International Journal of Agricultural Technology 2020 Vol. 16(2): 493-504
Available online http://www.ijat-aatsea.com
ISSN 2630-0192 (Online)

Effect of *Bacillus* sp. AAHMRU15 antagonist in combination with yeast extract product (Beta-Sac Plus®) on the inhibition of AHPND−causing *Vibrio parahaemolyticus* (Vp_{AHPND})

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Tep-Ubon, C., U-taynapun, K. and Chirapongsatonkul, N. (2020). Effect of *Bacillus* sp. AAHMRU15 antagonist in combination with yeast extract product (Beta-Sac Plus®) on the inhibition of AHPND−causing *Vibrio parahaemolyticus* (Vp_{AHPND}). International Journal of Agricultural Technology 16(2): 493-504.

Abstract Acute hepatopancreatic necrosis disease (AHPND) has caused huge losses in Pacific white shrimp (*Litopenaeus vannamei*) culture in Thailand since 2013. The causative agent of AHPND was originally reported to be a specific strain of *Vibrio parahaemolyticus* (Vp_{AHPND}). In this study, the *in vitro* antagonistic effect of *Bacillus* sp. AAHMRU15 against 4 strains of Vp_{AHPND} (VP01, VP02, VP03, and VP04) was clearly demonstrated. The effect of the yeast extract product named Beta-Sac Plus® (BS) on the inhibitory activity of AAHMRU15 was determined through agar well diffusion assay. The results suggested that BS increased the antibacterial activity. The real-time growth of Vp_{AHPND} cultured in the cell-free media containing extracellular products of AAHMRU15 alone (CFS) or in combination with Beta-Sac Plus® (CFS+BS) was monitored. The results indicated that the antagonistic *Bacillus* sp. AAHMRU15 and Beta-Sac Plus® were able to inhibit the growth of Vp_{AHPND}. The obtained data introduced the potential tool to control the pathogenic bacteria in white shrimp culture through biologically, environmentally friendly and safety practice. Our findings provide an alternative way to reduce the use of antibiotics in support to the concept of susceptible aquaculture and safety food.

Keywords: Antagonistic *Bacillus* sp., *Vibrio parahaemolyticus*–causing AHPND (Vp_{AHPND}), Beta-Sac Plus®, *Litopenaeus vannamei*

Introduction

Aquaculture industry has become an increasingly important food production sector. It has produced a large volume of products corresponding to the demands of customers worldwide. Given that, an intensive culture system became the trend to expand productivity. However, aquaculture has faced the problem of emergence or reemergence of several infectious diseases especially those which operates through the intensive culture system. Bacterial diseases are one of the emerging problems that cause losses in aquaculture. One

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example of bacterial diseases that has caused mortalities and economic losses in aquaculture is acute hepatopancreatic necrosis disease (AHPND). It has severely damaged the production of Pacific white shrimp (*Litopenaeus vannamei*) in Thailand since 2013. The causative agent of AHPND was originally a specific strain of *Vibrio parahaemolyticus* (Vp_{AHPND}) that carries plasmids with the genes encoding the Photorhabdus insect-related (Pir) A and Pir B toxins (Tran *et al*., 2013; Dangtip *et al*., 2015; Sirikharin *et al*., 2015; Xiao *et al*., 2017).

Antibiotics have been used as prophylactic and therapeutic agents; however, improper and extensive use of these substances has contributed to the selection, persistence and spread of antibiotic-resistant bacteria (Cabello *et al*., 2013). Several biological control strategies associated with the containment of pathogenic bacteria, promotion of health and welfare as well as improve the immunity of cultured or farmed aquatic animals have been investigated to replace the use of antibiotics (Akhter *et al*., 2015; Hai, 2015). Moreover, some practices have been developed addressing the concern on environmental-friendly regimen.

Probiotics have emerged as a good alternative measure instead of chemicals and antibiotics in aquaculture which assist in the prevention or protection of cultured species from diseases (Kuebutornye *et al*., 2019). However, the term "probiotics" as defined for aquaculture is considerably different from that for terrestrial animals and human in accordance with the intricate relationship of aquatic animals has with their ambient environment (Wang *et al*., 2019). The effective functions of probiotics in aquaculture have thus been focused on the aquaculture species and its ambient environment. Various probiotics, for instance, *Bacillus, Enterococcus, Lactobacillus, Lactococcus, Micrococcus, Enterobacter, Vibrio, Pseudomonas, Rhodopseudomonas, Roseobacter* and *Shewanella* have been reported as safe additive that are able to promote growth and enhance immunity of the aquaculture species (Nayak, 2010; Hai, 2015). The underlying functions of probiotics which are thought to be involved in health improvement of the host include provide nutrients, improve feed utilization, increase digestibility and digestive enzyme activities, modulate microbial colonization, improve immune responses, improve water quality, and control diseases (Selim and Reda, 2015).

Among the probiotics widely being used, *Bacillus* spp. have been a great interest in aquaculture because of their ability to produce various bioactive compounds that have antimicrobial activity and their sporulation capacity conferring their ability to survive and grow in diverse habitats (Ninawe and Selvin, 2009; Abriouel *et al*., 2011). Recent studies have shown the antagonistic effect of *Bacillus* spp. on the growth of pathogenic bacteria

belonging to *Vibrio* spp. (Tepaamorndech *et al*., 2019) and *Aeromonas hydrophila* (Kavitha *et al*., 2018). In addition, yeast has been generally used as probiotic in several animals, aquatic animals including humans due to its established health benefits. There has been reports on the beneficial effects of yeast in aquaculture in diverse aspects; improve animal growth, enhance survival rate of *Atremia* against *Vibrio* infection, and modulate cellular innate immune parameters (Vohra *et al*., 2016).

This present work aimed to evaluate the antagonistic efficiency of spore forming *Bacillus* sp. strain AAHMRU15 against the pathogenic Vp_{AHPND}. AAHMRU15 was tested for its antibacterial activity against 4 isolates of Vp_{AHPND}. In addition, the effect of yeast extract product named Beta-Sac Plus®, which has been typically used as a feed additive in shrimp farming, on the inhibitory efficacy of the test *Bacillus* sp. was determined.

Materials and methods

Bacterium, cultivation and chemicals

The *Bacillus* sp. AAHMRU15 was previously isolated from the gut of healthy Nile tilapia (*Oreochromis niloticus*) under sterile condition. It was preliminary tested for its antibacterial activity against some pathogenic bacteria. The pure AAHMRU15 was morphological characterized through Gram stain and endospore stain as well as molecularly identified based on 16S rRNA gene prior to being stored in 20% (v/v) glycerol broth at −80°C. For all experiments, AAHMRU15 was cultured in Tryptic Soy Broth (TSB, Difco) containing 1.5% (w/v) NaCl or TSB^{+}.

Beta-Sac Plus® (BS), the yeast extract product of Yeast Master Co., Ltd., was prepared as a stock at the concentration of 20% (w/v) in sterile distilled water. To increase the dissolution, the stock of Beta-Sac Plus® was crushed in the Stomacher® 400 Circulator (Seward) for 30 min. The mixed solution was then centrifuged at 8,000 × *g* for 15 min at 4 ℃. The supernatant was filtered through membrane filter pore size 0.45 μm (Whatman) then kept at −20 ℃ and used for further experiments.

AHPND−causing V. parahaemolyticus (Vp_{AHPND})

All Vp_{AHPND}: VP01, VP02, VP03 and VP04, used throughout this study were isolated from shrimps exhibiting clinical signs of AHPND. These pathogenic bacteria were confirmed for the species and the existence of the specific plasmid (pVA1) and gene encoding Pir toxin by multiplex PCR following the method described by Chirapongsatonkul *et al*. (2018).

Evaluation of antagonistic activity

The antagonistic activity of *Bacillus* sp. AAHMRU15 against 4 isolates of Vp_{AHPND} was determined by cross streak method following the methods of Ran *et al*. (2012) and Kavitha *et al*. (2018) with slight modification. The pure AAHMRU15 isolate, VP01, VP02, VP03, and VP04 were separately cultured in TSB^+ at 37 ℃, 200 rpm for 24 h. The test Vp_{AHPND} was streaked in the center of Tryptic Soy Agar (TSA, Difco) plate while *Bacillus* sp. AAHMRU15 was streaked perpendicularly near the line of Vp_{AHPND} and then incubated at 37 ℃. After 24 h, the growth of test bacteria on the plate was observed for irregular characteristics (in shape and/or margin).

Determination of the antimicrobial activity

The *in vitro* antimicrobial activity of AAHMRU15 against Vp_{AHPND} was measured by agar well diffusion according to the method of Ran *et al*. (2012) with slight modification. AAHMRU15 was grown in the TSB^+ medium at 37 ℃, 200 rpm for 24 h while it was cultured in TSB^+ containing 2% (w/v) Beta-Sac Plus® for the determination of the effect of this product on its antimicrobial activity. The media containing AAHMRU15 cells (CCM) and the cell-free supernatant (CFS), the medium which was collected after centrifugation at 10,000 $\times g$ for 10 min at 4 ℃ and filtered through membrane filter pore size 0.45 μm (Whatman), were used for antimicrobial activity assay.

Broth culture of each Vp_{AHPND} isolate, 24 h culture in TSB^+, was adjusted to a 0.5 McFarland turbidity standard and evenly swabbed onto Mueller-Hinton (MH, Difco) agar plates supplemented with 1.5% (w/v) NaCl. Six wells were punched from the Vp_{AHPND} swabbed agar plate and 100 μl of test media; culture media with 10^8 CFU/ml of AAHMRU15 cells and cell-free supernatant grown with 2% Beta-Sac Plus® (CCM•BS and CFS•BS, respectively) and those grown without 2% Beta-Sac Plus® (CCM and CFS, respectively) was added into each well. Sterile 1.5% NaCl and 2% Beta-Sac Plus® was also added in the well as control to verify the effect of the yeast extract product on Vp_{AHPND}. Inhibition zones were measured after 24 h incubation at 37 ℃.

Quantitative analysis of antimicrobial activity

Quantitative analysis of antimicrobial activity of AAHMRU15 was determined by detection the growth of the candidate Vp_{AHPND} strain according to the modified method of U-taynapun *et al*. (2018). Prior to testing, the test Vp_{AHPND} was subcultured twice then transferred into analytical medium and

adjusted to a final concentration of 2 x 10^6 CFU/ml. There were 3 different test media; (1) TSB and 1.5% (w/v) NaCl (1:1) as control, (2) TSB and 1.5% (w/v) NaCl (1:1) along with 10% (v/v) *Bacillus* cell-free supernatant (CFS) and (3) TSB and 1.5% (w/v) NaCl (1:1), 10% (v/v) *Bacillus* cell-free supernatant and 2% Beta-Sac Plus® (CFS+BS). Growth curve and specific growth rate of the test *Vp*$_{AHPND}$ was real-time monitored at 37^{o}C for 24 h using RTS-1C Personal bioreactor (Biosan).

Statistical analysis

Statistical analysis was done using SPSS 16.0 for Window (SPSS Inc.). Student's *t* test was used to evaluate the significance of differences in mean values of inhibition zone diameter produced by *Bacillus* sp. AAHMRU15 cells and the *Bacillus* cell-free supernatant with or without the effect of 2% (w/v) Beta-Sac Plus®. *P* value of <0.05 was considered statistically significant.

Results

Antagonistic activity of Bacillus sp. AAHMRU15

The antagonistic activity of AAHMRU15 against the pathogenic bacteria *Vp*$_{AHPND}$ was clearly demonstrated through cross streak technique. All test *Vp*$_{AHPND}$ isolates grew nearby AAHMRU15 exhibited irregular shape and margin as asymmetrical shaped line of growth as well as the indented growth and faded colony compared to those grew in the distance (Figure 1). However, it seemed that there was a variation of inhibitory effect of AAHMRU15 among *Vp*$_{AHPND}$ isolates.

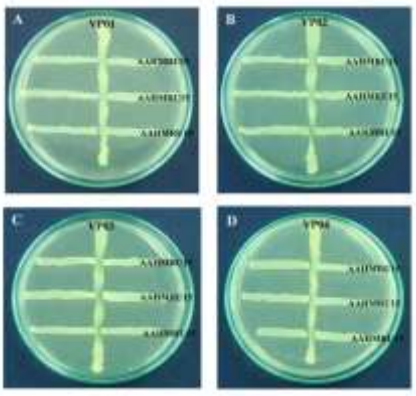

Figure 1. Antimicrobial activity of *Bacillus* sp. AAHMRU15 against *Vp*$_{AHPND}$ isolates; (A) VP01, (B) VP02, (C) VP03, and (D) VP04, conducted by cross streak technique

Effect of Beta-Sac Plus® on the antimicrobial activity of Bacillus sp. AAHMRU15

The effect of the yeast extract product, Beta-Sac Plus®, on the antimicrobial activity of the antagonist AAHMRU15 was evaluated in 2 different aspects. First, the effect on the production of extracellular substance associated with the containment of pathogenic bacteria *Vp*$_{AHPND}$ was determined by culturing AAHMRU15 in the medium containing 2% (w/v) Beta-Sac Plus®. The antimicrobial activity in the culture media with *Bacillus* cells (CCM) and cell-free media (CFS) were tested compared to those cultured in the media supplemented with Beta-Sac Plus® (CCM•BS and CFS•BS) by agar well diffusion method. The results showed that the inhibition zones produced by CCM•BS and CFS•BS were larger than those of CCM and CFS for all test *Vp*$_{AHPND}$ isolates (Figure 2). Control was also done by filling up the well with sterile NaCl (C) and Beta-Sac Plus® (BS) similar to the test culture media to verify the effect of the yeast extract product on *Vp*$_{AHPND}$. No clear zone was observed in the wells of sterile NaCl (C) and Beta-Sac Plus® (BS).

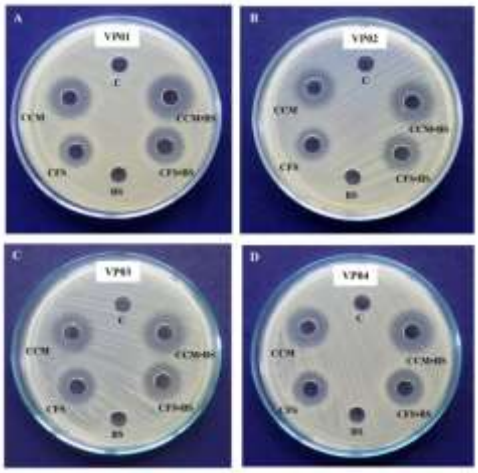

Figure 2. The antimicrobial activity of *Bacillus* sp. AAHMRU15 against the test *Vp*$_{AHPND}$ isolates; (A) VP01, (B) VP02, (C) VP03, and (D) VP04, revealed by agar well diffusion method. C: 1.5% (w/v) NaCl; BS: 2% (w/v) Beta-Sac Plus®; CCM; cell-containing medium of *Bacillus* sp. AAHMRU15; CCM•BS: cell-containing medium of AAHMRU15 grown in the culture medium supplemented with 2% (w/v) Beta-Sac Plus®; CFS: cell-free supernatant of AAHMRU15 culture medium; CFS•BS: cell-free supernatant of AAHMRU15 grown in the culture medium supplemented with 2% (w/v) Beta-Sac Plus®

Moreover, the diameters of inhibition zone were measured in all treatments and compared between the conditions with and without Beta-Sac Plus® (Table 1 and Table 2). For the cell-containing medium, the sizes of inhibition zone of the culture medium supplemented with the yeast extract product (CCM•BS) were significantly greater (P<0.05) than those observed in CCM for VP01, VP02 and VP04 while there was no significant difference for VP03 (Table 1). Similarly, the cell-free supernatant CFS•BS caused significantly larger inhibition zone than those of CFS for almost every test *Vp*$_{AHPND}$ isolates except VP03 (Table 2).

Table 1. Inhibition zone diameter (mm) of the test *Vp*$_{AHPND}$ isolates caused by cell-containing media of *Bacillus* sp. AAHMRU15 (CCM) and AAHMRU15 grown in the medium supplemented with 2% (w/v) Beta-Sac Plus® (CCM•BS)

***Vp*$_{AHPND}$ isolate**	**Inhibition zone (mm)**[1]		***P*-value**
	CCM	**CCM•BS**	
VP01	19.5±0.3	20.8±0.4*	0.00
VP02	15.8±0.4	16.9±0.3*	0.00
VP03	17.2±0.3	17.0±0.3	0.24
VP04	18.9±0.1	20.2±0.2*	0.00

* indicate statistical difference, P<0.05

1/: Mean±SD of inhibition zone diameters (n=6) determined by agar well diffusion method.

Table 2. Inhibition zone diameter (mm) of the test *Vp*$_{AHPND}$ isolates caused by cell-free supernatant of *Bacillus* sp. AAHMRU15 (CFS) and AAHMRU15 grown in the medium supplemented with 2% (w/v) Beta-Sac Plus® (CFS•BS)

***Vp*$_{AHPND}$ isolate**	**Inhibition zone (mm)**		***P*-value**
	CFS	**CFS•BS**	
VP01	15.3±0.3	18.6±0.2*	0.00
VP02	13.4±0.2	14.8±0.4*	0.00
VP03	15.5±0.3	15.2±0.3	0.26
VP04	14.7±0.3	17.3±0.4*	0.00

* indicate statistical difference, P<0.05

1/: Mean±SD of inhibition zone diameters (n=6) determined by agar well diffusion method.

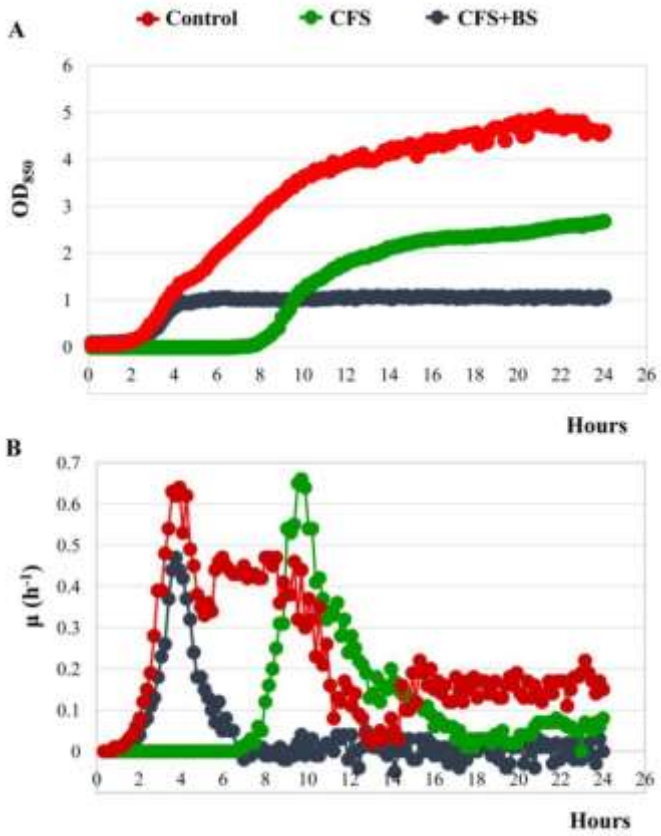

Figure 3. The real-time growth of *Vp*$_{AHPND}$ isolate VP03, (A) Growth curve and (B) Specific growth rate, grown in cell-free medium of *Bacillus* sp. AAHMRU15 (CFS) and in the combination of CFS and 2% (w/v) Beta-Sac Plus® (CFS+BS). VP03 cultured in TSB containing 1.5% (w/v) NaCl was observed as control

Secondly, the effect of Beta-Sac Plus® on the inhibitory activity of the *Bacillus* sp. AAHMRU15 was determined whether it impeded the action of the produced antimicrobial substance. The growth of VP03, a candidate of pathogenic *Vp*$_{AHPND}$, was real-time measured for 24 h and presented as a growth curve and specific growth rate. The inhibitory activity was found in both media containing cell-free supernatant of AAHMRU15 (CFS) alone and in combination with 2% (w/v) Beta-Sac Plus® (CFS+BS) (Figure 3). However, different inhibitory patterns were observed. VP03 cultured in CFS+BS attained the stationary phase quickly compared to those of control and CFS while the CFS caused longer lag phase duration compared to other treatments (Figure 3A). Moreover, the total bacteria represented by optical density (OD) in the

CFS+BS is lowest, followed by CFS and control. The specific growth rate was comparable to the obtained growth curve. In control, 3 peaks were observed throughout the experimental time. The peak of specific growth rate was delayed in the CFS+BS treatment while only one sharped peak was observed in the CFS treatment (Figure 3B). Specific growth rate in control was broad while it was narrower in the CFS treatment. Interestingly, there was no other peak observed at later time point in CFS treatment corresponding to the growth curve which reached a plateau or stationary phase.

Discussion

Shrimp farming is one of the most important aquaculture business and Pacific white shrimp or white leg shrimp has been reported as the main shrimp species being reared in Thailand. However, its production has decreased because of disease emergence. One of the diseases that cause economic losses in shrimp farming is AHPND which was first reported in China in 2009 and in Thailand in 2012 (Tinwongger *et al*., 2014). Many approaches have been applied to control *Vp*$_{AHPND}$, the causative agent of AHPND. In order to reduce the adverse results from improper use of antibiotics, development of probiotics is an alternative. *Bacillus* spp. have been proven for their efficiency in shrimp cultivation (Vaseeharan and Ramasamy, 2003; Shen *et al*., 2010; Zokaeifar *et al*., 2012; Tepaamorndech *et al*., 2019). Bacteria belonging to the genus *Bacillus*, *B. subtilis*, *B. velezensis*, *B. licheniformis*, *B. flexus*, and *B. aryabhattai,* have been shown to possess the antimicrobial activity against several pathogens in aquaculture (Interaminense *et al*., 2018; Kavitha *et al*., 2018; Yi *et al*., 2018; Cai *et al*., 2019; Kuebutornye *et al*., 2019; Tepaamorndech *et al*., 2019). In this present work, the antimicrobial activity of *Bacillus* sp. AAHMRU15 was demonstrated. The obtained results clearly indicated the potential antagonistic activity against the pathogenic *Vp*$_{AHPND}$. According to the result of agar well diffusion, we found that both cell-containing medium (CCM) and cell-free medium (CFS) of *Bacillus* sp. AAHMRU15 were able to inhibit the growth of all test *Vp*$_{AHPND}$ isolates. The antimicrobial activity of our *Bacillus* sp. might be the result of the produced antimicrobial compounds excreted into the culture medium. The real-time growth of *Vp*$_{AHPND}$ showed that the growth of *Vp*$_{AHPND}$ was inhibited by CFS. Therefore, this finding was the evidence supporting that the secreted antimicrobial substances contribute to the antimicrobial activity of *Bacillus* sp. AAHMRU15. Correspondingly, the members of *Bacillus* have been known to produce a wide range of extracellular substances and antimicrobial peptides against a variety of microorganisms (Abriouel *et al*., 2011; Sumi *et al*., 2015). Gao *et al*. (2017) have shown that *B. pumilus* strain H2 inhibited the growth of

Vibrio spp. through the produced bacteriocin amicoumacin A. Yi *et al*. (2018) have reported that there are 4 bacteriocin gene clusters in the genome of *B. velezensis* JW which exhibits the antimicrobial activity against fish pathogenic bacteria.

The effect of the yeast extract product, Beta-Sac Plus®, on the antimicrobial activity of *Bacillus* sp. AAHMRU15 was evaluated. The concentration of 2% (w/v) of Beta-Sac Plus® was used in this study since it was the recommended rate for practical application (mixed with shrimp feed). It seemed that Beta-Sac Plus®, at this concentration, increased the inhibitory efficacy since the inhibition zone diameters of Vp_{AHPND} caused by *Bacillus* sp. AAHMRU15 grown in the culture medium supplemented with Beta-Sac Plus® were larger than those grown without this product. These results suggested that there was no negative effect of Beta-Sac Plus® on either inhibitory activity or the produced antimicrobial compounds, on the other hand it could promote the inhibitory effect. The effect of Beta-Sac Plus® was additionally verified on the inhibitory activity of the antimicrobial compounds produced by *Bacillus* sp. AAHMRU15 to inhibit the growth of Vp_{AHPND}. The candidate isolate of Vp_{AHPND} was cultured in the media containing cell-free supernatant (CFS) alone (CFS) and in combination with Beta-Sac Plus® (CFS+BS). In comparison with the control, CFS and CFS+BS revealed the inhibitory activity against Vp_{AHPND}. Based on the observed growth curve, the lag phase was extended and the bacteria attained stationary phase quickly in the CFS treatment suggesting that the initial number of Vp_{AHPND} inocula was lower than that of the control. Moreover, the OD value represent the number of bacterial cells was lowest in CFS+BS and much lower than those of CFS and control indicating that there might be some factors which were able to restrict the number of Vp_{AHPND}. This result corresponded to the specific growth rate of which the peak was delayed and sharp suggesting that there was no bacteria grew in later time point.

Conclusion

In conclusion, this work demonstrated the potent antimicrobial activity of *Bacillus* sp. AAHMRU15 isolated from the gut of healthy Nile tilapia against the causative agent of AHPND, Vp_{AHPND}. In addition, the product Beta-Sac Plus® increased the inhibitory ability to restrict the number and growth of Vp_{AHPND}. Therefore this product could be applied together with *Bacillus* sp. AAHMRU15 to control the growth of Vp_{AHPND}. Our findings provided the evidence that *Bacillus* sp. strain AAHMRU15 and Beta-Sac Plus® are good biological agents to control the pathogen growth which can be applied in protection of infectious diseases caused by *Vibrio* spp. However, other important beneficial capability of *Bacillus* sp. AAHMRU15 to improve growth

performance, increase digestive enzyme activities, and enhance host immunity and disease resistance, should also be investigated to prove that this bacterium has a potential to be developed as a probiotic agent in aquaculture.

Acknowledgement

This work was supported by Research and Research Industries, RRI (grant number MSD62I0037).

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(Received: 11 August 2019, accepted: 20 February 2020)