African Journal of Biotechnology Vol. 10(22), pp. 4643-4650, 30 May, 2011
Available online at http://www.academicjournals.org/AJB
DOI: 10.5897/AJB10.2404
ISSN 1684–5315 © 2011 Academic Journals

Full Length Research Paper

In vitro evaluation of commercial probiotic products used for marine shrimp cultivation in Thailand

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Accepted 2 May, 2011

The objective of this study was to evaluate the effectiveness of 12 commercial probiotic products in Thailand, used in marine shrimp cultivation, based on two criteria namely, accuracy of the information on product labels as regards the number and types of microorganisms and acceptability of the number of probiotic microorganisms at 10^6 colony forming unit (CFU)/g in the products. Of the 12 products sampled, only two of them provided adequate information on the number and composition of microorganisms and their proper dosage. In addition, none of the probiotic products possessed the correct number and composition of microorganisms or qualitative extracellular enzymes, declared on their labels nor did they show any *in vitro* inhibitory activity on shrimp pathogenic *Vibrio harveyi.* However, a few products were capable of biosynthesis of amylase, protease and lipase with high capacities.

Key words: Commercial probiotic product, marine shrimp, *Vibrio harveyi*, amylase, protease, lipase.

INTRODUCTION

Marine aquaculture is one of the most important agricultural industries of Thailand. Black tiger shrimp (*Penaeus monodon*) culture has increased sharply in Thailand since 1972 (Lin and Nash, 1996; Wyban, 2007). Thailand, a world leader in shrimp production for a few decades, accounted for approximately 280,000 metric tons in 2001 (Klinbunga et al., 2006). However, production of this species has decreased dramatically since 2002, due to disease outbreaks and the introduction of Pacific white shrimp (*Litopenaeus vannamei*) to the compendium of cultured species. Currently, Pacific white shrimp is considered a commercially important crustacean species in Thailand (Wyban, 2007).

Marine shrimp cultivation in Thailand commonly follows the intensive system because it produces higher yields than other culture systems (Rosenberry, 1996). High mortality and low production of shrimp in Thailand resulting from environmental deterioration and the pathogen outbreaks were observed initially in the central part of Thailand in 1989 (Lin and Nash, 1996). Wastewater effluents from intensive shrimp farming generally contain a considerable amount of waste nutrients (ammonia, nitrate, nitrite and phosphate), organic matter and inorganic solid which contribute to eutrophication in receiving waters (Ziemann et al., 1992; Trott and Alongi, 2000; Jackson et al., 2003). Moreover, effluents and organic sludge from shrimp ponds may contain pathogenic microorganisms such as *Vibrio* and *Pseudomonas aeroginosa*. Consequently, a wide range of antimicrobial substances (oxytetracycline, ciprofloxacin, nitrofurantoin, furazolidone or chloramphenicol) have been applied to control and prevent disease outbreaks in hatcheries and farms. The negative effect of antibiotics in shrimp culture is the evolution of antibiotic-resistant microorganisms that can transfer resistance to pathogenic microorganisms, leading to a reduced efficiency of antibiotics in treating diseases (Kautsky et al., 2000).

Sustainability of shrimp production requires suitable cultivation practices (Phillips et al., 1993; Primavera, 1994). Presently, probiotics and/or bioremediators are gaining popularity as environmentally-friendly alternatives for antibiotics in improving shrimp health and minimizing disease (Gatesoupe, 1999; Senok et al., 2005). Probiotics

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Table 1. Number of total heterotrophic bacteria of tested commercial probiotic products.

Commercial probiotic products	Country of origin	Measured total aerobic heterotrophic bacteria (CFU/g)	Labeled total aerobic heterotrophic bacteria (CFU/g)
Product 1	Thailand	8.57×10^4	10^9
Product 2	Thailand	2.07×10^4	10^9
Product 3	China	1.26×10^4	10^9
Product 4	Thailand	8.23×10^3	10^9
Product 5	China	4.43×10^3	10^9
Product 6	Thailand	6.13×10^2	10^9
Product 7	Thailand	2.50×10^5	ND
Product 8	Thailand	1.26×10^8	ND
Product 9	Thailand	7.55×10^7	10^9
Product 10	Thailand	1.70×10^7	ND
Product 11	US	1.30×10^9	10^9
Product 12	Thailand	1.10×10^6	ND

ND, No data of heterotrophic bacterial number on the label.

are defined as microorganisms or microbial cell preparations with beneficial health effects on a host by controlling the balance of intestinal microorganisms, enhancing digestibility and absorption capacity as well as decreasing organic waste. Although probiotic supplies a putative alternative to antimicrobial agents for shrimp cultivation, probiotics should be specifically selected to optimize their effectiveness. Many probiotic products being distributed contain inappropriate bacterial species and/or unsuitable bacterial densities for aquatic species (Moriarty et al., 2005; Balcazar et al., 2006).

In Thailand, there are several types of commercial probiotic products for marine shrimp cultivation. However, major problems of probiotic usage are related to their ineffectiveness and high cost. Probiotic product effectiveness for marine aquaculture has been evaluated, based on a combination of the understanding of nature, their composition and user technology transfer (Kautsky et al., 2000).

The objective of this research was to determine the quality of commercial probiotic products in Thailand for marine shrimp based on 2 criteria namely, the accuracy of information on product labels as regards the number and types of microorganisms and the acceptability of the number of probiotic microorganisms in the products.

MATERIALS AND METHODS

Probiotic product samples

Twelve (12) probiotic product samples used for marine shrimp cultivation available in Thailand were purchased from a local aquaculture product retailer (distributor) as shown in Table 1. All samples were stored at 4°C before use.

Isolation and enumeration of bacteria in probiotic products

Probiotic product samples were diluted in 0.85% NaCl and analyzed by spread inoculation. An inoculum (0.1 ml) of each decimal dilution of samples was plated onto the surface of plate count agar (PCA, Difco, Detroit, MI, USA), which were incubated under aerobic condition and de Man, Rogosa and Sharpe (MRS) agar (Difco, Detroit, MI, USA) which were incubated anaerobically in anaerobic jar (BBL, GasPak Plus), for enumeration of viable bacteria and lactic acid bacteria, respectively. The bacterial counts were recorded after incubation at 30°C for 48 h. Representatives of bacterial colony types from PCA and MRS agar were isolated and identified to genus and species using conventional morphological and biochemical tests according to Bergey's manual of determinative bacteriology (Holt et al., 1994) and API test kit (Bio-Merieux, Marcyl'Etoile, France). Isolation and enumeration of bacteria were performed in triplicate.

Assay for extracellular protease, amylase and lipase

Preparation of commercial probiotic products

A final concentration of 10^4 CFU/g of each commercial probiotic product was prepared in 0.85% NaCl. The suspensions were serially diluted in 0.85% NaCl and bacterial numbers were determined using spread plating technique. Then, they were used for assay of extracellular enzymes.

Assay of extracellular qualitative enzymes

The assay of three extracellular enzymes, protease, amylase and lipase, was carried out by agar diffusion method based on Leonel Ochoa-Solano and Olmos-Soto (2006). Twenty microliters (20 µL) of 10^4 CFU/g suspension of each probiotic products were dropped onto skim milk agar, starch agar and tributyrin agar for assay of extracellular protease, amylase and lipase, respectively. All Petri dishes were incubated at 30°C in the dark for 48 h. The presence of clear zone around the colonies at the end of incubation was indicative of each enzymatic activity. Clear zone around the colonies of dropped commercial probiotic products was measured on skim milk agar and tributyrin agar. In case of starch agar, iodine solution was poured onto the tested Petri dishes for appearance of clear zones around the colonies and then the diameters of clear zone and spotted colony were measured. Efficiency of enzymatic activities for each commercial probiotic product was expressed

based on calculation of the ratio of clear zone diameter (mm) to the diameter of the colony of dropped commercial probiotic products (mm) following the protocol of Leonel Ochoa-Solano and Olmos-Soto (2006). Experiments were repeated in triplicate.

Inhibitory activity on *Vibrio harveyi*

The inhibitory effect of each probiotic product on *V. harveyi* was investigated by agar diffusion assay as described by Ruiz et al. (1996) with slight modification. *V. harveyi* was proliferated in a 125 ml flask containing 50 ml of alkaline peptone water (APW) pH 8.4 and incubated at 30°C for 48 h. The cell density of *V. harveyi* was adjusted to be 0.01 A.U at 600 nm with a spectrophotometer. The adjusted suspension was swabbed onto nutrient agar (NA) supplemented with 3% NaCl. The suspension of each probiotic product was prepared to obtain equal concentration of microbial cell with 0.85% NaCl and then divided into 2 sections. The first section was spotted onto NA supplemented with 3% NaCl swabbed with *V. harveyi* and the latter section was centrifuged at 8,000 rpm at 4°C for 10 min. Supernatant fluid of each suspension was harvested and filtered through a 0.45 µm membrane filter (Sartorius, Bedford, MA, USA). A small amount of filtered supernatant (20 ml) was spotted onto those media swabbed with tested bacteria. All Petri dishes were incubated at 30°C for 24 h. Inhibition zone around the spotted colony was gauged and calculated as the ratio of inhibition diameters (mm) to the diameters of spotted colony (mm) and this was expressed as inhibitory efficiency of probiotic products. Experiments were repeated in triplicate.

RESULTS AND DISCUSSION

Number and species composition of bacteria in commercial probiotic products

The total number and type of bacteria in commercial probiotic products sold in Thailand were verified and compared with the information on the products' label. Probiotic product numbers 1, 2, 4, 6 to 10 and 12 were produced in Thailand, numbers 3 and 5 were imported from China and number 11, from the United State (US) (Table 1). Culture viability was presumed to be a reasonable measure of product activity (Panigrahi et al., 2005).

The two criteria used for *in vitro* evaluation of commercial probiotic products were established based on Thai regulation for functional food used in animal feed products (Ministry of Agriculture and Cooperatives of Thailand, 1996; Ziaei-Nejad et al., 2006). The number of viable bacteria in the probiotic products was summarized in Table 1. Bacterial numbers in products 7, 10 and 12 were not provided on their respective labels (Table 1) although bacterial compositions were shown (Table 2). In contrast, products 2 and 5 provided only the total number of bacteria (Table 1) without information on the type of bacteria on the label (Table 2). Product 8 was without information on either bacterial numbers or composition (Tables 1 and 2).

Enumeration and identification of probiotic microorganisms were additionally performed to assess their agreement with label information. Eight (8) of the probiotic product labels declared bacterial numbers at 10^9 CFU/g of total heterotrophic bacteria (Table 1). However, only product 11 had an estimated number that was consistent with that on the label, while other products' estimated numbers were below those on their labels (Table 1). These findings were not surprising because probiotic microorganisms are living organisms and some microorganisms may have died during storage. Product users should be aware also of the correlation between probiotic microorganism activity and shelf life through label information as well as the date the product was manufactured. However, probiotic products must contain the acceptable viable number and composition of declared microorganisms.

Commercial probiotic product number 9 was labeled with more than 10 types of microorganisms (Table 2); however, only one strain of *Bacillus* was isolated from this product. Species identified in probiotic product number 10, 11 and 12 differed from those given on the labels (Tables 2 and 3). Probiotic product number 10 was labeled as containing *Bacillus* and *Micrococcus*. *Micrococcus* was absent but *Staphylococcus* non-*aureus* was present together with 3 *Bacillus* species, *Bacillus megaterium*, *Bacillus pasteurii* and *Bacillus sphaericus*. Probiotic product number 11 was consistent with 3 of the identified *Bacillus* species, however, 4 species were listed on the label, *B. licheniformis, B. subtilis, B. megaterium, B. polymyxa*.

All the tested commercial probiotic products were found to contain the genus *Bacillus* and major species (40 isolates in combination) such as *Bacillus mycoides*, *B. subtilis*, *Bacillus stearothermophilus*, *Bacillus lentus*, *Bacillus firmus*, *Bacillus macquariensis*, *Bacillus badius*, *B. megaterium*, *B. pasteurii* and *B. sphaericus* were identified as shown in Tables 2 and 3 which were in agreement with several reports (Rengpipat et al., 1998; Green et al., 1999; Shariff et al., 2001; Wang et al., 2005). For examples, Ziaei-Negad et al. (2006) reported that commercial probiotic products were made up of *B. subtilis*, *B. licheniformis*, *B. polymyxa*, *Bacillus laterosporus* and *Bacillus circulans*. Some probiotic products used in aquaculture in Australia comprises two bacterial genera, *Bacillus* and *Pseudomonas* (Hai et al., 2007). In most Asian and European countries, many probiotic products are composed mainly of *Bacillus*, *Clostridium*, *Pseudomonas putida*, *P. aeruginosa* (Green et al., 1999; Hai et al., 2007). In this study, *S.* non-*aureus*, *Streptococcus feacalis*, *Micrococcus* and *Corynebacterium* were also detected with less numbers (14 isolates in combination) from seven probiotic products (Table 2). They are commonly found in farmed shrimp and their environments (Ahmed et al., 1995; Sugumar et al., 2001; Harish et al., 2003; Wang et al., 2008). Indeed, based on the first criterion in terms of explanation of data in numbers and types of probiotic microorganisms on label of tested products, only six of the probiotic products met the first criterion as shown in Table 4.

Table 2. Species composition of bacteria in tested commercial probiotic products.

Commercial probiotic products	Composition of probiotic bacteria identified in this study						Composition of declared probiotic bacteria on the label	
	Bacillus	*S.* non-*aureus*	*S. feacalis**	*Micrococcus*	*Corynebacterium***	Total number of identified bacteria	Total number of declared probiotic bacteria on the label	Type of declared probiotic bacteria on the label
Product 1	6	-	-	1	-	7	2	*Bacillus* and *Micrococcus*
Product 2	3	1	-	2	-	6	ND	ND
Product 3	4	-	-	-	-	4	1	*Bacillus*
Product 4	3	1	-	1	-	5	3	*Bacillus*, *Micrococcus* and *Staphylococcus*
Product 5	6	-	-	2	1	9	ND	ND
Product 6	4	-	-	-	-	4	1	*Bacillus*
Product 7	2	-	-	-	-	2	1	*Bacillus*
Product 8	2	2	-	-	-	4	ND	ND
Product 9	1	-	-	-	-	1	More than 10	More than 10 types of microorganisms
Product 10	3	1	-	-	-	4	2	*Bacillus* and *Micrococcus*
Product 11	3	-	-	-	-	3	4	*Bacillus licheniformis, B. subtilis, B. megaterium, B. polymyxa*
Product 12	3	1	1	-	-	5	3	*Streptococcus faecalis, B. mesentericus, Clostridium butyricum*

-,Not found; ND, no data of bacterial type on the label;*, the other name is *Enterococcus feacalis*;**,it is not *Corynebacterium diphtheriae*, a human pathogenic bacterium, based on morphological and biochemical characteristics.

The second criterion, bacterial numbers at 10^6 CFU/g, was based on the recommended dosage for shrimp cultivation according to Ministry of Agriculture and Co-operatives of Thailand (1996) and Ziaei-Nejad et al. (2006). Five products (products 8 to 12) met this criterion (Table 4). The concentration of probiotic microorganisms must be higher than those of pathogen, since the degree of antagonistic activity is significantly increased with a higher concentration of the antagonist (Vaseeharan and Ramasamy, 2003).

Extracellular qualitative enzyme

Proteins, carbohydrates and lipids are essential aliments of human, livestock, fish, shellfish, mollusk including shrimp for the growth, function and structure. The commercial shrimp diet supplies the protein as a major component. Generally, the protein requirement of penaied shrimp varies from 28 to 57% depending on the shrimp species (Shiau, 1998). Furthermore, lipid levels in commercial shrimp diets fluctuate from 6 to 7.5% with a maximum of 10% recommended by Akiyama et al. (1991). Leonel Ochoa-Solano and Olmos-Soto (2006) suggested that *B. subtilis* and *B. megaterium* were good exoenzyme-producing bacteria. Additionally, Saha et al. (2006) found high values of protease and amylase activities in *B. circulans* and *B. megaterium*, isolated from tilapia (*Oreochromis mossambica*) and grass carp (*Ctenopharyngodon idella*). Extracellular enzymes such as amylase, cellulase, lipase, protease, lactase and catalase biosynthesized by promising probiotic bacteria can improve nutrient digestibility and overall animal health. Therefore, information on the enzymatic producing efficacy of the probiotic products should be helpful to the aquaculture industry. In order to rate the efficiency of enzymatic activity, products were graded from 0 to 10 after that developed by Leonel Ochoa-Solano and Olmos-Soto (2006); Table 5. Even though probiotic products consisted of various bacteria with the majority being *Bacillus* spp., production of protease, amylase and lipase differed

Table 3. Species of *Bacillus* in tested commercial probiotic products.

Commercial probiotic products	Total number of identified *Bacillus*	*B. mycoides*	*B. subtilis*	*B. stearothermophilus*	*B. lentus*	*B. firmus*	*B. macquariensis*	*B. badius*	*B. megaterium*	*B. pasteurii*	*B. sphaericus*	Composition of bacteria on the label of commercial probiotic products
Product 1	6	+	+ (3)	+	+	-	-	-	-	-	-	*Bacillus* and *Micrococcus*
Product 2	3	+	+	+	-	-	-	-	-	-	-	-
Product 3	4	-	-	+ (2)	+	+	-	-	-	-	-	*Bacillus*
Product 4	3	-	+ (2)	-	+	-	-	-	-	-	-	*Bacillus*, *Micrococcus* and *Staphylococcus*
Product 5	6	-	+ (2)	+	+	-	+	+	-	-	-	-
Product 6	4	-	-	+	+ (2)	-	-	+	-	-	-	*Bacillus*
Product 7	2	-	-	-	-	-	-	-	+	+	-	*Bacillus*
Product 8	2	-	-	-	-	-	-	-	+	+	-	-
Product 9	1	-	-	-	-	-	-	-	-	+	-	More than 10 types of microorganisms
Product 10	3	-	-	-	-	-	-	-	+	+	+	*Bacillus* and *Micrococcus*
Product 11	3	-	-	-	-	-	-	-	+	+	+	*Bacillus licheniformis, B. subtilis, B. megaterium, B. polymyxa*
Product 12	3	-	-	-	-	-	-	-	+	+	+	*Streptococcus faecalis, B. mesentericus, Clostridium butyricum*

Numbers in parenthesis mean the number of isolates found in commercial probiotic products.

among products, especially probiotic product number 7 which did not produce any of these enzymes (Table 5). Probiotic product number 5, consisting of 6, 2 and 1 isolates of *Bacillus*, *Micrococcus* and *Corynebacterium*, respectively, had similar degree of protease and amylase production with the highest lipase production (Table 5). However, probiotic product number 1 composing of 7 isolates of contained bacteria also showed higher enzymatic activities for protein, lipid and carbohydrate break down, compared with other tested products.

Table 4. Qualities of commercial probiotic products based on criteria set up in the present study.

Commercial probiotic products	The first criterion*		The second criterion**	Acceptable probiotic products
	Data of number	Data of composition		
Product 1	✓	✓	x	x
Product 2	✓	x	x	x
Product 3	✓	✓	x	x
Product 4	✓	✓	x	x
Product 5	✓	x	x	x
Product 6	✓	✓	x	x
Product 7	x	✓	x	x
Product 8	x	x	✓	x
Product 9	✓	✓	✓	✓
Product 10	x	✓	✓	x
Product 11	✓	✓	✓	✓
Product 12	x	✓	✓	x

✓Met criterion; x, did not meet criterion;*,the first criterion was established based on data of number and composition of contained microorganisms; **, the second criterion was established based on acceptable numbers of bacteria for 10^6 CFU/g according to Ministry of Agriculture and Cooperatives of Thailand (1996) and Ziaei-Nejad et al. (2006).

Table 5. Efficiency for enzymatic activities of commercial probiotic products.

Commercial probiotic products	Efficiency for enzymatic activities					
	Protease		Amylase		Lipase	
Product 1	1.72 ± 0.28	4+	1.42 ± 0.32	4+	2.22 ± 0.00	5+
Product 2	1.69 ± 0.00	4+	1.01 ± 0.53	4+	1.60 ± 0.00	4+
Product 3	1.63 ± 0.08	4+	1.16 ± 0.49	4+	1.88 ± 0.12	4+
Product 4	1.59 ± 1.32	4+	1.08 ± 0.11	4+	1.62 ± 1.41	4+
Product 5	1.52 ± 0.12	4+	1.14 ± 0.01	4+	4.52 ± 0.18	7+
Product 6	1.33 ± 0.00	4+	1.19 ± 0.13	4+	1.60 ± 0.00	4+
Product 7	-	-	-	-	-	-
Product 8	1.13 ± 0.13	4+	1.12 ± 0.21	4+	-	-
Product 9	-	-	1.09 ± 0.04	4+	-	-
Product 10	1.07 ± 0.05	4+	1.08 ± 0.11	4+	-	-
Product 11	1.22 ± 0.32	4+	1.00 ± 0.07	3+	-	-
Product 12	1.36 ± 0.15	4+	1.19 ± 0.03	4+	-	-

Enzymatic activities were expressed as ratio of clear zone diameter and the diameter of colony spotted commercial probiotic products (mean ± standard deviation). Effective degradation: - (0), 1+ (0.01- 0.5), 2+ (0.51- 0.7), 3+ (0.71-1), 4+ (1.01-2), 5+ (2.01-3), 6+ (3.01-4), 7+ (4.01-5), 8+ (5.01-6), 9+ (6.01-7), 10+ (7.01-8).

All the tested probiotic products comprised *Bacillus* genera as the main probiotic bacteria. There are several reasons for using *Bacillus* as the putative probiotics (Verschuere et al., 2000; Ziaei-Nejad et al., 2006). They are found normally in shrimp and their environments (Sharmila et al., 1996; Nimrat et al., 2005, 2008a). They are also commonly capable of producing a wide range of exoenzymes such as protease, amylase and lipase (Moriarty, 1998; Ghasemi et al., 2007). As a consequence they can improve food degradation by breaking large molecules of protein, carbohydrate and lipid into smaller units (Leonel Ochoa-Solano and Olmos-Soto, 2006) and enhance its nutritional value (Verschuere et al., 2000). Based on high enzymatic activities, *Bacillus* are able to degrade accumulated organic sludge in shrimp ponds (Verschuere et al., 2000; Nimrat et al., 2008b). In addition, *Bacillus* strains are capable of improving water quality, activating immune response, reducing the outbreak of pathogenic microbes and providing the essential nutrients as well as increasing the survival and growth rate of shrimp (Gatesoupe, 1999; Verschuere et al., 2000; Irianto and Austin, 2002; Balcazar et al., 2006).

Ability for *V. harveyi* resistance

Probiotic microorganisms provide many useful activities such as the production of digestive enzymes, competition for attachment site or nutrients and immunostimulation. The screening method by producing the inhibitory substances *in vitro* was identified as one of the good probiotics in aquaculture (Irianto and Austin, 2002; Lategan and Gibson, 2003). All commercial probiotic products in this study were explored by agar diffusion method for the production of an *in vitro* inhibitory effect against the pathogenic bacteria, *V. harveyi*. None showed any *in vitro* inhibitory activity. This suggests that all probiotic products did not produce or produced insufficient amounts of extracellular substances to inhibit the representation of pathogenic bacteria. This could be an important property for product selection. However, *in vitro* results are not always consistent with *in vivo* ones and this might prove to be a disadvantage for the shrimp farmer (Ruiz-Ponte et al., 1999; Gram et al., 2001). For example, Riquelme et al. (1997) reported that the expression of antagonism in *in vitro* is not a sufficient factor to select candidate probiotics or to rule the strain out.

Conclusion

The present investigation suggests that only two of the 12 tested commercial probiotic products (product 9 and 11) contained the numbers of probiotic microorganisms recommended for shrimp cultivation and provided the correct label containing information on product number as well as composition of microorganisms. Furthermore, some of the tested commercial probiotic products had high capacity for amylase, protease and lipase production and none showed any *in vitro* inhibitory activity against *V. harveyi*. However, the effectiveness of the tested commercial probiotic products in the present study was determined under *in vitro* conditions, however, the effectiveness of probiotic products when used in situations directly relevant to aquaculture condition may not be consistent with *in vitro* conditions.

ACKNOWLEDGEMENTS

We gratefully acknowledge Dr. F.W.H. Beamish for reading manuscript and all of our students for their helpful comments and contributions to this work. We also would like to thank Department of Microbiology and Department of Aquatic Science, Faculty of Science, Burapha University for providing equipments and facilities. This study was supported by the Thailand Research Fund (TRF), Thailand, grant no. RMU 5080020.

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