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# Evaluation of Antagonism Activity of Potential Malaysian Probiont Strains, *Bacillus* spp. JAQ04 and *Micrococcus* spp. JAQ07 in *in vitro* Condition and on *Artemia fransisca* against *Vibrio alginolyticus*

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## ABSTRACT

Vibriosis caused by *Vibrio alginolyticus* has become one of the most threatening diseases that could limit the production of marine fish in aquaculture industry. In this study, microbe strains *Micrococcu*s spp. (JAQ07) and *Bacillus* spp. (JAQ04) were used as potential probiotics. Both potential probionts were identified as gram-positive bacteria with different morphology. The antagonistic ability of each candidate probiotics towards *V. alginolyticus* (ATCC33839) were conducted in liquids modes via co-culture assay in three different concentrations of probiont ($10^2$, $10^4$ and $10^6$ CFU $mL^{-1}$) and each concentration was inoculated with $10^5$ CFU $mL^{-1}$ of *V. alginolyticus*. The effectiveness of antagonistic activity was measured by the reduction of *V. alginolyticus* colonies via plate count at 24 h interval for 120 h. The co-culture assays revealed the reduction of *V. alginolyticus* colonies by both probiont strains compared to the control (*V. alginolyticus* at $10^5$ CFU $mL^{-1}$). In *in-vivo* assay, JAQ04 was able to enhance the survival of *Artemia* compared to JAQ07 after challenged with *V. alginolyticus*. Results revealed that at seven days after inoculation, *Artemia* treated with at $10^6$ CFU $mL^{-1}$ cell density followed by challenged with *V. alginolyticus* showed 70% of survival, while *Artemia* challenged with only *V. alginolyticus* demonstrated a 20% survival rate. Since, both strains displayed excellent probiotic activities, they are indeed suitable probiont candidates for managing Vibriosis infecting marine fish.

**Key words:** Probiotics, *Bacillus* spp., *Micrococcu*s spp., *Vibrio alginolyticus*, *Artemia*

## INTRODUCTION

Aquaculture is one of the fast and rapid growing food industry in the world. According to FAO (2012), the commercialization of products derived from aquaculture sector has been increased from 78 091 908 t in 2010, to 83 729 313 t in 2011. However, disease outbreaks have been the most important constraint towards its commercialization. Several contributing factors such as weather, high stocking density or bad water management will subsequently lead to infection of viruses, fungi, bacteria and parasites on the cultured fish (Moriarty, 1997).

Vibriosis is a common bacterial fish disease caused by *Vibrio* species, which generally affect marine fish species to a greater loss. In most cases, marine fish infected with this disease become hemorrhage with superficial skin lesion and septicemia (Egidius *et al*., 1986; Colwell and Grimes, 1984).

Early solution of treatment for controlling fish diseases is by means of antibiotics. The use of antibiotics to manage diseases was widely accepted in the beginning, only to be discovered that they later initiated to the emergence of numerous antibiotic-resistant bacteria (Balcazar *et al*., 2008). As a remedy, an alternative solution by applying bacterial probiotics such as lactic acid bacteria was introduced to promote the development of antibiotic-resistant bacteria in fish and the environment (Villamil *et al*., 2003; Verschuere *et al*., 2000).

Probiotics are bacteria that enhance the health of other organisms (Balcazar *et al*., 2006). The mechanisms of the probiotics include pathogen inhibition through production of antagonistic compounds, competition for essential nutrients and attachment sites, alteration of enzymatic activity for a greater immunity response, feed digestibility and modulation of interactions within the environments (Gomez and Balcazar, 2008; Bomba *et al*., 2002; Verschuere *et al*., 2000). Literally, the selection of probiotics candidate was based on their *in vitro* antagonism ability, the adhesion results as well as colonization and growth in intestinal mucus (Vine *et al*., 2006; Verschuere *et al*., 2000).

Thus, the present study was carried out to elucidate the antagonistic ability of two probiont candidates, strains, *Bacillus* JAQ04 and *Micrococcus* JAQ07 against *V. alginolyticus* by performing the *in vitro* and *in vivo* tests.

## MATERIALS AND METHODS

**Bacterial cultures and growth conditions:** Two probiotics strains, previously identified by Nurhidayu *et al.* (2012) as *Bacillus* spp. (JAQ04) and *Micrococcus* spp. (JAQ07) and a pathogen strain, *Vibrio alginolyticus* (VA) were obtained from Aquatic Biotechnology Laboratory, Department of Aquaculture, UPM. All the bacteria were cultured using Tryptic Soy Agar (TSA; Oxoid, UK) with addition of 1.5% sodium chloride (NaCl). Both probionts and pathogen were incubated at 30 and 37°C, respectively. The strains were later cultured in Tryptic Soy Broth (TSB; Oxoid, UK) with addition of 1.5% NaCl and incubated overnight in an incubator shaker at 30°C of 120 rpm.

**Morphology observation:** Gram-staining procedure was performed for morphological identification of the probionts based on protocol by Gram (1884).

**Co-culture assay:** Each of the broth culture of probiotics treatments ($10^2$, $10^4$ and $10^6$ CFU $mL^{-1}$) was separately inoculated into 10 mL of TSB supplied with 1.5% NaCl. All combination was done in triplicates and the remaining broth culture (control) was incubated at optimum condition for 120 h. The total viable count of *V. alginolyticus* was estimated by withdrawing 100 μL of each 8 fold serial dilution of each treatment into triplicates. All treatment was spreaded onto Thiosulfate-Citrate-Bile Salts-sucrose (TCBS) agar plates using a glass hockey stick and incubated for 24 h. Colonies produced were counted under a colony counter (ROCKER galaxy 230, Taiwan) and all data was recorded for further assessment. Each of the procedure was performed at 0, 24, 48, 72, 96 and 120 h.

***Artemia* bacterial challenge:** Post-hatched *Artemia nauplii* were pre-incubated with probiont strains, JAQ04 and JAQ07 at three different concentrations ($10^2$, $10^4$ and $10^6$ CFU $mL^{-1}$) in triplicates. On the following day, *V. alginolyticus* was added to the respective treatments at $10^5$ CFU $mL^{-1}$ cell concentrations. All tubes containing treatments and control (VA only) was kept in shaker in order to provide aeration and was maintained at the room temperature. The mortality rate of each treatment and control tubes was observed and recorded daily. The observation was dismissed once the control tube achieved a 50% mortality rate.

The *Artemia* were separated from culture water of each treatment by passing over the *Artemia* using a sterile 100 µm mesh. *Artemia* culture trapped in the sieve were rinsed with Sterile Sea Water (SSW) and re-suspended in 1 mL SSW. In order to determine the count of *Vibrios* loaded into the *Artemia* and culture water, a 100 µL suspension from each sample was plated on TCBS agar in triplicates. The plates were incubated for 24 h at room temperature (26°C). During the next day, colonies produced were counted using a colony counter (ROCKER galaxy 230) and calculated as CFU $mL^{-1}$ using the formula:

$$\text{Concentration of bacteria} = \left( \frac{\text{No. of CFU}}{\text{Volume plated}} \right) \times \text{Total dilution}$$

**Statistical analysis:** All data was analyzed using a One-way Analysis of Variance (ANOVA). Multiple comparison tests (Duncan's and Tukey's-tests) were performed using SPSS Statistic 2.0 software. Results were denoted as Mean±Standard Error and the differences were considered significant at $p<0.05$.

## RESULTS

**Morphology observation:** The morphology results of probiont strains, JAQ04 and JAQ07 are presented in Table 1. Based on gram-staining, strain JAQ07 was classified as gram-positive with cocci shaped (Stackebrandt *et al*., 1980). Instead, another probiont strain, JAQ04 was also classified as gram positive but with rod shaped (Claus and Berkeley, 1986).

**Co-culture assay:** Both strains JAQ04 and JAQ07 were able to inhibit the growth of *V. alginolyticus* at every given concentration in broth culture assays (Fig. 1 and 2). However, when

Table 1: Morphological identification of probiont strains JAQ04 and JAQ07 based on gram staining

| Codes | Gram reaction | Cellular morphology | Description |
|---|---|---|---|
| A1 | Positive | Cocci | JAQ07 at 24 h incubation |
| A2 | Positive | Cocci | JAQ07 at 48 h incubation |
| A3 | Positive | Cocci | JAQ07 at 72 h incubation |
| B1 | Positive | Rods | JAQ04 at 24 h incubation |
| B2 | Positive | Rods | JAQ04 at 48 h incubation |
| B3 | Positive | Rods | JAQ04 at 72 h incubation |

A1-A3- JAQ07: *Micrococcus* spp., B1-B3-JAQ04: *Bacillus* spp.

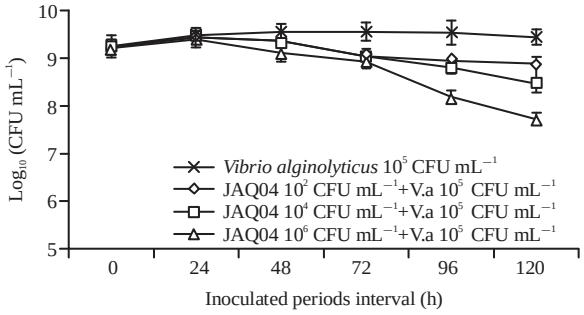


Fig. 1: Growth pattern of *Vibrio alginolyticus* $10^5$ CFU $mL^{-1}$ with *Bacillus* spp. JAQ04 at different cell densities ($10^2$, $10^4$ and $10^6$ CFU $mL^{-1}$) along with inoculation periods interval

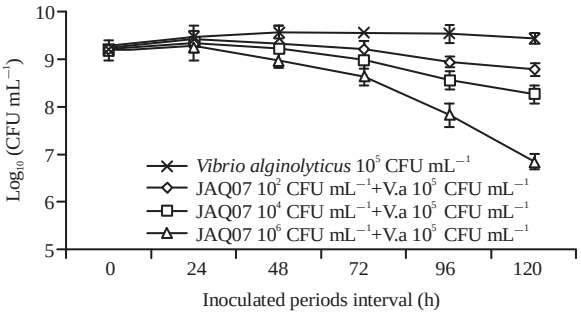


Fig. 2: Growth pattern of *Vibrio alginolyticus* $10^5$ CFU $mL^{-1}$ with *Micrococcus* spp. JAQ07 at different cell densities ($10^2$, $10^4$ and $10^6$ CFU $mL^{-1}$) along with inoculation periods interval.

there was *V. alginolyticus* alone with no probiont strain added, the cell count increased from $10^5$-$10^8$ CFU $mL^{-1}$ within 24 h of incubation. The cell count was consistent at $10^9$ CFU $mL^{-1}$ after 120 h incubation (48-120 h).

Multiple means comparison between different concentrations of treatments disclosed significant differences ($p<0.05$) for both probiotic strains (JAQ04 and JAQ07). At initial concentration of $10^5$ CFU $mL^{-1}$, the growth of *V. alginolyticus* was inhibited by strains JAQ04 and JAQ07 at all concentrations ($10^2$, $10^4$ and $10^6$ CFU $mL^{-1}$) within 120 h. Moreover, all bacterial concentrations of strains JAQ04 and JAQ07 allowed an initial increment of *V. alginolyticus* cell density at 0-24 h, followed by decrement of the pathogen cell density at 48, 72, 96 and 120 h, respectively. At lower concentrations ($10^2$ and $10^4$ CFU $mL^{-1}$), both probiotics allowed initial growth of *V. alginolyticus*. At higher concentrations ($10^6$ and $10^6$ CFU $mL^{-1}$), the probiotics offered the best result by rapidly decreasing *V. alginolyticus* cell densities.

These experiments demonstrated that within *in vitro* conditions, co-culture assay of JAQ04 and JAQ07 strains at various increasing concentrations and prolonged incubation periods successfully inhibited the growth of the *V. alginolyticus*.

**Bacterial challenge and pathogens count:** After 7 days of observation, no significant difference was found on the survival of *Artemia* treated with both probionts JAQ04 and JAQ07 and the control (Fig. 3a-b). Thus, our results demonstrated that both probionts were not harmful to the *Artemia*. Meanwhile, the survival rate of the *Artemia* was more than 60% after challenged, indicates the potential of these probiont strains in protecting the *Artemia* against *V. alginolyticus*. Indeed, as the concentrations of the probiotics were greater, the survival rate was also increased. In contrast, *Artemia* challenged with only *V. alginolyticus* showed significant differences ($p<0.05$) with other treatments, with the survival rate of 20%. The results indicated that *V. alginolyticus* was pathogenic to *Artemia*.

In this experiment, we also revealed that the highest concentration of the probiont strain JAQ07 was able to reduce *vibrios* load both in the *Artemia* and the culture water as presented in Fig. 4a-b. Concentration of JAQ07 strain at $10^6$ CFU $mL^{-1}$ (T11) significantly reduced the number of *vibrios* in *Artemia* and culture water, compared to *V. alginolyticus* only (T2) ($p<0.05$). Meanwhile, JAQ04 strain was not able to reduce the number of *vibrios* in both *Artemia* and culture water at any tested concentrations.

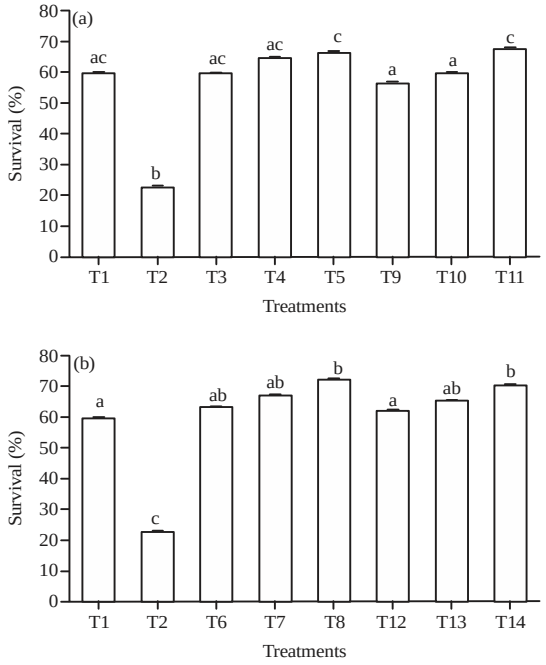


Fig. 3(a-b): Survival of *Artemia* with JAQ07 treatments after 7 days observation. (a) T1: Control, T2: VA $10^5$ CFU $mL^{-1}$, T3: JAQ07 $10^2$ CFU $mL^{-1}$, T4: JAQ07 $10^4$ CFU $mL^{-1}$, T5: JAQ07 $10^6$ CFU $mL^{-1}$, T9: JAQ07 $10^2$+VA $10^5$ CFU $mL^{-1}$, T10: JAQ07 $10^4$ +VA $10^5$ CFU $mL^{-1}$ and T11: JAQ07 $10^6$+VA $10^5$ CFU $mL^{-1}$ and (b) T1: Control, T2: VA $10^5$ CFU $mL^{-}$1), T6: JAQ04 $10^2$ CFU $mL^{-1}$, T7: JAQ04 $10^4$ CFU $mL^{-1}$, T8: JAQ04 $10^6$ CFU $mL^{-1}$, T12: JAQ04 $10^2$+VA $10^5$ CFU $mL^{-1}$, T13: JAQ04 $10^4$+VA $10^5$ CFU $mL^{-1}$ and T14: JAQ04 $10^6$+VA $10^5$ CFU $mL^{-1}$. Error bars indicate the Standard Error

## DISCUSSION

Probiotics have been widely used in fish culture to increase the survival and reduce the antibiotics usage. In these present studies, we have reported results that disclosed the potential of two non-pathogenic probiont strains, *Bacillus* spp. JAQ04 and *Micrococcus* spp. JAQ07 in controlling the growth of a destructive fish pathogen, *V. alginolyticus* within *in vivo* and *in vitro* conditions. Initially, both of the probiont strains, which were isolated from the intestines of 51 juvenile tiger grouper were preliminary evaluated as candidate bacterial probiotics by Nurhidayu *et al.* (2012).

Co-culture experiments revealed that the inhibitory activity of JAQ04 and JAQ07 increased along with the increasing density of the antagonist at 120 h after incubation. In our study, we found out that both of these antagonist strains must be presented at significantly higher levels than the fish pathogen *V. alginolyticus*, as the degree of inhibition elevated proportionally with the level of antagonistic activity. Our results were also in accordance with Nurhidayu *et al.* (2012), indicated that JAQ04 and JAQ07 were able to produce inhibitory substances only after 72 h of incubation in liquid mode.

Pre-incubation of probiotics in *Artemia* culture showed the ability of these probionts to increase the survival of the *Artemia* compared to the control culture without the probionts. On the other hand, both probionts were able to confer protection to the *Artemia* after challenged with

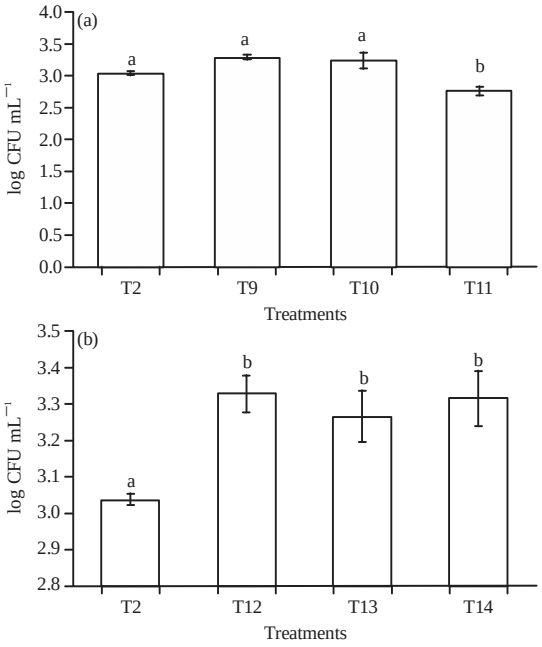


Fig. 4(a-b): Means of *Vibrio* spp. loaded in *Artemia* together with JAQ07 treatments. (a) T2: VA $10^5$ CFU $mL^{-1}$, T9: JAQ07 $10^2$+VA $10^5$ CFU $mL^{-1}$, T10: JAQ07 $10^4$+VA $10^5$ CFU $mL^{-1}$ and T11: JAQ07 $10^6$+VA $10^5$ CFU mL and (b) T2: VA $10^5$ CFU $mL^{-1}$, T9: JAQ07 $10^2$+VA $10^5$ CFU $mL^{-1}$, T10: JAQ07 $10^4$ +VA $10^5$ CFU $mL^{-1}$ and T11: JAQ07 $10^6$+VA $10^5$ CFU mL. Error bars indicated the Standard Error (SE)

*V. alginolyticus*. The level of protection was different depending on the relative concentrations of candidate probionts added. The results suggested that the probionts provided beneficial effects to the *Artemia*. Moreover, *Bacillus* and *Micrococcus* strains were proved not harmful and some of them could be beneficial probiotics for animals (Ryan *et al*., 2004).

Interestingly, we discovered that a cell density at $10^6$ CFU $mL^{-1}$ of probiont strain JAQ07 was required to inhibit the growth of *V. alginolyticus*. In contrast, probiont strain JAQ04 was not able to reduce *V. alginolyticus* loaded in both culture water and *Artemia* at any concentrations. These results suggested JAQ07 strain might potentially work by killing the pathogenic *V. alginolyticus,* perhaps with the combination of other probiotic mechanisms. In addition, JAQ04 strain acts by competing for essential nutrients and attachment sites, while the alteration of its enzymatic activity may lead to development of immunity response and increase feed digestibility and utilization (Verschuere *et al*., 2000).

Probiotics treatment offers a very promising alternative solution to combat diseases in fish and shrimp aquaculture. Our studies add into evidence that both JAQ04 and JAQ07 strains are safe as probiotics to be applied in aquaculture. However, further researches are needed to elucidate the exact mode of action to observe the beneficial effects and to understand the possibilities and limitations of microbial control in aquaculture.

## CONCLUSION

These two probiont strains have demonstrated efficient probiotic properties, thus they can be used as potential probionts in aquaculture system against Vibriosis.

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