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

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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 probiotics were Gram positive bacteria. Three probiotics were rod in shape and the fourth was cocci in shape and arranged in grape-like cluster. These 4 probiotics 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 probiotics 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 probiotics, P. barcinonensis strain D12 and Paenibacillus sp. strain D14, were safe for tilapia.

Key words: Paenibacillus barcinonensis, Bacillus megaterium, probiotics, 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 carp (Kevin, 2008; Abdelhadi, 2011). Fish diseases are major obstacles in aquaculture industry which cause high economic losses every year. According to Plumb (1999), Streploccoccus, 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; Balcacaz 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 (Baleazar 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 probiotics, their molecular characterization and antibacterial ability to other pathogens has not yet been tested. Therefore, this study was established to characterize 4 strains of probiotics using 16S ribosomal ribonucleic acid (16S rRNA) gene, to evaluate the antibacterial ability of the 4 probiotics 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 probiotics on tilapia.

MATERIALS AND METHODS

Bacterial strains (probiotics): Four bacterial isolates were previously identified biochemically (BBL crystal) as potential probiotics or probions (Khairi, 2010). The probiotics 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 probiotics were thawed from the glycerol stock. The probiotics 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 probiotics were also maintained in Trypticase™ Soy Broth (TSB) and glycerol stock (25% w/v) and stored at -80°C.

Molecular characterization of the 4 probions: This was conducted according to the method described by Zolgharnain et al. (2010). The primers used to amplify the 16S rRNA gene sequence sample were forward primer: E. coli 9' GAG TTT GAT CCT GGC TCA G 3'; and reverse primer: Loop 27re 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 probiotics were identified by comparison of the 16S rRNA sequence of the probiotics 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 probions 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 probiotics 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 $10^5$ CFU mL$^{-1}$ and the probiotics were adjusted to $10^7$ CFU mL$^{-1}$ before using for cross-streaking assay.

**In vivo experiment for safety of probiotics on tilapia:** Two out of 4 probiotics (*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 $10^5$ CFU mL$^{-1}$ of the 2 types of selected probiotics, 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 probiotics after the BLAST analysis of the 16S rRNA gene sequence. All of the identities of probiotics had 99% similarity of 16S rRNA gene sequence compared to the bacteria in Genbank.

![DNA ladder](image)

**Fig. 1(a-b):** Agarose gel (1.0%) of PCR-amplified DNA products of the 4 probiotics (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

<table>
<thead>
<tr>
<th>Probiotics</th>
<th>Closest relative</th>
<th>Accession No.</th>
<th>Similarity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>B11</td>
<td><em>Staphylococcus cohnii</em></td>
<td>JN129837.1</td>
<td>99</td>
</tr>
<tr>
<td>D12</td>
<td><em>Paenibacillus barcinonensis</em></td>
<td>FJ174655.1</td>
<td>99</td>
</tr>
<tr>
<td>D14</td>
<td><em>Paenibacillus sp.</em></td>
<td>HQ222849.1</td>
<td>99</td>
</tr>
<tr>
<td>E28</td>
<td><em>Bacillus megaterium</em></td>
<td>JF792082.1</td>
<td>99</td>
</tr>
</tbody>
</table>

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

<table>
<thead>
<tr>
<th>Probiotics</th>
<th>Estimated species from analysis of 16S rRNA gene sequence</th>
<th>Estimated species from biochemical test</th>
</tr>
</thead>
<tbody>
<tr>
<td>B11</td>
<td>Staphylococcus cohnii</td>
<td>Staphylococcus cohnii subsp. cohnii</td>
</tr>
<tr>
<td>D12</td>
<td>Paenibacillus barcinonensis</td>
<td>Bacillus circulans</td>
</tr>
<tr>
<td>D14</td>
<td>Paenibacillus sp.</td>
<td>Bacillus circulans</td>
</tr>
<tr>
<td>E28</td>
<td>Bacillus megaterium</td>
<td>Bacillus megaterium</td>
</tr>
</tbody>
</table>

Table 3: Antibacterial ability of probiotics against fish pathogens

<table>
<thead>
<tr>
<th>Probiotics</th>
<th>0 h</th>
<th>24 h</th>
<th>48 h</th>
<th>72 h</th>
</tr>
</thead>
<tbody>
<tr>
<td>Code</td>
<td>AH</td>
<td>AS</td>
<td>VA</td>
<td>AH</td>
</tr>
<tr>
<td>B11</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>D12</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>D14</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>E28</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

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)

<table>
<thead>
<tr>
<th>Group</th>
<th>Probiotic</th>
<th>Dose (mL)</th>
<th>RPS (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td><em>Paenibacillus barcinonensis</em> strain D12</td>
<td>0.3×10⁷ CFU mL⁻¹ saline</td>
<td>79.2±4.2°</td>
</tr>
<tr>
<td>2</td>
<td><em>Paenibacillus</em> sp. strain D14</td>
<td>0.3×10⁷ CFU mL⁻¹ saline</td>
<td>83.3±4.2°</td>
</tr>
<tr>
<td>3</td>
<td>Control (sterile saline)</td>
<td>0.3 mL saline</td>
<td>83.3±8.3°</td>
</tr>
</tbody>
</table>

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

In vivo safety of probiotics 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 probiotics 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 amplified 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 rDNA gene sequencing and phylogenetic analysis: The identity of probiotic B11 from analysis of the 16S rDNA gene sequence did not determine the subspecies of the probiotic as was determined in the biochemical test. This might be due to the sequence used in the analysis was partial sequence of 16S rDNA gene (Table 2). Gorkiewicz et al. (2003) found that partial sequence of 16S rDNA gene failed to discriminate bacteria among the taxa *Campylobacter jejuni, Campylobacter coli* and *Campylobacter lari* strains, which shared identical and nearly identical 16S rDNA sequences. Therefore, complete sequencing of 16S rDNA gene should be used in determining bacterial identity up to subspecies level due to its higher accuracy compared to the partial sequencing of 16S rDNA gene. On the other hand, identities of probiotics 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 probiotics’ identity (Fig. 2).

Compared to 16S rDNA 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, 2008). Because of probiotics D12 and D14 were also Gram-positive bacterial rods, so it has the possibility that biochemical test identified them incorrectly. Moreover, 16S rDNA 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 probiotics against fish pathogens: Table 3 demonstrated the results of antagonism ability of probiotics. These results could be attributed to the fact that all probiotics were able to produce antimicrobial substances. However, the production and effectiveness of these
microbial substances were dependent on period of incubation and species of probiotics. 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 probiotics 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 probiotics 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 IP route to test the safety of two probiotics, *Micrococcus luteus* and *Pseudomonas* sp., on tilapia and proved that both probiotics 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 probiotics as feed additives, on the growth parameters, blood chemistry and immune response of tilapia.

**REFERENCES**


