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Original article

Growth promoting activity of *Penaeus indicus* by secondary metabolite producing probiotic bacterium Bacillus subtilis isolated from the shrimp gut

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ABSTRACT

In this study, 104 bacteria were isolated from the gut of *Penaeus indicus*. The morphologically different bacterial isolates were screened for probiotic properties. The strain D5 showed potent activity against various bacteria. The isolated strain *Bacillus subtilis* showed 17 mm zone of inhibition against *Bacillus* sp., 19 mm against *Pseudomonas* sp., 22 mm against *Vibrio* sp., and 22 mm against *Micrococcus* sp. The bacterial growth and bacterocin production in relation with incubation time was evaluated. Bacteriocin production was found to be high at the stationary phase and reduced considerably after fifth day. Bacterial growth and bacterocin production was maximum at pH 7.0 and optimum between pH 6.0 and 8.0. Incubation temperature is one of the important factors which significantly influences on the growth and production of bacteriocin. Bacterial growth and bacterocin production (17 ± 2 mm) were maximum at 30 °C. Glucose stimulated growth and bacterocin production (21 ± 3 mm). Among the supplemented nitrogen sources, glycine positively influenced on growth and metabolite production. In this study, bacteriocin supplemented diet enhanced the growth than control shrimp Bacteriocin was administered at various doses ranged between 10 and 50 mg/100 gm level. Shrimp length was 3.3 ± 0.13 cm and shrimp weight was 9.7 ± 0.12 g in 100 mg. The present finding revealed enhanced growth at high bacteriocin concentration. At 2×10^2 CFU/100 g probiotics in feed, the juvenile shrimp attained 16.8 ± 0.11 g after 40 days. The weight gain was 16.8 ± 0.11 CFU/100 g at 10×10^2 CFU/100 g probiotic concentration.

1. Introduction

Aquaculture is a significant economic activity in many countries and is an important source of protein for human consumption (Sathyamoorthi et al., 2019; Kumaresan et al., 2019; Ravichandran et al., 2018;Sathyamoorthi et al., 2017). Based on Food and Agriculture Organization (FAO) report, the production of molluscs, fish and crustaceans in aquaculture increased rapidly from 4% to 27% in 2000. Also, this sector growing very rapidly than that of animal-food sector. In Asia, China plays a significant role than Asia Pacific region and the production was reported about 89% (FAO, 2005). In aquaculture, antibiotics have been frequently used; however, drug resistance is an important problem in aquaculture sector (Ravichandran et al., 2017;Arasu et al., 2017a, Arasu et al., 2017b; Ravichandran et al., 2016; Arasu et al., 2016a,b). The antibiotics administered in aquaculture easily transmitted to human beings through food chain. Also, antibiotics will inhibit or kill useful microorganisms from the gut of fishes and affect digestibility, immunity and fish nutrition (Maynard et al., 2012). There are many

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findings showing the transferral of highly antibiotic resistant genes among bacteria (Schwarz et al., 2001). Further, the antimicrobial resistance bacteria from the fish could slowly transfer plasmids to other non-resistant bacterium and this may affect human health (Phillips et al., 2004). To avoid the risk of antibiotics resistance, Government institutions implemented strict regulations for the usage of antibiotics in aquaculture practice. European Union (EU) earlier ban on the application of avoparcin in aquaculture practice, later ban on bacitracin, virginiamycin, tylosin and spiramcin, these were included with animal feed as growth promoters to improve the growth of fishes (Delsol et al., 2005).

In shrimp culture, antibiotics is widely used used to improve the production (Chaurasia et al., 2016a; Kumaresan et al., 2016; Chaurasia et al., 2016b; Kumaresan et al., 2015a; Arockiaraj et al., 2015a). The continuous application of these antibiotics in the aquaculture ponds enhane drug resistance among bacterial pathogens in shrimps. The usage of antibiotics significantly affected shrimps and also allows transmit of resistant genes in food chain (Grave et al., 1999). In general, Asian countries have limited guidelines and regulations to control the use of antibiotics in aquaculture sector. In Thailand, antibiotics such as, chloramphenicol has been widely used to control various bacterial diseases in 1990s, however very small quantity of this antibiotic is frequently detected from the cultured shrimp of Thailand. This leads to complete ban of shrimp export in Thailand (Heckman, 2004). Also, chloramphenicol has detected in shrimp farm in Myanmar, Vietnam and India.

To avoid the use of antimicrobial agents, probiotic bacteria were used in aquaculture farms to improve the fish health (Cruz et al., 2012; Palanisamy et al., 2015; Arockiaraj et al., 2015b; Chaurasia et al., 2015; Kumaresan et al., 2015b; Rao et al., 2015). These probiotic organisms significantly improved the growth of fishes and also involved in the production of various extracellulare enzymes. Recently, organisms suh as, bacteria and yeast were screened for the production of various antibiotics for aquaculture industry (Banerjee et al., 2016). Probiotic organisms showed antibacterial potentials and has the ability to alter the microbial flora in the intestine. Probiotic bacteria produce various natural antibacterial substances such as, organic acids and bacterocin (Aarti et al., 2018; Arasu and Al-Dhabi, 2017; Aarti et al., 2017; Arasu et al., 2014a; Arasu et al., 2014b). These agents readily stimulate the production of toxic substances and thus improve the growth (Ringo and Vadstein, 1998). Probiotics has also been used to enhance reproductive performance and stress tolerance in fishs (Mohapatra et al., 2014). The introduction of these probiotics in aquaculture sector significantly replaced the use of commercial synthetic antibiotics. Probiotics release various antibacterial agents with either bactericidal or bacteriostatic properties that inhibit colonization of pathogenic organism in the intestine of shrimps(Aarti et al., 2016; Arasu et al., 2016a,b; Arasu et al., 2014c; Arasu et al., 2014d). Probiotics have been applied along with feed in commercial aquaculture sector to control or prevent bacterial or fungal infections. The applied probiotics effectively control diseases in both fin fishes and shell fishes (Castex et al., 2014). Also, alter the microbial consortium in the gut, reduce microbial diversity and increase richness of beneficial microbes in the gut. Probiotic bacteria have the potential to produce various extracellular enzymes namely proteases, amylases and lipases and also produce the growth promoting substances such as, amino acids, vitamins and fatty acids (Dimitroglou et al., 2011).

2. Materials and methods

2.1. Isolation of probiotic bacteria from the gut of Penaeus indicus

In this study 20*P. indicus* was collected from the coastal region under disease free conditions. It was maintained in glass tank at 30 ± 2 ppt salinity, pH 7.5 and at 30 ± 2 °C temperature. From the experimental tank, healthy animals were sorted out and subjected for the analysis of intestinal microbial flora. Intestine was carefully removed using a sterile needle and washed with sterile seawater to eliminate non-adherant bacteria. The sample was homogenized using a glass homogenizer. Homogenized sample was serially diluted with sterile seawater and spreaded on Zobell Marine Agar (ZMA) plates by pour plate method. After 48 h incubation, the colonies appeared were purified by repeat straking on ZMA plates (Sivakumar et al., 2012).

2.2. Screneing of probiotic bacteria

Antibacterial potential of the morphologically distinct bacterial isolates were initially screened by cross streak method using indicator bacterium (*Pseudomonas* sp). Morphologically distinct bacterial isolates were streaked on ZMA on a straight line and incubated for 24 h at 37 °C. After 24 h incubation, indicator strain (*Pseudomonas* sp.) was streaked across the probiotic bacterium and further incubated for 24 h at 37 °C. Antibiotic producing ability of the isolate showed zone of inhibiton around the test organism (Karthik et al., 2013). Isolates showed inhibitory activity against indicator organism was further used for secondary screening.

2.3. Screnning of bacteriocin production by bacteria

Acid production test has been used to identify bacteriocin production by the selected bacteria. The bacterial isolates were cultured in submerged fermentation at 37 °C for 48 h. Then the culture was centrifuged (10,000×g) for 10 min and the cell free supernatant was obtained. It was subjected for acid production test using bromothymol blue indicator. Developement of blue colour into yellow colour indicated the production of acid. Bacterocin positive bacteria did not show any colour change.

2.4. Screening of antibiotic potentials by well diffusion method

The bacterial strains with acid production negative were confirmed bacteriocin production. Then quantitative assays of the selected culture were performed using agar well diffusion method. The selected bacterial isolate was inoculated in 250 ml Erlenmeyer flask containing nutrient broth (100 ml) and incubated at 37 °C for 48 h. The culture was centrifuged (10,000×g, 10 min) and loaded (10 μl) on Meuller Hinton Agar (MHA) plates. Four pathogenic bacterial isolates, namely, *Vibrio* sp., *Pseudomonas* sp., *Micrococcus* sp. and *Bacillus* sp. were selected and the zone of inhibition against these bacteria was registered.

2.5. Characterization of the probiotic bacterium

A probiotic bacterium was selected based on the above screening protocol and sub cultured periodically for every two months and stored at 4 °C. Morphological and biochemical identification of bacterium was performed by standard methods. 16S rDNA analysis was performed to identify the probiotic bacterium in molecular level.

2.6. Optimized production of bacteriocin by a traditional method

To find the effect of incubation period on the growth and the production of bacteriocin, the selected bacteria strain *Bacillus subtilis* was cultured in submerged fermentation for 7 days. To study the influence of pH on bacteriocin production, the culture medium pH was adjusted at various pH values (pH 4.0 – 9.0). After 72 h incubation, bacterial growth and bacteriocin production were determined from the cell free supernatant. To evaluate the impact

of temperature on the growth and the production of bacteriocin, the strain was incubated in culture medium for 20–45 °C. To analyze the impact of carbon source, glucose, starch, glycerol, maltose, lactose and sucrose were supplemented with the culture medium at 1% (w/v) level. To the control, carbon source was not incorporated. The nitrogen sources such as, sodium nitrate, ammonium chloride, peptone, yeast extract, beef extract and glycine were incorporated with the culture medium at a level of 1% (w/v) to explore best nitrogen sources. These experiments were performed in triplicates and Mean ± SD was calculated. One-way analysis of variance (ANOVA) was used to analyze the significance level.

2.7. Shrimp feed formulation

The experimental diet consists of protein (47%), lipid (8.2%), ash (13.2%), moisture content (8.1%) and fibre level was 2.1%. These ingredients were used for the formulation of artificial shrimp diet as suggested by Boonyaratpalin and New (1982). These feed ingredients were mixed with gelatine 5% (w/v) and required water was added. The pH of the feed was adjusted as 7.0 ± 0.20. The formulated diet consists of (g) fish (2 8 0), shrimp head waste (1 0 0), squid meal (20), squid liver (30), wheat (60), wheat flour (2 0 0), soy bean (1 0 0), broken rice (1 0 0), fish oil (25), vitamin (10), minerals (40) and gelatine (15). To the feed bacteriocin was added (10 mg–100 mg) and used for experiment (Swapna et al., 2015).

2.8. Effect of bacteriocin on the growth performance of P. Indicus

P. indicus (8 ± 2 g) was purchased from the shrimp farm and stock was maintained in culture tank (25 L). Continuous air flow was attached with the tank and the flow rate was maintained as 2.0 L/min. The experimental animal was fed with bacteriocin containing diet at various concentrations (10 mg–100 mg) for 40 days at the rate of 10% total body weight. The control and experimental group animals were fed twice in a day (morning and evening). Debris and uneaten pellet diet was removed every day. The other factors such as, salinity, temperature and pH was also monitiored continuously.

2.9. Effect of probiotic on the growth and survival of juvenile P. Indicus

The screened probiotic strain was used for the production of secondary metabolites. The probiotic bacterium was grown in nutrient broth medium for 48 h. It was centrifuged at 10,000 rpm and the cells were collected. The obtained pellet was washed with physiological saline (0.9% NaCl, w/v) and resuspended with the same. To the artificial diet, probiotic organism was sprayed and the feed was dried at room temperature. Probioic organism was sprayed at five different concentrations viz., 2×10^2, 4×10^4, 6×10^6, 8×10^8 and 10×10^{10} CFU/100 g of feed. Inoculum was not included with the control shrimp. 20 experimental animals were introduced into the experimental tank (12 ± 0.5 g each) and growth performance was analyzed (Ajitha et al., 2004).

3. Results and discussion

3.1. Gut probiotics

A total of 104 bacterial isolates were isolated from the gut of *P. indicus*. The morphologically different bacterial isolate were further screened for probiotic properties. Based on inital screening only seven bacterial isolates were tested against indicator bacterium. The zone of inhibition against the tested bacterial pathogens was tabulated (Table 1). The present finding revealed that *B. subtilis* showed potent ablity to control the growth of marine pathogens.

Table 1
Antibacterial activity of probiotic bacteria isolated from the shrimp gut of *P. indicus*.

Gut bacteria	Zone of inhibition (mm)			
	Bacillus sp.	*Pseudomonas* sp.	*Vibrio* sp.	*Micrococcus* sp.
D1	12 ± 2	12 ± 2	21 ± 3	12 ± 2
D2	10 ± 1	18 ± 3	–	18 ± 1
D3	11 ± 2	10 ± 2	15 ± 2	16 ± 3
D4	–	12 ± 1	10 ± 1	–
D5	17 ± 1	19 ± 1	22 ± 2	21 ± 1
D6	11 ± 2	–	–	20 ± 2
D7	–	11 ± 2	–	–

There are various bacterial strains from the genus *Bacillus* produce various bioactive molecules such as, polymyxin, bacitracin and colistin. *Bacillus* species are aerobic bacteria, Gram's-positive rod which are widely distributed. These organisms were found in gastrointestinal tracts, rocks and aquatic environment (Nicholson, 2002). Previously, many bacteriocin producing bacterial isolates have been screened from various sources. In a study, Schallmey et al. (2004) reported the ability of *Bacillus* sp. to produce bacitracin A.

The isolated probiotic bacteria showed activity against these bacteria. 16S rDNA sequencing was performed and identified as strain D5. The strain D5 showed potent activity against all tested organisms. The isolated strain D5 showed 17 mm zone of inhibition against *Bacillus* sp., 19 mm against *Pseudomonas* sp., 22 mm against *Vibrio* sp., and 22 mm against *Micrococcus* sp. Based on these experiments antibacterial potential of *B. subtilis* was studied and it was selected for further studies. The identified bacterial isolate was Gram –positive, motile, rod shaped, hydrolyzed casein and starch. It was not able to hydrolyze urea, nitrate reduction-negative, indole-negative and citrate-negative. Bacteriocins have potent activity against various bacterial pathogens. In a study, *Bacillus licheniformis* for bacillocin production and effectively inhibited the growth of *Micrococcus flavus*. Likewise, Mendo et al. (2004) isolated a probiotic *Bacillus licheniformis* strain from the extreme environment which inhibited the growth of various Gram-positive bacteria. Kugler et al. (1990) isolated the bacteria such as, *Bacillus subtilis*, *B. cereus*, and *B. licheniformis* for the production of seconday metabolites and the produced metabolites were found to be effective against both Gram-positive and Gram-negative bacteria.

3.2. Optimization of growth of bacteria and bacteriocin production in submerged fermentation

The influence of bacterial growth and bacterocin production in relation with incubation time was evaluated. Bacteriocin production was found to be high at the stationary phase and was detected only after 48 h and reduced considerably after fifth day (Table 2). Likewise, Eppelmann et al. (2001) found that *Bacillus* sp. produce secondary metabolites after 48 h incubation and reached maximum level after 96 h. In *L. brevis* FPTLB3 and *W. paramesenteroides* DFR-8 bacteriocin synthesis were found to be maximum after 96 h of incubation (Banerjee et al., 2013). However, in *B. cereus* Bc7, production of bacteriocin was maximum before stationary phase (Oscáriz and Pisabarro, 2000).

Bacterial growth and bacterocin production was maximum at pH 7.0, however secondary metabolite production was found to be optimum between pH 6.0 and 8.0 (Table 3). Hydrogen ion concentration (pH) of the culture medium is one of the significant factors for the production of bacteriocin because it significantly affects the adsorption of secondary metabolites to their cell surface and attachment or aggregation of the newly produced cells. Also, the pH of the culture medium critically plays significant role in

Table 2
Effect of incubation time on the growth and bacteriocin production from *B. subtilis*. Data represent mean ± SD (n = 3).

Incubation time (days)	Optical Density (600 nm)	Zone of inhibition (mm)
1	0.329 ± 0.021	0 ± 0
2	1.572 ± 0.057	7 ± 2
3	2.195 ± 0.081	15 ± 1
4	1.986 ± 0.038	21 ± 3
5	1.822 ± 0.042	18 ± 5
6	1.712 ± 0.061	12 ± 2
7	0.713 ± 0.049	10 ± 1

Table 3
Effect of pH on the growth and bacteriocin production from *B. subtilis*. Data represent mean ± SD (n = 3).

pH	Optical Density (at 600 nm)	Zone of inhibition (mm)
4	0.126 ± 0.033	0 ± 0
5	0.572 ± 0.039	7 ± 1
6	1.893 ± 0.057	12 ± 0
7	1.972 ± 0.064	27 ± 5
8	1.426 ± 0.092	18 ± 3
9	1.172 ± 0.074	11 ± 2
10	0.724 ± 0.059	± 0

the degradation of bacteriocins by proteases (Cheigh et al., 2002). The influence of pH on bacteriocins production was analyzed from *L. mesenteroides* FR52, *Bacillus* spp. P11 and *L. plantarum* 17.2b and reported earlier (Delgado et al., 2007). The present findings are in accordance with observation made previously with other *Bacillus* sp. In *Bacillus subtilis* KIBGE IB-17, bacteriocin snynthesis was optimum at pH 7.0 (Ansari et al., 2012). Also, in *B. licheniformis*, bacteriocins production was found to be high from 7.0 to 8.0 (Martirani et al., 2002).

Incubation temperature is one of the important factors which significantly influences on growth and production of bacteriocin. Bacterial growth and bacterocin production (17 ± 2 mm) were maximum at 30 °C (Table 4). Fermentation temperature is a critical factor which significantly influenced on bacterocin production. In our study, bacteriocin production was maximum at 30 °C. However, various temperatures have been reported for the production of secondary metabolites by bacteria. Kim et al. reported that secondary metabolite production was maximum at 37 °C in the case of *Micrococcus* sp. GO5.

The influence of various carbon sources on the growth and bacteriocin production were analyzed. Glucose stimulated growth and bacteriocin production (21 ± 3 mm) (Table 5). In the case of bacteria, glucose is one of the important nutrients and it significantly influenced on bacteriocin production. In a study, Todorov and Dicks (2005) reported maximum production of bacteriocin in the medium containing glucose. Likewise, in *E. faecium* ST311LD glucose stimulated the production of bacteriocin and has been reported by Todorov (2008). The present results suggest that the

Table 4
Effect of temperature on the growth and bacteriocin production from *B. subtilis*. Data represent mean ± SD (n = 3).

Temperature (°C)	Optical Density (600 nm)	Zone of inhibition (mm)
20	1.127 ± 0.057	14 ± 1
25	1.565 ± 0.033	19 ± 2
30	1.946 ± 0.029	22 ± 3
35	1.707 ± 0.032	17 ± 2
40	1.632 ± 0.044	13 ± 2
45	0.501 ± 0.024	10 ± 1
50	0.305 ± 0.019	10 ± 0

Table 5
Effect of carbon sources on the growth and bacteriocin production from *B. subtilis*. Data represent mean ± SD (n = 3).

Carbon source (1%)	Optical Density (600 nm)	Zone of inhibition (mm)
Glucose	2.208 ± 0.101	21 ± 3
Glycerol	1.529 ± 0.068	12 ± 2
Starch	1.796 ± 0.031	20 ± 2
Lactose	1.097 ± 0.042	14 ± 1
Maltose	1.927 ± 0.062	18 ± 3
Sucrose	1.508 ± 0.079	12 ± 3
Control	2.042 ± 0.051	17 ± 4

glucose moiety of sucrose was very essential for the production of bacteriocin. Todorov and Dicks also found the positive regulation of glucose on bacteriocin production by *Lactobacillus pentosus* ST151. Among the supplemented nitrogen sources, glycine positively influenced on growth and metabolite production than other sources (Table 6). Our findings are in accordance the results reported previoiusly with many bacteria. In lactic acid probiotic bacteria and *B. cereus* XH25, glycine significantly stimulated bacteriocin production (Meera and Devi, 2012). Nitrogen sources enhance bacterial biomass and bacteriocin production in *Lactococcus lactis* (Kim et al., 2007) (Tables 7 and 8).

3.3. Influence of bacteriocin and probiotics on the growth and survival of P. Indicus

In the present study, bacteriocin supplemented diet enhanced the growth than control shrimp. Bacteriocin was administered at various doses ranged between 10 and 50 mg/100 gm level. Shrimp length was 3.3 ± 0.13 cm and shrimp weight was 9.7 ± 0.12 g in 100 mg bacteriocin group. The present finding revealed enhanced growth at high bacteriocin concentration. The administered bacteriocin enhanced the immunity and induced immune modulation. Previously, Chiu et al. (2007) reported increased superoxide dismutase and phenoloxidase activity in probiotic administered diet. In a study Itami et al. (1998) reported immuno stimulatory effect of peptidoglycans of *L. plantarum* in *Penaeus japonicas* and were effective against *Vibrio penaeicida*. Probioic organism was administered at five different concentrations (2×10^2, 4×10^4, 6×10^6, 8×10^8 and 10×10^{10} CFU/100 g) with feed and growth performance was analyzed. Probiotic feed stimulated the growth of *P. indicus*. At 2×10^2 CFU/100 g feed, the juvenile shrimp attained 16.8 ± 0.11 g weight after 40 days. The weight gain was 16.8 ± 0.11 g at 10×10^2 CFU/100 g probiotic concentration. In a study, Rengpipat et al., 1998 used *Bacillus* S11 as a probiotic isolate in shrimp culture and reported better survival rate in probiotic treated animals than control. The application of probiotics with feed enhanced digestibility. The administered probiotics significantly improved the growth, survival and Feed Conversion Ratio (FCR) in shrimps (Hai et al., 2009).

Table 6
Effect of nitrogen sources on the growth and bacteriocin production from *B. subtilis*. Data represent mean ± SD (n = 3).

Nitrogen source (1%)	Optical Density (at 600 nm)	Zone of inhibition (mm)
Ammonium chloride	1.629 ± 0.072	13 ± 2
Sodium nitrate	1.803 ± 0.052	17 ± 1
Peptone	1.554 ± 0.042	13 ± 2
Beef extract	1.992 ± 0.039	18 ± 2
Yeast extract	1.982 ± 0.041	16 ± 4
Glycine	2.109 ± 0.038	19 ± 3
Control	1.434 ± 0.061	16 ± 2

Table 7
Administration of bacteriocin with formulated diet and its effect on the growth and survival of *P. indicus*. Data represent mean ± SD (n = 10).

Bacteriocin (mg)	Shrimp length (cm)	Shrimp weight (g)	Survival (%)
10	3.3 ± 0.13	9.7 ± 0.12	98
20	4.9 ± 0.15	11.3 ± 0.15	98
30	5.7 ± 0.2	12.2 ± 0.19	100
40	5.3 ± 0.2	13.2 ± 0.13	100
50	4.8 ± 0.15	12.8 ± 0.11	100

Table 8
Effect of probiotics on the growth of *P. indicus*. Data represent mean ± SD (n = 10).

Probiotics (CFU/100 g of feed)	Shrimp length (cm)	Shrimp weight (g)
2×10^2	4.4 ± 0.13	13.7 ± 0.12
4×10^4	5.9 ± 0.12	14.3 ± 0.15
6×10^6	6.1 ± 0.21	15.2 ± 0.19
8×10^8	6.2 ± 0.23	16.2 ± 0.13
10×10^{10}	6.2 ± 0.16	16.8 ± 0.11

4. Conclusions

Intestinal microbial flora may significantly influence the diversity of the gut microbiota in shrimps. The present finding shows bacteria isolated from *Penaeus indicus* have the ability to produce secondary metabolites and these metabolites showed activity against various bacteria. *P. indicus* fed with probiotic diet enhanced growth rate in juvenile shrimps. The applied proiotic organism could colonize and synthezise various secondary metabolites in the gut. Also, probiotic may involve in the synthesis of various enzymes to enhance the digestibility of shrimp feed.

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References

Aarti, C., Khusro, Ameer, Varghes, Rakesh, Arasu, Mariadhas Valan, Paul AgastianAl-Dhabi, N.A., Ilavenil, Soundharrajan, Choi, Ki Choon, 2018. *In vitro* investigation on probiotic, anti-Candida, and antibiofilm properties of *Lactobacillus pentosus* strain LAP1. Arch. Oral Biol. 89, 99–106.

Aarti, C., Khusro, A., Rakesh, V., Arasu, M.V., Agastian, P., Al-Dhabi, N.A., Ilavenil, S., Choi, K.C., 2017. *In vitro* studies on probiotic and antioxidant properties of *Lactobacillus brevis* strain LAP2 isolated from Hentak, a fermented fish product of North-East India. LWT – Food Sci. Technol. 86 (2017), 438–446.

Aarti, C., Khusro, A., Arasu, M.V., Agastian, P., & Al–Dhabi, N.A. (2016). Biological potency and characterization of antibacterial substances produced by Lactobacillus pentosus isolated from Hentak, a fermented fish product of North–East India. SpringerPlus, 5, Article Number: 1743.

Ajitha, S., Sridhar, M., Sridhar, N., Bright Singh, I.S., Varghese, V., 2004. Probiotic effects of lactic acid bacteria against *Vibrio alginolyticus* in *Penaeus* (*Fenneropenaeus*) *indicus* (H. Milne Edwards). Asian Fish. Sci. 17, 71–80.

Ansari, A., Aman, A., Naveed Siddiqui, N., Iqbal, S., Ali ul Qader, S., 2012. Bacteriocin (BAC IB17): screening, isolation and production from *Bacillus subtilis* KIBGE IB 17. Pak. J. Pharm. Sci. 25 (1), 195–201.

Arasu, A., Kumaresan, V., Ganesh, M.R., Pasupuleti, M., Arasu, M.V., Al-Dhabi, N.A., Arockiaraj, J., 2017a. Bactericidal activity of fish galectin 4 derived membrane-binding peptide tagged with oligotryptophan. Dev. Comp. Immunol. 71, 37–48.

Arasu, A., Kumaresan, V., Palanisamy, R., Arasu, M.V., Al-Dhabi, N.A., Ganesh, M.R., Arockiaraj, J., 2017b. Bacterial membrane binding and pore formation abilities of carbohydrate recognition domain of fish lectin. Dev. Comp. Immunol. 67, 202–212.

Arasu, A., Kumaresan, V., Sathyamoorthi, A., Arasu, M.V., Al-Dhabi, N.A., Arockiaraj, J., 2016a. Coagulation profile, gene expression and bioinformatics characterization of coagulation factor X of striped murrel Channa striatus. Fish Shellfish Immunol. 55, 149–158.

Arasu, M.V., Jung, M.-W., Ilavenil, S., Kim, D.H., Park, H.S., Park, J.W., Al-Dhabi, N.A., Choi, K.C., 2014a. Characterization, phylogenetic affiliation and probiotic properties of high cell density *Lactobacillus* strains recovered from silage. J. Sci. Food Agric. 94, 2429–2440.

Arasu, M.V., Al-Dhabi, N.A., 2017. *In vitro* antifungal, probiotic, and antioxidant functional properties of a novel *Lactobacillus paraplantarum* isolated from fermented dates in Saudi Arabia. J. Sci. Food Agric. https://doi.org/10.1002/jsfa.8413.

Arasu, M.V., Al-Dhabi, N.A., Ilavenil, S., Choi, K.C., Srigopalram, S., 2016b. *In vitro* importance of probiotic *Lactobacillusplantarum* related to medical field. Saudi J. Biol. Sci. https://doi.org/10.1016/j.sjbs.2015.09.022.

Arasu, M.V., Al-Dhabi, N.A., Rejiniemon, T.S., Lee, K.D., Huxley, V.A.J., Kim, D.H., Duraipandiyan, V., Karuppiah, P., Choi, K.C., 2014b. Identification and characterization of *Lactobacillus brevis* P68 with antifungal, antioxidant and probiotic functional properties. Indian J. Microbiol. 55, 19–28.

Arasu, M.V., Jung, M.-W., Kim, D.H., Ilavenil, S., Jane, M., Park, H.S., Al-Dhabi, N.A., Jeon, B.T., Choi, K.C., 2014c. Enhancing nutritional quality of silage by fermentation with *Lactobacillus plantarum*. Indian J. Microbiol. 54, 396–402.

Arasu, M.V., Kim, D.H., Kim, P.I., Jung, M.W., Ilavenil, S., Jane, M., Lee, K.D., Al-Dhabi, N.A., Choi, K.C., 2014d. *In vitro* antifungal, probiotic and antioxidant properties of novel *Lactobacillusplantarum* K46 isolated from fermented sesame leaf. Ann. Microbiol. 64, 1333–1346.

Arockiaraj, J., Bhatt, P., Harikrishnan, R., Arasu, M.V., Al-Dhabi, N.A., 2015a. Molecular and functional roles of 6C CC chemokine 19 in defense system of striped murrel Channa striatus. Fish Shellfish Immunol. 45 (2), 817–827.

Arockiaraj, J., Bhatt, P., Kumaresan, V., Dhayanithi, N.B., Arshad, A., Harikrishnan, R., Arasu, M.V., Al-Dhabi, N.A., 2015b. Fish chemokines 14, 20 and 25: A comparative statement on computational analysis and mRNA regulation upon pathogenic infection. Fish Shellfish Immunol. 47 (1), 221–230.

Banerjee, G., Nandi, A., Dan, S.K., Ghosh, P., Ray, A.K., 2016. Mode of association, enzyme producing ability and identification of autochthonous bacteria in the gastrointestinaltract of two Indian air-breathing fish, murrel *(Channa punctatus)* and stinging catfish *(Heteropneustes fossilis)*. Proc. Zool. Soc. 1 (1), 20–25.

Banerjee, G., Ray, A.K., Askarian, F., Ringo, E., 2013. Characterization and identification of enzyme-producing autochthonous bacteria from the gastrointestinal tract of two Indian air-breathing fish. Benef. Microbes. 4, 277–284.

Castex, M., Daniels, C., Chim, L., 2014. Probiotic applications in crustaceans, in aquaculture nutrition: gut health, probiotics and prebiotics. John Wiley & Sons Ltd, Chichester, UK.

Chaurasia, M.K., Nizam, F., Ravichandran, G., Arasu, M.V., Al-Dhabi, N.A., Arshad, A., Elumalai, P., Arockiaraj, J., 2016a. Molecular importance of prawn large heat shock proteins 60, 70 and 90. Fish Shellfish Immunol. 48, 228–238.

Chaurasia, M.K., Ravichandran, G., Nizam, F., Arasu, M.V., Al-Dhabi, N.A., Arshad, A., Harikrishnan, R., Arockiaraj, J., 2016b. In-silico analysis and mRNA modulation of detoxification enzymes GST delta and kappa against various biotic and abiotic oxidative stressors. Fish Shellfish Immunol. 54, 353–363.

Chaurasia, M.R., Palanisamy, R., Harikrishnan, R., Arasu, M.V., Al-Dhabi, N.A., Arockiaraj, J., 2015. Molecular profiles and pathogen-induced transcriptional responses of prawn B cell lymphoma-2 related ovarian killer protein (BOK). Fish Shellfish Immunol. 45 (2), 598–607.

Cheigh, C.I., Choi, H.J., Park, H., 2002. Influence of growth conditions on the production of a nisin-like bacteriocin by *Lactococcus lactis* subsp. *lactis* A164 isolated from kimchi. J. Biotechnol. 95, 225–235.

Chiu, C.H., Guu, Y.K., Lui, C.H., Pan, T.M., Cheng, W., 2007. Immune response and gene expression in white shrimp, Litopenaeus vannamei, induced by Lactobacillus plantarum. Fish Shellfish Immun. 23, 364e77.

Cruz, P.M., Ibanez, A.L., Hermosillo, O.A.M., Saad, H.C.R., 2012. Use of probiotics in aquaculture. ISRN Microbiol., 96845

Delgado, A., López, F.N.A., Brito, D., 2007. Optimum bacteriocin production by *Lactobacillus plantarum* 17.2b requires absence of NaCl and apparently follows a mixed metabolite kinetics. J. Biotechnol. 130, 193–201.

Delsol, A.A., Randall, L., Cooles, S., Woodward, M.J., Sunderland, J., Roe, J.M., 2005. Effect of the growth promoter *avilamycin* on emergence and persistence of antimicrobial resistance in enteric bacteria in the pig. J. Appl. Microbiol. 98, 564–571.

Dimitroglou, A., Merrifield, D.L., Carnevali, O., Picchietti, S., Avella, M., Daniels, C., Güroy, D., Davies, S.J., 2011. Microbial manipulations to improve fish health and production a Mediterranean perspective. Fish Shellfish Immunol. 30 (1), 1–16.

Eppelmann, K., Doekel, S., Marahiel, M.A., 2001. Engineered biosynthesis of the peptide antibiotic bacitracin in the surrogate host *Bacillus subtilis*. J. Biol. Chem. 276, 34824–34831.

FAO. In: Serrano PH, ed. 2005. Responsible Use of Antibiotics in Aquaculture. Rome: FAO; FAO Fisheries Technical Paper. 98, 469.

Grave, K., Lingaas, E., Bangen, M., Rønning, M., 1999. Surveillance of the overall consumption of antibacterial drugs in humans, domestic animals and farmed fish in Norway in 1992 and 1996. J. Antimicrob. Chemother. 43, 243–252.

Hai, N.V., Buller, N., Fotedar, R., 2009. The use of customised probiotics in the cultivation of western king shrimps (*Penaeus latisulcatus* Kishinouye, 1896). Fish Shellfish Immunol. 27, 100–104.

Heckman, R., 2004. What else can happen? Other problems for fish production. Aquacult. Magaz. 30 (3), 27–40.

Itami, T., Asano, M., Tokushige, K., Kubono, K., Nakagawa, A., Tekeno, N., et al., 1998. Enhancement of disease resistance of kuruma shrimp, Penaeus japonicus, after oral administration of peptidoglycan derived from Bifidobacterium thermophilum. Aquaculture 164, 277–288.

Karthik, R., Gobalakrishnan, S., Hussain, A.J., Muthezhilan, R., 2013. Efficacy of bacteriocin from *Lactobacillus* sp. (AMET 1506) as a biopreservative for seafood's

under different storage temperature conditions. J. Modern Biotechnol. 2 (3), 59–65.
Kim, D.H., Brunt, J., Austin, B., 2007. Microbial diversity of intestinal contents and mucus in rainbow trout (*Oncorhynchus mykiss*). J. Appl. Microbiol. 102, 1654–1664.
Kugler, M., Loeffler, W., Rapp, C., Kern, A., Jung, G., 1990. Rhizocticin an antifungal phosphono-oligopeptide of *Bacillus subtilis* ATCC 6633: biological properties. Arch. Microbiol. 153, 276–281.
Kumaresan, V., Bhatt, P., Ganesh, M.R., Harikrishnan, R., Arasu, M.V., Al-Dhabi, N.A., Pasupuleti, M., Marimuthu, K., 2015a,. Arockiaraj J.A novel antimicrobial peptide derived from fish goose type lysozyme disrupts the membrane of Salmonella enterica. Mol. Immunol., 421–433
Kumaresan, V., Gnanam, A.J., Pasupuleti, M., Arasu, M.V., Al-Dhabi, N.A., Harikrishnan, R., Arockiaraj, J., 2015b. Comparative analysis of CsCu/ZnSOD defense role by molecular characterization: gene expression-enzyme activity-protein level. Gene 564, 53–62.
Kumaresan, V., Pasupuleti, M., Arasu, M.V., Al-Dhabi, N.A., Arshad, A., Nurul Amin, S. M., MdYusoff, F., Arockiaraj, J., 2019. A comparative transcriptome approach for identification of molecular changes in Aphanomycesinvadans infected Channastriatus. Mol. Biol. Rep. 45, 2511–2523.
Kumaresan, V., Ravichandran, G., Nizam, F., Dhayanithi, N.B., Arasu, M.V., Al-Dhabi, N.A., Harikrishnan, R., Arockiaraj, J., 2016. Multifunctional murrel caspase 1, 2, 3, 8 and 9: Conservation, uniqueness and their pathogen-induced expression pattern. Fish Shellfish Immunol. 49, 493–504.
Martirani, L., Varcamonti, M., Naclerio, G., De-Felice, M., 2002. Purification and partial characterization of *bacillocin* 490, a novel bacteriocin produced by a thermophilic strain of *Bacillus licheniformis*. Microb. Cell Factories. 1, 1–5.
Maynard, C.L., Elson, C.O., Hatton, R.D., Weaver, C.T., 2012. Reciprocal interactions of the intestinal microbiota and immune system. Nature 489, 231–241.
Meera, N.S., Devi, M.C., 2012. Partial characterization and optimization of parameters for Bacteriocin production by Probiotic Lactic acid bacteria. J. Microbiol. Biotechnol. Res. 2 (2), 357–365.
Mendo, S., Faustino, N.A., Sarmento, A.C., Amado, F., Moir, A.J., 2004. Purification and characterization of a new peptide antibiotic produced by a thermotolerant *Bacillus licheniformis* strain. Biotechnol. Lett. 26, 115–119.
Mohapatra, S., Chakraborty, T., Prusty, A.K., PaniPrasad, K., Mohanta, K.N., 2014. Beneficial effects of dietary probiotics mixture on hemato-immunology and cell apoptosis of *Labeo rohita* fingerlings reared at higher water temperatures. PLoSONE 9 (6). 100929.
Nicholson, W.L., 2002. Roles of *Bacillus* endospores in the environment. Cell Mol. Life Sci. 59, 410–416.
Oscáriz, J.C., Pisabarro, A.G., 2000. Characterization and mechanism of action of cerein 7, a bacteriocin produced by *Bacillus cereus* Bc7. J. Appl. Microbiol. 89 (2), 361–369.
Palanisamy, R., Kumaresan, V., Harikrishnan, R., Arasu, M.V., Al-Dhabi, N.A., Arockiaraj, J., 2015. Functional roles and gene regulation of tumor necrosis factor receptor 1 in freshwater striped murrel. Mol. Immunol. 66, 240–252.
Phillips, I., Casewell, M., Cox, T., De Groot, B., Friis, C., Jones, R., Nightingale, C., Preston, R., Waddell, J., 2004. Does the use of antibiotics in food animals pose a risk to human health? A critical review of published data. J. Antimicrob. Chemother. 53, 28–52.
Rao, V., Marimuthu, K., Kupusamy, T., Rathinam, X., Arasu, M.V., Al-Dhabi, N.A., Arockiaraj, J., 2015. Defense properties in the epidermal mucus of different freshwater fish species. Aquacult., Aquar., Conservat. Legisl. 8, 184–190.
Ravichandran, G., Kumaresan, V., Arasu, M.V., Al-Dhabi, N.A., Ganesh, M.R., Mahesh, A., Dhayalan, A., Pasupuleti, M., Arockiaraj, J., 2016. Pellino-1 derived cationic antimicrobial prawn peptide: bactericidal activity, toxicity and mode of action. Mol. Immunol. 78, 171–182.
Ravichandran, G., Kumaresan, V., Bhatt, P., Arasu, M.V., Al-Dhabi, N.A., Arockiaraj, J., 2017. A cumulative strategy to predict and characterize antimicrobial peptides (AMPs) from protein database. Int. J. Pept. Res. Ther. 23, 281–290.
Ravichandran, G., Kumaresan, V., Mahesh, A., Dhayalan, A., Arshad, A., Arasu, M.V., Al-Dhabi, N.A., Pasupuleti, M., Arockiaraj, J., 2018. Bactericidal and fungistatic activity of peptide derived from GH18 domain of prawn chitinase 3 and its immunological functions during biological stress. Int. J. Biol. Macromol. 106, 1014–1022.
Rengpipat, S., Phianphak, W., Piyatiratitivorakul, S., Menasveta, P., 1998. Effects of a probiotic bacterium on black tiger shrimp *Penaeus monodon* survival and growth. Aquaculture 167, 301–313.
Ringo, E., Vadstein, O., 1998. Colonization of *Vibrio pelagius* and *Aeromonas caviae* in early developing turbot (*Scophthalmus maximus* L.) larvae. J. Appl. Microbiol. 84 (2), 227–233.
Sathyamoorthi, A., Bhatt, P., Ravichandran, G., Kumaresan, V., Arasu, M.V., Al-Dhabi, N.A., Arockiaraj, J., 2017. Gene expression and in silico analysis of snakehead murrel interleukin 8 and antimicrobial activity of C-terminal derived peptide WS12. Vet. Immunol. Immunopathol. 190, 1–9.
Sathyamoorthi, A., Kumaresan, V., Palanisamy, R., Pasupuleti, M., Arasu, M.V., Al-Dhabi, N.A., Marimuthu, K., AminSM, Nurul, Arshad, A., MdYusoff, F., Arockiaraj, J., 2019. Therapeutic cationic antimicrobial peptide (CAP) derived from fish aspartic proteinase cathepsin D and its antimicrobial mechanism. Int. J. Pept. Res. Therapeut. 25, 93–105.
Schallmey, M., Singh, A., Ward, O.P., 2004. Developments in the use of *Bacillus* species for industrial production. Can. J. Microbiol. 50, 1–17.
Schwarz, S., Kehrenberg, C., Walsh, T.R., 2001. Use of antimicrobial agents in veterinary medicine and food animal production. Int. J. Antimicrob. Agents. 17, 431–437.
Sivakumar, N., Sundararaman, M., Selvakumar, G., 2012. Probiotic effect of Lactobacillus acidophilus against vibriosis in juvenile shrimp (*Penaeus monodon*). Afr. J. Biotechnol. 11 (91), 15811–15818.
Swapna, B., Venkatrayulu, C., Swathi, A.V., 2015. Effect of probiotic bacteria *Bacillus licheniformis* and *Lactobacillus rhamnosus* on growth of the Pacific white shrimp *Litopenaeus vannamei* (Boone, 1931). Eur. J. Exp. Biol. 5 (11), 31–36.
Todorov, S.D., Dicks, L.M.T., 2005. Optimization of bacteriocin ST311LD production by *Enterococcus faecium* ST311LD, isolated from spoiled black olives. J. Microbiol. 43, 370–374.