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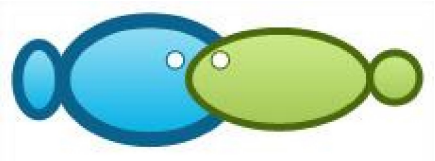

Identification of *Vibrio* sp. as cause of white feces diseases in white shrimp *Penaeus vannamei* and handling with herbal ingredients in East Lampung Regency, Indonesia

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Abstract. White feces disease (WFD) is one of the diseases that attack white shrimp (*Penaeus vannamei*). This disease causes cultivation failure and huge loss for shrimp farmers. The cause of this disease is thought to be due to the abundance of the *Vibrio* bacteria population in cultivation media. This study was aimed to determine the abundance of *Vibrio* sp. and the presence of *Vibrio* sp. as a trigger for WFD in *P. vannamei* ponds and to study the use of several herbal ingredients in suppressing the growth of *Vibrio* sp. Samples were taken from secondary canals, tertiary canals, primary canals, shrimp pond waters that were not infected with WFD, shrimp pond water infected with WFD, and shrimp infected by WFD. Samples taken from those locations were then inoculated; the total population of the bacteria was calculated, identified and tested for anti-bacterial activity using several herbal products. According to the results of the study, *Vibrio* abundance was obtained as follows: water sample was positive of WFD by $3.5\pm0.9\times10^5$ CFU mL^{-1}, shrimp intestine was positive of WFD by $4.4\pm0.1\times10^5$ CFU mL^{-1}, primary canal of $3.9\pm2.\times10^4$ CFU mL^{-1}, secondary canal of $1.0\pm0.1\times10^5$ CFU mL^{-1}, tertiary canal $3.2\pm1.1\times10^5$ CFU mL^{-1}, shrimp pond 1 of $2.2\pm0.3\times10^5$ CFU mL^{-1}, shrimp pond 2 of $1.3\pm0.3\times10^5$ CFU mL^{-1}, shrimp pond 3 of $5.2\pm1.0\times10^4$ CFU mL^{-1}, and healthy shrimp intestine of $\leq2.5\pm0,5\times10^4$ CFU mL^{-1}. The type of *Vibrio* identified and suspected of triggering WFD disease were *V. vulnificus*, *V. parahaemolyticus*, and *V. alginolyticus*. Antibacterial test showed that mangrove leaf extract (*Rhizophora apiculata*) had the best inhibitory effect on *V. parahaemolyticus* (zone of inhibition of 5.61 mm), followed by ketapang leaf extract (4.9 mm zone of inhibition) and papaya leaf extract (zone of inhibition of 4.5 mm). The best concentration of mangrove leaf extract in suppressing the growth of *Vibrio parahaemolyticus* was 700 mg L^{-1}.

Key Words: *Vibrio* abundance, WFD triggers, antibacterial activity, *Vibrio parahaemolyticus*, *Rhizophora apiculata*.

Introduction. White shrimp *Penaeus vannamei* is a type of shrimp that is widely cultivated in Indonesia. This is because these shrimp have promising prospects and profits (Babu et al 2014). However, disease attacks often occur in *P. vannamei* cultivation. Diseases are commonly caused by bacteria, parasites, viruses, and fungi. This disease attack occurs when environmental conditions, pathogens, and cultivan are not balanced (Engering et al 2013). Pathogens that often attack *P. vannamei* are *Vibrio* (Khamesipour et al 2014; Widowati et al 2018).

One of the diseases that appear in shrimp farming is white feces disease (WFD). This disease is characterized by the appearance of white feces on the surface of the culture medium. WFD is allegedly caused by an abundance of *Vibrio* sp. on cultivation media (Jayadi et al 2016). The increase in the number of *Vibrio* sp. is due to adverse environmental conditions, especially high organic matter content. According to Taslihan et al (2015), increasment of the shrimp production can be maintain by monitoring the quality of water, providing the proper feed, and appropriate administration of probiotic or antibiotic doses. However, the use of antibiotics is not recommended in shrimp farming because it leaves a residue that endangers consumers. Safe ingredients that have antibacterial potential can be obtained from some plants. Herbal ingredients or medicinal

plants are all types of plants that can be used as medicinal herbs, both singly and in a mixture that is considered and believed to cure a disease or can have an influence on health. Herbal ingredients are used because they are safe and do not produce residues in cultivated organisms so that they are safe for consumption and friendly to the environment (Yuhana et al 2008; Aminzare et al 2015; Ventola 2015).

Some herbal ingredients that can be used include papaya (*Carica papaya*), ketapang (*Terminalia catappa*), and mangrove (*Rhizophora apiculata*) leaf extracts. *C. papaya* leaf extract contains flavonoids, tannins, alkaloids, vitamin C, karpain, cyanogenic, glucosides, and papain enzymes so that it has the potential as an antioxidant and antiseptic (Eleazu et al 2012). While *T. catappa* leaf extract has an effective antibacterial chemical content (bactericidal).

One area of shrimp farming in Indonesia that faces an epidemic of WFD is Pasir Sakti District, East Lampung Regency, Lampung Province. This region is one of the shrimp producing regions in Lampung Province. The condition of the shrimp ponds in that location has been attacked by White feces disease (WFD) which causes a huge loss for shrimp farmers. The study was aimed to determine the cause of WFD and its treatment needs to be conducted to reduce the incidence of WFD and prevent failure of shrimp farming using herbal ingredients.

Material and Method

Study site. The research was conducted in East Lampung Regency, Indonesia. Samples were taken from secondary canals, tertiary canals, primary canals, shrimp pond waters that were not infected with WFD, shrimp pond water infected with WFD, and shrimp infected by WFD. Research location can be seen in Figure 1.

Figure 1. Research location.

Sample collection. Sampling was carried out by taking 100 mL water samples using a sample bottles. Samples were taken from 6 locations with 3 replicates for each location. Taken Samples were directly inoculated on TCBS media. Samples also were collected from healty and WFD infected *P. vannamei*.

Total bacterial count. After incubation for 24 hours, a growing bacterial colony was calculated by placing a petri dish on top of the Colony Counter device and the bacterial colony was then calculated and counted using the formula:

$$N = C\ x\ \frac{1}{P} x \frac{1}{V}$$

Where:
N = Number of colonies (CFU mL^{-1})
C = Number of calculated of bacterial colonies on the petri dishes
P = Dilution Factor
V = Sample Volume

Bacterial identification. Identification of *Vibrio* was carried out by a quadrant stroke method with several stages to obtain 1 pure isolate. The isolate were then identified. Identification of *Vibrio* sp. was carried out using MICROBACTTM 24 E Gram Negative Identification System (OXOID) and read using software microrobact 2000. Observation of cell morphology includes gram staining, cell shape, and motility test. Physiological properties include catalase test, indole test, MR-VP test, Citrate Simmons test, and TSIA test.

Bacterial medium. A total of 3.2 g of Nutrient Agar (NA) media was weighed and then placed into erlenmeyer and diluted with 160 mL of sea water. Erlenmeyer tube containing nutrient agar was heated using a hot plate until the solution was homogeneous, then autoclaved for 15 minutes with a temperature of 121°C under a pressure of 1 atm. A total of 20 mL of sterile nutrient agar was poured into a petri dish and leaved until it solidified. The pouring was done in the laminar air flow to prevent contamination.

Bacterial cultivation. Nutrient Broth (NB) media was weighed as much as 0.09 g then transferred into erlenmeyer and 90 mL of sea water was added. The erlenmeyer tube containing media NB was heated using a hot plate until the solution was homogeneous and heated by autoclaving for 15 minutes at a temperature of 121°C under a pressure of 1 atm. Pure culture of *Vibrio parahaemolyticus* was inoculated aseptically into 2 test tubes containing 15 mL sterile Nutrient Broth (NB) media. Test tube was incubated in shaking incubator for 4 to 5 hours (Tampemawa et al 2016).

Antibacterial activity test of herbal ingrediennts. A total of 20 µL of liquid isolate *V. parahaemolyticus* with a density of 10^7 CFU mL^{-1} were dropped on Nutrient Agar (NA) media and was leveled with spreader. Disc paper was placed on the media containing a spread of bacteria with a little pressure. A total of 40 µL of *C. papaya* leaf extract, *T. catappa* leaf extract, and *R. apiculata* leaf extract with a concentration of 500 mg L^{-1} in each extract were dripped on a paper disc with a diameter of 6 mm. The petri dish was then incubated for 24 hours. Furthermore, measurement of the zone of inhibition of the extracts against bacteria was carried out. The ability to inhibit bacteria is characterized by the formation of a clear zone around the tested disc papers.

Determination of the best concentration. A total of 20 µL of liquid isolate *Vibrio parahaemolyticus* with a density of 10^7 CFU mL^{-1} were dropped on Nutrient Agar (NA) media and was leveled with spreader. Disc paper was placed on the media containing a spread of isolate with a little pressure. A total of 40 µL of *R. apiculata* leaf extract was dropped on paper disks measuring 6 mm with concentrations of 300, 400, 500, 600, and 700 mg L^{-1}. Positive control was represented by giving paper discs containing chloramphenicol antibiotics while negative control was neutral disc paper (water disinfectant only). All treatments were incubated for 24 hours. After the incubation period, the diameter of the zone of inhibition formed around the disc paper was observed and measured.

Statistical analysis. The data of *Vibrio* sp. abundance in the study field were presented in table form and analyzed descriptively according to the research objectives. Data of antibacterial activities were analyzed using Anova followed by least significant difference (significance of differences at $p<0.05$).

Results. In the primary canal or the main canal, abundance of *Vibrio* sp. was $3.9\pm2.1\times10^4$ CFU mL^{-1} so that this canal is still at safety level for shrimp culture (Taslihan et al 2015). The total abundance of *Vibrio* sp. on secondary canals was $1.0\pm0.1\times10^5$ CFU mL^{-1} so that it exceeds the safe limit in the waters. While in the tertiary canal which is the inlet and outlet canal, the total abundance of *Vibrio* sp. was $3.2\pm1.1\times10^5$ CFU mL^{-1}. Moreover, the total abundance of *Vibrio* sp. in shrimp pond 1 was $2.2\pm0.3\times10^5$ CFU mL^{-1} and $1.3\pm0.3\times10^5$ CFU mL^{-1} in shrimp pond 2. While total abundance of *Vibrio* sp. in shrimp pond 2 was $5.2\pm1.0\times10^4$ CFU mL^{-1}. The abundance of *Vibrio* sp. in all study sites is presented in Table 1.

Table 1

The abundance of *Vibrio* sp. in all study sites

Sample	*Abundance of Vibrio (CFU mL^{-1})*
Water (+)	$3.5\pm0.9\times10^5$
Primary canal	$3.9\pm2.1\times10^4$
Secondary canal	$1.0\pm0.1\times10^5$
Tertiary canal	$3.2\pm1.1\times10^5$
Shrimp pond 1 (+)	$2.2\pm0.3\times10^5$
Shrimp pond 2	$1.3\pm0.3\times10^5$
Shrimp pond 3	$5.2\pm1.0\times10^4$
Shrimp intestine (+)	$4.4\pm0.1\times10^5$
Healthy sShrimp intestine	$\leq2.5\pm0.5\times10^4$

+ = infected with WFD.

Color of bacterial colony in TCBSA media. TCBSA media is a selective medium that can inhibit the growth of undesirable bacteria and can distinguish *Vibrio* into 2 groups, namely a group that ferments sucrose characterized by yellow colonies and a group that does not ferment sucrose characterized by green colonies.

The characteristics of the colonies of *V. parahaemolyticus* and *V. vulnificus* on the TCBSA media included round, green or bluish green colonies in the middle of colonies with a diameter of 2-3 mm and did not ferment sucrose. Colonies of *V. parahaemolyticus* and *V. vulnificus* were found in secondary, tertiary, shrimp pond 1 and shrimp pond 2 samples. Primary canal and shrimp pond 3 samples showed morphological characteristics of yellow colonies and the ability of the colony to ferment sucrose (Figure 2). This was seen from colony changes from green to yellow on TCBSA media. The characteristics of these colonies are typical *V. alginolyticus*, *V. fluvialis*, *Listonella anguillara*, and others.

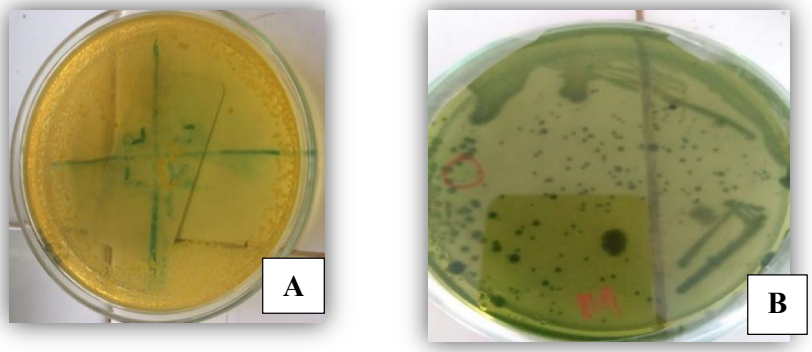

Figure 2. Color of *Vibrio* sp. in the media. A - Yellow-colored *Vibrio* sp. colony, B - Green-colored *Vibrio* sp. colony.

Bacterial identification. Identification of *Vibrio* sp. at the study sites was carried out by taking the most formed colonies on TVC (Total Vibrio Count). Identification was carried out using the MICROBACTTM 24 E Gram Negative Identification System (OXOID) and the results were read by the microbact 2000 software. The results of the identification carried out are presented in Table 2.

Table 2

Identification Results of *Vibrio* sp. at the study sites

Sample	*Kind of Vibrio*
Feces (+)	*V. parahaemolyticus*
Primary canal	*V. alginolyticus*
Secondary canal	*V. vulnificus*
Tertiary canal	*V. vulnificus*
Shrimp pond 1 (infected with WFD)	*V. parahaemolyticus*
Shrimp pond 2	*V. alginolyticus*
Shrimp pond 3	*V. alginolyticus*
Shrimp intestine (+)	*V. parahaemolyticus*

Antibacterial activity of herbal ingredients. The results of the measurement of antibacterial activity from several extracts of herbal ingredients with a concentration of 500 mg L^{-1} incubated for 24 hours can be seen in Table 3.

Table 3

Observation of antibacterial activity of herbal ingredients

Tested material	*Inhibition zone diameter (mm)*			*Average (mm)*
	I	*II*	*III*	
C. papaya leaf extract	3.57	4.68	5.25	4.5±0.85
T. catappa leaf extract	3.64	5.58	5.46	4.9±1.08
R. apiculata leaf extract	4.11	6.28	6.44	5.6±1.30

Concerning the antibacterial activity of herbal ingredients tested, based on statistical analysis, there is no significant diffrenece among treatments.

The best concentration of mangrove leaf extract. The results of the measurement of antibacterial activity were indicated by the presence of zone of inhibitions, namely zone of inhibitions where bacteria did not grow around paper discs of growth of *V. parahaemolyticus* after 24 hours incubation can be seen in Table 4.

Table 4

Observation of antibacterial activity of mangrove leaf extract

Concentration of R. apiculata leaf extract (mg L^{-1})	*Inhibition zone diameter (mm)*			*Average (mm)*
	Sample I	Sample II	Sample III	
300	4.87	4.98	5.44	5.09±0.30
400	5.26	5.14	5.79	5.39±0.35
500	6.42	6.50	7.57	6.83±0.64
600	7.11	7.19	7.36	7.22±0.13
700	8.25	8.11	8.16	8.17±0.07

In the antibacterial testing of herbal leaf extract in inhibiting the growth of *V. parahaemolyticus*, positive controls were used in the form of chloramphenicol antibiotics and negative controls in the form of distilled water. Antibacterial testing using chloramphenicol showed that the diameter of the zone of inhibition produced was 10.98

mm which was categorized as medium. Desinfected water do not form zone of inhibition because it is a are pure water without the content of active compounds that have antibacterial properties (Tampemawa et al 2016). Thus, each concentration shows a different zone of inhibition. The greater the concentration of extract the greater the zone of inhibition formed. The higher the concentration of *R. apiculata* leaf extract used, the more antibacterial content will be, thus it can inhibit the growth of *V. parahaemolyticus.* Statistical analysis showed that different concentration of *R. apiculata* leaf extract affected on inhibition toward *V. parahaemolyticus* growth.

Discussion. Secondary and tertiary canals are generally closed canals that are not crossed by heavy irrigation canals. In addition, both canals have a level of organic matter that tends to be higher than of the primary canal due to waste from previous crop. This causes a buildup of the amount of bacteria and total organic matter (TOM) in the canal at low tide. Total organic matter will trigger the growth of *Vibrio* sp. (Eiler et al 2003). The high total organic matter in tertiary and secondary canals was due to waste from shrimp pond which cannot be released totally into the sea. In this study area, inlet and outlet of pond were in one canal. Pond it is filled when high tide occurs and emptyed when at low.

The condition of shrimp pond 2 and shrimp pond 3 have not shown clinical symptoms of WFD attack, however, total abundance of *Vibrio* $\geq 10^4$ CFU mL^{-1} can trigger WFD attacks. This can be seen from the results obtained in WFD positive water samples and WFD positive shrimp intestines which have a total abundance of *Vibrio* sp. of $3.5\pm0.9\times10^5$ CFU mL^{-1} and $4.4\pm0.1\times10^5$ CFU mL^{-1} respectively. At the location of the farm, previously were WFD and WSSV (White Spot Syndrome Virus) outbreaks, so that it did not rule out the possibility of WFD disease attacks again. Healthy shrimp intestines have *Vibrio* bacteria with a total abundance of $\leq2.5\pm0.5\times10^4$ CFU mL^{-1}, which indicates that *Vibrio* presence in healthy shrimp does not become a pathogen because the nature of the bacteria is opportunistic, but can be pathogenic in certain conditions. Research conducted by Kharisma & Manan (2012) reported that, in vaname shrimp, *Vibrio* abundance exceeding 10^4 CFU mL^{-1} was susceptible to attack by *Vibrio*sis. According to Taslihan et al (2015) the presence of *Vibrio* bacteria which exceeds 10^4 CFU mL^{-1} can cause mass death in cultivated shrimp.

Low population of pathogenic bacteria in cultivation media will provide better shrimp health conditions so that growth of shrimp is better (Nurbaya et al 2010). According to Kharisma & Manan (2012), total bacteria that exceeds the threshold has the potential to increase disease infection which in turn causes mass mortality of cultured shrimp. Increased population of *Vibrio* sp. can be caused by high dissolved organic matter from the rest of the feed and shrimp feces (Paena et al 2018).

Identification results showed several types of *Vibrio*, namely *V. parahaemolyticus*, *V. vulnificus*, and *V. alginolyticus*. The group that was successfully isolated at the study site showed a low variety of species. This indicates that the shrimp pond waters do not contain many types of *Vibrio*.

Vibrio is the dominant flora in eggs, larvae and post-larvae of shrimp (Hameed et al 2003). According to Otta & Karunasagar (2001), an increase in organic material sourced is derived from feed and feces encourage the microflora to develop into opportunistic pathogens. Limsuwan (2010) reported that *V. harveyi*, *V. vulnificus*, *V. parahaemolyticus*, *V. alginolyticus*, and *Vibrio* sp. are pathogenic bacteria that are always found in hatcheries and shrimp ponds. According to Taslihah et al (2015) and Anjaini et al (2018), WFD is caused by microsporidia (from the *Enterocytozoon* group) and gregarin (allegedly derived from the Nematopsis sp.) incorporated with *Vibrio*. Some *Vibrio* are identified in WFD-infected shrimp, including *V. vulnificus*, *V. fluvialis*, *V. parahaemolyticus*, *V. alginolyticus*, *V. mimicus*, *V. cholerae*, and *Listonella damsela.* Ralalage et al (2017) found *V. alginolyticus* and *V. fluvialis* as the causes of white feces diseases in *P. monodon* grow-out ponds in Sri Lanka.

The results of the measurement of the antibacterial activity of *C. papaya*, *T. catappa* and *R. apiculata* leaf extracts showed the showed the antibacterial potential againts *Vibrio* sp. They had different zone of inhibition values of which *C. papaya* leaf

extract was 4.5 mm, *T. catappa* leaf extract was 4.9 mm, and *R. apiculata* leaf extract 5.61 mm. *R. apiculata* leaf extract had the highest average zone of inhibition compared to *C. papaya* leaf and *T. catappa* leaf extracts. The difference in the average zone of inhibition of *C. papaya*, *T. catappa*, and mangrove leaf extract was due to the antibacterial compounds contained in each of the herbal leaf extracts having different amounts of active compounds.

Marshall et al (2015) reported that antibacterial compounds found in *C. papaya* leaf extract are alkaloid of 0.05 g, flavonoids of 2.80 g, saponin of 0.07 g, and tannin of 1.05 g. Whereas *T. catappa* leaf extract contains antibacterial compounds such as alkaloid of 1.20 g, flavonoid of 0.93 g, saponin of 2.67 g, tannin of 0.50 g. According to Okpako et al (2017), the leaf of *T. catappa* contains saponin, tannin, phenol and flavonoid as phytochemical compounds, which can surpress the growth of bacteria. A study conducted by Ekwueme et al (2015) showed that *R. apiculata* leaf extract contained active compounds in the form of alkaloid of 3.43 g, flavonoid of 2.67 g, saponins of 1.97 g, tannin of 4.75 g, steroid of 0.86 g, and terpenoids of 0.87 g.

Antibacterial activity compounds found in herbal leaf extracts have different mechanisms to suppress bacterial growth. According to Darsana et al (2012), the mechanism of action of alkaloids as antibacterial is to disrupt the constituent components of peptidoglycan in bacterial cells so that the cell wall layer is not formed intact and causes cell death. Flavonoid compounds serve to inhibit cell membrane function (Kumar et al 2012). While saponins serve to reduce surface tension so that permeability will rise or cell leakage will occur so that intracellular compounds will come out (Nuria et al 2009). Tanin works by inhibiting the synthesis of peptidoglycan so that the bacteria are unable to divide and cells are lyses due to osmotic and physical pressure which in turn bacterial cells die (Ngajow et al 2013).

The antibacterial activity test of *R. apiculata* leaf extract showed its ability as an antibacterial. Antibacterial testing of *R. apiculata* leaf extract can be seen in Table 4 above which is characterized by the formation of a clear zone which shows that at concentrations of 300 mg L^{-1}, 400 mg L^{-1}, 500 mg L^{-1}, 600 mg L^{-1}, and 700 mg L^{-1}, *R. apiculata* leaf extract was able to inhibit the growth of *V. parahaemolyticus.*

The measurement results of zone of inhibition diameter of *R. apiculata* leaf extract at a concentration of 300 mg L^{-1} and 400 mg L^{-1} were categorized as weak of which 5.09 mm and 5.39 mm, respectively. *R. apiculata* leaf extract at concentrations of 500 mg L^{-1}, 600 mg L^{-1}, and 700 mg L^{-1} were categorized as moderate of which 6.83 mm, 7.22 mm; and 8.17 mm, respectively. The value of antibacterial activity at a concentration of 700 mg L^{-1} had the highest zone of inhibition with an average value of the diameter of 8.17 mm which was categorized as moderate.

Conclusions. According to the research results, it can be concluded that:

1. The abundance of bacteria in the study site varied. The abundance of *Vibrio* sp. as a trigger for WFD is around 10^5 CFU mL^{-1} or can be said exceeding the safe limit of 10^4 CFU mL^{-1}.
2. Identification on positive control and positive shrimp intestine of WFD, type of *Vibrio* sp. found in the study sites were *V. parahaemolyticus*, *V. vulnificus*, and *V. alginolyticus.* The type of *Vibrio* sp. as a trigger for WFD is *V. parahaemolyticus*.
3. The best concentration of mangrove (*R. apiculata*) leaf extract in suppressing the growth of *V. parahaemolyticus* was 700 mg L^{-1}.

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