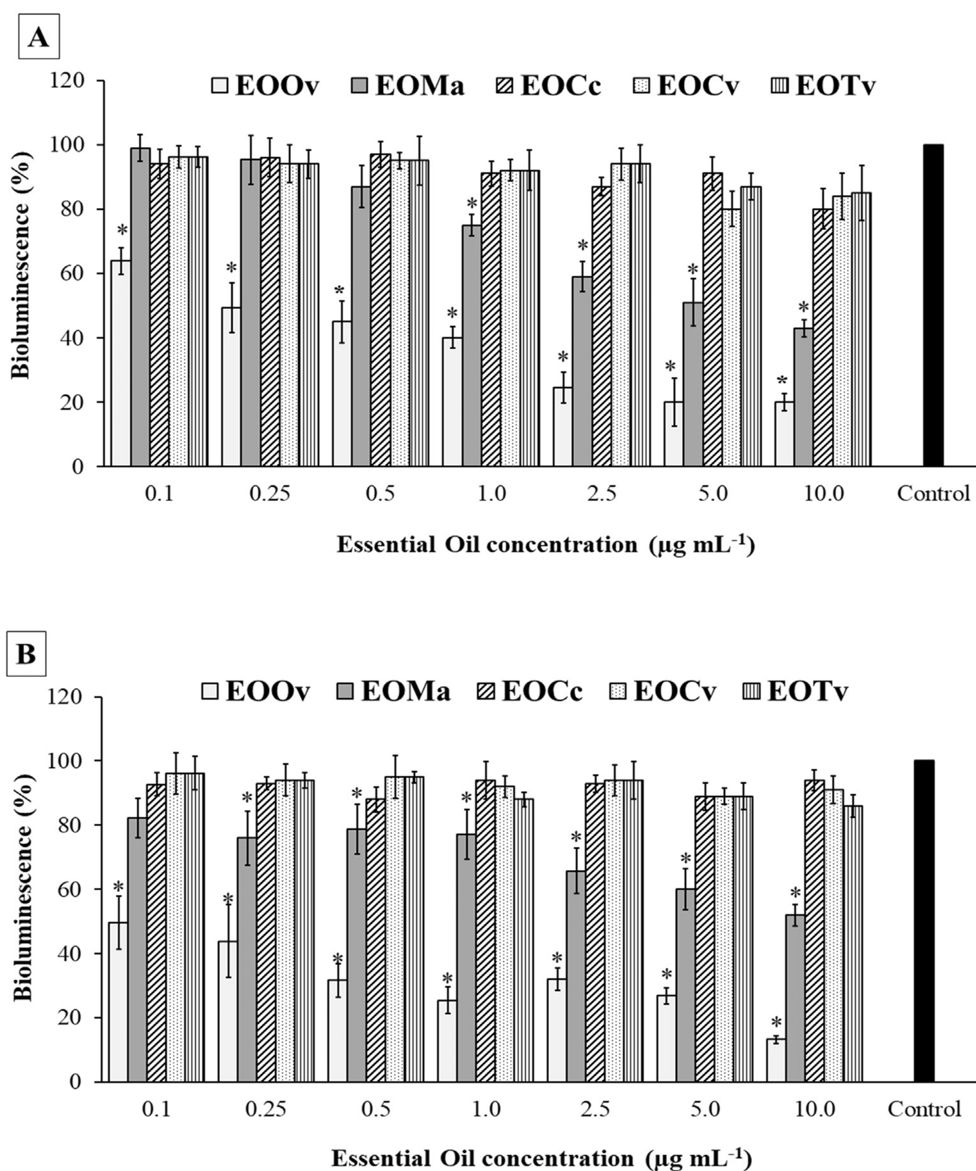

Fig. 2. Effect of various concentrations of five essential oils on *Vibrio* strains bioluminescence. (A) *V. harveyi* and (B) *V. campbellii*. Luminescence measurements were performed 12 h after the addition of the essential oil. All bioluminescence measurements were normalized against the average value recorded in control samples. Error bars represent the SD of six replicates. Asterisks (*) denote statistical differences ($P < 0.05$) compared to the control treatment.

the untreated vibrios.

In addition, the effect of EOs on swarming motility in the presence of the antibiotic, was also evaluated. Using the antibiotic oxytetracycline (T, 30 µg, oxoid), antimicrobial susceptibility disks were used (diameter 6 mm). Petri dishes LB medium supplemented with 0.6% agar and 2% NaCl for each EO at two final concentrations (0.5 and 1.0 µg mL^{-1}) and without EO (control) were prepared. Immediately, 5 µL of *V. vulnificus* suspension (2 × 10^{8} CFU mL^{-1}) was inoculated in the center of the Petri dishes, and an antibiotic disc was placed by each plate. The Petri dishes were incubated at 26 °C for 96 h in a humid chamber. The diameter of the inhibition halo produced by the antibiotic was measured in mm, and the sizes of the halos of the plates treated with EO were compared with respect to the control group.

2.7. In vitro and in vivo toxicity of EOs

Initially, the toxicity of the EOs was determined *in vitro* by MTT reduction assay, following the methodology described by Domínguez-Borbor et al. (2018). First, hemolymph was extracted from healthy shrimp. Then, a primary culture of hemocytes was carried out at a concentration of 10^{6} cells mL^{-1} in 96-well plates with Hanks salts (Gibco 14185-052) supplemented with 13 mM Cl_2Ca and 26 mM Cl_2Mg. The primary cultures were incubated for 75 min at 26 °C. The supernatant was then discarded and 100 µL of Hanks salts supplemented with 12 mM Cl_2Ca and 6 mM Cl_2Mg were immediately placed on the plates. Hanks salts were used as vehicle to dilute EOs to various concentrations (0.1, 0.5, 1.0, 2.5, 5.0, 10.0 µg mL^{-1}). There were six replicates for each concentration evaluated. A control of hemocytes without EO was included. After 120 min of exposure, 10 µl of MTT (5 mg/mL MTT in Hanks) was added to all wells and was incubated for 120 min at 26 °C. The supernatant was removed, and the formazan crystals were dissolved with 150 µL of 0.04 N isopropanol HCL. This colorimetric reaction read OD at 620 nm. The results were transformed into percentages of cell viability using the following formula.

$$\text{Cell viability OD} = (\text{OD exposed cells}/\text{OD control cells}) \times 100\%.$$

To determine the safety of EOs *in vivo*, *P. vannamei* larvae were used in three larval stages, zoea 1 (Z-1), mysis 1 (M-1), and post larva (PL-3).

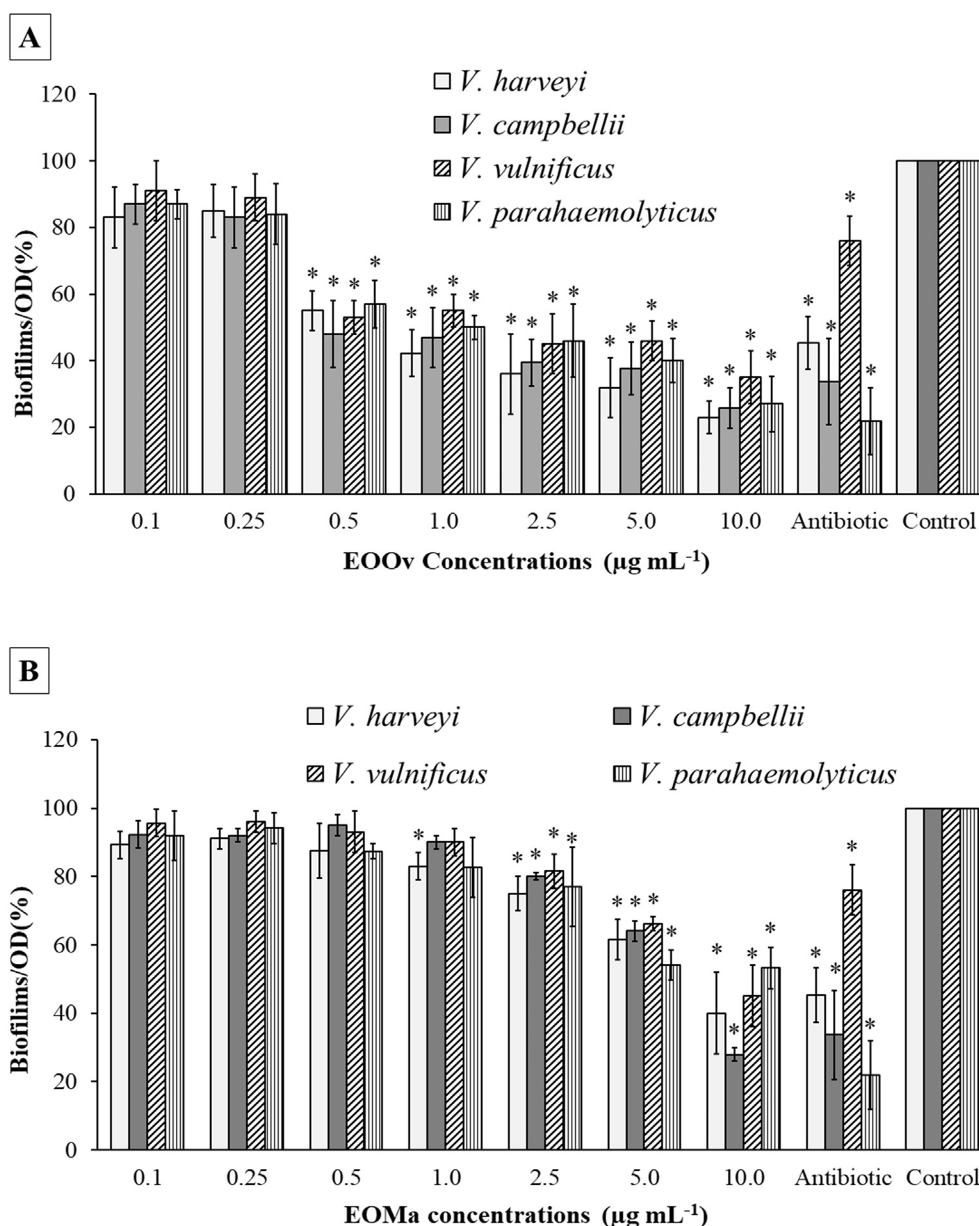

Fig. 3. Effect of various concentration of two essential oils on *Vibrio* strains biofilm formation. (A) Essential oil of oregano (EOOv) and (B) essential oil of tea tree (EOMa). Data points are represented as mean ± SD of six replicates and correspond to the percentage of biofilm formation normalized against the average value recorded in control samples. Asterisks (*) denote statistical differences ($P < 0.05$) compared to the control treatment.

Shrimp larvae were provided by a commercial hatchery. Each trial was carried out independently. The water used in the tests was filtered and sterilized in an autoclave. For the Z-1 and M-1 tests, with a density of 1000 ind. L^{-1}, six replicates were used for each evaluated concentration of EOs (0.1, 0.25, 0.5, 1.0, 2.5, 5.0, 10.0 µg mL^{-1}). The EOs were applied every eight hours (8 h) in relation to the total volume of water of each experimental unit. As a control, larvae were included under the same conditions but without exposure to EOs. The larvae were monitored for 96 h. The data were transformed to survival percentages, considering 100% as the survival of larvae that did not receive EOs. Additionally, with the data obtained were determined the dose causing 50% mortality (LD_{50}) for each larval stage of shrimp.

2.8. In vivo antivirulence effect of EOs

The antivirulence effect of the EOs was verified by a challenge test using healthy *P. vannamei* larvae of stage (PL8). The PL8 were fed with a commercial diet every 4 h throughout the bioassay for a duration of four days. The bacterial inoculum was prepared following the methodology described by Domínguez-Borbor et al. (2019), with slight modifications. First, a fresh culture of *V. campbellii* grown for 10 h was adjusted to 10^8 UCF mL^{-1}. One mL of the adjusted inoculum was transferred to three flasks containing 1000 mL of fresh broth LB + 2% NaCl. Immediately, the EOs were added at sublethal concentrations (1.0 and 2.5 µg mL^{-1}) in the vibrios cultures. As control, a culture of *V. campbellii* without EO was performed. The culture flasks were incubated overnight at 30 °C with constant movement. Subsequently, the cultures were centrifuged (3500*g*, 10 min, 25 °C), the supernatants were discarded, and the pellet cells were resuspended in SS-2% NaCl adjusted at 10^7 CFU by mL and immediately inoculated to each treatment assigned. Water exchange (50%) was performed at 12 post exposure hours (hpe) and the survival was determined by counting the PL8 every 4 h until 96 hpe. This bioassay considered 100% of the virulence to the inoculum of *V. campbellii* cultivated without EO. Each treatment had six

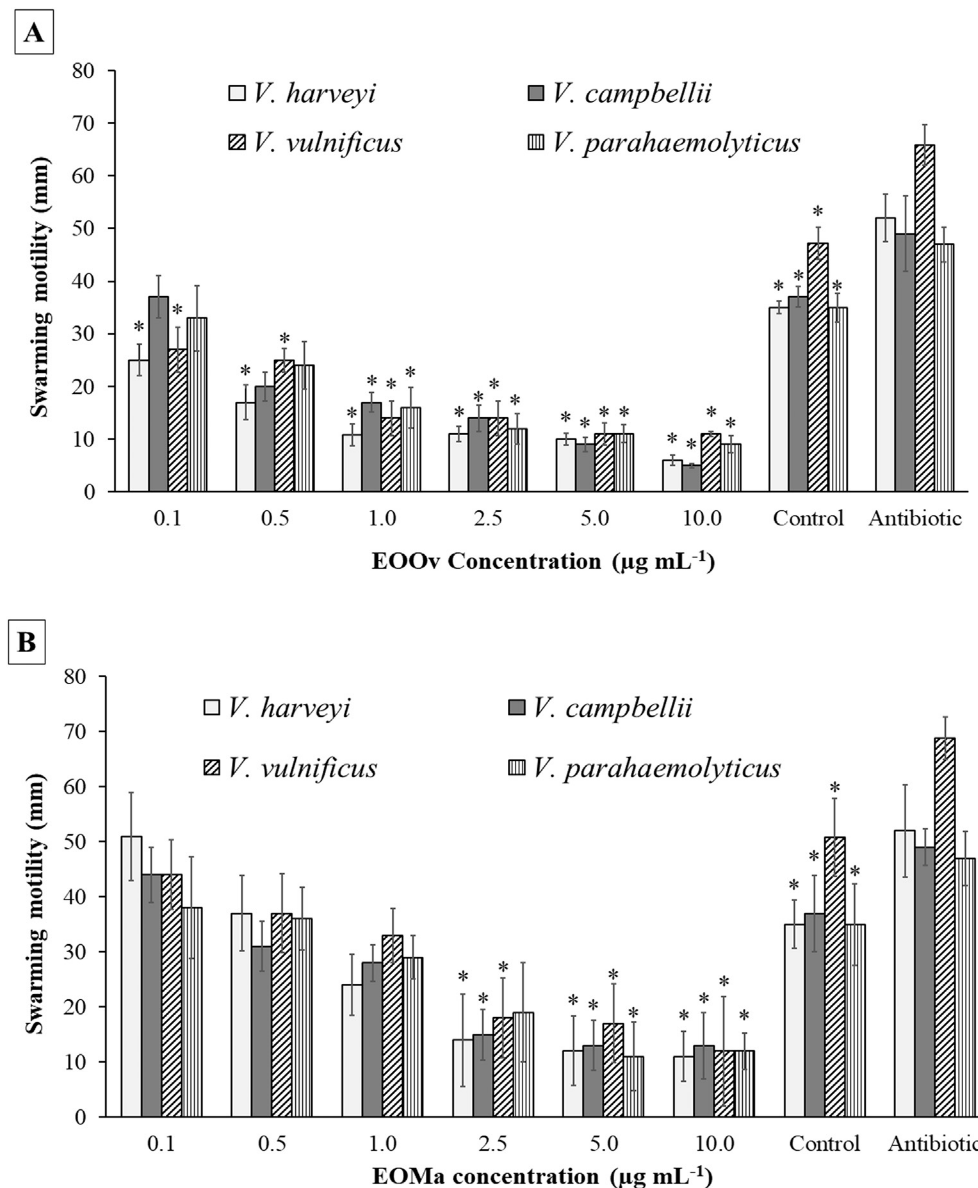

Fig. 4. Effect of various concentration of two essential oils on *Vibrio* strains swarming motility. (A) Essential oil of oregano (EOOv) and (B) essential oil of tea tree (EOMa). Data points are represented as mean ± SD of six replicates of the swarming motility diameters recorded in each *Vibrio* species. Asterisks (*) denote statistical differences ($P < 0.05$) compared to the control treatment.

repetitions with 100 post-larvae. Seawater filtered and sterilized with UV was used, continuous aeration was provided, and the temperature was set at 30.5 ± 0.5 °C.

2.9. Effect of EOs application in P. vannamei grow-out ponds

The potential effect of two EOs in shrimp production grow-out systems was evaluated. Twelve 400 m^2 earthen ponds of CENAIM's Experimental Station (Santa Elena Province, Ecuador) were used for this purpose. A control group without EO application was included. Each treatment had four replicates. A total of 3200 shrimp post-larvae PL12 stage were stocked in each pond (stocking density of 12 post-larvae per square meter). The EOs were applied at two daily doses in the feed at a concentration of 2.5 and 5.0 mg kg^{-1} for the entire production cycle of 102 days. Each day, the EOs were incorporated into the commercial pelleted feed (28% protein) and were immediately supplied to the assigned ponds. Daily feeding was set initially to approximately 3% of the average body weight of the shrimp and adjusted weekly based on observed feed consumption and growth. Environmental parameters such as temperature, dissolved O_2, and salinity were monitored daily. Final shrimp survival (%), average weight (g), production yields (kg/ha) and feed conversion ratio (FCR) were evaluated at harvest time.

2.10. Statistical analysis

All experiments were done in six replicates, except in the bioassay in grow-out ponds that four replicas were used. The results were expressed as an average (± standard deviation) of the replicates. Statistical analyses were performed to determine significant differences ($P \leq 0.05$) using one-way ANOVA, after verification of the normality and variance homogeneity assumptions. When significant differences were detected a Dunnett's analysis was applied (control and treated groups). The data expressed in percentages were transformed (using arcsine), and the assumptions were fulfilled before performing the statistical analysis. The dose causing 50% mortality (LD_{50}), was

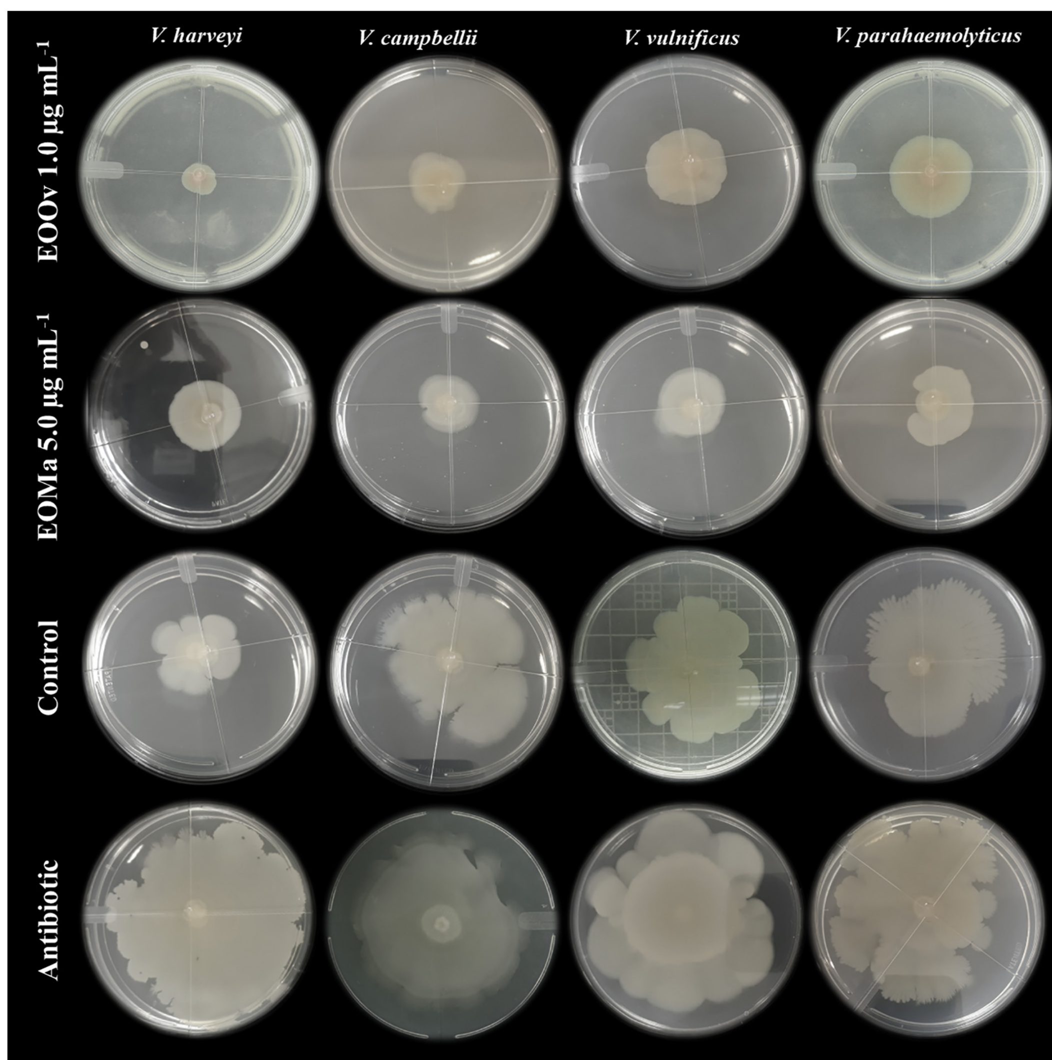

Fig. 5. Effect of essential oils and oxytetracycline on *Vibrio* strains swarming motility. All pictures were recorded after 72 h of incubation. Essayed concentrations were: Essential oil of oregano (EOOv) 1.0 µg mL^{-1}; essential oil of tea tree (EOMa) 5.0 µg mL^{-1}; antibiotic (oxytetracycline) 10.0 µg mL^{-1}; control: no essential oil or antibiotic added.

estimated by Probit regressions. All analyses were performed using the SPSS statistical software (version 21).

3. Results

3.1. EOs sublethal doses determination

EOs exhibited different MIC and MBC values against the four *Vibrio* strains evaluated (Table 1). MIC and MBC values were lowest for EOOv and EOMa, showing that their inhibitory and bactericidal activities were stronger compared to the other essential oils evaluated. MIC and MBC values were used as references for subsequent tests, and only sublethal concentrations below the MIC were assayed in each case. Results shown in (Fig. 1) indicate that none of the EOs affected the growth of the four pathogenic vibrios at the highest concentration assayed (10.0 µg mL^{-1}).

3.2. Bioluminescence inhibition

Only EOOv and EOMa significantly reduced ($P < 0.05$) the bioluminescence of *V. harveyi* (Fig. 2A) and of *V. campbellii* (Fig. 2B). The percentage of bioluminescence inhibition in each bacterial strain showed a marked concentration dependency for the case of EOOv and EOMa (Fig. 2). EOOv was the most efficient oil to inhibit bioluminescence. At a concentration of 1.0 µg mL^{-1}, EOOv inhibited more than 50% of the bioluminescence of both vibrios.

3.3. EOs effect on biofilm formation

EOCc, EOCv and EOTv did not inhibit biofilm formation in the four pathogenic vibrios evaluated, so they were not further considered for the swarming test and *in vivo* trials. EOOv and EOMa significantly reduced the biofilms of the four vibrios in a concentration dependent manner (Fig. 3). EOOv was the most efficient oil in reducing the biofilms of the four pathogenic vibrios in more than 50% in each case starting from a concentration of 1.0 µg mL^{-1} (Fig. 3A). For the EOMa, the lowest active concentration that significantly reduced ($P < 0.05$) the biofilm formation in the four vibrios was of 2.5 µg mL^{-1} (Fig. 3B). The antibiotic oxytetracycline inhibited vibrios biofilm formation less efficiently than the EOs especially in *V. parahaemolyticus* (Fig. 3).

3.4. EOs effect on swarming motility

The swarming motility of the four pathogenic vibrios was significantly ($P < 0.05$) affected by the EOs in a concentration dependent manner (Fig. 4). When 1.0 µg mL^{-1} EOOv was added, swarm motility

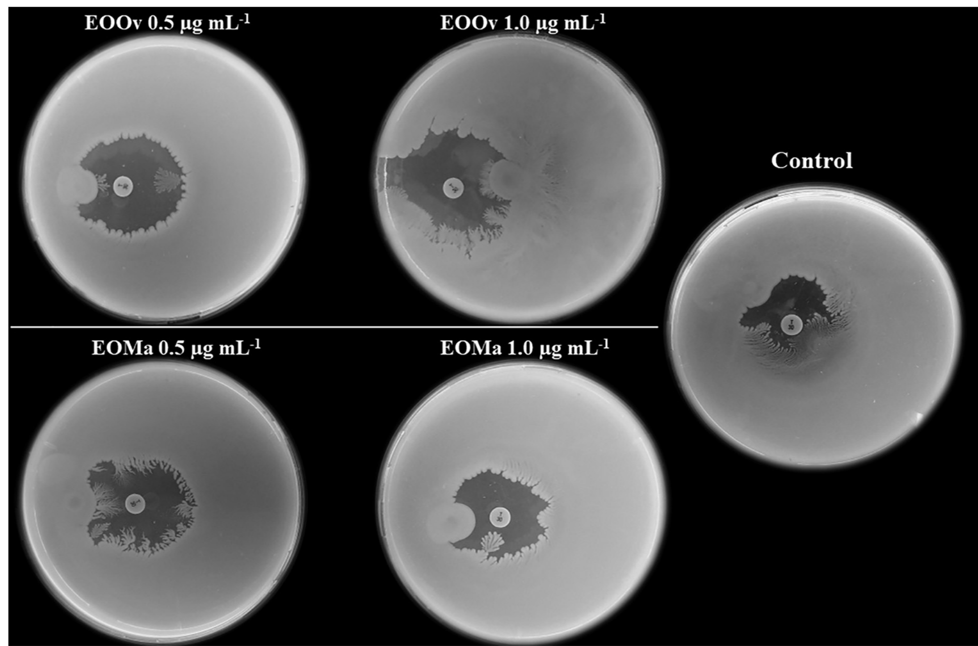

Fig. 6. Essential oil attenuating effect on the swarming motility of *V. vulnificus* in the presence of oxytetracycline antibiotic. After 96 h.

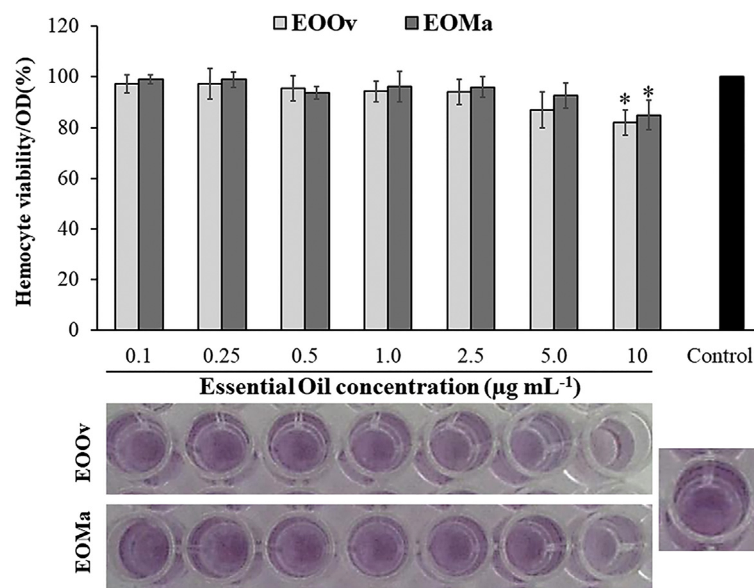

Fig. 7. Toxicity test performed with essential oils on shrimp hemocytes (after 4 h). The viability of the hemocytes in the control treatment was established at 100% and the other treatments were normalized accordingly. Asterisks (*) denote statistical differences ($P < 0.05$) compared to the control treatment.

migration diameters of ~20 mm were recorded for the four vibrios (Fig. 4A). This is significantly smaller than the swarming motility migration diameters recorder in the control group (~50 mm). EOMa significantly reduced ($P < 0.05$) the swarming motility at a dose of 2.5 µg mL^{-1}, registering swarm motility migration diameters of ~30 mm (Fig. 4B). In the case of EOMa, a dose of 5.0 µg mL^{-1} was necessary EOMa to reduce swarm motility migration diameters in around 20 mm for the four vibrios assessed (Fig. 5). Additionally, it was observed that swarming motility increased significantly ($P < 0.05$) when the antibiotic oxytetracycline was added (Fig. 4).

For the oxytetracycline swarming motility test, we selected the *V. vulnificus* strain due to its swarming ability of covering the entire Petri dish in 96 h. In this trial, we found that EOs also affect antibiotic resistance. Since EOs decreased the swarm motility of *V. vulnificus*, oxytetracycline inhibition halos in presence of EOs were kept until the end of the experiment 96 h (Fig. 6). In the control group, inhibition halos were reduced as the hours passed (Fig. 6).

3.5. *In vitro and in vivo toxicity of EOs*

EOs *in vitro* toxicity tests showed that EOs did not affected hemocytes viability at concentrations lowers than 5.0 µg mL^{-1} (Fig. 7). Hemocytes viability was affected by a concentration of 10.0 µg mL^{-1} of the EOs. EOs *in vivo* toxicity tests revealed that the earlier the larval stage, the greater their susceptibility to the EOs. In the zoea stage, the two EOs significantly affected ($P < 0.05$) the survival in most of the doses evaluated, except for the doses below 0.25 µg mL^{-1} (Fig. 8). Regarding the mysis stage, EOs only showed a negative effect on survival at the highest doses evaluated (5.0 and 10.0 µg mL^{-1}). Regarding the PLs, EOOv decreased survival only at a concentration of

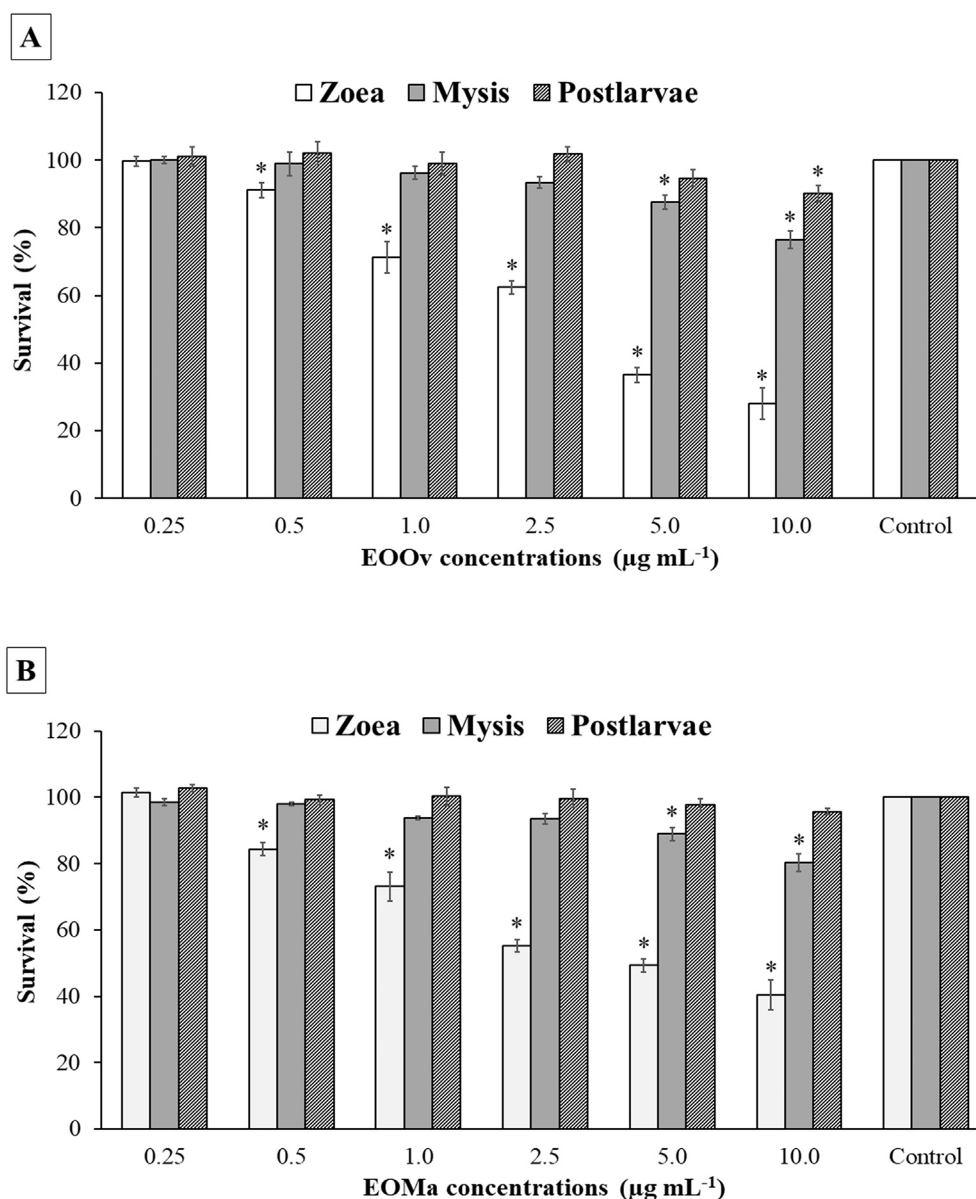

Fig. 8. Survival results of *P. vannamei* larvae exposed to different concentrations of essential oils in three developmental stages. (A) Effect of essential oil of oregano (EOOv) and (B) Effect of essential oil of tea tree (EOMa). Results correspond to cumulative survival after 72 h of exposure. Data points are presented as average values ± SD of six replicates. Asterisks (*) denote statistical differences ($P < 0.05$) compared to the control treatment.

Table 2
Estimation of the lethal dose (LD_{50}) by probit analysis for the two most active essential oil and per larval stage of *P. vannamei*. Values are shown in (μg mL^{-1}) and 95% confidence intervals (IC 95%) displayed for each larval stage of *P. vannamei*. Essential oil of oregano (EOOv) and essential oil of tea tree (EOMa).

Essential oil	LD_{50} per larval stages of *P. vannamei*					
	Zoea		Mysis		PL	
	Valor	IC 95%	Valor	IC 95%	Valor	IC 95%
EOOv	2.5	1.7–3.4	18.5	15.3–32.1	54.4	32.7–103.3
EOMa	3.4	1.7–5.4	39.7	22.7–99.4	91.7	65.5–146.7

10.0 μg mL^{-1} (Fig. 8A), and EOMa did not affect the survival of the PLs at any of the concentrations evaluated (Fig. 8B). Estimated LD_{50} values are shown for the two EOs in each larval stage of *P. vannamei* in (Table 2).

3.6. EOs effect on *V. campbellii* virulence

Significant differences ($P < 0.05$) were recorded between the cumulative mortality rates of *P. vannamei* PLs, challenged with *V. campbellii* grown in the presence and absence of EOs. When 1.0 μg mL^{-1} of EOOv was used, the cumulative mortality rate was reduced to 62.3 ± 7.5%. A greater effect was obtained when the dose was increased to 2.5 μg mL^{-1}, which resulted in a mortality of 53.7 ± 9.1% (Fig. 9). For EOMa, only at doses of 2.5 μg mL^{-1}, the mortality rate of PLs (61.5 ± 7.1%) was significantly reduced ($P < 0.05$) compared to the control group (93.7 ± 5.3%) (Fig. 9).

3.7. Beneficial effects of EOs in *P. vannamei* grow-out ponds

Cumulative survival and yield improved significantly ($P < 0.05$) in ponds treated with EOOv at both doses evaluated, compared to the control group. Regarding EOMa, only at the highest dose (5.0 mg kg^{-1}) were survival (89.2 ± 4.5%) and production performances

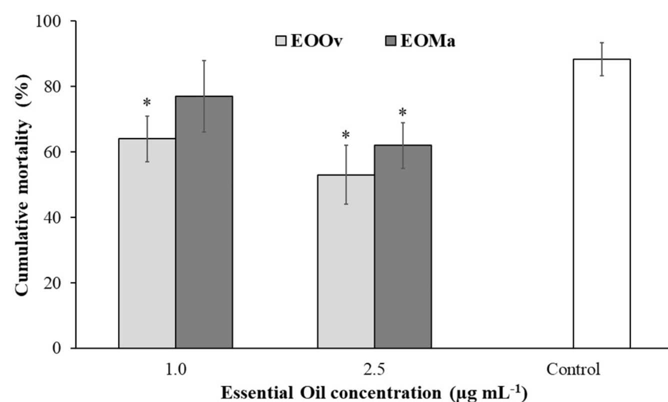

Fig. 9. Effect of two essential oils on the virulence of *V. campbellii*. Results correspond to the cumulative mortality (%) of *P. vannamei* PLs 96 h post exposition to the pathogen *V. campbellii*. The pathogen *V. campbellii* was grown in a medium supplemented with an essential oil at two sublethal doses prior the challenge test. Error bar represents the SD of the mean of six replicates. Asterisks (*) denote statistical differences ($P < 0.05$) compared to the control treatment.

(941.4 ± 66.4 kg/ha) significantly higher ($P < 0.05$) compared to the control group. For the control group final survival and the yield were 72.0 ± 8.7% and 715.9 ± 96.4, respectively (Table 3).

4. Discussion

The low efficacy of common disinfectants to control vibriosis in shrimp farming, and the risk of resistance to antibiotics make necessary to look for new alternatives. Antivirulence strategies aimed at inhibiting QS, have been proposed as a tool for the control of pathogenic vibrios (Defoirdt et al., 2011; Zhao et al., 2015; Torres et al., 2019). Particularly, EOs have a well-reported ability to inhibit QS in human (Swamy et al., 2016; Qaralleh, 2019; Sun et al., 2019) and animal pathogenic bacteria (O'Bryan et al., 2015; Ferro et al., 2016). The results we obtained showed that EOOv and EOMa are able to inhibit QS mediated processes in four pathogenic vibrios related to shrimp farming. Observations from the *in vitro* assays allowed us to determine actives doses for *in vivo* tests, in which EOOv and EOMa significantly increased survival of shrimp challenged with the *V. campbellii* pathogen. EOOv and EOMa also showed encouraging results when used feed supplements in shrimp ponds.

EOOv and EOMa were able to inhibit the bioluminescence of *V. harveyi* and *V. campbellii*. Bioluminescence production is positively regulated by the QS and is involved in the establishment of the pathogen in the host (Niu et al., 2006; Wang et al., 2013). Luminescent vibrios are widely used as models in the search for anti-QS products (Defoirdt et al., 2005; Brackman et al., 2008; Vikram et al., 2011; Kiran et al., 2016; Naik et al., 2018), because this phenotype is only expressed when bacteria reach the quorum. For example, Kiran et al. (2016) used the inhibition of luminescence of *V. campbellii* to test the antivirulence features of polyhydroxy butyrate. Vibrios utilize a three-channel system for QS detection. Channel 1 consists of the LuxM-dependent autoinducer HAI-1 and the HAI-1 sensor, LuxN. Channel 2 consists of the LuxS-dependent autoinducer AI-2 and the AI-2 detector, LuxPQ. Channel 3 consists of the CqsA-dependent autoinducer CAI-1 and a sensor called CqsS (Yang et al., 2011; Wadsworth and Cockell, 2017). In an *in vivo* study, Defoirdt et al. (2005) described that the virulence of *V. harveyi* in *Artemia franciscana* is mediated by the AI2 and CAI-1 channels. In fact, when they inactivated the luxS AI-2 synthase or the AI-2 luxP receptor gene, the virulence of *V. harveyi* was abolished. In the present study, we did not determine whether the EOs affected the autoinducer 2 (AI-2) and/or the cholerae autoinducer 1 (CAI-1) channels for virulence related gene activation. But we assessed the effect of EOs on others QS-mediated processes, such as biofilm formation and swarming motility. The two EOs (EOOv and EOMa) that negatively affected the bioluminescence production also shown to have effects on biofilm inhibition in the four studied pathogenic vibrios.

Biofilm formation is generally associated with colonization and subsequent pathogenesis of vibrios in hosts from marine environments (Faruque et al., 2006; Nadell et al., 2008; Yildiz and Visick, 2009; Rothenbacher and Zhu, 2013). Vibrios form biofilms on the surfaces of a cement slab, plastic, and steel coupons (Karunasagar et al., 1996; Manilal et al., 2010), elements widely used in shrimp farming systems. Adhesion and proliferation within the biofilm are established mechanisms of pathogenesis and infection of some *Vibrio* species in shrimp (Karunasagar et al., 1996; Manilal et al., 2010; Vanmaele et al., 2015). Several studies indicate that biofilms are important for survival, virulence, and resistance to stress in *Vibrio* species (Faruque et al., 2006; Milton, 2006; Dang and Lovellc, 2015). When biofilm formation capacity is reduced, antibiotic resistance and pathogenesis potential are also reduced in the population of free-living vibrios. Once a mature biofilm is established, it is very difficult to eliminate, since the bacteria embedded in the biofilm exhibit a 1000-fold increased resistance to conventional antimicrobial agents (Karunasagar et al., 1996; King et al., 2008; Gupta and Birdi, 2017), in this way limiting the possibilities of treatment (Thompson et al., 2004). The efficacy of EOs in preventing the formation of biofilms in bacteria of clinical and veterinary interest has been well documented (Vikram et al., 2011; Alvarez et al., 2014; Zhang et al., 2018; Kerekes et al., 2019). To date, only few studies have been carried out on biofilm inhibition pathogenic vibrios associated to shrimp farming. These studies focused on molecules such as thiophenones (Yang et al., 2015), polyhydroxy butyrate (Kiran et al., 2016), indol (Yang et al., 2017), catecholamine (Suong et al., 2017) and 2,6-di-tert-butyl-4-methylphenol (Santhakumari et al., 2018). In the present study, EOOv and EOMa showed a clear effect on preventing biofilm formation in the four vibrios evaluated. To our knowledge, it is the first report where EOOv is evaluated as an agent that prevents biofilm formation in pathogenic vibrios of *P. vannamei*.

In addition to biofilms, another aspect that must be considered in tissue colonization is swarming motility, through which pathogenic vibrios can move collectively. Vibrios are highly motile bacteria due to the rotation of the flagella that facilitate movement. It has been proven that the swarming motility of several pathogenic vibrios of aquaculture interest is also positively regulated by QS, such is the case of *V. harveyi* (see Yang and Defoirdt, 2015), *V. campbellii* (see Kiran et al., 2016), *V.*

Table 3
Effect of essential oils on shrimp culture parameters at harvest. Results are presented as mean ± SD of four replicates. Different lowercase letters indicate significant differences ($P < 0.05$). SGR: specific growth rate; FCR: feed conversion ratio; EOOv: essential oil of oregano; EOMa: essential oil of tea tree; Control: no essential oil added.

Treatments	Concentration Eos (mg kg^{-1})	Stocking density (shrimp/m^2)	SGR (% day^{-1})	Average weight (g)	Survival (%)	Yield (kg/ha)	FCR
EOOv	2.5	12	8.4 ± 0.2 a	12.4 ± 1.3 a	92.2 ± 5.6 b	953.7 ± 88.1 bc	1.06 ± 0.13 a
	5.0	12	8.5 ± 0.4 a	13.3 ± 1.9 a	93.1 ± 4.8 b	1041.4 ± 89.5 bc	1.03 ± 0.09 a
EOMa	2.5	12	8.2 ± 0.2 a	11.1 ± 1.1 a	87.1 ± 6.8 ab	883.4 ± 77.2 ab	1.14 ± 0.28 a
	5.0	12	8.5 ± 0.3 a	12.1 ± 1.5 a	89.2 ± 4.4 b	941.4 ± 66.4 b	1.03 ± 0.04 a
Control	–	12	8.3 ± 0.4 a	11.5 ± 2.0 a	72.0 ± 8.7 a	715.9 ± 96.4 a	1.27 ± 0.37 a

alginolyticus (see Liu et al., 2020). The swarming motility of the vibrios allows them to develop a colonial bacterial population both inside and outside the host (Wolfe et al., 2004), form biofilms (Dang and Lovellc, 2015), and to become resistant to antibiotics (Tesdale et al., 2009). Interfering with vibrios swarming motility is essential to affect their virulence. EOOv, and EOMa significantly reduced the swarming motility of the four pathogenic vibrios with respect to the control group. Greater swarming motility migration zones of the four vibrios were recorded when they were exposed to oxytetracycline antibiotic at doses of 10 μg mL^{-1}, with respect to the control. While many reports indicate the negative effect of antibiotics on swarming motility, results similar to ours have been reported by Sun et al. (2018) who evaluated vibrios swarming motility in the presence of several antibiotics. It seems that swarming motility generates resistance to antibiotics since it facilitates close contact of the bacteria with antibiotics which ultimately results in a greater acquired resistance. In consequence, swarming motility is greater in the presence of antibiotics. We observed EOs ability to inhibit swarming motility even in the presence of the antibiotic oxytetracycline, a result that indicates an additional application of EOs, potentiating antibiotics effectiveness (Fig. 6).

In this last decade, EOs have aroused the interest of several scientists for their anti-Quorum activity, attributed to several molecules present in the EOs such as, carvacrol (Burt et al., 2014; Joshi et al., 2016; Tapia-Rodriguez et al., 2019), thymol (Singh et al., 2017; Ghafari et al., 2018), linalool (Ahmad et al., 2015; Mukherji and Prabhune, 2015), citral (Sun et al., 2019; Liu et al., 2020), cinnamaldehyde (Niu et al., 2006; Brackman et al., 2008; Jia et al., 2011; Khan et al., 2017). In the present study, the EOOv was the most efficient to inhibit QS mediated processes it the four vibrios evaluated. Most likely, the anti-QS activity observed is related to the considerable proportions of carvacrol (45.6%) and of thymol (5.2%) present in this EOOv. Burt et al. (2014), document that 0.8 mM of carvacrol effectively inhibited the biofilms of *Chromobacterium violaceum*, *Salmonella enterica* and *Staphylococcus aureus*. Mith et al. (2015), document that carvacrol present in the EO of *Origanum heracleoticum* is the active compound that affects toxin production in *E. coli*.

EOs have been widely used in food preparation and as a food preservative for human consumption for several decades (Prakash et al., 2015; Pandey et al., 2017). EOs display a low toxicity and are considered as safe (GRAS) substances by the USA Food and Drug (FDA, 2016). To date there is no prohibition for the use of EOs as feed supplements to breed animals for human consumption. In the present study, EOOv and EOMa at doses below 2.5 μg mL^{-1}, affected the virulence factors of vibrios without toxic effects for mysis and PLs of *P. vannamei*, with EOOv being the most effective. In earlier larval stages, such as *P. vannamei* zoea, the two EOs affected survival, so it is suggested to start feed supplementation from mysis at doses below 2.5 μg mL^{-1} and in PLs up to a maximum of 10 μg mL^{-1}. Both EOOv and EOMa were tested in shrimp grow-out ponds, and greater survival and production yields were obtained. In both *in vivo* trials, better results were obtained with the EOOv. This result matches results obtained *in vitro*, in which the EOOv was more effective to arrest QS indicators.

EOs are aromatic and limpid substances that can be obtained from different parts of the plants, and their effectiveness is given by the proportions of the bioactive molecules (Cunha et al., 2018; Zhang et al., 2018), being able to vary in a wide and diverse spectrum of action within the same plant genus. In addition, EOs from the same plant species can vary in their chemical composition, depending on the environmental and climatic conditions in which they grow (Burt, 2004), their maturity and the extraction method. Although we did not find anti-QS activity in EOCc, EOCv and EOTv, we did not rule out their benefits and plan to run experiments accounting for inter batch variability. In this sense, it is important to mention that a disadvantage of the use of EOs to control vibriosis in shrimp farming is that they do not have a standard composition. It is advisable to obtain EOs from guaranteed providers and to evaluate the quality of each batch by means of controlled *in vitro* tests.

5. Conclusion

The use of EOs as antivirulence tools to control vibriosis in shrimp farming is very promising. Out of the five EOs evaluated, EOOv and EOMa were effective in inhibiting QS mediated processes in four pathogenic vibrios at sublethal concentrations. EOOv and EOMa included in the diet of *P. vannamei* shrimps in culture ponds, improved survival, and yield performances. The low levels of EOOv and EOMa (5.0 mg kg^{-1}) needed to achieve these results make them attractive to replace antibiotics during shrimp farming.

CRediT authorship contribution statement

Cristóbal Domínguez-Borbor: Methodology, Visualization, Investigation, Data curation, Writing - original draft. **Aminael Sánchez-Rodríguez:** Supervision, Writing - review & editing. **Stanislaus Sonnenholzner:** Investigation, Writing - review & editing. **Jenny Rodríguez:** Conceptualization, Visualization, Investigation, Supervision, Writing - review & editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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