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Bacillus cereus; JAQ04 Strain as a Potential Probiotic for Red Tilapia; *Oreochromis* Species

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ABSTRACT

The present study evaluated the inhibitory effect of *Bacillus cereus* strain JAQ04 against several pathogenic bacteria for tilapia under *in vitro* conditions. *B. cereus* strain JAQ04 was tested against *Vibrio alginolyticus*, *Aeromonas hydrophila* and *Pseudomonas putida* in a cross streaking assay, where *B. cereus* showed a strong antagonistic effect against the three tested bacterial fish pathogens. In the following test, *B. cereus* strain JAQ04 at 10^3, 10^5 and 10^7 CFU mL^{-1} was then tested against *V. alginolyticus* at 10^3 CFU mL^{-1} for 6 days in co-culture assay. The results revealed that higher concentrations (10^5 and 10^7 CFU mL^{-1}) of *B. cereus* strain JAQ04 had higher inhibitory effect against pathogenic *V. alginolyticus*. *Bacillus cereus* strain JAQ04 was administered to juvenile tilapia to determine its safety for tilapia. The results showed that the Relative Percent Survival (RPS, %) was 70.83+18.2 which was not significantly different from the RPS of fish in the control group (78.50+3.5%).

Key words: *Bacillus cereus*, probiotic, *Vibrio*, *Aeromonas*, *Pseudomonas*, tilapia

INTRODUCTION

Aquaculture is considered to be an important economic activity in many countries. Mass production by intensive culture systems has led to a variety of complications. High stocking density induces stress to the cultured fish making them more susceptible to diseases which may lead to severe economic loss (Wang *et al*., 2008). The utilization of antibiotics and disinfectants to control bacterial diseases has resulted in resistant strains (Moriarty, 1999; Barker, 2000; Subasinghe, 2009). Jackson *et al*. (2010) reported about the growing problems caused by evolution and mutation of bacteria to pathogenic bacteria.

Aquaculturists are now resorting to the use of probiotics that have been proved to be better option as they are environmentally sound (Gatesoupe, 1999; Vine *et al*., 2006; Wang *et al*., 2008). FAO/WHO (2001) and Reid *et al*. (2003) defined probiotics as "live microorganisms which when administered in adequate amounts confer a health benefit on the host". Studies have proved that several Gram-positive spore forming *Bacillus* species have the potential to serve as probiotics as well as biocontrol agents in aquaculture (Hong *et al*., 2005). They are able to exhibit competitive exclusion of pathogenic bacteria via producing inhibitory compounds, enhancing the immune response of host, improving water quality and producing supplemental digestive enzymes that improve nutrition of the host species (Thompson *et al*., 1999; Verschuere *et al*., 2000; Qi *et al*., 2009; Sun *et al*., 2010).

According to Nicholson (2002), *Bacillus* species are saprophytic Gram-positive bacteria that can be easily found in soil, water, dust and air. This means that it can be found in almost anywhere since it is dispersible in dust and water. *Bacillus cereus* has been known to be used as probiotics in farmed animals such as swine, calves, poultry and rabbits as well as for human use (Hong *et al.*, 2005). Although, there have been reports that *B. cereus* is pathogenic towards human but there are strains that are deemed safe as health food supplements or novel foods (Hong *et al.*, 2005; Mietke *et al.*, 2010).

Recently, *Bacillus cereus* has been demonstrated to be able to inhibit the pathogenic *Aeromonas hydrophila* in an *in vitro* study (Lalloo *et al.*, 2007, 2008). *B. cereus* strain JAQ04 was isolated from the intestine of tiger grouper, cultured in a farm in Kelantan, Malaysia (Nurhidayu *et al.*, 2012). Using the cross-streaking method, antagonism test was done against several common pathogens, where *B. cereus* strain JAQ04 exhibited an inhibitory effect against *V. alginolyticus* ATCC33839, *V. parahaemolyticus* ATCC43996, *V. harveyi* ATCC35084 and *A. hydrophila* ATCC35654. These vibrios are common pathogens found to infect marine fish while *A. hydrophila* is a common pathogen infecting freshwater fish species, especially carps. Haemolysis test showed that it is also safe for the host fish. The strain is susceptible to 6 different antibiotics (chloramphenicol, tetracycline, streptomycin, kanamycin, gentamicin and ampicilin) (Nurhidayu *et al.*, 2012). This study was done to study the antimicrobial activity of *B. cereus* strain JAQ04 against *V. alginolyticus*, *A. hydrophila* and *P. putida*, to detect the effect of different concentrations of *B. cereus* strain JAQ04 against pathogenic *V. alginolyticus* and to determine the safety of *B. cereus* strain JAQ04 on juveniles of the freshwater red tilapia hybrid.

MATERIALS AND METHODS

Bacterial strain and culture conditions: *Bacillus cereus* JAQ04, used in this study, was isolated from the gut of a healthy adult tiger grouper (Nurhidayu *et al.*, 2012). The bacterium was confirmed using the BBL CRYSTAL™ identification system and 16S rRNA gene sequencing. *Aeromonas hydrophila* and *Pseudomonas putida* were isolated from infected tilapia. The pathogenic *Vibrio alginolyticus* ATCC33839, *A. hydrophila* and *P. putida* were also identified using standard molecular methods. *Bacillus cereus* strain JAQ04 was picked from glycerol stock and grown on Tryptic Soy Agar (TSA) plate for 24 h at 25°C. A colony from the TSA plate was then picked and cultured aerobically in Tryptic Soy Broth (TSB) supplemented with 1.5% NaCl (w/v) in a shaking incubator for 24 h at 25°C. *V. alginolyticus*, *A. hydrophila* and *P. putida* were cultured using the same method and same culture condition.

Antimicrobial activity assay: *B. cereus* strain JAQ04, *V. alginolyticus*, *A. hydrophila* and *P. putida* were grown in TSB medium separately at 25°C in an incubator shaker for 24 h. Through plate counting, the concentration of *V. alginolyticus*, *A. hydrophila* and *P. putida* were adjusted to 10^6 CFU mL^{-1} while *B. cereus* strain JAQ04 was adjusted to 10^5 CFU mL^{-1}. The cross streaking method was done according to Annuk *et al.* (2003) and Hill *et al.* (2009) with slight modifications. Cross-streaking for testing the antimicrobial activity of probiont, *B. cereus* strain JAQ04 was carried out at 0, 24, 48 and 72 h pre-incubation. A sterile cotton swab was dipped into the inoculated probiont broth and streaked vertically down the center of TSA plate to form a single line with a width of 1 cm. This was done to all 4 plates simultaneously. The first plate (0 h pre-incubation) was

cross-streaked with *V. alginolyticus*, *A. hydrophila* and *P. putida* from the center to the edge of the plate. The rest of the plates were pre-incubated at 24, 48 and 72 h, respectively before being crossly-streaked. Upon cross-streaking, each plate was then incubated at 25°C for 24 h. Three replicates were maintained for each plate. After 24 h of incubation, the clear zones of inhibition were measured from the edge of the vertical streak to the closest edge of the first neighboring colony of the cross-steak to the nearest mm and then recorded.

Co-culture assay: The probiont and pathogenic bacteria, *V. alginolyticus* were pre-cultured separately in TSB at 25°C for 24 h. *V. alginolyticus* was inoculated into TSB at an initial level of 10^3 CFU mL^{-1} while the initial concentrations of *B. cereus* were 10^3, 10^5 and 10^7 CFU mL^{-1} . The co-culture tubes were then incubated at 25°C and 180 rpm in an incubator shaker. During incubation, samples were taken at 0, 4.5, 24, 48, 72, 96 and 120 h for observation of the competitive relationship between candidate probiotic and the pathogenic *Vibrio*. All treatments were triplicates.

Safety test of *B. cereus* strain JAQ04 on tilapia juveniles: The experimental design was adapted and modified according to Guo *et al.* (2009). The probiont was grown separately in TSB at 25°C for 24 h. Tilapia juveniles with average total length of 8 cm were obtained from a fish hatchery in Selangor. The fish were stocked in plastic tanks and fed with a commercial starter pelleted feed for 5 days of acclimatization. The juveniles were then randomly selected and distributed into nine plastic tanks (10 L) at a rate of 8 fish per tank in a Complete Randomized Design (CRD). The probionts were then added into the water of 3 tanks at an initial concentration of 10^5 CFU mL^{-1}. Another 3 tanks had commercial probiotics added according to the given instructions. Nothing was added into the remaining 3 tanks as control. Fish were fed twice daily at a feeding rate of 5% body weight. Daily 20% water change was conducted during the experiment period. Dead fish were recorded daily for duration of 7 days upon start of the biological experiment.

Determination of cumulative mortality and relative percent survival (RPS): The cumulative mortality was tabulated at the end of the experiment and the RPS was calculated.

Statistical analysis: The results were analyzed using one-way analysis of variance (ANOVA). All statistics were performed using SPSS 16.0.

RESULTS

Antimicrobial ability: The cross-streaking assay of *B. cereus* strain JAQ04 exhibited inhibitory activity against *V. alginolyticus*, *A. hydrophila* and *P. putida* (Fig. 1). The inhibitory zone range was 15-25 mm at 24 h post-incubation and 20-25 mm at 48 h post-incubation and onward. There was no inhibition by JAQ04 at 0 h and only slight inhibition at 4.5 h pre-incubation.

Co-culture assay: Co-culture of potential probiotic JAQ04 at 10^5 and 10^7 CFU mL^{-1} with pathogenic *V. alginolyticus* under *in vitro* conditions, showed an initial growth of pathogen in the first 24 h followed by a decrease in number of cells (Fig. 2). JAQ04 at concentration of 10^3 CFU mL^{-1} allowed initial growth of pathogen until 96 h before inhibition occurred.

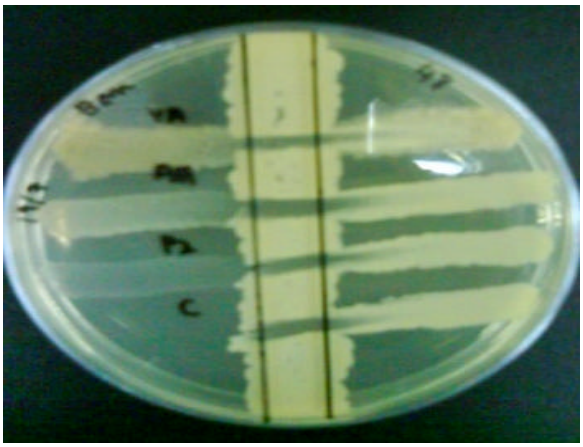

Fig. 1: Agar plate cross-streaking of *B. cereus* JAQ04 with inhibition zone against *V. alginolyticus* (VA), *A. hydrophila* (AH), *P. putida* (P2) and sterile TSB as control (C)

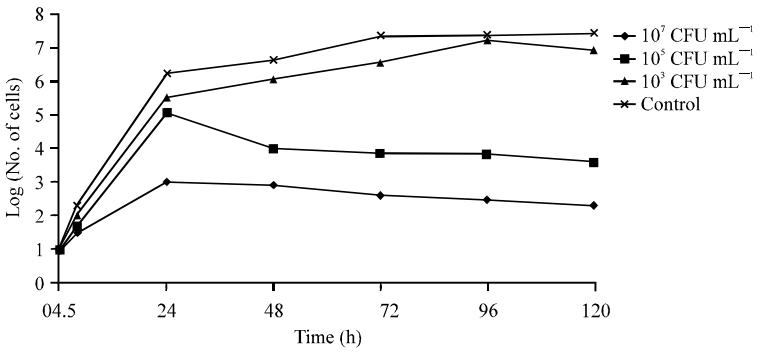

Fig. 2: Growth of *Vibrio alginolyticus* concurrently grown with JAQ04 at different concentrations via co-culture

Table 1: Relative percent survival (RPS) of tested juvenile tilapia tested with and without probiotics added to water

Treatment	Concentration (CFU mL^{-1})	RPS (%)
Bacillus cereus JAQ04	10^5	70.83±18.2
Commercial probiotics	10^5	95.83±4.20
Sterile saline (control)	0	78.50±3.50

Safety of *B. cereus* strain JAQ04 for tilapia juveniles: There was no significant difference between the Relative Percent Survival (RPS) of juvenile tilapia in the immersion test using *B. cereus* strain JAQ04, commercial probiotics and control (Table 1) indicating the safety of this probiont on tilapia.

DISCUSSION

Antimicrobial ability: Higher pre-incubation hours resulted in stronger inhibition ability by strain JAQ04 and this may suggest that it takes at least 24 h for strain JAQ04 to colonize the agar and prevent growth of pathogenic bacteria. This may also be in accordance to early application of

probiotics to enable colonization of gut microflora to increase competitive exclusion ability by probiotic against pathogenic bacteria (Balcazar *et al*., 2006). The inhibition effect may also be contributed by the production of extracellular products such as antibiotics, bacteriocins, lysozymes, proteases, hydrogen peroxide and/or alteration of pH values by production of organic acids (Verschuere *et al*., 2000).

Co-culture assay: The results showed that an increase in concentration of *B. cereus* strain JAQ04 resulted in a higher inhibition of the pathogen; *V. alginolyticus*. Similar results were reported (Vaseeharan and Ramasamy, 2003).

Safety of *B. cereus* strain JAQ04 for tilapia juveniles: *B. cereus* strain JAQ04 was found safe to be used in tilapia aquaculture. RPS of juveniles treated with strain JAQ04 was less (70.83+18.2%) than that of the control group (78.50+3.5%) and commercial probiotics (95.83+4.2%) (but with no significant difference). These results may be due to the fact that commercial probiotics consist of a mixture of several probiotic strains of *Bacillus* sp. that could have a synergistic effect (Chapman *et al*., 2011).

It was also observed that juveniles under treatment of strain JAQ04 showed reduced appetite, 24 h post-treatment but remained actively swimming. Aggressive behavior of tilapia was one of the noticeable problems of juvenile tilapia that repeatedly attack each other. This could also be one of the causes of death of some tilapia. There was also noticeable reduction in fecal matter in tanks treated with strain JAQ04. This could have been a result of reduced appetite as well as organic waste degradation by the potential probiotics. Previous research showed that *Bacillus* spp. are known to break down organic wastes (Verschuere *et al*., 2000).

It could be concluded that *B. cereus* strain JAQ04 showed an antagonistic activity against the 3 tested fish pathogens. It exhibited a strong inhibition effect against *V. alginolyticus* beginning from 48 h pre-incubation and onwards, at concentrations of 10^5 and 10^7 CFU mL^{-1}. *B. cereus* was found safe to be used for tilapia aquaculture.

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