IDENTIFICATION OF A LOCAL PROBIOTIC BACTERIUM USING 16S rRNA GENE SEQUENCE THAT WAS USED FOR FIELD TRIAL TO ENHANCED WHITELEG SHRIMP (*Litopenaeus vannamei)* SURVIVAL

Tb. Haeru Rahayu[*)] and Ketut Sugama[**)]

[*)] Department of Aquaculture, Jakarta Fisheries University

[**)] Center for Aquaculture Research and Development

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ABSTRACT

The use of local probiotics in the culture of aquatic organisms is increasing with the demand for more environmental-friendly aquaculture practices. The local bacterium isolate considered as a probiotic was added into the water of whiteleg shrimp (*Litopenaeus vannamei*) culture in a field trial. Four rectangular plastic ponds (ca. 20 m x 30 m per pond) were used for 100 days experimentation for six consecutive crops in two years experiment. Survival, harvest size, feed conversion ratio (FCR) and *Vibrio* bacterial count was compared with those of shrimp receiving and none of local isolate. Identification based on 16S rRNA gene sequence shown those isolate was *Bacillus pumilus* strain DURCK14 with 99% homology. Water shrimp pond added a local isolate had significantly higher survival at about 10.0% to 11.7% than shrimp without added the isolate ($p<0.05$), and better FCR, but no significant different in shrimp harvest size. *Vibrio* bacterial was undetected by total plate count. Moreover, it shown better projected yields on an annual basis (three crops per year).

KEYWORDS: **shrimp pond, *Litopenaeus vannamei*, local probiotic, *Bacillus pumilus*, *Vibrio*, survival rate, harvest size, FCR**

INTRODUCTION

Microbes are being considered as both beneficial and detrimental roles in aquaculture ponds (Rheinheimer, 1992; Laurencin & Vigneulle, 1994; Valiela, 1995; Moriarty, 1997). On the beneficial side, they are important and essential components for the nutrient and elemental cycling required to maintaining water quality suitable for cultivation (Valiela, 1995; Moriarty, 1997). Conversely, bacteria and viruses can cause serious disease problems, with viral pathogens having the most serious economic impact on shrimp farming. White spot syndrome virus (WSSV), Taura Syndrome Virus (TSV), and *Vibrio harveyi* (cause of luminescent bacterial disease) are the three pathogens that account for the majority of losses in Indonesia shrimp culture by causing sudden and massive shrimp mortality (Flegel *et al*., 1992; Spaargaren, 1996; Lightner & Redman, 1998).

Successful shrimp culture requires a combination of factors, including larvae free from pathogens, application of nutritious feeds, physical exclusion of disease organisms and maintenance of proper aeration and suitable pond water quality (Boyd, 1998). Prophylactic, probiotic microbes are now being used widely for treatment of poultry, swine and other land animals to protect against pathogenic microbes (Fuller, 1997; Holzapfel *et al*., 1998). It is now being applied too in aquaculture and believed can improve the survival and growth (Staley & Stanley, 1986; Gatesoupe, 1999; Verschuere *et al*., 2000).

We previously isolated the local bacterium and demonstrated its probiotic properties with whiteleg shrimp *Litopenaeus* in small laboratory aquaria (Rahayu, 2009). *L. vannamei* receiving of a proper bacterium in the water had better survival after bacterial challenge tests and showed a high immune response compared with control shrimp without administered of *Bacillus* (Rahayu, 2009). Here we described field trials in earthen ponds in order to test efficacy of T28 isolate in conditions of commercial grow out ponds.

Correspondence: Department of Aquaculture, Jakarta Fisheries University. Jakarta Selatan 12520, Indonesia, Indonesia.
E-mail: *ketut_sugama@yahoo.com*

MATERIALS AND METHODS

Bacterium

Isolate T28 was taken from Jakarta Fisheries University culture collection (JFUCC). It was previously isolated from gastrointestinal tract of *Litopenaeus vannamei* and demonstrated its efficacy as a probiotic for *L. vannamei* in laboratory trials (Rahayu, 2009). The isolate maintained in marine agar and stored at 4°C and were grown in medium tryptic soy broth (TSB) for 24 hours at 30°C. The incubated isolate was mixed with sterilized fine bran and formed of pellets using pellet machine. The amount of isolate was determined by standard solution McFarland No. 6 (1.8 x 10^9 cfu/mL). Culture purity and identity were routinely checked during preparation by monitoring the unique and specific physical appearance of isolate on tryptic soy agar (TSA) (Rengpipat *et al*., 1998).

Pond Trial 1

Hatchery-reared *Litopenaeus vannamei* of 0.01-0.02 g body weight were stocked into four (20 m x 30 m) mini shrimp pond at 100 shrimps per m^2. Ponds were lined by high density polyethylene plastic with 90 cm depth. Salinity was 22‰ at first crop, in January 2012 and increased to 24‰ at second crop in April and eventually 25‰ in third crop in August 2012. Water aerated using two units of 1 HP paddle wheel. All shrimps were fed four times daily at 15% body weight at first month, and 3% body weight on the next following months. Treated shrimp pond (three ponds) received routine administration of a local isolate probiotic which was added into the water culture every other days at a concentration of 1 mg/L since the beginning at first stocking continuously until the end of the trial (100 days), and none for the control. Shrimp survival and weights were measured every 10 days beginning at Day 50^{th} by random sampling using lift net to check the shrimp health and feed determination. During the trial, water temperature and pH, were measured daily, while salinity, dissolved oxygen, ammonium, nitrite, nitrate, and alkalinity were measured every 10 days starting day 50 as described by Rengpipat *et al*. (2000). Total plate count was done at beginning and the end of the culture to examine the standing of *Vibrio* bacteria (van Stappen, 2006). There was no water exchange during the trial, except adding the fresh water due to evaporation.

Pond Trial 2

Conditions were nearly identical to those in Trial 1 with respect to shrimp stocking densities, tested parameters, feeding, monitoring, and experiment phase.

Molecular Isolate Identification

This step was initiated by preparation isolate for DNA isolation and template DNA for PCR. Isolate was grown in medium of marine agar (Difco) and incubated at 30°C to log phase stages for 48 hours. Template DNA was prepared by boiling method (Sjamsuridzal & Oetari, 2003). Amplification of 16S ribosomal RNA gene followed PCR protocol of Yuwono (2006) using universal bacterium primer, *Escherichia coli* bacterium in the position 9F and 1510R. Electrophoresis of PCR products was done according the protocol described by Lightner (1996) and visualized by using the gel documentation ultra violet trans-illuminator. Next following step was purifying PCR products by ethanol precipitation method and sodium acetate to remove excess primer. Suspended RNA , as a template RNA was used for cycle sequencing reactions followed procedures of Applied Bio Systems Inc., consisting of the big dye terminator ready reaction. Amplification product was purified to remove excess dye, primary, and minerals using ethanol precipitation method before sequenced. The last step, 16S ribosomal RNA gene sequence obtained was compared with the database of Gen-Bank using the Basic Local Alignment program Search Tool (BLAST) (Macrae, 2000) to obtain the identity of bacterium isolate (Saitou & Nei, 1987; Holmes, 2003).

RESULTS AND DISCUSSION

Whiteleg shrimp (*Litopenaeus vannamei*) added by local isolate T28 had significantly higher survival (Figure 1), i.e. 96.3% (first year/trial 1) and 95.7% (second year/trial 2) respectively compared to 85.7% and 84,0% respectively for the control. Significant survival differences began at day 60 after the start of both trials (Figure 2). After 100 days, mean individual weights (Figure 3) of the treated shrimp were 16.69 ± 0.1 g and 16.67 ± 6.1 g respectively. Meanwhile the control were 16.84 ± 0.1 g and 16.84 ± 0.1 respectively and showed no significant difference to the probiotic treatment ($P>0.05$). This represents a 10.6% and 11.7% higher survival than the control for Trial 1 and Trial 2, respectively. Projected yields per crop were 964.34 kg and 957.19 kg for treated pond in Trial 1 and Trial 2, respectively, and 865.91 kg and 848.73 kg for the controls in both trials.

The ammonium, nitrite, and nitrate concentrations in the treated ponds were about at maximum value of 0.05, 0.25, and 1.0 mg/L respectively for both trials, whereas the non-treated ponds shown over the standard limit i.e. 1.5, 1.3, and 3 mg/L respectively for both trials. The pH ranged from 7.4 to 7.8 respectively for treated ponds in Trial 1 and Trial 2 com-

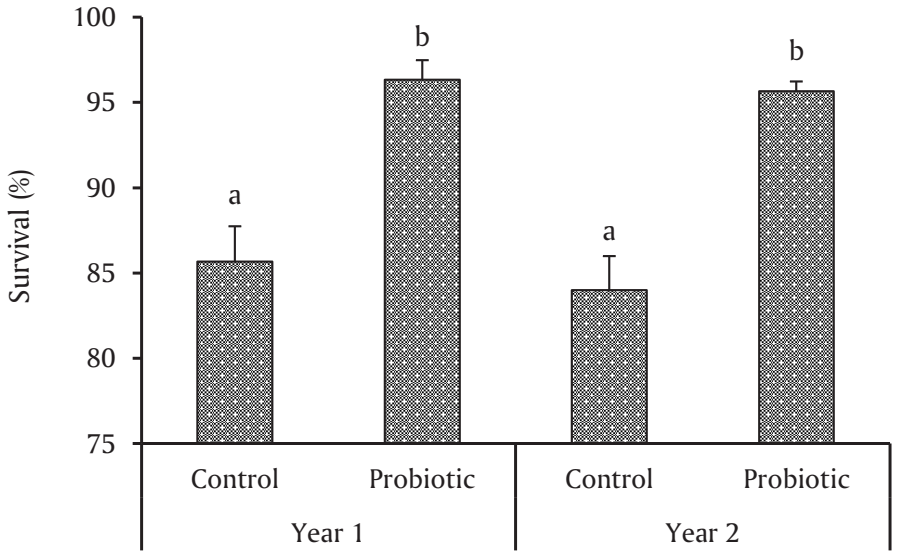

Figure 1. Shrimp survival (100 days culture) during the experiment

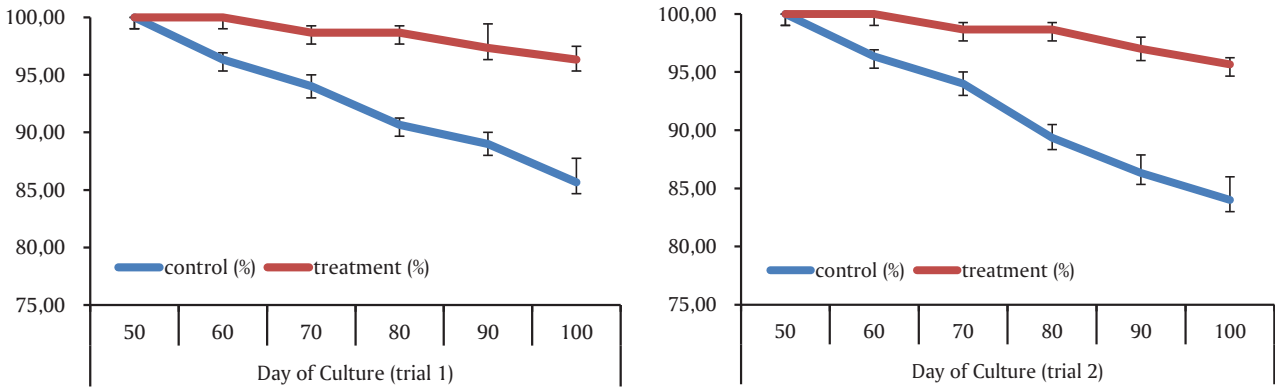

Figure 2. Shrimp mortality (100 days culture) during field experiment

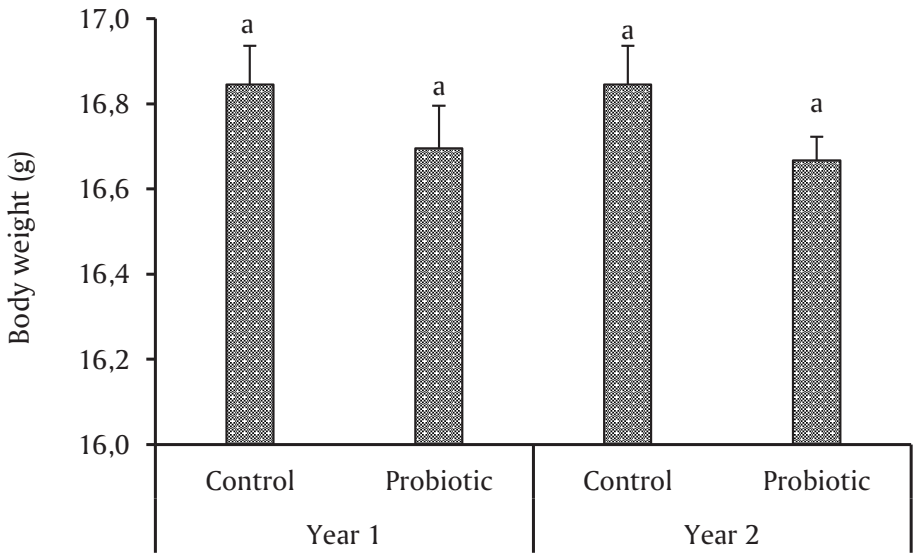

Figure 3. Individual shrimp body weight (100 days culture) during experiment

pared to 7.0 to 8.0 respectively for non-treated ponds during both trial. Whereas, water temperatures and other water quality values were essentially the same between ponds in each trial. Water temperatures ranged from 27°C to 30°C, respectively, for Trial 1 and Trial 2. Salinity ranged between 2°C and 4‰ for Trial 1 and Trial 2. Dissolved oxygen from both trials was never less than 4.0 mg/L during the day and night and total alkalinity ranged from 95 to 140 mg/L for both trials. Total plate count on *Vibrio* (Figure 5) was not detected on treated pond, while the control was about 1.0 x 10^3 cfu/mL.

Feed conversion ratio showed linear result that indicated a better value on treated pond (Figure 4) i.e. 1.28 ± 0,03 and 1.26 ± 0.04 as compared to 1.48 ± 0.03 and 1.49 ± 0.04 respectively for the control. This indicated a 15.62% to 18.25% more efficient use of feed in the probiotic treatments.

BLAST analysis showed that the isolate was from genus *Bacillus*. The species was *Bacillus pumilus* strain DURCK14 with 99% homology.

These results indicated that the benefits of a *Bacillus pumilus* supplementation seen in laboratory tests (Rahayu, 2009) could also be obtained in field trials conducted under normal commercial farming conditions. *Bacillus* known is the type of bacteria used for probiotics in aquaculture (Verschuere *et al*., 2000; Rengpipat *et al*., 2000; Balcazar *et al*., 2006; Geovanny *et al*., 2007). The genus of *Bacillus* is considered as cosmopolitan bacteria (can live in many areas). *Bacillus* has wide physiological tolerance to heat, acidity, and salinity (Holt *et al*., 1994). *Bacillus pumilus* was

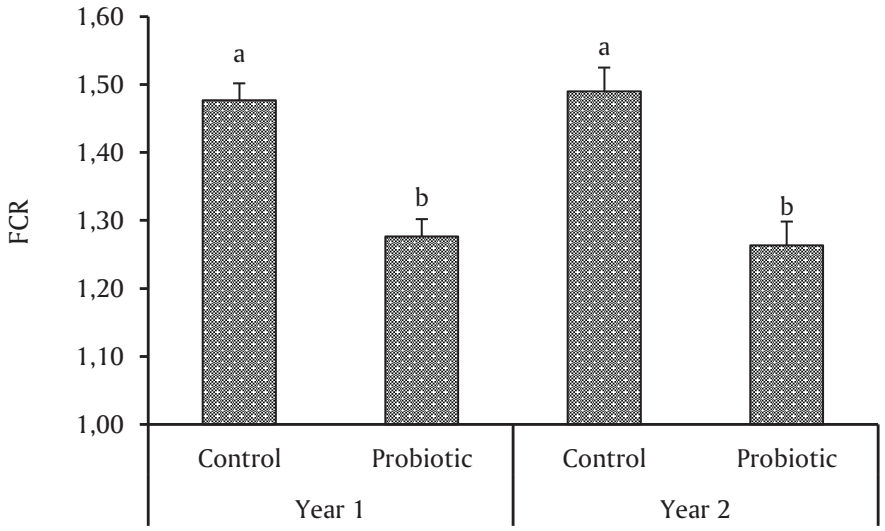

Figure 4. Feed conversion ratio (FCR) of shrimp (100 days culture) during experiment

Figure 5. Presenting of *Vibrio* on un-treated pond at the end of the shrimp culture (arrow shown *Vibrio* colony after 24 hours incubation)

considered having performance as mentioned above during the experiment as can be seen by the better production performance.

Better shrimp survival and no different on shrimp size showed that *Bacillus pumilus* managed on maintaining the water quality in 100 days experiment. This bacterium predicted conducting the nitrifying cycle and converting the organic matter into the tolerable matter (Verschuere, 2000). Whereas in control ponds where no bacterium was added, showed some increase in ammonia, nitrite, and nitrate concentrations. Stress can increase the shrimp susceptibility to pathogens even in low virulence (Song *et al*., 1993) and indeed suppressed the shrimp survival.

In addition, the effectiveness of *Bacillus pumilus* addition was not only considered as physical and chemical water purifier agent water, however, it supported the biological aspect as well, especially the microbial performance on the water (Verschuere, 2000). *Bacillus*, in general, can suppress the *Vibrio* population in the water (Marques *et al*., 2006). It was very important, since *Vibrio* is considered as opportunistic bacteria that may be pathogenic in shrimp culture (Balcazar *et al*., 2006). *Bacillus* was considered to be able to compete with the *Vibrio* sp. for chemicals, nutrients, and space (Verschuere *et al*., 2000; Geovanny *et al*., 2007; Rengpipat *et al*., 2003).

CONCLUSION

In conclusion, *Bacillus* contribution was not only improved the water quality, but also improved the shrimp performance and indeed improved the metabolism performance as well. Shrimp shown a more efficient on feed given during the trial.

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