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Isolation, characterization of *Vibrio* and *Pseudomonas* spp from infected fresh water ornamental fishes and evaluation of potential agents for its control

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The present work aims at comparing the antibacterial effect of probiotics, plant extracts of *Vitex negundo* and *Clitoria ternatae* and antibiotics against the disease causing bacteria isolated from infected ornamental fishes. The molecular characterization of isolated pathogens was performed by randomly amplified polymorphic DNA (RAPD) technique. The antibacterial effects of probiotic and plant extracts were analyzed by well diffusion method and antibiotic disc was performed by disc diffusion method. Isolated probiotics such as *Bacillus* sp. showed maximum antibacterial activity of 18 mm and 16 mm zone against *Vibrio* sp (V_2) and *Pseudomonas* sp (Ps_1) respectively. Leaf extract of *Vitex negundo* displayed zone of inhibition of 20 mm on *Pseudomonas* sp (Ps_3) and 16 mm on *Vibrio* sp (V_1). Among the antibiotics, chloramphenicol and ampicillin showed maximum inhibitory activity against *Vibrio* sp (V_3) (25mm) and *Pseudomonas* sp (Ps_2) (25mm) respectively. Even though, antibiotics showed higher inhibitory activity than the isolated *Bacillus* bacteria and plant extract (*V. negundo* and *C. ternatae*). This study concluded that the use of probiotics as an alternative strategy to the use of antibiotics because of its effective antibacterial activity and growing concern in violence of disinfectants and antibacterial agents.

Key words: Probiotics, antibacterial activity, ornamental fish pathogens, antibiotics, *Vitex negundo*.

INTRODUCTION

Disease outbreak is being increasingly recognized as one of the major constrain in aquaculture production which affect the trade and economic development of the sector. Virus, bacteria and parasitic diseases cause immense damage in host metabolic process by producing toxic substances. So far, conventional approaches similar to disinfectants, vaccines, antibiotics and chemotherapeutics are continued to be an important disease control measures in ornamental fishes. Massive use of synthetic antimicrobial drugs encourages the natural emergence of bacterial resistance (David *et al*., 2007) and also antibiotics pose threats to consumer health, non-target organisms and the environment (Jose *et al*., 2006, Parker, 1974). In addition to that, over dose of chemical or antibiotics spoil the fish physical appearance and colour which ultimately affect the sale price of ornamental fishes. However, conventional approaches have limited success in prevention or cure of aquatic diseases, it might be necessarily to introduce

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alternative strategies to the use of antimicrobials in disease control have been proposed.

The use of beneficial bacteria (probiotics) to displace pathogens by competitive processes is being used in the animal industry as a better remedy than administering antibiotics and is now gaining acceptance for the control of pathogens in aquaculture (Havenaar *et al*., 1992, Moriarty, 2000). Many investigators demonstrated positive effects of probiotics for various fish species, including rainbow trout (*Oncorhynchus mykiss*) (Dugenci *et al*., 2003, Aubin *et al*., 2005, Brunt and Austin, 2005, Panigrahi *et al*., 2004, 2005, 2007, Staykov *et al*., 2005, 2005a, 2005b, 2006, 2006a, 2007, 2009, Bagheri *et al*., 2008, Denev, 2008, Sealey *et al*., 2008), common carp (*Cyprinus carpio*) (Yanbo and Zirong, 2006, Staykov *et al*., 2005, 2005c, 2006b, 2007, 2007a, Denev, 2008) and Indian major carp (*Labeo rohita*) (Nayak *et al*., 2007). Their mechanism of action is unclear but may compete with pathogenic bacteria and produce inhibitory substances. Probiotics can make provision of essential nutrients for the cultured animal and provision of digestive enzymes and enhance direct uptake or decomposition of water-borne organic matter (Rodgers and Furones, 2009). Most of the probiotics proposed as biological control agents in aquaculture belong to the lactic acid bacteria, *Bacillus* sp. and few *Pseudomonas* sp. (Laurent *et al*., 2000).

Traditional herbal medicines seem to have the potential immunostimulation. Many studies have proved that herbal additives enhance the growth of fishes and also protect from the diseases (Francis *et al*., 2000, Jassim and Naji, 2003, Sasmal *et al*., 2005, Shalaby *et al*., 2006, Johnson and Banerji, 2007). It has been reported that Indian almond leaves used as an alternative of antibacterial agents and chemical substances (Nantarika and Nongnut, 2008). The growth of *Aeromonas hydrophila* which cause infection in fins, gills and in the caudal region of goldfish *Carassius auratus* was restricted by the administration of *Phyllanthus niruri* and *Aloe vera* (Ahilan *et al*., 2010). Hakan *et al*., (2009) discussed the antibacterial activity of plants *Nelumbo lutea* and *Vinca minor* against fish pathogens namely *A. hydrophila, Yersinia ruckeri*, *Lactococcus garvieae*, *Streptococcus agalactiae* and *Enterococcus faecalis*. The present study describes the evaluation of antibacterial effect of probiotics and plant extract, compared with antibiotics and also to study the molecular characterization (RAPD) of isolated fish pathogens.

MATERIALS AND METHODS

Isolation of pathogenic bacteria

Infected ornamental fish samples (Fig. 1 a, b) were aseptically excised and the gills, body surface were examined microscopically. Spleen, gill, liver and kidney of the fishes were aseptically removed. Homogenized samples were platted on thiosulphate citrate bile salts sucrose (TCBS) media (Hi Media, Mumbai) for *Vibrio* sp. isolation. Kings medium with the pH - 7.2 was used to isolate *Pseudomonas* sp. After incubation, predominant bacterial colonies were sub cultured in the nutrient medium to check purity of the isolate. The selected pathogenic *Vibrio* spp and *Pseudomonas* spp were cultured individually on Luria bertani (LB) broth at 37° C for 24 hrs before assay. 100 µl of broth culture, which contain 10^6 – 10^8 numbers of bacteria per millilitres was streaked on Muller hinton agar (MHA) medium for further utilization.

DNA extraction and RAPD analysis of pathogenic bacteria

Extraction of genomic DNA from all the isolated pathogens were carried out by following Sambrook *et al*. (1989) method. PCR-RAPD reaction was done with primer RBA-5 (5'TTCCCCGAC3'). The template DNA was amplified by PCR (Eppendorf - Germany) with cocktail of standard PCR buffer 2.5 µl, 10 mM dNTP 0.5 µl, primer 2.0 µl, template DNA 2.0 µl, Taq polymerase 0.2 µl. The amplification conditions were 1 min at 94° C, followed by 35 cycles of 1 min at 94° C, 1 min at 32° C and 1 min at 72° C

and a final extension at 72° C for 10 min. PCR products were analysed in 1.4% agarose gel electrophoresis.

Isolation of probiotic bacteria

Probiotic bacteria were isolated from healthy fish samples (gut, spleen, gill, liver and kidney) and water samples of fresh water ponds and reservoir from Sivagangai district. MRS-*Lactobacillus* agar was used to isolate *Bacillus* sp. After incubation at 37° C for 24 hrs (Bushra *et al*., 2007), predominant bacterial colonies were sub cultured in the nutrient medium to check the purity of isolate.

Preparation of cell free extract of probiotic bacteria

Overnight culture of isolated probiotic strain was raised in the LB broth. The culture in the broth was centrifuged for 10 mins at 8500rpm. Cell-free extract was filtered using a syringe with 0.2 µm filter and again filtered with 0.2 µm acetate cellulose filter. The cell free extract was stored for the purposes of further bacterial screening (Mitsunobu and Nadine, 1987).

Preparation of plant extract

The medicinal plant *Vitex negundo* and *Clitoria ternatae* were rinsed with sterile distilled water and cut into small pieces. The leaves pieces were homogenized by adding chloroform and ethanol in 1:1 ratio and the extract was separated by centrifugation. This process was repeated for many times to get more amounts of extract (Gary *et al*.).

Screening the antibacterial activity of probiotic and plant extracts

20 µl of overnight raised culture of all the pathogens in LB broth was swabbed on the MHA plates. Sterile cork borer were used to make 6 mm wells in MHA plates and inoculated with the overnight culture of the pathogens (*Pseudomonas* spp and *Vibrio* spp). About 100 µl of probiotics cell free extract and plant extract of *V. negundo* and *C. ternatae* were loaded in the wells. The plates were incubated at 37° C for 24 hrs. After incubation the zone of inhibition was measured and tabulated.

Antibacterial activity of antibiotics

Commercially available antibiotic disc chloramphenicol, gentamycin, ampicillin, nalidixic acid were used to find out the antibacterial activity against the isolated pathogenic *Vibrio* spp and *Pseudomonas* spp by disc diffusion method.

RESULTS

Isolation and identification of pathogenic bacteria

Totally seven pathogenic bacteria of different genus (*Pseudomonas* and *Vibrio*) were isolated from the infected ornamental fishes (Fig.1 a, b). Among the seven, four of them were *Vibrio* spp (V_1, V_2, V_3 and V_4) and three of them were *Pseudomonas* spp (Ps_1, Ps_2 and Ps_3).

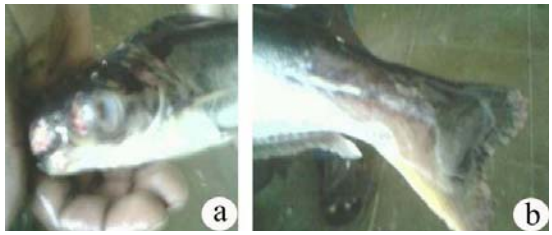

Figure 1. Infected ornamental fish collected from aquarium, a - Infected mouth parts, b - Tail rot disease

Molecular characterization of the bacterial pathogens

Primer RBA-5 (5′ TTCCCCGAC 3′) was used to distinguish the isolated *Pseudomonas* and *Vibrio* spp by RAPD method. RAPD profile showed reproducible banding patterns of *Pseudomonas* spp (Ps_1, Ps_2 and Ps_3) and *Vibrio* spp (V_1, V_2, V_3 and V_4) (Fig. 2). Primer RBA-5 showed band patterns ranged from 500 – 3000bp which was common to all the isolated pathogens. Furthermore, *Vibrio* spp V_2 and V_3 produced a band pattern with the size of 3500bp and 2500bp respectively. The result showed that *Pseudomonas* spp and *Vibrio* spp isolated from fresh water

ornamental fishes produced unique RAPD profile.

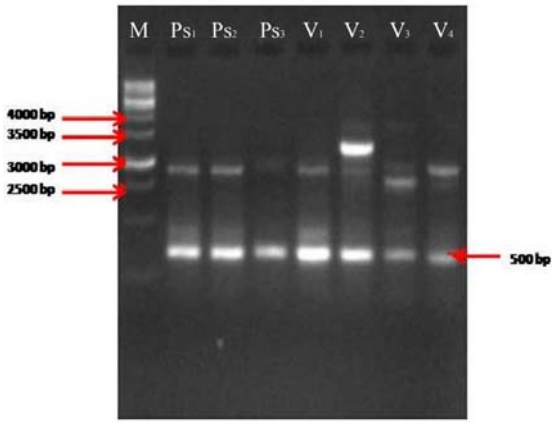

Figure 2. RAPD profile of primer RBA-5 of *Vibrio* spp and *Pseudomonas* spp isolated from infected regions of ornamental fish. M – 1Kb Ladder

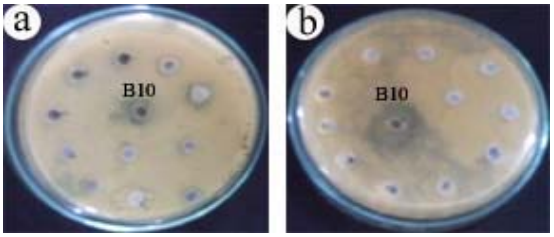

Figure 3. Antibacterial activity of probiotics on *Vibrio* spp and *Pseudomonas* spp a - Antibacterial activity of probiotics (B_{10}) against the Ps_1, b - Antibacterial activity of probiotics (B_{10}) against V_2.

Antibacterial activity of probiotic strains

Ten probiotics belong to the genus *Bacillus* sp. were isolated from different environment and named as *Bacillus*$_1$-*Bacillus*$_{10}$ (B_1-B_{10}). Among the ten *Bacillus* spp, only B_{10} showed inhibitory activity against *Vibrio* spp with the zone of inhibition of 12 to 18 mm and maximum of 18mm against V_2 and minimum of 12 mm was noticed against V_3. 16 mm of inhibition was noticed against *Pseudomonas* sp (Ps_1) (Table.1) (Fig.3 a, b).

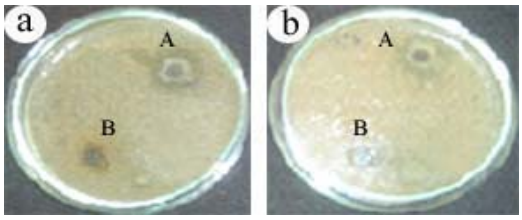

Figure 4. Antibacterial activity of plant extract on *Vibrio* spp and *Pseudomonas* spp a - Antibacterial activity of plant extracts *V. negundo* and *C. ternetae* against Ps_3, b- Antibacterial activity of plant extracts *V. negundo* and *C. ternetae* against V_1 [A-ethanol chloroform extract of *V. negundo*, B- ethanol chloroform extract of *C. ternetae*].

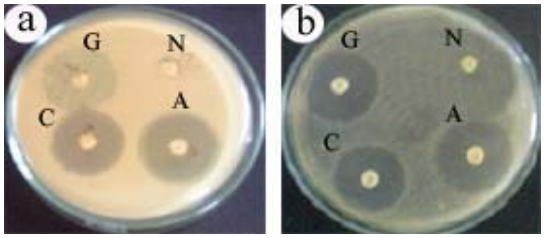

Figure 5. Antibacterial activity of antibiotics on *Vibrio* spp and *Pseudomonas* spp a - Antibacterial activity of antibiotic discs against Ps_2, b- Antibacterial activity of antibiotic discs against V_3 [G- Gentamycin, C- Chloramphenicol, N- Nalidixic acid, A- Ampicillin].

Antibacterial activity of plant extracts

V. negundo and *C. ternatae* extract were used for testing antibacterial effect against the isolated *Vibrio* spp and *Pseudomonas* spp *V. negundo* showed the inhibition on both the *Vibrio* spp and *Pseudomonas* spp (Table. 2). The ethanol chloroform leaf extract of *V. negundo* showed 20 mm zone of inhibition (Fig. 4 a, b) on *Pseudomonas* Ps_3 and 16 mm on V_1. No activities were noticed with the rest of the pathogens. *C. ternatae* did not show any inhibitory effect on any of the pathogens.

Effect of antibiotic against *Vibrio* spp and *Pseudomonas* spp

Among the selected antibiotics, chloramphenical and ampicillin showed the maximum zone of inhibition (25 mm) against V_3 and Ps_2 (Fig.5 a, b). Nalidixic

acid showed no activity against any pathogens except Ps_2 (10 mm). The minimum activity of 5 mm was noticed in ampicillin against V_1. The results were tabulated (Table 3).

Table 1. Antibacterial activity (zone of inhibition in mm) of probiotics against fish pathogens

Name of the pathogens	Name of the Probiotics									
	B_1	B_2	B_3	B_4	B_5	B_6	B_7	B_8	B_9	B_{10}
Ps_1	-	-	-	-	-	-	-	-	-	16
Ps_2	-	-	-	-	-	-	-	-	-	
Ps_3	-	-	-	-	-	-	-	-	-	-
V_1	-	-	-	-	-	-	-	-	-	-
V_2	-	-	-	-	-	-	-	-	-	18
V_3	-	-	-	-	-	-	-	-	-	12
V_4	-	-	-	-	-	-	-	-	-	15

Table 2. Antibacterial activity of plant extracts *Vitex negundo* (nochi) and *Clitoria ternetae* against fish pathogens

Pathogens	Plant extracts (zone of inhibition in mm)	
	Vitex negundo	*Clitoria ternetae*
Ps_1	-	-
Ps_2	-	-
Ps_3	20	-
V_1	16	-
V_2	-	-
V_3	-	-
V_4	-	-

Table 3. Antibacterial activity of commercially available antibiotics on fish pathogens

Pathogens	Antibiotics (zone of inhibition in mm)			
	Chloram-phenicol	Genta-mycin	Ampi-cillin	Nalidixic acid
Ps_1	16	11	-	-
Ps_2	18	20	25	10
Ps_3	18	15	-	-
V_1	19	11	5	-
V_2	14	12	-	-
V_3	25	15	12	-
V_4	12	16	-	-

DISCUSSION

During the last decades, there is better improvement in search of antibiotics used as traditional strategy for fish diseases management and growth. For that reason, the present study designed to perform a comparative analysis of antibacterial activity of probiotics, plant extracts and commercially available antibiotics. This study has also done with the RAPD profile for the molecular characterization of isolated fish pathogens. RAPD-PCR has been shown in recent years to be useful for classifying a number of bacterial species (Madico *et al*., 1995, Williams *et al*., 1990). The results indicated that all of the strains from the same type of sampling source produced bands of same molecular weight. The sharing of common bands indicates the presence of highly conserved genomic regions in diverse strains of *Vibrio* and *Pseudomonas*. This assumes significance as amplification of common fragments by RAPD-PCR with a particular primer has been shown to be useful in genetic amplifications and hybridization assays for diagnostic purpose (Dalla *et al*., 2002). Thus, it can be concluded that RAPD-PCR which is a rapid and simple tool could be used to differentiate a large number of bacterial strains, which could be useful in studying the epidemiology and distribution for implementing appropriate measures for controlling disease caused by the bacteria.

Previous studies in the area of probiotics revealed its antibacterial activity against bacterial fish pathogens. Out of 170 strains, 101 strains were found active against three major fish pathogens include *Vibrio parahaemolyticus, Vibrio harveyi,* and *Aeromonas hydrophila* (Prem *et al*., 2011). Also reported that different probiotics were isolated from a variety of marine samples such as algae, sponge, biofilm, sediment, sea cucumber, sea urchin, jellyfish, gut microflora of gastropods, crab, ascidian and corals (Prem *et al*., 2011). This study explored that probiotics are better antibacterial agents against fish pathogens. Probiotic *Streptococcus phocae* and *Enterococcus faecium* isolated from shrimp and fish intestine showed broad spectrum of antimicrobial effect on more than eighteen gram positive and gram negative pathogenic strains including *Vibrio* and *Pseudomonas* sp. with the zone of inhibition ranging from 13 to 16 mm in diameter (Paulraj *et al*., 2010). In the present study, out of seven pathogens, four showed susceptibility in the presence of probiotic bacteria (*Bacillus* sp.) with the observed zone of inhibition 12 to 18 mm. The observed results indicated that the B*acillus* sp. isolated from fish samples showed a good antibacterial activity.

There are large numbers of studies already been done with the medicinal effects of plants which enhanced the growth of fish and protection from diseases (Francis *et al*., 2002, Jassim and Naji, 2003, Shalaby *et al*., 2006, Johnson and Banerji, 2007). Ethanol extract of neem leaf exhibited minimum inhibitory concentration of 75 to 250 g ml^{-1} on *Pseudomonas aeruginosa* and *Vibrio cholerae* strains isolated from infected regions of the clown fishes *Amphiprion sebae* and *A. ocellaris* (Dhayanithi *et al*., 2010). The ethanol leaf extract of *V. negundo* showed the spectrum of inhibition on *V. cholera* (Merlin and Cathrine, 2011). In our study, *V. negundo* showed antibacterial activity of 20 mm against Ps_3 and 16 mm against V_1. By considering the range of zone of inhibition, *V. negundo* possesses maximum inhibition on *Pseudomonas* sp. isolated from infected fish. Recent reports have shown that methanolic extract of *C. ternetae* affected the growth of *Bacillus cereus, Klebsiella pneumoniae, Proteus vulgaris* and *Salmonella typhi* (Anand *et al*., 2011). Also aqueous, methanol and chloroform extracts exhibited antibacterial activity against *Pseudomonas aureginosa* (Babu *et al*., 2009). But no data were published on the antibacterial effect of *C. ternetae* on *Vibrio* sp. In our study, no antibacterial activity of *C. ternetae* was observed against any of the isolated pathogens.

This present investigation performed a comparative antibacterial activity of probiotics, plant extract and antibiotics on disease causing pathogens isolated from infected parts of fishes. From this study, it was observed that chloramphenicol, a commercially available antibiotic, showed high inhibitory activity on isolated fish pathogens with the zone of inhibition ranging from 12 to 25 mm in diameter, which is higher than the inhibitory activity of plant extract and probiotics. When compared to the antibacterial effect of plant extracts, probiotics showed inhibitory activity against isolated *Vibrio* spp (V_2, V_3 and V_4) and *Pseudomonas* sp. (Ps_1). Plant extract (*V. negundo*) showed inhibition against less number of isolated pathogens (Ps_3 and V_1). Although the antibiotics exhibit higher activity, there is a growing concern about the abuse of antimicrobial drugs not only in human medicine and agriculture but also in aquaculture. By keeping the instructions of World Health Organization in mind (fact sheet 194 web site), it could be necessitate reducing the overuse and inappropriate use of antimicrobials. According to the results of this study, *Bacillus* sp (B_{10}) showed effective inhibitions (16 - 18 mm) against isolated fish pathogens which may be consider for probiotic strain and could be used as a safer alternative strategy for the control of ornamental fish diseases. This study can be a better supportive to ornamental fish farmers and aquarium

owners as an alternative strategy in the use of antibacterial in disease control.

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REFERENCES

Ahilan B, Nithiyapriyatharshini A, Ravaneshwaran K. 2010. Influence of certain herbal additives on the growth, survival and disease resistance of goldfish, *Carassius auratus*. (linnaeus). Tamilnadu Journal of Veterinary & Animal Sciences 6(1): 5-11.

Anand SP, Doss A, Nandagopalan V. 2011. Antibacterial studies on leaves of *Clitoria ternatea* linn. - A high potential medicinal plant. International journal of applied biology and pharmaceutical technology 2(3): 453-456.

Aubin J, Gatesoupe FJ, Laurent L, Lebrun L. 2005. Trial of probiotics to prevent the vertebral column compression syndrome in rainbow trout (*Oncorhynchus mykiss* Walbaum). Aquacul. Res. 36(8): 758-767.

Babu Uma, Kesani Prabhakar, Sadayappan Rajendran. 2009. Phytochemical analysis and antimicrobial activity of *Clitorea ternatea* Linn against extended spectrum beta Lactamase producing enteric and urinary pathogens. Asian Journal of Pharmaceutical and Clinical Research 2(4): 94-96.

Bagheri T, Hedayati S, Yavari V, Alizade M, Farzanfar A. 2008. Growth, survival and gut microbial load of rainbow trout (*Onchorhynchus mykiss*) fry given diet supplemented with probiotic during the two months of first feeding. Turkish J. Fisheries Aquatic. Sci. 8: 43-48.

Brunt B, Austin B. 2005. Use of a probiotic to control lactococcosis and streptococcosis in rainbow trout, *Oncorhynchus mykiss* (Walbaum). J. Fish Diseases 28: 693-701.

Bushra Jamil, Fariha Hasan, Hameed A, Safia Ahmed. 2007. Isolation of *Bacillus subtilis* mh-4 from soil and its potential of polypeptidic antibiotic production. Pak. J. Pharm. Sci. 20(1): 26-31.

Dalla VL, Zanella L, Belvedere P, Colombo L. 2002. Use of random amplification to developed a PCR detection method for the causative agents of fish pasteurellosis. *Photobacterium damselae* subsp. piscicida (Vibrionaceae). Aquaculture 207: 187-202.

David G Lalloo, Delane Shingadia, Geoffrey Pasvol, Peter L Chiodini, Christopher J Whitty, Nicholas J Beeching, David R Hill, David A Warrell, Barbara A Bannister. 2007. UK malaria treatment guidelines. Journal of Infection 54: 111-121.

Denev SA. 2008. Ecological alternatives of antibiotic growth promoters in the animal husbandry and aquaculture. DSc. Thesis, Department of Biochemistry Microbiology, Trakia University, Stara Zagora, Bulgaria; 294.

Dhayanithi NB, Ajith Kumar TT, Kathiresan K. 2010. Effect of neem extract against the bacteria isolated from marine fish. Journal of Environmental Biology 31: 409-412.

Dugenci SK, Arda N, Candan A. 2003. Some medicinal plants as immunostimulant for fish. Journal of Ethnopharmacology 88: 99-106.

Francis F, Sabu A, Nampoothiri KM, Szakacs G, Pandey A. 2002. Synthesis of alpha-amylase by *Aspergillus oryzae* in solid-state fermentation. J. Basic Microbiol. 42: 320-326.

Gary D Foster, Sally C Taylor (Eds). Methods in molecular biology. Plant virology protocols. Humana press. Volume 81.

Hakan T, Arzu BY, Fatma PK. 2009. Sensitivity of bacteria isolated from fish to some medicinal plants. Turk. J. Fish Aqua. Sci. 9: 181-186.

Havenaar R, Ten Brink B, Huis int Veld JHJ. 1992. Selection of strains for probiotic use. In: *Probiotics*: the scientific basis, Eds. Fuller R. Chapman and Hall, London, pp. 209-224.

Jassim SAA, Mazen A Naji. 2003. Review/ Novel antiviral agents: A medicinal plant perspective. Journal of Applied Microbiology (UK) 95: 412-427.

Johnson C, Banerji A. 2007. Influence of extract isolated from the plant sesuvium portulacastrum on growth and metabolism in freshwater teleost, *Labeo rohita* (Rohu). Fishery Technology 44(2): 229-234.

Jose Luis Balcazar, Ignacio de Blas, Imanol Ruiz-Zarzuela, David Cunningham, Daniel Vendrell, Jose Luis Muzquiz. 2006. The role of probiotics in aquaculture. Veterinary Microbiology 114: 173–186.

Madico G, Akopyants NS, Berg DE. 1995. Arbitrarily primed PCR DNA fingerprinting of *Escherichia coli* O157:H7 strains by using templates from boiled cultures. J. Clin. Microbiol. 33: 1534-1536.

Merlin Rose C, Cathrine L. 2011. Preliminary phytochemical screening and antibacterial activity on *Vitex negundo*. International Journal of Current Pharmaceutical Research 3(2): 99-101.

Mitsunobu Sakajoh, Nadine A Solomon, Arnold L Demain. 1987. Cell-free synthesis of the dipeptide antibiotic bacilysin. Journal of Industrial Microbiology 2: 201-208.

Moriarty DJW. 2000. Disease control in shrimp aquaculture with probiotic bacteria. Microbial Interactions in Aquaculture. In: *Microbial Biosystems*: New Frontiers. Eds. Bell CR, Brylinsky M, Johnson-Green P. Proceedings of the 8th International symposium on microbial ecology. Atlantic Canada society for microbial ecology, Halifax, Canada, pp. 237-243.

Nantarika Chansue, Nongnut Assawawongkasem. 2008. The in vitro antibacterial activity and ornamental fish toxicity of the water extract of Indian almond leaves (*Terminalia catappa* Linn.). KKU Vet. J. 18(1): 36-45.

Nayak SK, Swain P, Mukherjee SC. 2007. Effect of dietary supplementation of probiotic and vitamin C on the immune response of Indian major carp, *Labeo rohita* (Ham.) Fish Shellfish Immunol. 23: 892-896.

Panigrahi A, Azad IS. 2007. Microbial intervention for better fish health in aquaculture: the Indian scenario. Fish Physiology Biochem. 33: 429-440.

Panigrahi A, Kiron V, Kobayashi T, Puangkaew J, Satoh S, Sugita S. 2004. Immune responses in rainbow trout *Oncorhynchus mykiss* induced by a potential probiotic bacteria *Lactobacillus rhamnosus* JCM 1136. Veterin. Immunol. Immunopath. 102: 379-388.

Panigrahi A, Kiron V, Puangkaew J, Kobayashi T, Satoh S, and Sugita H. 2005. The viability of probiotic bacteria as a factor influencing the immune response in rainbow trout *Oncorhynchus mykiss*. Aquaculture 243: 241-254.

Parker GA. 1974. Assessment strategy and the evc zution of fighting behaviour. J. Theor. Boil. 47: 223-243.

Paulraj Kanmani R, Sathish Kumar N, Yuvaraj KA, Paari V Pattukumar, Venkatesan. 2010. Comparison of Antimicrobial activity of probiotic bacterium *Streptococcus phocae*, *Enterococcus faecium* MC13 and *Carnobacterium divergens* against fish pathogen. World journal of Dairy & food sciences 5(2): 145-151.

Prem Anand T, Chellaram C, Kumaran S, Felicia Shanthini C. 2011. Screening for antibiotic producing marine bacteria against fish pathogens. International journal of Pharma and Bio sciences 2(1).

Rodgers CJ, Furones MD. 2009. Antimicrobial agents in aquaculture: Practice, needs and issues. Options Mediterraneennes; A / no. 86: 41-59.

Sambrook, J, Fritsch EF Maniatis T. 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory Press.

Sasmal D, Babu CS, Abraham TJ. 2005. Effect of garlic (*Allium sativum*) extract on the growth and disease resistance of *Carassius auratus* (Linnaeus, 1758). Indian J. Fish 52(2): 207-214.

Sealey WM, Barrows FT, Hang A, Johansen KA, Overturf K, LaPatra SE. 2008. Evaluation of the ability of barley genotypes containing different amounts of ß-glucan to alter growth and disease resistance of rainbow trout *Oncorhynchus mykiss*. Animal Feed Sci. Technol. 141: 115-128.

Shalaby SM, Zakora M, Otte J. 2006. Performance of two commonly used angiotensin-converting enzyme inhibition assays using FA-PGG and HHL as substrates. J. Dairy Res. 73: 178-186.

Staykov Y, Denev SA, Spring P. 2005a. The effect of dietary mannan oligosacharides (bio-mos®) on the growth rate and immune function of rainbow trout (salmo gairdneri irideus g.) Growth in netcages. In: Eds. B. Howell and R. Flos. Lessons from the past to optimise the future - Aquaculture Europe 2005, August 5-9th, Tronheim, Norway, European Aquaculture Society, Special Pub No 35. pp. 427-428.

Staykov Y, Denev SA, Spring P. 2005b. The effect of dietary mannan oligosaccharides (bio-mos®) on the growth rate and immunity status of rainbow trout (*Salmo Gairdneri Irideus* G.) Grown in Raceways. In: Eds. Howell B and Flos R. Lessons from the past to optimise the future - Aquaculture Europe 2005, Tronheim, Norway, European Aquaculture Society, Special Pub No 35. pp. 429-430.

Staykov Y, Denev SA, Spring P. 2005c. The influence of dietary (bio-mos®) on the growth rate and immune function of common carp (*Cyprinus Carpio* L.). In: Lessons from the past to optimise the future - Aquaculture Europe. Eds. B. Howell and R. Flos, August 5-9th, Tronheim, Norway, European Aquaculture Society; Special Pub No 35. pp. 431-432.

Staykov Y, Spring P, Denev SA. 2005. Influence of dietary Bio-Mos® on growth, survival and immune status of rainbow trout (*Salmo gairdneri irideus* G.) and common carp (*Cyprinus carpio* L.). In: Eds. Lyons TP, Jackues K. Nutritional Biotechnology in the Feed and Food Industries. Nottingham University Press, Nottingham, UK; 333-343.

Staykov Y, Spring P, Denev SA. 2006. Effects of dietary Bio-Mos® on the growth rate and immunity of rainbow trout (*Salmo gairdneri irideus* G.) grown in raceways. Proceedings of aquaculture meeting. Improving stock performance and efficiency the natural way, 7th November, Dunboyne, Ireland; 21.

Staykov Y, Spring P, Denev SA. 2006a. Influence of dietary Bio-Mos® on the growth rate and immune function of rainbow trout (*Salmo gairdneri irideus* G.). Proceedings of aquaculture meeting. Improving stock performance and efficiency the natural way, 7th November, Dunboyne, Ireland; 23.

Staykov Y, Spring P, Denev SA. 2006b. Influence of dietary Bio-Mos® on the growth rate and immune function of common carp (*Cyprinus carpio* L.). Proceedings of aquaculture meeting. Improving stock performance and efficiency the natural way, 7th November, Dunboyne, Ireland; 35.

Staykov Y, Spring P, Denev SA, Sweetman J. 2007. Effect of a mannan oligosaccharide on the growth performance and immune status of rainbow trout (*Oncorhynchus mykiss*). Aquacul. Int. 2: 153-161.

Staykov Y, Spring P, Denev SA, Sweetman J. 2009. Effect of a mannan oligosaccharide on the growth performance and immune status of rainbow trout (*Oncorhynchus mykiss*). Book of Abstracts of the 60th Annual Meeting of the European Association for Animal Production, Barcelona, Spain, 524.

Staykov Y, Spring P, Sweetman J, Denev SA. 2007a. The influence of 2 and 4% Nu-Pro® on the growth performance Common carp (*Cyprinus carpio* L.), raised in net cages. In: Eds. T. P. Lyons and K. A. Jacques. Proceedings of the

22nd annual symposium "nutritional biotechnology in the feed and food industries" (Suppl. Abstracts of Poster Presented), Lexington, Kentucky, USA: 84.

Williams JGK, Kubelik AR, Livak KJ, Rafalski JA, Tingey SV. 1990. DNA polymorphisms amplified by arbitrary primers are useful as genetic markers. Nucleic Acids Res. 18: 6531-6535.

Yanbo W, Zirong X. 2006. Effect of probiotics for common carp (*Cyprinus carpio*) based on growth performance and digestive enzyme activities. Animal Feed Sci. Technol. 127: 283-292.