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In vitro EVALUATION OF PROBIOTIC PROPERTIES OF *Bacillus licheniformis* STRAIN UMTK003 FROM GUT OF WILD ORANGE MUD CRAB, *Scylla olivacea* FROM SETIU WETLANDS, TERENGGANU, MALAYSIA

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Abstract: Mud crabs are among the commercially important aquatic animals because of high demand in Asian countries. Currently, one of the main constraints for expansion of mud crab aquaculture is the inconsistent and low hatchery production of crab juveniles. The use of beneficial bacteria offers a promising approach in aquaculture to prevent infections by pathogenic bacteria, and has been shown to help reduce mortality after infection. The goals of this study were to isolate, identify and evaluate potential probiotic bacteria from the gut of wild orange mud crab from Setiu Wetlands, Terengganu. Isolate was identified using BBL™ Crystal Gram Positive (GP) Identification System. Probiotic properties were evaluated including antibiotic susceptibility, tolerances to pH, bile salt, trypsin and pepsin, autoaggregation and coaggregation with *Vibrio* pathogens. Isolate UMTK003 was identified as *Bacillus licheniformis*. The isolate was able to withstand pH 4.5 to 9.5. Bile salt, pepsin and trypsin tolerances were 0.1-0.4%, 0.1-0.7% and 0.4-1%, respectively. Antibiotic susceptibility profiling showed multiple antibiotic resistance (MAR) index of 0.15. Autoaggregation was 79.83±0.0015%. Coaggregation varied from 57.71±0.0050% (*Vibrio harveyi*), 52.41±0.0011% (*Vibrio metschnikovii*), 50.07±0.0011% (*Vibrio cholera*) to 43.25±0.0010% (*Vibrio parahaemolyticus)*. These results suggested probiotic potential of *B. licheniformis* strain UMTK003 for mud crab aquaculture in Malaysia.

Keywords: Mud crab, probiotic, *Scylla olivacea*, Setiu Wetlands

Introduction

Orange mud crab, *Scylla olivacea* is widely found throughout Asian countries. As the global demands for aquaculture products are rising, the interest in mud crab as a candidate aquaculture species has also increased. Generally, mud crab farming highly depends on collection of crablets from the wild. Until recently, low production and inconsistent survival of larvae remain a major problem in the mud crab aquaculture industry (Holme *et al*., 2008). Many previous studies have reported that microbial infections in mud crab aquaculture may be a cause of failure in seed production. Jithendran *et al*. (2010) have observed several chitinolytic bacteria (Gram negative rods) in mud crab larvae such as *Vibrio* spp., *Pseudomonas* spp., *Aeromonas* spp. and *Spirillium* spp. *Vibrio Harveyi* is known to be pathogenic to the zoea stages of mud crab

at 10^2-10^3cfu/mL. Vibriosis affects a diverse range of marine and estuarine shellfish species, and is frequently secondary to some inciting factors *e.g.*, poor water quality, stress and poor nutrition.

Currently, antibiotics are used extensively in mud crab larval culture to improve survival and control vibriosis outbreaks due to *V. harveyi* (Liessmann, 2005). Overuse of antibiotic may however cause some deformities in mud crab larvae as reported by Pates Jr. *et al.* (2016). Furthermore, inappropriate uses of antibiotics in treatment and prevention of bacterial infections have led to growing concern on emergence and spread of antibiotic resistant bacteria (Balcázar *et al*., 2006). Recent study by Aftabuddin *et al.* (2013) has reported six *Vibrio* isolates highly resistant to ampicillin from mud crab *S. seratta*. Therefore, there is an increasing need for research into less harmful alternative disease control strategies, and the application of probiotics is one of the considerations (Macey & Coyne, 2005).

Verschuere *et al*. (2000) proposed that a probiotic is defined as a "live microbial adjunct which has a beneficial effect on the host by modifying the host-associated or ambient microbial community, by ensuring improved use of the feed or enhancing its nutritional value, by enhancing the host response towards disease, or by improving the quality of its ambient environment". Whereas, an expert panel convened in 2002 by the Food and Agriculture Organization of the United Nations (FAO) and the World Health Organization (WHO), probiotics are "live microorganisms administered in adequate amounts that confer a beneficial health effect on the host" (FAO/WHO, 2002). Probiotic bacteria have been shown to stimulate the immune system and confer certain levels of protection against pathogens, as well as compete for space and food within the gut (Maeda and Liao, 1992; Moriarty, 1998; Rengpipat *et al*., 1998). Liessmann (2005) also revealed the protective effect of *V. alginolyticus* LLB2 probiotic in *S. serrata* larvae towards challenge of virulent *V. harveyi*. Other study by Candelaria *et al.* (2010) reported that the use of probiotics in *S. serrata* significantly improved the water quality by reducing the ammonia and nitrite levels, and resulted in higher survival rates in probiotic treated larvae challenged with *V. harveyi*. Lavilla-Pitogo *et al*. (2001) stated that with continuous research, probiotics might serve as good alternatives to antibiotics for mud crab larval culture in the future. Although the application of probiotics in aquaculture is rapidly growing due to their environment-friendliness, probiotics have not been used extensively in mud crab aquaculture.

The present study was undertaken to isolate, identify and evaluate potential probiotic bacteria from the gut of wild *S. olivacea*.

Materials and Methods

Sampling

The sampling was carried out at Pulau Stopa, Setiu Wetlands, Terengganu, Malaysia (N 05˚40.574' E 102˚42.962') during dry season. Six live female and male *S. olivacea* weighing 100-300 g were collected using crab traps. The crabs were kept alive in sterile plastic bags and transported to Fish Disease Laboratory, Universiti Malaysia Terengganu within an hour for further works.

Bacterial Isolation and Identification

The crabs were sacrificed according to RSPCA Guidelines (2006). Briefly, the crabs were chilled in refrigerator and then sacrificed by spiking. All samples were washed with running tap water for several minutes before rinsed with sterile de-ionized water to remove loosely attached bacteria. Then, all samples were aseptically dissected for their guts. The gut samples were rinsed twice with sterile de-ionized water, and then pulverized with sterile mortar and pestle.

Sterile physiological saline (0.85% NaCl) was added to the samples at a ratio of 9:1 for homogenization. The homogenates were serially diluted up to 10^{-10}, and 0.1 mL of the dilutions was spread-plated on trypticase soy agar (TSA), and incubated for 3 to 7 days at 30°C. The plates were observed from the 3rd day onwards up to 7th day for unique colonies. The colonies were purified by sub-culturing on TSA. The colonies were Gram-stained to select Gram positive isolates. The Gram positive isolates were identified using BBL Crystal Gram Positive (GP) Identification System (Becton Dickinson, USA).

Antibiotic Susceptibility Test

Antibiotic susceptibility test (AST) was conducted by Kirby–Bauer disc diffusion method using Mueller-Hinton agar (MHA) (Bauer *et al*., 1966) against erythromycin (E30, 30 µg), oxytetracycline (T30, 30 µg), ciprofloxacin (CIP5, 5 µg), kanamycin (K30, 30 µg), ampicilin (AM10, 10 µg), penicillin (P10, 10 µg), rifampin (RA5, 5 µg), colistin sulphate (CT25, 25 µg), chloramphenicol (C30, 30 µg), furantoin (F50, 50 µg), lincomycin (MY15, 15 µg) and oleandomycin (OL15, 15 µg). Standardized bacterial suspension (McFarland 0.5) was spread on MHA, and antibiotic discs were placed on the agar plate. Incubation was done overnight at 30°C. The results were interpreted as sensitive (S), intermediate (I) and resistant (R) according to Clinical and Laboratory Standards Institute (CLSI) (2014), and multiple antibiotic resistance (MAR) index was generated according to Sarter *et al.* (2007).

pH Tolerance

The bacterial suspensions were serially diluted in sterile PBS, and 0.1 mL was spread on TSA at pH 1.5, 2.5, 3.5, 4.5, 5.5, 6.5, 7.5, 8.5 and 9.5, respectively. TSA pH 7.5 was used as control. The plates were incubated for 24 h at 30°C. After incubation, the colonies on every plate were enumerated as viable count. The assay was carried out in triplicate (Zhang *et al*., 2013).

Bile Salt Tolerance

Bacterial suspensions were prepared with sterile PBS containing 0, 1, 2, 3 and 4 g/L of bile, and incubated for 6 h at 30 °C in an anaerobic condition (Zhang *et al*., 2013). After incubation, the suspensions were serially diluted in sterile PBS, and plated on TSA for viable count (De Man et al., 1960). The assay was carried out in triplicate.

Trypsin and Pepsin Tolerances

These assays evaluated the tolerance of isolate to the simulated gastric and intestinal juices. The bacterial suspensions were prepared with sterile PBS containing 1, 3, 5 and 7 g/L of trypsinase at pH 6.8, respectively. Pepsin tolerance was determined using bacterial suspension in sterile PBS containing 0, 4, 6, 8 and 10 g/L of pepsin at pH 3.0, respectively. After 6 h incubation at 30°C, 0.1 mL of each bacterial suspension was spread on TSA for viable count (Zhang *et al*., 2013). The assays were carried out in triplicate.

Autoaggregation Assay

Autoaggregation ability was measured as autoaggregation percentage. The selected isolate was cultured in TSB for 24 h at 30°C, and harvested by centrifugation at 5,000 g for 15 min. The pellet was washed three times in sterile PBS, and re-suspended in sterile PBS, and adjusted to 0.25±0.05 at OD 600 (Collado *et al*., 2008). Four mL of bacterial suspension was mixed for 10s and incubated at room temperature for 0, 2, 4, 6, 8, 10 and 20h. The bacterial suspensions were measured for OD600. The autoaggregation percentage is expressed as: $A\% = (A0 - At)/A0 \times 100$, where At represents the absorbance at time (2, 4, 6, 8, 10 and 20 h) and A0 represents the absorbance at 0 h. (Zhang *et al.,* 2013).The assay was carried out in triplicate.

Coaggregation Assay

Cell suspensions for coaggregation assay were prepared as above. The bacterial pathogens used in this study were *V. parahaemolyticus, V. harveyi, V. metschnikovii* and *V. cholera.* An equal volume (2 mL) of the isolate and pathogen suspensions were mixed for 10 s. OD600 was measured after 0, 2, 4, 6, 8, 10 and 20 h incubation at room temperature. The coaggregation percentage is expressed as: A% = (1 – Amix/A0) × 100, where A mix represents the absorbance at 2, 4, 6, 8, 10 and 20 h, whereas A0 represents the absorbance at 0 h. The assay was performed in triplicate (Zhang *et al*., 2013).

Results and Discussion

Bacterial Isolation and Identification

A Gram positive rod-shaped isolate was successfully obtained from the gut of wild orange mud crab *S. olivacea,* and designated as UMTK003. The isolate appeared white, irregular, undulate, convex, opaque and sticky with hair-like structure strongly attached to agar TSA surface (Table 1).

Table 1: Colony and cell morphologies of isolate UMTK003 from wild orange mud crab, *Scylla olivacea*.

Criteria	Cell morphologies
Colour	White
Form	Irregular
Margin	Undulate
Elevation	Convex
Size	1.1 cm
Optical property	Opaque
Texture	Sticky
Gram staining	Positive, rod

The isolate was identified as *Bacillus licheniformis* (90.94%) (Table 2). The morphologies of this isolate corresponded precisely with the descriptions by Bisset and Street (1973) for *B. lichenifromis* including active swarming on agar, resembling lichen, surrounded by bulging droplets of mucilaginous growth, and fringed with rhizoid filaments. *B. licheniformis* is a catalase and oxidase positive endospore forming non-pathogenic Gram positive bacterium belonging to the genus Bacillus (De Boer *et al.,*1994; Pasnik *et al.*, 2008). Recent studies have reported that *B. lichenformis* produces potential probiotics that enhance the growth performance and feed utilization in many aquatic animals such as Pacific white shrimp, *Litopenaeus vannamei* (Swapna *et al.*, 2015), Nile tilapia, *Oreochromis niloticus* (Hassaan *et al*., 2014) and juvenile sea bream (Avella *et al.,* 2010). Dietary supplementation with *B. licheniformis* can also manipulate gut microbiota, and increase the growth and immune response of *Macrobrachium rosenbergii* (Kumar *et al.,* 2013).

Li *et al.* (2007) also indicated that administration of *B. licheniformis* can improve the intestinal microflora and immune ability of white shrimp, *Litopenaeus vannamei*. While, study by Cladera-Olivera *et al*. (2004) reported that probiotic *B. licheniformis* strain P40 from teleost fish *Leporinus* sp. can produce broad spectrum antibacterial peptide against pathogenic and spoilage organisms. To the best of our knowledge, the present study is the first to report probiotic properties of *B. licheniformis* isolated from gut of wild *S. olivacea* from Setiu Wetlands, Terengganu, Malaysia.

Antibiotic Susceptibility Test

Isolate UMTK003 was sensitive to erythromycin E30, oxytetracycline T30, ciprofloxacin CIP5, kanamycin K30, ampicilin AM10, penicillin P10 and rifampin RA5 but resistant to colistin sulphate and lincomycin (Table 3), resulting in a MAR index of 0.15. The isolate showed intermediate susceptibility to chloramphenicol C30, furantoin F50 and oleandomycin OL15. On the contrary, *B. licheniformis* has been reported to be resistant to chloramphenicol and clindamycin (Sorokulova *et al*., 2008). MAR

Table 2: Biochemical and enzymatic reaction patterns of isolate UMTK003 based on BBL Crystal™ Gram-Positive (GP) Identification (ID) System, and the identity determined

Biochemical and enzymatic reaction	Code	Result	Identity and confidence level
Fluorescent negative control	FCT	0	
4 MU-β-D-glucoside	FGC	+	
L-valine-AMC	FVA	-	
L-phenylalanine-AMC	FPH	+	
4MU-α-D-glucoside	FGS	+	
L-pyroglutamic acid-AMC	FPY	+	
L-tryptophan-AMC	FTR	+	
L-arginine-AMC	FAR	-	
4MU-N-acetyl-β-D-glucosaminide	FGA	+	
4MU-phosphate	FHO	-	
4MU-β-D-glucuronide	FGN	-	
L-isoleucine-AMC	FIS	-	
Trehalose	TRE	-	
Lactose	LAC	-	
Methyl-α & β glucoside	MAB	-	*Bacillus*
Sucrose	SUC	+	*licheniformis*
Mannitol	MNT	-	(90.94%)
Maltotriose	MTT	-	
Arabinose	ARA	-	
Glycerol	GLR	-	
Fructose	FRU	-	
p-n-p-β-D-glucoside	BGL	+	
p-n-p-β-D-cellobioside	PCE	+	
Proline & Leucine-p-nitroanilide	PLN	+	
p-n-p-phosphate PHO	PHO	-	
p-n-p-α-D-maltoside	PAM	+	
ONPG & p-n-p-α-D-galactoside	PGO	+	
Urea	URE	+	
Esculin	ESC	+	
Arginine	ARG	+	

index 0.15 suggested that those antibiotics were seldom or never been used in the crabs. The antibiogram indicated low probability of antibiotic resistance in the isolate. The susceptibility profile of this isolate is of clinical importance because it implicates minimum risk of disseminating the antibiotic resistance genes to bacterial pathogens as suggested by Beyan *et al*. (2011).

pH Tolerance

pH is one of the most important factors affecting the survival of bacteria in gastric juice. Tolerance to gastric acid in the host stomach is one of the essential characteristics of probiotic candidates because they will have to go through the host stomach to reach the intestine (Kesarcodi-

Watson *et al.,* 2008). Isolate UMTK003 was tolerant to pH 4.5 to 9.5 (Figure 1). The viability was significantly reduced at pH ≤ 3.5 compared with pH 7.5 (control). Ghani *et al.* (2013) have reported pH tolerances of 3.0 to 11.0 in *B. licheniformis.* This is in agreement with the pH tolerance of isolate UMTK003. The pH of foregut fluid in most crustaceans has been reported in the ranges of 5.0 to 7.0 and 6.0 to 7.0, respectively by Van Weel (1970) and Brown (1995). Moreover, Cebeci and Gurakan (2003) mentioned that probiotics that tolerate low pH

Table 3: Antibiotic susceptibility profile of *Bacillus licheniformis* UMTK003

Antibiotic disc	Disc potency	Susceptibility
Erythromycin E30	30 µg	S
Oxytetracycline T30	30 µg	S
Ciprofloxacin CIP5	5 µg	S
Kanamycin K30	30 µg	S
Ampicilin AM10	10 µg	S
Penicillin P10	10 µg	S
Rifampin RA5	5 µg	S
Colistin sulphate CT25	25 µg	R
Chloramphenicol C30	30 µg	I
Furantoin F50	50 µg	I
Lincomycin MY15	15 µg	R
Oleandomycin OL15	15 µg	I

are able to transit through stomach, and remain active in the intestine. Spore forming *Bacillus* spp. also make gut environment less conducive to colonization by pathogenic bacteria, and compete with them for mucosal attachment and nutrients, as well as improve nutrient uptake through villi development (Lee *et al.,* 2010).

Bile Salt Tolerance

The viable count of isolate UMTK003 reduced inversely with increment of bile salt concentration from 5.7×10^7cfu/mL at 0 g/L (control) to 0.7×10^7cfu/mL at 4 g/L (0.4 %) (Figure 2). Previously Wang *et al.* (2010) reported bile salt tolerance of 0.6% (6 g/L) in *B. licheniformis* IMAUB1002 isolated from traditional fermented food in Inner Mongolia of China. Tolerance to bile salt is among the essential characteristics of probiotic candidates because they will encounter the alkaline bile salt before moving further down the intestine (Kesarcodi-Watson *et al.,* 2008). Acid fermentation of *Bacillus* spp. can lower gut pH and create a positive environment for beneficial bacteria such as *Lactobacilli* which in turn reduce the amount of pathogenic bacteria (Kemmett, 2014). Bile salt tolerances of 0.3 and 0.5% are considered good and very good for probiotics, respectively (Charteris *et al.,* 1998; Hyronimus *et al.,* 2000).

Trypsin and Pepsin Tolerances

The viability of isolate UMTK003 was inversely correlated with trypsin concentration after 6h incubation. The viable count was decreased from 9.88×10^7 cfu/mL at 0 g/L to 1.55×10^7cfu/mL at 7 g/L (Figure 3). The viability was also inversely correlated with pepsin concentration.

The viable count was reduced from 6.15 × 10^7cfu/mL at 0 g/L to 2.05 × 10^7 cfu/mL at 10 g/L (Figure 4). Digestive enzymes in crustaceans play fundamental roles such as assimilating substance and energy from the food (Jones *et al.,* 1997). Digestive enzymes such as pepsin, protease, trypsin, carboxy-peptidase, amino-peptidase, chymotrypsin, elastase, collagenase, carbohydrase, lipase, amylase and laminarinase can be found in decapods. Like in other animals, trypsin serves a function in digestion of dietary protein in mud crab. Similar patterns have also been reported in decapod species such as *S. serrata* (Johnson *et al*., 2002).

Autoaggregation Assay

The autoaggregation varied directly with the duration of interaction. The autoaggregation was 29.43± 0.0010% for 2 hours compared with 79.83± 0.0015% for 20 hours (Figure 5). The high autoaggregation capacity suggested that isolate UMTK003 is good at forming biofilms and/or GI tract colonization, which are the ways of forming barrier against colonization by pathogenic microorganisms (Schachtsiek *et al*., 2004). Autoaggregation of probiotic is related to adhesion to intestinal cells (Boris *et al*., 1997). The ability to adhere to the intestinal mucus is considered as one of the main criteria in the selection of potential probiotic bacteria as adhesion prolongs their permanence in the

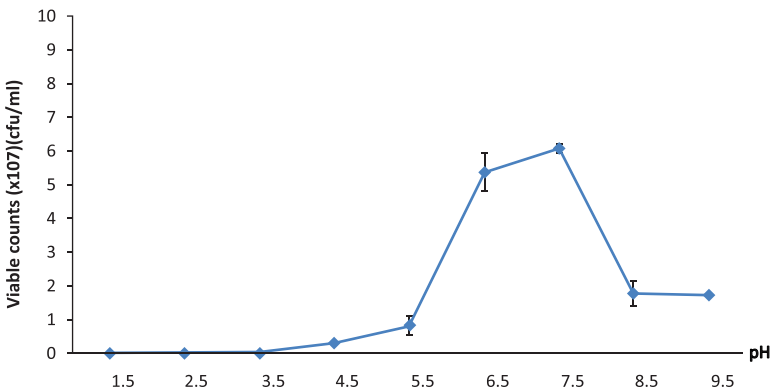


Figure 1: Survival of isolate UMTK003 at different pH based on viable counts. Error bar represents standard deviation of the mean values of results from replicate experiments.

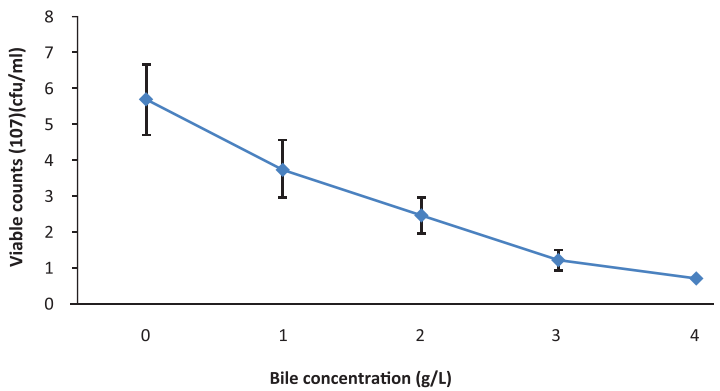


Figure 2: Survival of isolate UMTK003 at different bile salt concentrations based on viable counts. Error bar represents standard deviation of the mean values of results from replicate experiments.

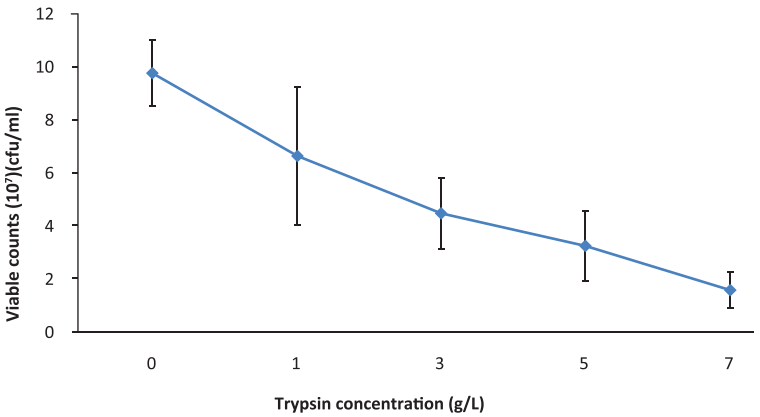


Figure 3: Survival of isolate UMTK003 at different trypsin concentrations based on viable counts. Error bar represents standard deviation of the mean values of results from replicate experiments.

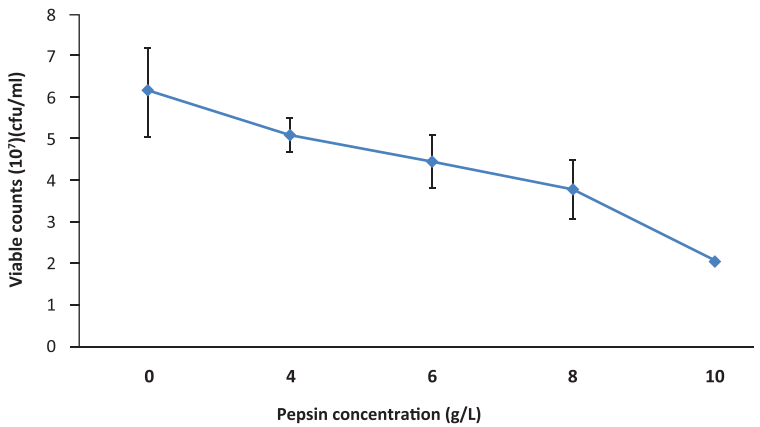
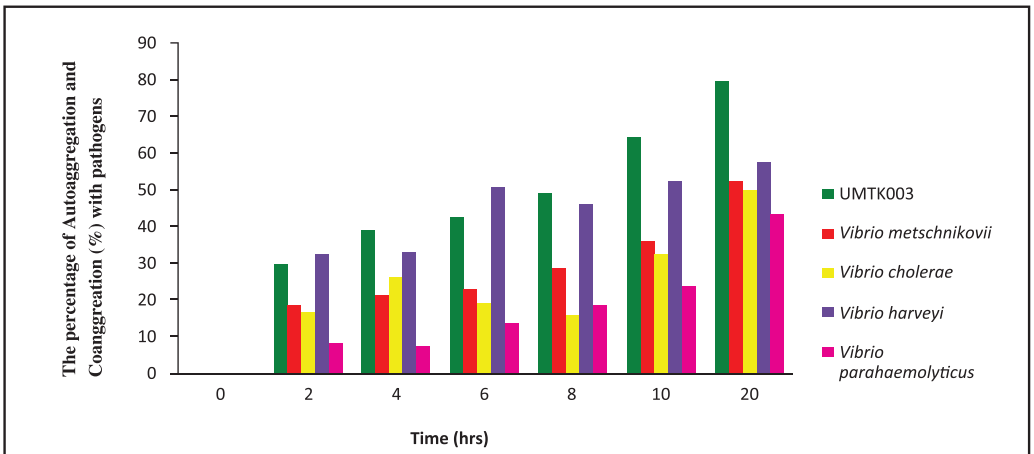


Figure 4: Survival of isolate UMTK003 at different pepsin concentrations based on viable counts. Error bar represents standard deviation of the mean values of results from replicate experiments.

intestine, which in turn allows them to exert a healthful effect (Apostolou *et al.,* 2001). In a study of cell surface adherence and aggregative capacity in *B. licheniformis,* Collado *et al*. (2007) reported correlation of autoaggregative capacity with adherence, which is prerequisite for GI tract colonization and infection by pathogens. Furthermore, by attaching to the intestinal mucosa, probiotics can extend their time within the gut thereby influencing the gastrointestinal microflora of the host. In general, probiotic strains show higher autoaggregation abilities than pathogenic strains (Collado *et al*., 2008). Adhesion of probiotic bacteria to intestinal mucosa has been shown to enhance their antagonistic activities against pathogens (Coconnier *et al*., 1993).



Time (hour)	2	4	6	8	10	20
UMTK003	29.43±0.0010	39.11±0.0011	42.74±0.0020	49.19±0.0005	64.51±0.0271	79.83±0.0015
Vibrio metschnikovii	18.38±0.005	21.26±0.0015	23.31±0.0015	28.94±0.0015	35.81±0.0020	52.41±0.0011
Vibrio cholerae	16.54±0.0028	26.04±0.0041	18.99±0.0055	15.82±0.0095	32.66±0.0020	50.07±0.0011
Vibrio harveyi	32.44±0.0050	32.92±0.0010	50.42±0.0010	45.92±0.0023	52.36±0.0011	57.71±0.0050
Vibrio parahaemolyticus	8.06±0.0057	7.28±0.0011	13.48±0.0010	18.91±0.0037	23.87±0.0032	43.25±0.0010

Figure 5: Percentage of autoaggregation and coaggregation of isolate UMTK003 with four *Vibrio* pathogens. Error bar represents standard deviation of the mean values of results from replicate experiments.

Coaggregation assay

Isolate UMTK003 showed varying degree of coaggregation with *V. harveyi* (57.71±0.0050%), *V. metschnikovii* (52.41±0.0011%), *V. cholera* (50.07±0.0011%) and *V. parahaemolyticus* (43.25±0.0010%) (Figure5).

Coaggregation is a process by which genetically distinct bacteria attach to each other via specific molecules (Rickard *et al*., 2003). Coaggregative and autoaggregative capacities can be used for preliminary selection of probiotic bacteria for potential applications in humans and animals.

Coaggregation may constitute an important host defense mechanism against infection (Rickard *et al.,* 2003). Similar results were obtained by (Balcázar & Rojas-Luna 2007), where *B. subtilis* UTM126 was found to inhibit *V. parahaemolyticus* PS-107. Decamp *et al.* (2008) also reported inhibition of *Vibrio* spp. in *Litopenaeus vannamei* and *Penaeus monodon* larvae by *B. licheniformis* and *B. subtilis*, and increased the survival rate of shrimp. Zhang *et al.* (2013) also reported coaggregation by marine lactic acid bacterium, *Leuconostoc lactis* isolated from the intestinal tract of marine black porgy fish, *Sparus macrocephalus*. In the aquaculture environment, the composition of bacterial community has a strong influence on the internal bacterial flora of farmed animals, which is vital for their nutrition, immunity and disease resistance (Balcázar & Rojas- Luna,

2007). Intestinal microbiota in cultured aquatic organisms has been found to be important for health maintenance, either by preventing pathogen colonization, degradation of food, and production of antimicrobial compounds and nutrients, or maintaining normal mucosal immunity (Ringø *et al*., 2015).

Conclusion

The present *in vitro* evaluation showed the good potential of *B. licheniformis* strain UMTK003 as a probiotic for mud crab aquaculture. As probiotics are progressively gaining acceptance for eco-friendly and sustainable aquaculture, it can potentially serve as an economical alternative for disease control in mud crab aquaculture, which in turn helps promote sustainable development of mud crab aquaculture in Setiu Wetlands and other parts of the country. Further *in vivo* evaluation of the isolate on safety and inhibitory potential is necessary.

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