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Use of an essential oil blend formulation (EOBF) as an effective disinfectant against pathogenic luminescent *Vibrio* bacteria

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Abstract
Vibrio, mainly *Vibrio harveyi*, *V. campbellii*, *V. parahaemolyticus* and *V. splendidus*, especially in their luminescent form are considered highly pathogenic to shrimp larvae. An effort was made to minimize the luminescent *Vibrio* load using an essential oil blend formulation (EOBF) consisting of *Eucalyptus* oil, *jasmine* oil, and *gardenia* oil in equal proportions. An eighteen-day small hatchery-scale trial was initiated, starting from the nauplii stage. The shrimp were distributed into three groups and four replicates each: a negative control, positive control with four replicates, and an EOBF treatment group. The shrimp at the mysis stage were challenged using a sublethal dose of 10^3 CFU/mL luminescent *Vibrio harveyi*.
The obtained results indicated that the presence of harmful *Vibrio* in tank water, was almost one log lower in the EOBF group than in the positive control. The presence of pathogenic luminescent *Vibrio* in the positive control was 45%, whereas it was 19% in the treatment group. In addition, the EOBF-treated had better performance, and productivity. The results of this trial suggest that EOBF can reduce pathogenic *Vibrio* in hatchery environments and can increase productivity.

Keywords: Pathogenic *Vibrio*, Luminescent *Vibrio*, *Vibrio parahaemolyticus*, *Vibrio harveyi*, Essential Oil Blend Formulation, Disinfectant. Shrimp hatchery, *Penaeus vannamei*

Introduction
Early fish development is the most important thing that should be known before producing any seeds, especially in the new cultured species or strain. Generally, early development is divided into egg, larvae and juvenile, whereas mostly fish embryos develop from transparent eggs [1]. According to Kendall *et al.* [2], the egg stage is divided into early, middle and late subdivisions, which end with blastopore closure, freeing of the tail bud from the yolk, and hatching. After hatching, the intestine is considered as the most necessary organ in supporting their life due to the digestion and absorption process of nutrients [3, 4].
Even though, the the embryonic period mostly depends on temperature and oxygen supply to support their survival rate, yet, the availability of food also playing a big role [5, 6]. For the exogenously feeding larvae, the functional of food acquisition and growth is decisive [5]. Thus, it is important to know when the yolk sac is exhausted and when the larvae start to feed actively, because this period is recognized as a critical period which determine the survival of the next stages [7]. Furthermore, Southgate and Lucas [8] stated when larvae first hatch, they usually have sufficient energy reserves in yolk sac to support development for a day or more before they need to be fed. Therefore, it is suggested that food must be available, given it early and abundant during this periods [9].
Considering that digestive system during the larval stage is still very poor, therefore, understanding the mouth gape is important in relating to larvae first feeding. Laven and Sorgeloos [10], and Petkam and Moodie [11], stated that during the first feeding avtivity, fish larvae mostly depend on small live prey which is easy to be ingested. In this situation, diatom, flagellate, rotifer, and Artemia seem to meet the suitable size [12].
It is wellknown that digestive system in larvae is different from juvenile and adult. Therefore, the nutrition requirements of early life stages are distinct from those of older fish [13].

Luminescent or glowing bacteria is the common terminology for harmful *Vibrio* bacteria in shrimp hatcheries. The colonies of this group of bacteria appear green on TCBS media. The most pathogenic species in the luminescent group is *Vibrio harveyi*, which is infectious to cultured shrimp, such as *Penaeus monodon* and *Penaeus vannamei* in hatcheries [1]. Apart from *Vibrio harveyi*, *V. campbellii* and *V. splendidus* can also produce luminescence and infect the larval, juvenile, and adult stages of cultured shrimp [2, 3]. *Vibrio parahaemolyticus* has quorum factors that can stimulate luminescence in *Vibrio harveyi* quorum-sensing mutants [4], a result confirmed in this study. Some reports are available indicating that glowing *Vibrio harveyi* can infect juvenile shrimp in culture ponds [3]. Heavy mortality, even up to 100% in shrimp, can be caused by pathogenic *Vibrio* in combination with environmental stress [1, 5, 6, 7]. Luminescence is a source of communication in bacteria, making them more pathogenic and aggressive [8, 9].

After the complete ban and prohibition of antibiotics in hatcheries, as pathogenic bacteria quickly become resistant to commercial probiotics, there is a need to obtain a remedy with minimal side effects that are equally effective against luminescent *Vibrio*. Essential oil (EO) with antimicrobial properties should be considered a compelling candidate against luminescent bacteria. Essential oil is recognized as safe for human and animal consumption, as it has been granted GRAS status by the U.S. Food and Drug Administration [10]. EOs, including eucalyptus oil, jasmine oil, and gardenia oil, were selected for testing against *Vibrio*. The presence of flavonoids and biophenols in Eucalyptus results in microbicidal activity against bacteria, such as *E. coli*, *P. aeruginosa*, *Streptococcus*, *Lactobacillus*, and *S. aureus* [11, 12]. Leaf extracts of gardenia possess antibacterial properties [13, 14]. Jasmine oil is effective against several bacteria, such as *Escherichia coli* [15, 16] and fungi [16, 17].

A successful effort was made to develop a blend of essential oils, eucalyptus oil, gardenia oil, and jasmine oil to establish a safe and effective disinfectant to minimize luminescent pathogenic *Vibrio* species such as *V. harveyi*, *V. campbellii* and *V. parahaemolyticus* for shrimp hatcheries.

Materials and Methods

Trial Station

The trial was conducted from December 21, 2020, to January 7, 2021, at the Marine Research Centre, PT. Central Proteina Prima, Lampung, Indonesia.

Preparation and composition of essential oil blend formulation (EOBF)

Eucalyptus globulus, *jasmine* and *gardenia* oils were obtained from vendors that comply with the strictest industry practices. Each essential oil was obtained through the steam distillation process and underwent thorough checking for quality and chemical composition based on the European Pharmacopeia. After the essential oils were declared to pass quality checking, the EOBF mixture was created with the following sequence and percentages: *Eucalyptus globulus*, *jasmine* and *gardenia* oils were added in equal quantities to form an oil mixture and then mixed with potable water.

Trial set up

The trial was designed to start with the nauplii stage to postlarvae stage 10, the latter of which is the harvesting stage of white shrimp (*L. vannamei*) in hatcheries. The negative control, positive control, and treatment groups each had four replicates, and the treatment group was stocked in 2 different rooms, called T-3 and T- 4, with four replicates each in 300-litre tanks. The stocking density of nauplii was 125 per litre. The treatment group had eight replicates subdivided into two rooms: room C, called T-3, and room D, called T-4, with 4 replicates each. The negative control and positive control had four replicates in room A and room B, respectively. The trial design is illustrated diagrammatically and described in Figures 1 and Figure 2. The animals were fed both artificial and natural food at 4 kilograms per million postlarvae (PL). The trial was terminated on day 18 at the PL -10 stage, and the harvest performance was measured.

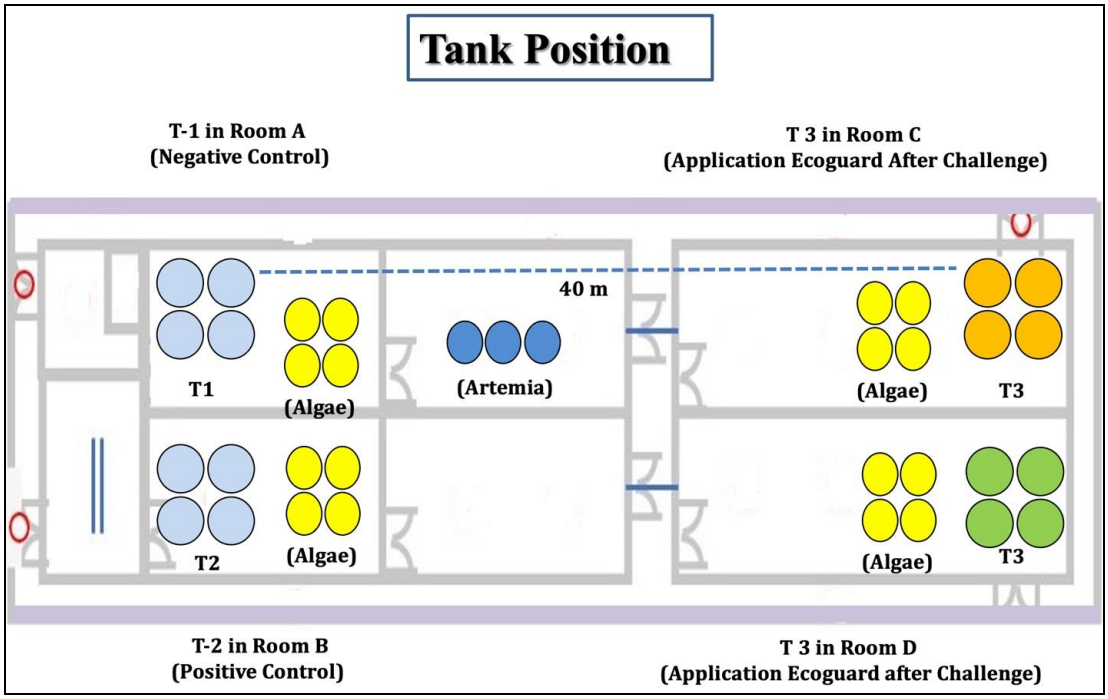

Fig 1: Trial illustration in four rooms, negative control in room A with four replicates, positive control in room B with four replicates, EOBF group subdivided into two subgroups: Treatment 3 in room C and Treatment 4 in room D.

Treatment	Replication	Challenge
T1 (Negative Control)	4	Sterile Tryptic Soy Broth (TSB) + 2% NaCl
T2 (Positive Control)	4	Luminescent Green *Vibrio* 10^3 CFU/mL
T3 (EOBF application after challenge)	4	Luminescent Green *Vibrio* 10^3 CFU/mL
T4 (EOBF application after challenge)	4	Luminescent Green *Vibrio* 10^3 CFU/mL

Fig 2: Trial illustration in four rooms, negative control in room A with four replicates, positive control in room B with four replicates, EOBF group subdivided into two subgroups: Treatment 3 in room C and Treatment 4 in room D.

Study and measurement of parameters

The water quality parameters of the tank water were measured, and the dissolved oxygen, saturation level, pH, temperature, and density of the remaining plankton were calculated daily. The total *Vibrio* count in tank water and shrimp bodies was measured at the following life stages: nauplii, zoea 2, mysis 2, and PL 1, 4 and 8. The luminescent *Vibrio* count was determined in two ways, visual and wet mount on stages, at the following life stages: nauplii, zoea 2, mysis 2, PL 1, 4 and 8. Postlarval performance was measured using data such as survival rate, postlarval length, coefficient of variation (CV) length, mean body weight (MBW) and biomass at PL 10 during harvest. The details are described in Figure 3. Tryptose soy agar (DIFCO, USA) and thiosulfate-citrate-bile salt-sucrose agar (DIFCO, USA) were used for bacterial culture.

Parameter	Methods	Frequency
Stage Development and Activity	Visual	Daily
Temperature (°C)	In build Thermometer in DO meter YSi Pro-20	Daily (7 am and 4 pm)
Dissolved Oxygen (mg/L)	DO meter YSi Pro-20	Daily (7 am and 4 pm)
Saturation (%)	DO meter YSi Pro-20	Daily (7 am and 4 pm)
pH	pH meter WTW 3310	Daily (7 am and 2 pm)
Remain Algae in tank (cell/ml)	Haemocytometer	Daily (7 am and 2 pm)
Total Vibrio Count at water and body (CFU/Individual)	TCBS Agar	N, Z2, M2, PL1, PL4 and PL8 (Harvest)
Luminescent Bacteria	Wet mount, TSA and TCBSA	Z2, M2, PL1, PL4 and PL8 (Harvest)
	Visual	Every day after inoculation of lumbact
Survival Rate, Post Larvae length, Coefficient of variation (CV) length, Mean Body Weight (MBW) and Biomass		PL 10 Stage (Harvest)

Fig 3: Details of the physical and performance parameters measured and frequency in the tank water.

Challenge procedure

The pathogenic luminescent bacterium *Vibrio harveyi* grown on TCBS medium as green colonies were used for the challenge. *Vibrio* was isolated from the commercial hatchery of PT. Central Protein Prima, Lampung. A sublethal dose, 10^3 CFU/mL *Vibrio*, was poured into the experimental tanks, except for the negative control at the mysis-2 stage.

Essential Oil Blend Formulation (EOBF) application

EOBF at a dose of 3 mL/ton of water was applied at stage mysis-3 in the experimental tanks. The EOBF was applied after a day of the challenge. EOBF was applied at a dose of 5 mL/ton from the PL-1 stage to the PL-9 stage.

Statistical Analysis

The general linear model (GLM) was used for statistical analysis during the trial.

Results

***Vibrio* count measurements in shrimp bodies**

The total bacteria and *Vibrio* count measurements on TSA and TCBS media present in the different shrimp body stages

are described in Figure 4. The data show that there was a decrease in pathogenic *Vibrio* (green colonies) in the shrimp bodies of the treatment groups (T-3 and T-4) compared to the positive control (T-2). The negative control (T-1) was free from pathogenic *Vibrio*. More emphasis was placed on the screening of green and glowing *Vibrio* bacteria, as they are considered highly pathogenic to shrimp in hatcheries [1, 3].

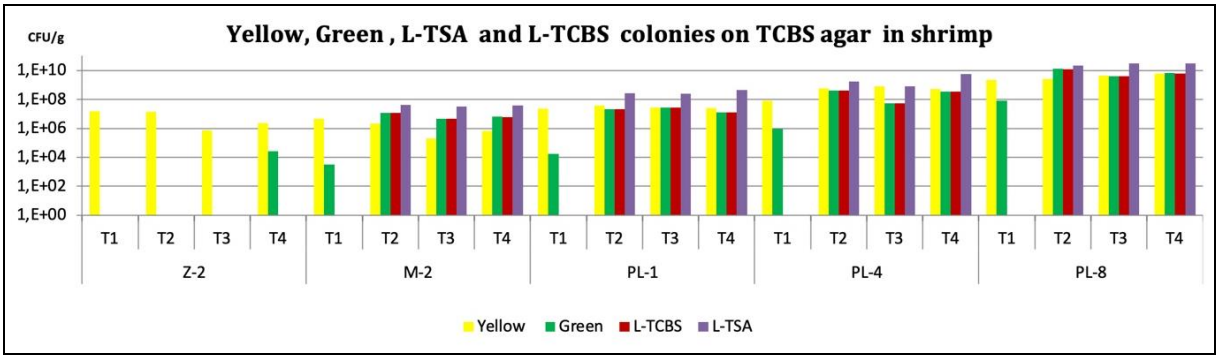

Fig 4: *Vibrio* colonies measured on TSA and TCBS media in shrimp bodies. The results of sampling for each tank at four stages, zoea 2, mysis 2, and postlarvae 1, 4 and 8, are described in yellow (non-pathogenic) and green (pathogenic), and total bacteria counts on TSA agar and total *Vibrio* counts on TCBS agar are shown.

Vibrio count measurements in tank water

The measurements of total bacteria and *Vibrio* count present in shrimp bodies at different stages on TSA and TCBS media are described in Figure 5. The data show that there was a decrease in pathogenic *Vibrio* (green colonies) in the shrimp bodies of the treatment group (T-3 and T-4) compared to the positive control (T-2). The negative control (T-1) was free of pathogenic *Vibrio*.

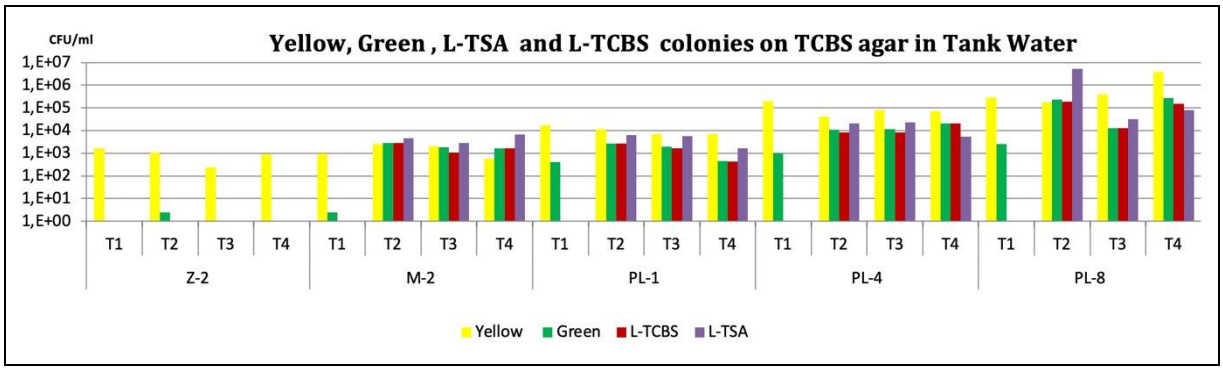

Fig 5: *Vibrio*-measured TSA and TCBS media in the water tank of each treatment, i.e., T-1, T-2, T-3, and T-4. The results of sampling each tank at four stages, zoea 2, mysis 2, and postlarvae 1, 4 and 8, are described in yellow (non-pathogenic) and green (pathogenic), and total bacteria counts on TSA agar and total *Vibrio* counts on TCBS agar were measured.

Visual observation of Luminescent Bacteria

The data showed fewer glowing bacteria in the EOBF-treated groups. T-3 and T-4 were assigned as undetected (-), light (+) and medium (++) compared to a positive control (T-2), which was medium (++) to severe (+++). No glowing bacteria (-) were detected in the negative control (T-1), as described in Figure 6. The appearance of total luminescent bacteria in treatments T-3 and T-4 was 9.53 and 10 times that of the positive control (10.75 times). This strongly suggests that EOBF has a positive role in reducing luminescent pathogenic green *Vibrio* bacteria.

Stage	Luminescent Bacteria visual observation															
	T-1 Negative Control				T-2 Positive Control				T-3 EOBF				T-4 EOBF			
	A1	A2	A3	A4	B1	B2	B3	B4	C1	C2	C3	C4	D1	D2	D3	D4
N																
Z1-1																
Z1-2																
Z-2																
Z-3																
M-1																
M-2	Inoculation Green Lumbact 10^3 cfu/ml at M-2 Stage (9 am)															
	-	-	-	-	+	+	-	+	+	++	+	+	+	+	+	++
M-3	-	-	-	-	++	++	++	++	++	+	+	++	+	++	-	+
PL-1	-	-	-	-	+	++	++	++	++	++	+	+	++	++	+	++
PL-2	-	-	-	-	++	++	+	++	+	++	+	+	+	++	+	++
PL-3	-	-	-	-	++	++	+	++	+	++	+	+	+	++	+	++
PL-4	-	-	-	-	+	+	+	+	+	++	+	++	+	++	+	+
PL-5	-	-	-	-	++	+	+	+	+	+	+	+	+	++	+	+
PL-6	-	-	-	-	+	+	+	+	+	-	+	-	+	++	-	+
PL-7	-	-	-	-	+	+	+	+	+	+	+	-	+	+	-	-
PL-8	-	-	-	-	+	+	+	+	+	+	-	-	+	++	+	+
PL-9					+++	++	+	+	++	+	-	+	+	+	+	+
PL-10																

Fig 6: Visual observation of luminescent bacteria. Luminescent bacteria were observed daily from the mysis-2 stage to the postlarval-9 stage. The presence of luminescent bacteria was categorized as absent (-), light (+), medium (++) and severe (+++ or above).

Wet Mount observations of Luminescent Bacteria

Wet mount analysis was used to observe the percentage of luminescent bacteria in the trial groups. The data shown in Figure 7 indicate that the positive control T-2 had the highest percentage of glowing bacteria, which was 45%. Compared to that, EOBF subgroups T-3 and T-4 exhibited 14% and 25%, respectively, and on average, the EOBF group had 19.5% glowing bacteria. This strongly suggests that EOBF has a positive role in reducing luminescent pathogenic green *Vibrio* bacteria. The essential oil components present in EOBF [13, 14, 15, 16] helped to minimize the bacterial load.

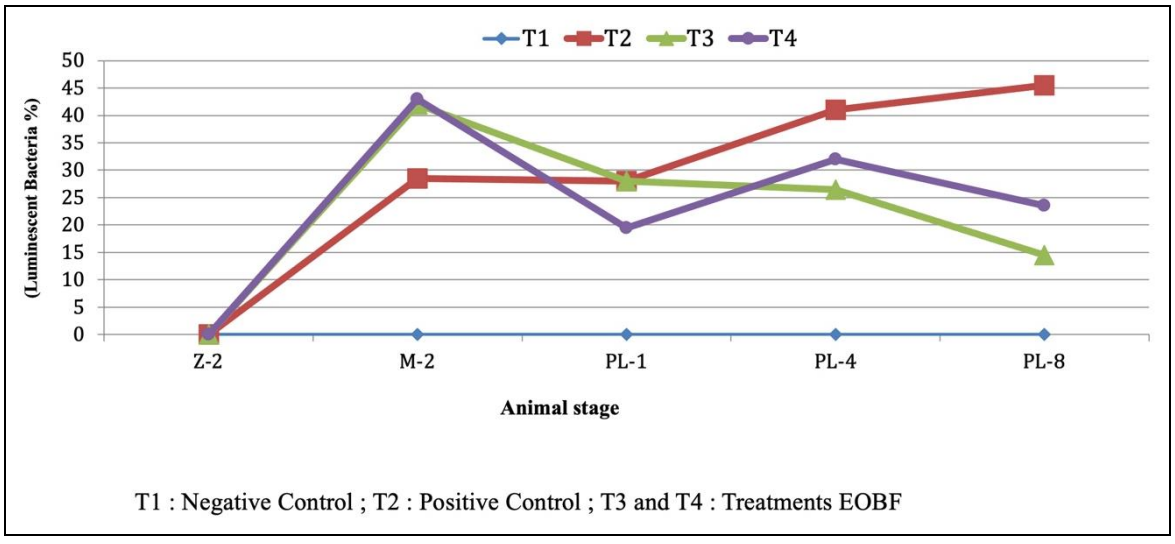

Fig 7: Wet mount observation of luminescent bacteria. Luminescent bacteria were observed daily from the mysis-2 stage to the postlarval-8 stage. The presence of glowing bacteria was the highest and showed increasing trend in the positive control (T-2), whereas a decreasing trend was observed in the EOBF treatment groups (T-3 and T-4).

Harvest performance

Treatment	Rep	Harvest Performance				
		Survival Rate (%)	PL-10 Length (mm)	Size Variation	MBW (mg)	Biomass (g)
T-1 Negative Control	4	71.13 ± 13.33^{b}	9.76 ± 0.19^{ab}	11.28 ± 0.61^{ab}	6.14 ± 0.33^{ab}	163.01 ± 22.32^{bc}
T-2 Positive Control	4	67.57 ± 6.79^{b}	9.01 ± 0.54^{a}	12.61 ± 0.27^{b}	4.51 ± 1.57^{a}	116.36 ± 33.71^{a}
T-3 EOBF Room C	4	42.31 ± 9.45^{a}	10.34 ± 0.09^{bc}	10.64 ± 2.41^{ab}	8.47 ± 0.77^{c}	129.45 ± 32.91^{ab}
T-3 EOBF Room D	4	70.37 ± 12.40^{b}	9.85 ± 0.61^{ab}	9.79 ± 2.52^{a}	6.96 ± 1.64^{bc}	181.32 ± 24.97^{c}
P value		0.072	0.133	0.060	0.056	0.036

Values are means ± SD

Fig 8: Harvest performance of experimental groups T-1, T-2, T-3 and T-4 at the end of the trial (PL-10). Glowing or luminescent bacteria were observed daily from the mysis stage.

The treatment group, especially T-3, had the best performance, as shown in Figure 8. The harvest performance of the positive control T-2 was as follows: survival rate 67.57%, length 9.01 mm, size variation 12.62, mean body weight 4.51 mg and biomass 116.36 g. The performance of EOBF groups T-3 and T-4 at the time of harvest (PL 10 stage) was as follows: survival rate 56.34%, length 10 mm, size variation 10.21, mean body weight 7.71 mg and biomass 155.38 g. The EOBF group was better in terms of length, size variation, mean body weight and biomass. The average survival rate of EOBF was lower due to the poor survival of T-3, i.e., 42.3%. In terms of luminescent *Vibrio* and productivity, the EOBF group performed better than the positive control. Pathogenic green *Vibrio* and glowing bacteria have a negative impact on survival [1, 2], which is minimized by the presence of antibacterial components of essential oil [14, 15] and ultimately leads to higher survival and better productivity.

Discussion

*Vibrio*s are the natural habitat of the ocean [18]. Any shrimp hatchery gets a continuous supply of *Vibrio* in the intake water [19]. Several chemical disinfectants of chlorine origins are reported to have a significant effect against *Vibrio* [20, 22, 22]. The mechanical and electrical ways of controlling *Vibrio* are Carbon filtration, Ozone treatment, UV- treatment and Ultrafiltration [20, 22, 22]. All these methods are cost-effective and with limitations. The limitations are due to side effects on the growth and development of animals [23]. In that scenario, identifying essential oil blends as a substitute for chemical and mechanical disinfectants in the hatchery environment is a revolutionary step. It reduces the risk of deformity and size variations due to strong disinfectants [24].

Vibrio grown on TCBS agar mainly appears in two colours, green and yellow. The green-coloured colonies are pathogenic to the shrimp larvae, whereas yellow colonies are non-pathogenic [25, 26]. The younger the shrimp, the more susceptible it is to the *Vibrio* [26]. The shrimp start getting more resistant to *Vibrio* with age. That is why we selected the youngest shrimp at the most susceptible age to challenge the *Vibrio harveyi* [27]. The two-nutrient media, Tryptose Soy Agar (TSA) and Thiosulfate-citrate-bile salts-sucrose agar (TCBS), are widely used in both researches in commercial laboratories. The TCBS media is an integral part of commercial hatchery worldwide [25, 26] and used as a base media in this research trial. Both yellow and green colonies of *Vibrio* glowed at dark and are named commonly luminescent bacteria [26]. It is challenging for any technicians to differentiate between pathogenic and non-pathogenic glowing bacteria [26]. So, there should not be any luminescing and gloominess in the packaging box at the time of receiving the Postlarvae. It is found that most of the glowing bacteria in the larva rearing tanks are attached to the dead and moribund animals [28]. The proper selection method and having enough water exchange could reduce a load of luminescent bacteria in a tank [27, 28].

It was explained by Lee Ventola [29] that bacterial gene can change and have mutations during exposure which makes species like *Vibrio* unbeatable by antibiotics. It can be inherited from relatives or transferred among different species of bacteria (horizontal gene transfer). The essential oil recently gained importance due to its multifunctional characteristics. It acts as antimicrobial, antioxidative, and anti-inflammatory effects, feeding palatability enhancement and improving gut growth and health [30]. Essential oil is widely used in animal feed to minimise the load of harmful bacteria [31]. Terpenes, one of the primary compounds in essential oils, targets the biosynthetic machinery of bacterial cell walls [32, 33]. First, the terpenes destroy the cell wall and cytoplasmic membranes of a bacteria [34]. Then the lipophilic structure, carvacrol and thymol, enters the bacterial membranes among the fatty acid chains and cause them to expand and become more fluid [35]. The thick outer membrane of gram-negative bacteria makes it less permeable for the essential oil [36, 37]. It can be seen in the trial results, where the application of the essential oil blend was able to reduce the luminescent bacteria incidence but also better productivity.

The blend formulation was developed from three well-known oils, Jasmine, Gardenia and Eucalyptus oil, with various properties. Jasmine oil constitutes benzyl acetate and is used in dermatology as an antibacterial agent [38, 39]. Gardenia is affectively damaging the DNA of bacteria like *Salmonella* spp [40]. Gardenia oil is widely used as antidiabetic, anti-inflammatory, anti-depression, and antioxidant properties and improves the sleep quality in Chinese medicine [41]. Eucalyptus contains potent phenolic compounds with antioxidant and antimicrobial properties, which helps enhance appetite and improve the health and growth performance of the animal [41, 42]. The essential oil group is reported to work against cell-to-cell communication among bacteria (quorum sensing), used to control group behaviours [43, 44, 45].

Conclusion

The obtained results demonstrated that essential oil blend

formulation (EOBF) is a potential disinfectant against pathogenic luminescent *Vibrio*, especially *V. harveyi* and *V. parahaemolyticus* in shrimp.

Conflict of interest statement
We declare that we have no conflicts of interest.

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