aJava

Asian Journal of Animal and Veterinary Advances

Academic
Journals Inc.

www.academicjournals.com

Asian Journal of Animal and Veterinary Advances 8 (1): 116-123, 2013
ISSN 1683-9919 / DOI: 10.3923/ajava.2013.116.123

Antibacterial Ability and Molecular Characterization of Probionts Isolated from Gut Microflora of Cultured Red Tilapia

[1]Lim Char Yee, [1]M.Y. Ina-Salwany and [2]Yasser M. Abdelhadi
[1]Department of Aquaculture, Faculty of Agriculture, UPM 43400, Serdang, Selangor, Malaysia
[2]The Central Laboratory for Aquaculture Research (CLAR), Abbassa, Sharkia, Egypt

Corresponding Author: Yasser M. Abdelhadi, Department of Aquaculture, Faculty of Agriculture, UPM 43400, Serdang, Selangor, Malaysia

ABSTRACT

Four bacterial isolates, which proved to have the potential to be used as probiotics, were used to study their genotypic characterization using analysis of 16S rRNA gene sequence, antagonistic ability and safety when applied on tilapia via immersion. Gram staining showed that all 4 probionts were Gram positive bacteria. Three probionts were rod in shape and the fourth was cocci in shape and arranged in grape-like cluster. These 4 probionts were also identified using 16S rRNA gene sequencing and their identities were *Paenibacillus barcinonensis* strain D12, *Paenibacillus* sp. strain D14, *Staphylococcus cohnii* strain B11 and *Bacillus megaterium* strain E28, respectively. All of the probionts were examined for their antagonistic ability against pathogenic bacteria (*Vibrio alginolyticus* ATCC 33839, *Aeromonas salmonicida* and *Aeromonas hydrophila* ATCC 35654) under *in vitro* conditions by using cross-streaking method. *P. barcinonensis* strain D12 and *Paenibacillus* sp. strain D14 have shown stronger antagonistic ability than *S. cohnii* strain B11 and *B. megaterium* strain E28 in the antagonism test. *P. barcinonensis* strain D12 and *Paenibacillus* sp. strain D14 were chosen to test their safety on tilapia due to their better performance in antagonism test. Both probionts, *P. barcinonensis* strain D12 and *Paenibacillus* sp. strain D14, were safe for tilapia.

Key words: *Paenibacillus barcinonensis*, *Bacillus megaterium*, probionts, tilapia

INTRODUCTION

World tilapia production has been increasing in the last decade and currently exceeded 3 million ton to become the second worldwide cultured species, second only to carps (Kevin, 2008; Abdelhadi, 2011). Fish diseases are major obstacles in aquaculture industry which cause high economic losses every year. According to Plumb (1999), *Streptococcus*, *Enterococcus*, *Aeromonas*, *Pseudomonas*, *Vibrio*, *Flexibacter* and *Edwardsiella* are common opportunistic pathogens that can easily infect tilapia especially if the fish is under stress condition such as high density of fish and poor water condition. Currently, vaccination and chemotherapeutic treatment are commonly used to protect fish from bacterial diseases. However, both tools have brought unfavorable results, at which the ineffectiveness of vaccination when applied to immunologically immature fish and development of pathogenic bacterial resistance caused by the excessive use of chemotherapeutics are the disadvantages of these treatments (Angulo, 2000; Balcazar *et al.*, 2006, 2008). Hence, other control measures should be developed to overcome these problems such as probiotics as immunostimulants.

Probiotics can reduce the incidence and duration of disease by ways such as enhancement of colonization and direct inhibitory effect to pathogens. Besides, probiotic strains have shown their ability to inhibit pathogenic bacteria both *in vivo* and *in vitro* through different mechanisms (Balcazar *et al.*, 2006). In a previous study, there were 135 bacterial strains isolated from gut microflora of red tilapia and 4 types of bacterial strains; *Bacillus circulans* 1, *Bacillus circulans* 2, *Bacillus megaterium* and *Staphylococcus cohnii* subsp. *cohnii* were determined to have a great potential as probiotics in aquaculture (Khairi, 2010). However, the safety of these probionts, their molecular characterization and antibacterial ability to other pathogens has not yet been tested. Therefore, this study was established to characterize 4 strains of probionts using 16S ribosomal ribonucleic acid (16S rRNA) gene, to evaluate the antibacterial ability of the 4 probionts isolated from gut microflora of cultured red tilapia against some common fish pathogens using cross-streaking inhibition assay and to investigate the safety of the isolated probionts on tilapia.

MATERIALS AND METHODS

Bacterial strains (probionts): Four bacterial isolates were previously identified biochemically (BBL crystal) as potential probiotics or probionts (Khairi, 2010). The probionts were identified as *S. cohnii* subsp. *cohnii*, *B. circulans* 1, *B. circulans* 2 and *B. megaterium*. In this study, *S. cohnii* subsp. *cohnii*, *B. circulans* 1, *B. circulans* 2 and *B. megaterium* were coded as B11, D12, D14 and E28, respectively. At the beginning of the study, the probionts were thawed from the glycerol stock. The probionts were streaked on Trypticase™ Soy Agar (TSA) plate and incubate for 24 h at 25°C. Pure and single colony was isolated and maintained in TSA for every two weeks. During the study, these 4 probionts were also maintained in Trypticase™ Soy Broth (TSB) and glycerol stock (25% w/v) and stored at -80°C.

Molecular characterization of the 4 probionts: This was conducted according to the method described by Zolgharnein *et al.* (2010). The primers used to amplify the 16S rRNA gene sequence sample were forward primer: *E. coli* 9 5' GAG TTT GAT CCT GGC TCA G 3'; and reverse primer: Loop 27rc 5' GAC TAC CAG GGT ATC TAA TC 3'. The primers amplified approximately 750 to 800 base pairs (bp) of the 16S rRNA gene (Sfanos *et al.*, 2005).

16S rRNA gene sequence analysis: The identities of probionts were identified by comparison of the 16S rRNA sequence of the probionts to GenBank using the Basic Local Alignment Search Tool (BLAST) program accessible at the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov). Bacteria with 99 to 100% similarity of 16S rRNA sequences in Genbank are members of the same species and 97 to 99% are members of the same genus (Drancourt *et al.*, 2000).

Phylogenetic analysis: Phylogeny model Kimura 2-parameter neighbor joint tree were constructed using the MEGALIGN computer program (DNAStar, Madison, WI, USA). Phylogenetic tree was based on comparative analysis of 16S rRNA sequence aligned with 10 of its closest match by BLAST analysis using CLUSTAL algorithm (Clarridge, 2004).

Antibacterial ability of probionts against fish pathogens: Cross streaking method described by Hill *et al.* (2009) with slight modification were used. Three pathogenic bacteria strain *Aeromonas hydrophila* ATCC 35654, *Aeromonas salmonicida*, previously isolated and identified from red tilapia gut (Khairi, 2010) and *Vibrio alginolyticus* ATCC 33839, were cross-streaked against the pre-incubated probionts on TSA plates (4 replicates) at 25°C for 0, 24, 48 and 72 h, respectively. The concentration of each pathogen culture was adjusted to 10^8 CFU mL^{-1} and the probionts were adjusted to 10^7 CFU mL^{-1} before using for cross-streaking assay.

***In vivo* experiment for safety of probionts on tilapia:** Two out of 4 probionts (*P. barcinonensis* strain D12 and *P.* sp. strain D14, which performed well in the antagonism test) were chosen to test their safety on tilapia. The method described by Irianto and Austin (2003) was adapted with slight modification. Seventy two apparently healthy tilapias (20-22 g/fish) were used, where they were acclimatized for one week in indoor tanks. The fish then were divided into 3 equal groups with three replicates per each. Fish of the first and second groups were intra-peritoneal inoculated by 0.3 mL of saline containing 10^7 CFU mL^{-1} of the 2 types of selected probionts, respectively. On the other hand, fish of the third group were intra-peritoneal inoculated by 0.3 mL of saline as control. All groups were kept under observation for 14 days and the mortality rate was recorded. The fish were subjected to laboratory examination and bacterial re-isolation. The mortality rate of each group was analyzed by using SPSS program where one-way ANOVA was used.

RESULTS

Molecular characterization: The bands appeared in Fig. 1 indicated that the DNA was amplified at 750 to 800 bp of 16S rRNA.

16S rRNA gene sequencing and phylogenetic analysis: Results of Table 1 showed the identities of probionts after the BLAST analysis of the 16S rRNA gene sequence. All of the identities of probionts had 99% similarity of 16S rRNA gene sequence compared to the bacteria in Genbank.

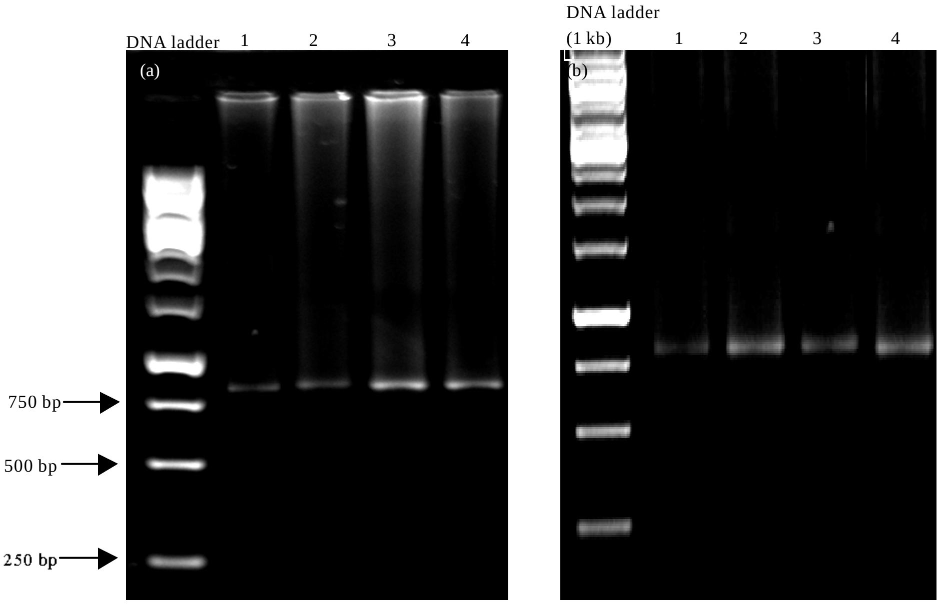

Fig. 1(a-b): Agarose gel (1.0%) of PCR-amplified DNA products of the 4 probionts (B11, D12, D14 and E28) in lanes (1, 2, 3 and 4) (a) Before and (b) After purification

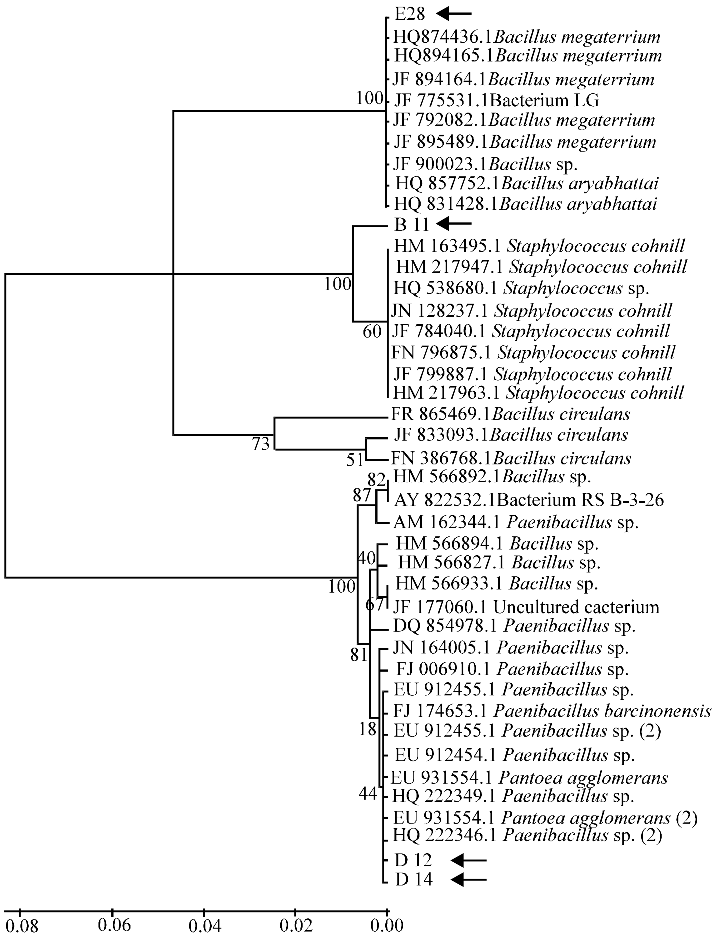

Fig. 2: Phylogenetic tree showing the position of the gene sequence of probionts

Table 1: Blast analysis of the 16S rRNA gene sequence

Probionts	Closest relative	Accession No.	Similarity (%)
B11	*Staphylococcus cohnii*	JN128237.1	99
D12	*Paenibacillus barcinonensis*	FJ174653.1	99
D14	*Paenibacillus* sp.	HQ222349.1	99
E28	*Bacillus megaterium*	JF792082.1	99

Therefore, the bacterial species shown in Table 1 were the identities of the probionts. Table 2 showed the identities of probionts obtained from analysis of 16S rRNA gene sequence. Phylogenetic analysis confirmed the probionts' identity (Fig. 2).

Antibacterial ability of probionts against fish pathogens: Table 3 demonstrated the results of antagonism ability of probionts. At 0 h pre-incubation of the probionts, there was no antagonism ability observed. All of the probionts showed their antibacterial ability after 24 h pre-incubation. However, the antibacterial ability of probionts was different. *S. cohnii* strain

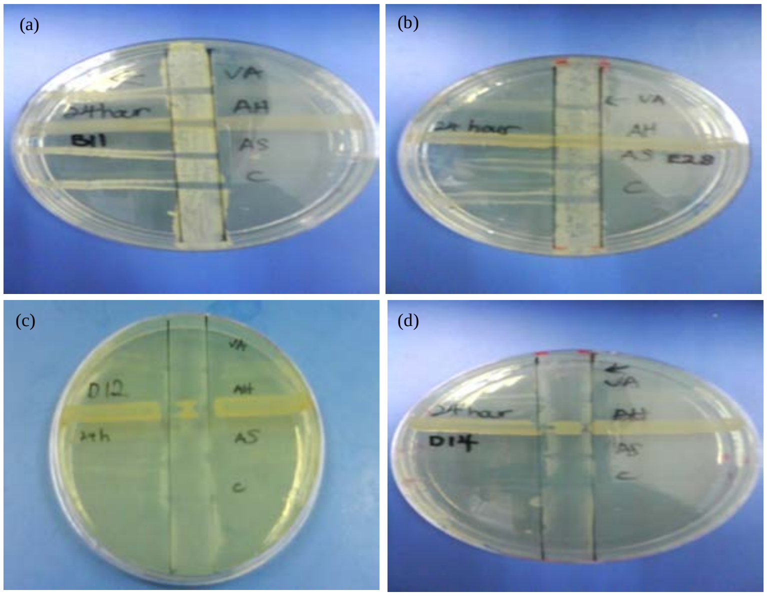

Fig. 3(a-d): Inhibition zones of (a) B11, (b) D12, (c) D14 and (d) E28 after 24 h pre-incubation

Table 2: Estimated species from 16S rRNA gene sequencing and biochemical identification

Probionts	Estimated species from analysis of 16S rRNA gene sequence	Estimated species from biochemical test
B11	*Staphylococcus cohnii*	*Staphylococcus cohnii* subsp. *cohnii*
D12	*Paenibacillus barcinonensis*	*Bacillus circulans*
D14	*Paenibacillus* sp.	*Bacillus circulans*
E28	*Bacillus megaterium*	*Bacillus megaterium*

Table 3: Antibacterial ability of probionts against fish pathogens

Probionts	0 h			24 h			48 h			72 h		
Code	AH	AS	VA	AH	AS	VA	AH	AS	VA	AH	AS	VA
B11	-	-	-	+	+	+	+	+	+	+	+	+
D12	-	-	-	++	++	++	++	++	++	++	++	++
D14	-	-	-	++	++	++	++	++	++	++	++	++
E28	-	-	-	+	+	+	+	+	+	+	+	+

AH: *Aeromonas hydrophila* ATCC 35654, AS: *Aeromonas salmonicida*, VA: *Vibrio alginolyticus*. ATCC 33839, +: Weak antagonism ability, ++: Strong antagonism ability, -: No antagonism ability

B11 and *B. megaterium* strain E28 weakly inhibited all three pathogenic bacteria (Fig. 3). The measurements of inhibition zones for *S. cohnii* strain B11 and *B. megaterium* strain E28 ranged from 0.1 to 0.3 cm at 24, 48 and 72 h pre-incubation. On the other hand, *P. barcinonensis* strain D12 and *Paenibacillus* sp. strain D14 strongly inhibited three pathogenic bacteria (Fig. 3). The measurements of inhibition zones for *P. barcinonensis* strain D12 and *Paenibacillus* sp. strain D14 ranged from 0.7 to 1.0 cm for *V. alginolyticus* ATCC 33839, *A. salmonicida* and *A. hydrophila* ATCC 35654 at 24, 48 and 72 h pre-incubation.

Table 4: Challenge test results for safety evaluation of probiotic bacterial isolates on red tilapia in each, using intra-peritoneal injection (I/P)

Group	Probiotic	Dose (mL)	RPS (%)
1	*Paenibacillus barcinonensis* strain D12	0.3×10^{7} CFU mL^{-1} saline	79.2 ± 4.2^{a}
2	*Paenibacillus* sp. strain D14	0.3×10^{7} CFU mL^{-1} saline	83.3 ± 4.2^{a}
3	Control (sterile saline)	0.3 mL saline	83.3 ± 8.3^{a}

Means having the same letter in the same column are not significantly different at $p<0.05$

***In vivo* safety of probionts on tilapia:** The Intra-peritoneal (IP) challenge of fish with *P. barcinonensis* strain D12 and *Paenibacillus* sp. strain D14 didn't induce any abnormal signs or mortalities. Thus, the 2 probionts were safe for tilapia (Table 4). Therefore, they have the potential to be used as probiotics for tilapia aquaculture.

DISCUSSION

Molecular characterization: The bands appeared in Fig. 1 indicated that the DNA was amphified at 750 to 800 bp of 16S rRNA since the primer was designed to amplify at 750 to 800 bp of 16S rRNA (Sfanos *et al*., 2005). The purified PCR product as shown in plate 1 looked like PCR products but smear under each band was reduced compared to PCR products. Molecular techniques are fast and effective technology for microbial diversity identification in different environment (Hatamoto *et al*., 2008). Genetic diversity can identify individual organisms from some unique part of DNA or RNA providing definitive information on its biodiversity (Hafez and Elbestawy, 2009).

16S rRNA gene sequencing and phylogenetic analysis: The identity of probiont B11 from analysis of the 16S rRNA gene sequence did not determine the subspecies of the probiont as was determined in the biochemical test. This might be due to the sequence used in the analysis was partial sequence of 16S rRNA gene (Table 2). Gorkiewicz *et al*. (2003) found that partial sequence of 16S rRNA gene failed to discriminate bacteria among the taxa *Campylobacter jejuni*, *Campylobacter coli* and *Campylobacter lari* strains, which shared identical and nearly identical 16S rRNA sequences. Therefore, complete sequencing of 16S rRNA gene should be used in determining bacterial identity up to subspecies level due to its higher accuracy compared to the partial sequencing of 16S rRNA gene. On the other hand, identities of probionts D12 and D14 were not matched with identities given via biochemical test. They were *P. barcinonensis* and *Paenibacillus* sp. instead of *B. circulans* (Table 2). Phylogenetic analysis confirmed the probionts' identity (Fig. 2).

Compared to 16S rRNA gene sequence analysis, biochemical test is a time consuming method and either fails to identify some Gram-positive bacterial rods entirely or at least fail to do so in some clinical situation (Mignard and Flandrois, 2006). Because of probionts D12 and D14 were also Gram-positive bacterial rods, so it has the possibility that biochemical test identified them incorrectly. Moreover, 16S rRNA gene sequence analysis can discriminate far more finely among strains of bacteria than possible with biochemical test. It can allow a more precise identification of poorly described, phenotypically aberrant, or rarely isolated strains (Clarridge, 2004).

Antibacterial ability of probionts against fish pathogens: Table 3 demonstrated the results of antagonism ability of probionts. These results could be attributed to the fact that all probionts were able to produce antimicrobial substances. However, the production and effectiveness of these microbial substances were dependent on period of incubation and species of probionts. These results were supported by those reported by Verschuere *et al.* (2000) and Balcazar *et al.* (2006). Ravi *et al.* (2007) reported that *Paenibacillus* sp., *Bacillus cereus* and *Paenibacillus polymyxa* were effective in inhibiting pathogenic Vibros (*Vibrio* sp., *Vibrio harveyi* and *Vibrio vulnificus*) in the post larvae of *Penaeus monodon.*

***In vivo* safety of probionts on tilapia:** The Intra-peritoneal (IP) challenge of fish with *P. barcinonensis* strain D12 and *Paenibacillus* sp. strain D14 didn't induce any abnormal signs or mortalities. Thus, the 2 probionts were safe for tilapia (Table 4). Therefore, they have the potential to be used as probiotics for tilapia aquaculture. Similar results were obtained by Abd El-Rhman *et al.* (2009) who used the same I/P route to test the safety of two probionts, *Micrococcus luteus* and *Pseudomonas* sp., on tilapia and proved that both probionts were safe for tilapia.

CONCLUSION

Thus, it could be concluded that *Paenibacillus barcinonensis* strain D12 and *Paenibacillus* sp. strain D14, have the potential to be used as probiotics for tilapia culture as a sustainable aquaculture practice. However, further biological studies are required to examine the effects of using these 2 probionts as feed additives, on the growth parameters, blood chemistry and immune response of tilapia.

REFERENCES

Abd El-Rhman, A.M., Y.A. Khattab and A.M. Shalaby, 2009. *Micrococcus luteus* and *Pseudomonas* species as probiotics for promoting the growth performance and health of Nile tilapia, *Oreochromis niloticus.* Fish Shellfish Immunol., 27: 175-180.

Abdelhadi, Y.M., 2011. Tilapia: From the Nile to the World. J. Agric. Sci. Technol. USA., 5: 45-51.

Angulo, F., 2000. Antimicrobial agents in aquaculture: Potential impact on public health. APUA Newslett., 18: 1-14.

Balcazar, J.L., I. de Blas, I. Ruiz-Zarzuela, D. Cunningham, D. Vendrell and J.L. Muzquiz, 2006. The role of probiotics in aquaculture. Vet. Microbiol., 114: 173-186.

Balcazar, J.L., D. Vendrell, I. de Blas, I. Ruiz-Zarzuela, J.L. Muzquiz and O. Girones, 2008. Characterization of probiotic properties of lactic acid bacteria isolated from intestinal microbiota of fish. Aquaculture, 278: 188-191.

Clarridge, J.E., 2004. Impact of 16S rRNA gene sequence analysis for identification of bacteria on clinical microbiology and infectious disease. Clin. Microbiol. Rev., 17: 840-862.

Drancourt, M., C. Bollet, A. Carlioz, R. Martelin, J.P. Gayral and D. Raoult, 2000. 16S ribosomal DNA sequence analysis of a large collection of environmental and clinical unidentifiable bacterial isolates. J. Clini. Microbiol., 38: 3623-3630.

Gorkiewicz, G., G. Feierl, C. Schober, F. Dieber, J. Kofer, R. Zechner and E.L. Zechner, 2003. Species-specific identification of campylobacters by partial 16S rRNA gene sequencing. J. Clin. Microbiol., 41: 2537-2546.

Hafez, E.E. and E. Elbestawy, 2009. Molecular characterization of soil microorganisms: Effect of industrial pollution on distribution and biodiversity. World J. Microbiol. Biot., 2: 215-224.

Hatamoto, M., H. Imachi, Y. Yashiro, A. Ohashi and H. Harada, 2008. Detection of active butyrate-degrading microorganisms in methanogenic sludges by RNA-based stable isotope probing. Applied Environ. Microbiol., 74: 3610-3614.

Hill, J.E., J.C.F. Baiano and A.C. Barnes, 2009. Isolation of a novel strain of *Bacillus pumilus* from penaied shrimp that is inhibitory against marine pathogen. J. Fish Dis., 32: 1007-1016.

Irianto, A. and B. Austin, 2003. Use of dead probiotic cells to control furunculosis in rainbow trout, *Oncorhynchus mykiss* (Walbaum). J. Fish Dis., 26: 59-62.

Kevin, F., 2008. Tilapia production, innovations and markets. Proceedings of the 4th International Symposium on Tilapia in Aquaculture, August 12, 2008, Orlando, FL., USA.

Khairi, A., 2010. Characterization of some bacteria isolated from gut microflora of red tilapia and their potential as probiotics. M.Sc. Thesis, University Putra, Malaysia.

Mignard, S. and J.P. Flandrois, 2006. 16S rRNA sequencing in routine bacterial idenftification: A 30-month experiment. J. Microbiol. Methods, 67: 574-581.

Plumb, J.A., 1999. Tilapia Bacterial Diseases. In: Health Maintenance and Principal Microbial Diseases Cultured Fishes, Plumb, J.A. (Ed.). Iowa State University Press, Ames, IA., USA., pp: 297-305.

Ravi, A.V., K.S. Musthafa, G. Jegathammbal, K. Kathiresan and S.K. Pandian, 2007. Screening and evaluation of probiotics as a biocontrol agent against pathogenic *Vibrios* in marine aquaculture. Lett. Applied Microbiol., 45: 219-223.

Sfanos, K., D. Harmody, P. Dang, A. Ledger, S. Pomponi, P. McCarthy and J. Lopez, 2005. A molecular systematic survey of cultured microbial associates of deep-water marine invertebrates. Syst. Applied Microbiol., 28: 242-264.

Verschuere, L., G. Rombaut, P. Sorgeloos and W. Verstraete, 2000. Probiotic bacteria as biological control agents in aquaculture. Microbiol. Mol. Biol. Rev., 64: 655-671.

Zolgharnein, H., K. Karami, M.M. Assadi and A.D. Sohrab, 2010. Molecular characterization and phylogenetic analyses of heavy metal removal bacteria from the Persian gulf. Biotechnology, 9: 1-8.