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# The Diversity Of Gut Bacteria Associated With Milkfish (*Chanos chanos Forsksal)* From Northern Coast of Central Java, Indonesia

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**Abstract**

A total of 30 bacterial isolates associated with milkfish gut were collected from traditional ponds at Northern Coast of Central Java, Indonesia. The study was conducted to isolate and identify the milkfish gut bacteria, and evaluate their potential for probiotics. Based on rapid grouping by using rep-PCR resulted in six groups. The six group represented by isolates of BS 11, BPI 01, BPL 10, BPi 03; BPi 08 and BPL 06 were then further selected for subsequent DNA sequencings. According to DNA sequencing it was demonstrated that the diversity of bacteria associated with milfish gut collected from Northern Coast of Central Java, revealed closely similar to *Shewanella upenei, Basillus* sp, *Vibrio fluvialis, Shewanella algae, Shewanella* sp. *and Photobacterium ganghwense.* Further sensitivity test among six baterial isolates demonstrated that *Shewanella upenei* and *Shewanella algae* were able to prevent the growth of *Vibrio fluvialis* and *Photobacterium ganghwense*. The six selected isolates were also successfully demonstrated antibacterial activity against pathogenic bacteria *Vibrio alginoliticus* and *Vibrio parahaemolitycus.* From this study, it can be concluded that the six bacteria isolated from milkfish intestine were potentially developed for probiotics.

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*Keywords:* milkfish, gut bacteria, rep-PCR, probiotics

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## 1. Introduction

Aquaculture production in Central Java in 2012 reached 167,191.8 tonnes. Around 65.37% (10,303 tonnes) was produced from brackhiswater aquaculture. Milkfish contributing more than 58% (63,630 tonnes) of the total brackhiswater aquaculture production[1]. This indicated that milkfish was widely produced and become an important brackhiswater fish species that most traditionally culture by the fish farmers in Central Java. Moreover, milkfish in the last 15 years become an icon for northern part of Central Java, especially Semarang Regency.

Milkfish has very large salinity tolerance, therefore, it can be cultured in various environments such as freshwater, brackhishwater, and seawater[2]. Milkfish has a tendency to disease resistance due to great non-specific immune system associated with gut microflora. Futthermore, grouper gut associated bacteria such as *Lactococcus sp, Carnoacterium sp, Staphylococcus sp, sp basillus, Eubacterium sp, Pseudomonas sp, Lactobacillus sp, Micrococcus sp, and Bifidobacterium sp.* have an important role in immunity mechanism and could provide a positive influence on grouper[1]. Other researchers added that intestine bacteria of milkfish (*Chanos chanos*) such as *Lactobacillus fermentum, Lactobacillus gasseri, Lactobacillus delbrueckii* and *Micrococcus lylae* biochemically were potentially used as a probiotics[3]. Similar studied has been conducted in the digestive tract of rainbow trout (*Salmo gairdneri*) [4]. They identified bacterial species namely *Acinetobacter calcoaceticus, Aeromonas hydrophila, Bacillus circulans, Bacillus megaterium, Flavobacterium sp., Kurthia sp., Microhacterium sp., Providencia stuartii, Pseudomonas spp., Psudomonas fluorescens and Pseudomonas alcaligenes* that swoed antibacterial activities.

The disease outbreaks nor mass mortality in the milkfish aquaculture was very rare and almost never been reported. This fenomena encouraged the researcher to isolate, molecularly identified, and further tested for their performance to control other gut bacterial growth and bacterial pathogens.

The variety methods of molecular biology has progressed in the framework to identify bacteria such as restriction fragment length polymorphism (RFLP)[5], Denaturing Gradient Gel electrophoresis (DGGE) and sequenced repetitive-based polymerase chain reaction (rep-PCR)[3]. This method has been used for the identification of marine bacteria[6], and deep-sea bacteria[7]. While the rep-PCR has been used for fast grouping on various marine microorganisms[8,9,10], Rep PCR has been applied to group bacterial agents causing vibriosis in fish grouper[11]. Therefore, identification of gut bacterial associated with milkfish was also using 16S rDNA sequences.

## 2. Material and Methods

### *2.1. Milkfish Sample and Bacterial Isolation*

The 15 milkfish, (*Chanos chanos* Forsksal) with weight of 100-200 g and length of 15-30 cm, were collected from extensive brackishwater ponds of Tambak Lorok village, Semarang regency, Juwana village, Pati District and Comal village, Pemalang District. Exploratory research using purposive sampling was applied. Necropsy and gut bacteria isolation were carried at Fish Quarantine, Quality Control and Safety of Fishery Products Agency, Semarang.

Isolation of gut bacterial of the milkfish samples were cultured using Zobell 2216E half-strength medium. Purification was done by spreading 1 ml of $10^{-1}$, $10^{-3}$ and $10^{-5}$ bacterial serial solution [4,12,13]. Then, colonies were picked and repurified by streak plating. Pure isolates was stored in NA media.

### *2.2. Repetitive – PCR*

The procedure was commenced according to Radjasa[9]. In the rep–PCR, BOX AIR ( 5'-CTACggCAAggCgACgCTgACg-3') was used[3]. The REP 1R-I and REP 2-I primers contain the nucluetide inosine (I) at ambigious positions in the REP consensus. PCR reaction contained of 1 µl DNA template (diluted 100x) , 1 µl primer, 7.5 µl Megamix Royal and sterile water up to total volume of 15 µl.

Amplifications were performed with a thermal cycler model Gene Amp PCR system 9700 with the following temperature conditions: initial denaturation at 95ºC for 5 minutes ; 30 cycled of denaturation (92°C for 1 minutes), annealing (50ºC for 1,5 minutes), extension (68ºC for 8 minutes) and final extension at 68ºC for 10 minutes. Five microliter aliquot PCR products were run using electrophoresis on 1 % ethidiumbromade gel by using 1X TBE buffer.

### *2.3. Grouping of Isolates*

Grouping was carried out according to a method of Radjasa[10] by making matrixes from the position of bands on the gel which were then analyzed by using Free Tree program using UPGMA method for constructing the tree. Resampling was performed by bootstrapping with 1000 replications.

### *2.4. PCR Amplification and Sequencing of 16s rRNA Gene Fragments*

PCR amplification was carried out according to method of Rad[asa[7]. Two primers, GM3F (5'AGAGTTTGATCMTGGC-3') and GM4R (5'-TACCTTGTTACGACTT-3') were used to amplify nearly complete 16S rRNA gene [14]. Genomic DNA of gut bacteria associated with milkfish (*Chanos chanos* Forsksal) strains for PCR analysis were obtained from cell materials taken from agar plate, suspended in sterile water (Sigma, Germany) and subjected to five cycles of deep freeze (-80ºC) and thawed at 95ºC. PCR amplification of partial 16S rRNA gene of bacteria, purification of PCR products and subsequent sequencing analysis were performed according to the method of Radjasa [9]. Sequencing was carried out to gain the alkaline composition that made up DNA sequences. The sequencing was performed using the Big Dye Terminator 3.1 dyes and automated DNA sequencer ABI3130 Genetic Analyzer XL Apllied Biosystemsat Macrogen Korea. The determined DNA sequences of strains were then compared for homology to the BLAST database [15].

### *2.5. Bacterial Sensitivity Test*

Selected bacterial isolates were tested for their potency against microorganisms in vitro. Inhibition of the growth of microorganisms by specific bacterial isolates was demonstrated by a clear zone in the bacterial colones area. Clear zone is an indication of the ability of bacterial isolates to prevent other bacterial growth. In addition, the diameter of clear area reflected the power and speed of the isolates to penetrate diffuse medium [16].

## 3. Results and Discussion

### *3.1. Gut bacterial isolation*

Isolation of milkfish gut bacteria from Semarang Regency, Pati and Pemalang Districs, morphologically revealed into 30 isolates (Table1).

Table 1. Morphological Feature of Milkfish Gut Bacterial Colonies from Pati, Pemalang Districts, and Semarang Regency

| No | Color | Form | Character | Isolates |
|---|---|---|---|---|
| 1 | Transparant | Rounded | Flat | BPi 01 |
| 2 | White turbide | Rounded | Flat | BPi 02 |
| 3 | Tranparant | Rounded | Flat | BPi 03 |
| 4 | White turbide | Rounded | Convex | BPi 04 |
| 5 | Transparant | Rounded | Flat | BPi 05 |
| 6 | White turbide | Rounded | Convex | BPi 06 |
| 7 | White turbide | Rounded | Flat | BPi 07 |
| 8 | White turbide | Rounded | Flat | BPi 08 |
| 9 | White turbide | Rounded | Flat | BPi 09 |

| | | | | |
|---|---|---|---|---|
| 10 | White turbide | Rounded | Flat | BPL01 |
| 11 | White turbide | Rounded | Convex | BPL02 |
| 12 | White turbide | Rounded | Flat | BPL03 |
| 13 | Transparant | Rounded | Convex | BPL04 |
| 14 | White turbide | Rounded | Convex | BPL05 |
| 15 | White turbide | Rounded | Convex | BPL06 |
| 16 | White | Rounded | Convex | BPL07 |
| 17 | White | Rounded | Convex | BPL08 |
| 18 | Transparant | Rounded | Convex | BPL09 |
| 19 | White | Rounded | Convex | BPL10 |
| 20 | White | Rounded | Convex | BS01 |
| 21 | White turbide | Rounded | Flat | BS02 |
| 22 | White turbide | Rounded | Flat | BS03 |
| 23 | White | Rounded | Convex | BS04 |
| 24 | Transparant | Rounded | Flat | BS05 |
| 25 | Transparant | Rounded | Convex | BS06 |
| 26 | White | Rounded | Flat | BS07 |
| 27 | Transparant | Rounded | Convex | BS08 |
| 28 | White turbide | Rounded | Convex | BS09 |
| 29 | Transparant | Rounded | Flat | BS10 |
| 30 | White | Rounded | Flat | BS11 |

Table 1 shows that based on colour, colony form, and character of colonies there were found 9, 10, 11 isolates from Pati, Pemalang and Semarang districts respectvely.Two characters were flat and convex whilst the colour were trasparent, white and white cloudy.

### 3.2. Repetitive-PCR Analysis

Based on the repetitive-PCR results and constructed into dendogram it was found 6 groups of isolates (Fig.1). The six isolates namely BS 11, BPL 01, BPL 10, BPi 03, BPi 08, BPL 06 were selected as representative of six groups for further investigation.

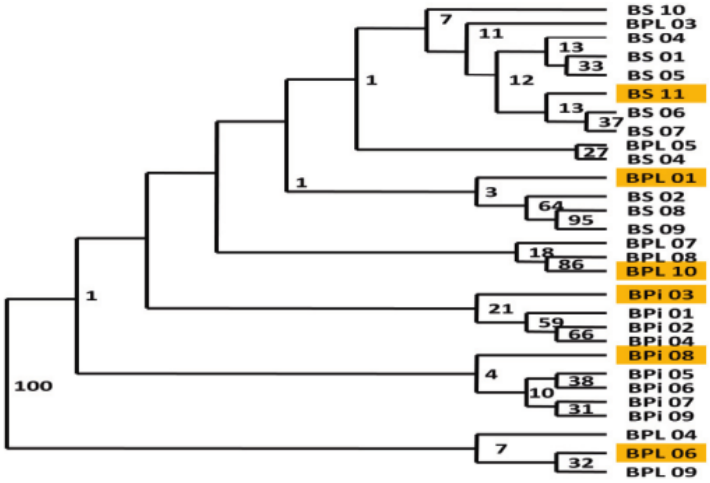


Figure 1. *Results Tree view* Dendogram Bacterial Grouping with rep PCR.

Rep-PCR rapid bacterial groupings results above were then followed by characterization. In this study, bacteria derived from the intestinal tract of milkfish revealed into 6 groups and to be sequenced with 16S rDNA. Group I, II, III, IV, V, VI were represented by BS11,BPL01, BPL10, BPi03, BPi08 and BPL06 respectively. This rep-PCR method was widely used for its ability to differentiate bacterial isolates to the species level, sub-species and strain specific and quickly for classification of bacteria (rapid grouping) [7,9].

### *3.3. Sequensing DNA and Analysis DNA Squence*

The DNA sequence of six isolates were confirmed with the world gen data base. Iit was found that they were closley related to 3 species namely *Shewanella sp, Shewanella algae, and Photobacterium ganghwense* and other three species were related to *Shewanella upenei, Vibrio fluvialis* and *Basilus sp.*(Table 2).

Table 2. Molecular Characteristics of Isolates and Their Homology to Gen Bank Reference

| No | Isolates | Strain | Homology | Access code |
|---|---|---|---|---|
| 1 | BS 11 | *Shewanella upenei* | 89% | gb\|JQ670746.1\| |
| 2 | BPL 01 | *Basillus* sp | 79% | gb\|GQ9800246.1\| |
| 3 | BPL 10 | *Vibrio fluvialis* | 88% | gb\|DQ296636.1\| |
| 4 | BPi 03 | *Shewanella* sp | 97% | emb\|X81622.1\| |
| 5 | BPi 08 | *Shewanella algae* | 98% | gb\|JQ670728.1\| |
| 6 | BPL 06 | *Photobacterium ganghwense* | 99% | gb\|AY960847.1\| |

Based on the results of the molecular characteristic and bacterial diversity. It were shown in Table 2 and 3, that BS11, BPL01, BPL10, BPI03, BPI08 and BPL06 were homolog 89% to *Shewanella upenei*, 79% to *Basillus* sp, 88% to *Vibrio fluvialis,* 97% to *Shewanella* sp, 98% to *Shewanella algae,* and *99%* similar to *Photobacterium ganghwense* respectiively. According to Hagstrom [17] , the isolates had 16S rDNA sequence similarities of more than 97% represented the same species. While the sequence similarities between 93% -97% may represented the identitical of the bacteria at genus level but might different species. Therefore in the present study it was found that isolates BS 11 and BPL01 were identified as *Shewanella upenei* with homology of 79% and *Basillus* sp, with homology of *88%* respectively. These indicated that the last two isolates potentialy a new species that needs further investigation.

Previous studies on the genetic biodiversity of intestinal tract bacteria from marine fish were reported by Chaiyapechara[18] and Martin-Antonio [19]. Bacteria group γ-Protobacteriaie, *Vibrio* and *Shewanella* sp and *Bacillus*was found in the digestive gut of reef fish, *Sebates* sp [18]. Martin-Antonio [19] also found *Vibrio* and *Shewanella*s pp in gastro intestinal of flatfish (*Solea senegalensis*). Navarrete [20] reported that *Shewanella* spp also found in digestive tract of the Atlantic salmon (*Salmo salar* L.). Whereas, *Photobacterium* is one of the 12 groups of bacteria found in the digestive tract of salmon (*Salmo salar* L.) [21].

The genus *Photobacterium* as *Photobacterium phosphoreum* and *Photobacterium leignathi*is a luminous bacteria and has an internal light organs origin from the gastro intestinal tract and associated livefish intestinal tract[22]. Gram-positive such as *Bacillus circulans*, *Bacillus. megaterium, Bacillus coryneforms* were also found in the intestinal tract of rainbow trout (*Salmo gairdneri*) [4].

Tabel 3.Bacterial Diversity in The Intestine Tract of Milkfish

| No. | Realeted Species | Homology | Total Isolates | % |
|---|---|---|---|---|
| 1 | *Shewanellaupenei* (BS11) | 89% | 10 | 25,64 |
| 2 | *Basillus*sp (BPL 01) | 79% | 4 | 10.26 |
| 3 | *Vibrio fluvialis* (BPL 10) | 88% | 3 | 7.69 |
| 4 | *Shewanella* sp (BPi 03) | 97% | 4 | 10.26 |
| 5 | *Shewanella algae* (BPi 08) | 98% | 5 | 12.82 |
| 6 | *Photobacteriumganghwense* (BPL 06) | 99% | 3 | 7.69 |

13

In order to determine the level of similarity of the 6 bacteria isolated from intestinal organs of milkfish, phylogenetic tree of thus bacteria were presented in the Figure 1.


Figure 1. Phylogenetic Tree Bacterial Isolates from Milkfish Intestinal Organ

### 3.4 Bacterial Sensitivity Test

The results of the anti bacterial activity among the intestinal bacterial isolates were presented in table 4. Table 4 indicated that *Shewanella upenei* BS11was able to prevent bacterial growth of *Vibrio fluvialis* BPL 10 and *Photobacterium ganghwense* BPL 06 at both $10^7$ and $10^9$ cfu/ml whilst *Shewanella algae* BPi 08 was only able to prevent both bacterial above at concentration of $10^9$ cfu/ml.

Based on the sensitivity test revealed that bacteria associated to digestive organs of milkfish was found in this study supposedly have the ability to prevent diseases caused by pathogenic bacteria ie. *Vibrio alginolyticus* and *V. parahamolyticus*. The microflora in the digestive tract has an important metabolic and specific role, and protective function of the antagonistic activity of bacterial pathogens and immune modulators [23,24]. One of the mechanisms involved to prevent the colonization of pathogenic bacteria in the host was by inhibited pathogenic bacteria in dominating the attachment[25]. In the present study also found that the two isolates (BS 11 and BPi08 ) were potentially exhibited as a probitic candidate candidate for marine and brackhiswater fish culture.

Tabel 4. Inhibition Zone (*mm)* of Tested Bacteria at Different concentration

| Isolates | Bact. Conc Cfu/ml | *Shewanella upenei* BS 11 | | *Shewanella algae* BPi 08 | |
|---|---|---|---|---|---|
| | | Sensitivity | Clear Zone (mm) | Sensitivity | Clear Zone (mm) |
| *Vibrio fluvialis* BPL 10 | $10^7$ | + | 11 | - | - |
| | $10^9$ | + | 13 | + | 13 |
| *Photobacterium ganghwense* BPL06 | $10^7$ | + | 11 | - | - |
| | $10^9$ | + | 14 | + | 14 |

Further study of the performance of the 6 bacterial isolates to the two marine bacterial pathogen was presented in the Table 4 and 5. Table 4 demonstrated that *Sehwanella upenei* BS11were able to inhibit the growth of *Vibrio fluvialis* BPL10 and *Photobacterium* ganghwense BPL06. Table 5 demonstrated that all 6 isolates had a capability to inhibit bacterial pathogen, V*ibrio parahaemolyticus* and *Vibrio alginolyticus*.

The sensitivity tests and inhibition zone in table 5, shown a strong inhibition because they able to produced clear ring between 9-14 mm. An antibacterial activity that able to produced inhibitation zone between 10-20 mm classified as strong category[26]. Thus intestinal bacteria found in milkfish has a strong ability against bacterial pathogens. So the association of several bacteria in the intestinal tract of milkfish have the ability to defend itself against disease. This results were similar with several other studies that has been published [27,28,29,30].

Table 5 : Diameter Inhibitation Zone (mm) of *Vibrio parahaemolyticus* and *Vibrio alginolyticus* Against 6 Bacterial Isolate*s* after 48 hours.

| Isolates | *Vibrio parahaemolyticus* | *Vibrio alginolyticus* |
|---|---|---|
| *Shewanella upenei* BS 11 | 12 | 10 |
| *Basillus sp* BS 01 | 12 | 12 |
| *Vibrio fluvialis* BPL 10 | 14 | 11 |
| *Shewanella* sp BPi 03 | 14 | 9 |
| *Shewanella algae* BPi 08 | 14 | 12 |
| *Photobacterium ganghwense* BPL 06 | 11 | 9 |

The criteria of bacterial probiotics candidate should has the ability to inhibit pathogenic bacteria and able to compete with pathogenic bacteria in the intestinal tract. Besides that, they also capable to produce antimicrobial to inhibit the growth of pathogenic microbes in the digestive tract of fish[31]. Therefore *Shewanella algae* and *Shewanella* sp, could be developed as a probiotic consortium formation. This results inline with previous

researches[32,33] that *Shewanella* sp could be used as a probiotics candidate. Similarly, *Bacillus* sp. S01 was also good candidate for probiotics in fish culture. The similar result was also reported in grouper[34], in the black tiger shrimp (*Penaeus monodon*) and white shrimp (*Litopenaeus vannamei*) [35,36, 37]. *Bacillus* was also reported to able to increase survival rate post larvae of *Penaeus monodon*, that was challenged with *Vibrio harveyi*. It may due to the probiont provided cellular and humoral immune defence responses [21].

The most probiotics proposed in aquaculture belongs to the lactic acid bacteria (*Lactobacillus* and *Carnobacterium*), genusVibrio (*V. alginolyticus*), Bacillus, or Pseudomonas, although other genera have also been mentioned (Aeromonas and Flavobacterium)[12]. They further explained that probiotic has different ways of out-competing pathogens in order to grow. These include (1) production of antagonistic compounds, (2) advantageous growth characteristics, i.e. a short lag period and a short doubling time, (3) attachment ability to intestinal mucus and (4) production of other compounds beneficial to the host.

## 4. Conclusion

1. The diversity of bacteria associated with milkfish gut collected from Northern Coast of Central Java, demonstrated closest similarity to *Shewanella upenei, Basillus* sp, *Vibrio fluvialis, Shewanella algae, Shewanella* sp. *and Photobacterium ganghwense.*
2. The six selected isolates were also successfully demonstrated antibacterial activity among the gut bacteria and against pathogenic bacteria *V. alginolyticus* and *V. parahaemolyticus.*
3. Milkfish gut bacteria namely *Shewanella upenei, Basillus* sp, *Vibrio fluvialis, Shewanella algae, Shewanella* sp. *and Photobacterium ganghwense.*have a pottential for probiotics.

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