Impacts of Probiotics on Water Quality and Milkfish Production (*Chanos chanos*) Grown in Polluted Ponds of Marilao and Meycauayan, Bulacan

John Vincent R. Pleto*
Environmental Biology Division, Institute of Biological Sciences
College of Arts and Sciences
University of the Philippines Los Baños

Mark Dondi M. Arboleda
School of Environmental Science and Management
University of the Philippines Los Baños

Veronica P. Migo
Department of Chemical Engineering
College of Engineering and Agro-Industrial Technology
University of the Philippines Los Baños

Jessica F. Simbahan
Institute of Biology, College of Science
University of the Philippines Diliman

ABSTRACT

The Marilao-Meycauayan-Obando River System is known to be heavily polluted with organics and heavy metals, thus affecting the ecosystem. This study used probiotics as an ecological approach to improve environmental quality, with a focus on determining the impacts of probiotics on fish health and survival as well as water quality. Probiotics are microbial feed supplements that can improve the survival and health of organisms. Probiotics were applied at the start and after two months of culture period. Physico-chemical water quality parameters were recorded. Growth parameters such as fish body weight, feed conversion ratio (FCR), and survival rate were determined. Polymerase chain reaction-denaturing gradient gel electrophoresis (PCR-DGGE) method was used to determine the microbial community present in the guts of milkfish *(Chanos chanos)* grown in polluted water treated with probiotics. The results showed that ponds treated with probiotics had higher dissolved oxygen and lower biochemical oxygen demand (BOD) and nitrate and phosphate levels, which are beneficial for the growth of milkfish. However, higher ammonia and chemical oxygen demand (COD) were observed in the probiotic ponds. Higher survival rate (95.3%) was obtained in treated ponds compared to non-treated ponds (74.1%). The FCR

* *Corresponding Author*

was less in probiotic-treated ponds (0.74) than non-treated ponds (1.35), which is beneficial for fish production. The study showed that the probiotic strains (*Bacillus*) were not able to establish in the milkfish gut. Instead, strains related to *Cetobacterium, Clostridium, Conexibacter, Cyanobium, Cyanothece, Cylindrospermum, Helicobacter, Romboutsia, Synechococcus,* and *Vibrio* were detected in the guts of milkfish. Overall, the probiotics had an impact on water quality and fish health through improvement of growth and survival rate.

Keywords: probiotics, water quality, *Chanos chanos*, PCR-DGGE (polymerase chain reaction-denaturing gradient gel electrophoresis), microflora

INTRODUCTION

The Marilao-Meycauayan-Obando River System (MMORS) is one of the most polluted rivers in the Philippines. In 2007, MMORS was included in the list of "dirty thirty" rivers in the world by then Blacksmith Institute (now Pure Earth), an environmental group in New York (Blacksmith Institute 2007). The river system was found to be heavily polluted with organic matter and heavy metals coming from different sources such as untreated municipal wastewater and industries in used lead acid battery recycling and gold refineries. Unfortunately, there are aquaculture ponds along the river system that are considered to be one of the primary sources of livelihood of fisherfolk in Bulacan. Since water from aquaculture ponds come from the river system, the ponds' ecosystem and people who consume the fishes are at high health risk. The ecosystem, including microorganisms, is greatly affected due to the pollutants present in the environment. In order to restore the ecosystem and make it conducive for fish growth, the use of probiotics was explored. The first application of probiotics was to test the ability to increase the growth of organisms that live in water. Then, it was used to improve water quality and to treat bacterial infections (Cruz et al. 2012). The aquaculture industry employs microorganisms, termed probiotics, as live adjunct to the diet of fishes. These probiotics have beneficial effects on the host by boosting the utilization of feeds or enhancing their nutritional value, strengthening host response against diseases or improving the quality of the environment (Verschuere et al. 2000). The application of probiotics is an eco-friendly bioremediation technology for aquaculture that can improve water quality. During the culture period, water quality has the tendency to deteriorate due to the accumulation of metabolic wastes, decomposition of unused feeds, and decay of biotic materials. Probiotics can utilize or decompose the organic matter and toxic material in water, thus improving water quality. The use of probiotics in aquaculture can also inhibit pathogens and promote the growth of farmed fish. It controls pathogens through a variety of mechanisms that serve as an alternative

to antibiotics (Padmavathi et al. 2012). In this study, probiotics were applied in fishponds in two sites located along the MMORS to assess their performance in improving water quality and growth and survival of milkfish (*Chanos chanos*). The diversity of microflora in the fish gut was also assessed. To date, no study has been undertaken to determine the impact of probiotics on the survival of fishes growing in heavily polluted sites in the Philippines.

The composition and identification of fish microflora has been investigated using culture-dependent methods that relied on phenotypic and biochemical characteristics. Also, only a low percentage of intestinal microflora can be cultured in laboratory media and identified using phenotypic and biochemical approaches. The analysis of polymerase chain reaction (PCR)-amplified 16S rRNA genes from gut samples through denaturing gradient gel electrophoresis (DGGE) allows for the visualization of the bacterial community through the DNA fingerprint of the presumed dominating microorganisms present in the sample. Microbial identification and clustering through PCR-DGGE without the need for classical culturable substrates allowed the direct use of the samples and a faster and simpler protocol (Manzano et al. 2012). PCR-DGGE has become a popular method for studying gut microbiota of humans and animals because it provides a rapid survey of the microbial community regardless of their ability to grow in cultivation media (Possemiers et al. 2004; Kim et al. 2007). However, one of the main limitations of DGGE is that it does not have the resolution to detect organisms that represent less than 1% of the overall community (Muyzer et al. 1993).

In this study, probiotics were tested on fishponds in two sites located along the MMORS to assess their performance in improving water quality and fish health. There have been no studies conducted on the use of probiotics in aquaculture ponds that get water from a polluted source. The use of probiotics has been proven to be positive promoters of aquatic animal growth, survival and health (Hai 2015). The objective of the study was to determine the impacts of probiotics on milkfish growth and survival and water quality. The study also determined the microflora diversity of milkfish gut using the PCR-DGGE method.

MATERIALS AND METHODS

Study site

Fishponds along the Marilao and Meycauayan Rivers of MMORS were the sites for this study. The two sampling sites were located in Barangay Nagbalon, Marilao, Bulacan and Barangay Liputan, Meycauayan, Bulacan. Three replicates of probiotic-

treated and non-treated ponds were prepared in each site. The ponds used in the study are those owned by farmers who agreed to participate in the study. Table 1 shows the physical characteristics of the ponds used in the study.

Table 1. Physical characteristics of the ponds used in the study.

Pond	Location	Treatment	Size (sq.m.)	Depth (m)
1	Nagbalon	Treated with probiotics	1400	1.0
2	Nagbalon	Treated with probiotics	780	1.5
3	Liputan	Treated with probiotics	300	1.0
4	Nagbalon	Non-treated	670	1.5
5	Nagbalon	Non-treated	300	1.0
6	Liputan	Non-treated	300	1.0

Pond treatment, preparation and application of probiotics

The probiotic used was purchased from Charoen Phokphand (CP) Feeds in Samal, Bataan and consisted of Super Biotic® and pH Fixer®. Super Biotic and pH Fixer both consist of a strain of *Bacillus* microorganism with a concentration of 10^9 CFU/mL. Super Biotic was applied once during pond preparation and pH Fixer was also applied once after two months of culture. Super Biotic was applied at a dosage of 3 L per ha and pH Fixer at a dosage of 150 g per ha. Super Biotic was mixed with molasses and water at a rate of 500 mL molasses and 100 L of water for every 1 L Super Biotic and was incubated for 24 hours in a drum before application to the ponds. The viability of probiotics was not conducted since the counts were already given on the product label and the probiotics were already tested and validated by the manufacturer.

Monitoring of physico-chemical parameters

Dissolved oxygen (DO), temperature, pH, salinity, ammonia, phosphates, biochemical oxygen demand (BOD), and chemical oxygen demand (COD) of the ponds were monitored throughout the four months of culture. The *in situ* parameters such as DO, temperature, pH, and salinity were determined on a daily basis during the morning and the afternoon. The DO, temperature, and pH were measured using a DO meter (HACH HQ30d) and Ultrameter III 9P. Salinity was determined using the Atago S-Mill refractometer. *Ex situ* parameters such as ammonia, phosphates, BOD, and COD were determined monthly.

Stocking of milkfish

Milkfish fingerlings were obtained from a farm in Binmaley, Pangasinan. These were conditioned and acclimatized for one week in the reservoir pond before stocking at 1.5 fish/m^2. A 27% allowance of milkfish was added for mortality and destructive

sampling every month. The average initial weight of fingerlings was 4.4 g for the Nagbalon site and 5.2 g for the Liputan site. The average initial length of fingerlings were 7.7 cm and 8.3 cm for Nagbalon and Liputan, respectively. The feeding program and the feeds used for the milkfish were adopted from CP. Feeding of milkfish was done every morning. Table 2 shows the feeding management program from CP Feeds.

Table 2. Feeding program for the milkfish using CP Feeds.

Day of Culture	Average Body Weight (ABW) (g)	% Feed	Feed Code
1	2	10.0	CP 9041
30	35	6.0	CP 9910s
60	90	4.0	CP 9991
90	170	2.7	CP 9991
120	300	2.3	CP 9992

Sample preparation

Six milkfish were randomly collected from each replicate pond during the second and fourth months of culture. Milkfish samples were collected after feeding. The milkfish were placed in an ice chest and transported to the laboratory. The exterior of each fish was cleaned with 95% ethanol prior to dissection. Dissection of the gut was done using a sterile scalpel/blade. Microbes attached to the intestinal wall were part of the natural gut microflora (Ringo et al. 2001), thus the whole intestine and gut contents were used for all extractions. Pooled gut samples for each pond were homogenized in a blender in TE Buffer then kept in -80 °C until further use.

Total bacterial count

Samples from the homogenized gut were obtained and dilutions of these samples were spread on Nutrient Agar plates. Drops of Nystatin were added to the agar to prevent fungal growth. The plates were incubated at room temperature (28–30 °C) for 24–48 hours. Colonies that developed on the plates were counted and reflected as colony forming units per gram of sample.

DNA extraction and DNA amplification by polymerase chain reaction

DNA was extracted from the homogenized sample using the protocol of ZR 96 Soil Microbe DNA kit™ and analyzed by electrophoresis for confirmation.

The 16S rDNA were amplified by PCR using the bacterial primers PRBAC-338fGC (5'-CGCCCGCCGCGCGCGGCGGGCGGGGCGGGGGCACGGGGGGACTCCTACGGGAG GCAGCAG-3') and 518r (5'ATTACCGCGGCTGCTGG) (Muyzer et al. 1993). Each mixture

(final volume of 50 µL) contained about 3.0 µL of template DNA, forward and reverse primers (PRBAC-338fGC and 518r) at 0.2 µM, the deoxyribonucleotide triphosphate (dNTPs) and $MgCl_2$ at 200 µM each, 5 µl of 10X PCR buffer $MgCl_2$ free and 0.4 µl of Taq polymerase.

The PCR conditions were an initial denaturation at 94 °C for 1 min, then annealing at 65 °C (with an increasing temperature 1 °C per cycle) for 1 min and extension at 72 °C for 3 min followed by 20 cycles of 94 °C for 1 min, 55 °C for 1 min, and 72°C for 3 min.

PCR products were analyzed in 1% (w/v) agarose gel in 0.5x TAE buffer using Mupid gel electrophoresis set-up and observed on a UV transilluminator (DCode™).

Denaturing Gradient Gel Electrophoresis (DGGE)

PCR products were analyzed using the DCode™ Universal Mutation Detection System (Bio-Rad Laboratories) using the method described by Muyzer et al. (1993) and improved by Leesing (2005). Samples containing approximately equal quantities of amplicons (DNA concentration was not determined) were loaded into 8% w/w polyacrylamide gel with a denaturing gradient urea formamide spreading 35 to 65%. Electrophoresis was done at 60 °C in TAE Buffer (2M sodium-acetate, 0.05M EDTA, pH 8.3) at 20 V for 10 min and then 80 V for 12 hours. After electrophoresis, the gels were stained with ethidium bromide (50 mg/l) for 1 hour and rinsed in distilled water for 20 min. The gels were observed under a UV transilluminator.

The bands were excised, sliced into small pieces and incubated in PCR grade water 37 °C for 30 min before storing at -80 °C until further use.

Sequencing and identification

The excised bands were subjected to PCR for amplification. The same conditions were used except for the annealing temperature of 62 °C. Each mixture contained about 0.5 µL of template DNA, the primers (PRBAC-338fGC and 518r) at 0.2 µM each, the deoxyribonucleotide triphosphate (dNTPs) and $MgCl_2$ at 200 µM each, 5.0 µl of 10X PCR buffer $MgCl_2$ free and 0.4 µL of Taq polymerase. PCR products were analyzed by electrophoresis as previously mentioned.

The PCR amplicons were sent to AITbiotech, Singapore for sequencing. The results were viewed using FinchTV. The identities of the organisms were determined using Basic Local Alignment Tool (BLAST). Multiple sequence alignment was done using the software BioEdit™ version 7.1 (Hall 2011). The phylogenetic tree was constructed using the Phylip software (Felsenstein 2005).

RESULTS

Physico-chemical water quality parameters of ponds

Table 3 shows the mean physico-chemical values of pond water for the non-treated and treated ponds for the whole monitoring period. The DO is relatively higher in ponds treated with probiotics for morning and afternoon monitoring. The water temperature varied from 29.33 ± 0.52 to 31.93 ± 0.15 ˚C. The pH of the ponds was slightly alkaline. The ammonia levels of treated ponds have a higher mean at 2.72 ± 1.33 ppm compared to non-treated ponds at 1.98 ± 0.98 ppm. The ammonia levels exceeded the recommended level of DENR at 0.5 ppm. The nitrate and phosphate levels of ponds treated with probiotics were lower than those of the non-treated ponds. The nitrate levels of ponds were within the recommended level of 7 ppm while phosphate levels exceeded the 0.5 ppm recommended level. The BOD of non-treated ponds (12.17 ± 1.61 ppm) were higher than the levels in treated ponds (10.67 ± 1.76 ppm). The COD of treated ponds (650.93 ± 97.93 ppm) were much higher than those in non-treated ponds (502.00 ± 65.98 ppm). Both BOD and COD exceeded the DENR recommended limits of 7 ppm and 100 ppm, respectively.

Table 3. Mean physico-chemical parameters of the ponds for the whole monitoring period.

Treatment Ponds	Monitoring Time	DO (mg/L)	Temp. (˚C)	pH	Ammonia (ppm)	Nitrates (ppm)	Phosphates (ppm)	BOD (ppm)	COD** (ppm)
Non-Treated Ponds	AM	6.34 ± 1.23	29.36 ± 0.63	8.05 ± 0.28	1.98 ± 0.98	0.42 ± 0.13	1.81 ± 1.49	12.17 ± 1.61	502.00 ± 65.98
	PM	11.85 ± 0.85	31.71 ± 0.20	8.65 ± 0.15					
Treated Ponds	AM	7.13 ± 0.51	29.33 ± 0.52	8.03 ± 0.06	2.72 ± 1.33	0.30 ± 0.10	0.85 ± 0.05	10.67 ± 1.76	650.93 ± 97.93
	PM	12.76 ± 0.71	31.93 ± 0.15	8.44 ± 0.20					
Recommended Level*		5.0 mg/L	25 - 31°C	6.5 – 8.5	0.05 ppm	7 ppm	0.5 ppm	7 ppm	100 ppm

* The recommended level for each parameter is based on the Water Quality Guidelines and General Effluent Standards of 2016 (DAO 16-08) by the Department of Environment and Natural Resources (DENR)

**There was a significant difference for the chemical oxygen demand (COD) of the non-treated and treated ponds ($P<0.05$).

Average body weight, total bacterial count and percent survival of milkfish

The average body weight and the gut bacterial counts were determined from milkfish gathered from all the ponds in the Nagbalon and Liputan sites.

The initial microbial counts of the fingerlings and probiotics used were 4.67 x 10^4 and 3.05 x 10^5 CFU/mL, respectively. Table 4 shows the average body weight of milkfish and the total viable count of bacterial isolates from the guts of the samples after two and four months of culture in the ponds. Ponds treated with probiotics during the second month have milkfish with relatively higher body weight compared to those in the non-treated ponds. During the harvest, the average body weight of milkfish from treated ponds was 169.17 g while that in the non-treated ponds was only 129.57 g. The feed conversion ratio (FCR) is the rate measuring the efficiency by which fish convert feed into the desired output. The lower the FCR, the less feed is being used to produce 1 kg of fish. In terms of the survival rate, it was noted that fish grown in ponds treated with probiotics have higher survival rate (95.3%) compared to those in non-treated ponds. Based on the results, treated ponds have relatively higher microbial population during the 4th month.

Table 4. Average body weight, feed conversion ratio, and total viable count of bacterial isolates of milkfish from the two ponds.

Treatment ponds	Second Month		Fourth Month		Feed Conversion Ratio (FCR)	Percent Survival*
	Average Body Weight (g)	Total Bacterial Count (CFU/ml)	Average Body Weight (g)	Total Bacterial Count (CFU/ml)		
Non-treated ponds	48.97 ± 19.03	3.08 x 10^5 ± 3.15 x 10^5	129.57 ± 68.00	2.21 x 10^5 ± 2.80 x 10^5	1.35 ± 1.31	74.1%
Treated ponds	61.33 ± 14.99	5.19 x 10^4 ± 2.62 x 10^4	169.17 ± 54.05	3.48 x 10^5 ± 2.75 x 10^5	0.74 ± 0.10	95.3%
P value	0.403	0.481	0.536	0.724	0.22	

*based on the number of milkfish that survived

Denaturing Gradient Gel Electrophoresis, sequencing and identification

DGGE analysis was conducted through the amplification of the V3 variable region of bacterial 16S rDNA. Figure 1 shows the DGGE pattern of the amplified DNA from milkfish gut during the 2nd and 4th months for both sites as well as those of the fingerlings and probiotics. The identities of the marker organisms are as follows: A - *Lactobacillus plantarum LP1;* B - *Lactobacillus casei 4E5;* C - *Pediococcus acidilactici;* D – *Bacillus* sp.*;* and E - *Lactobacillus acidophilus 1900W.*

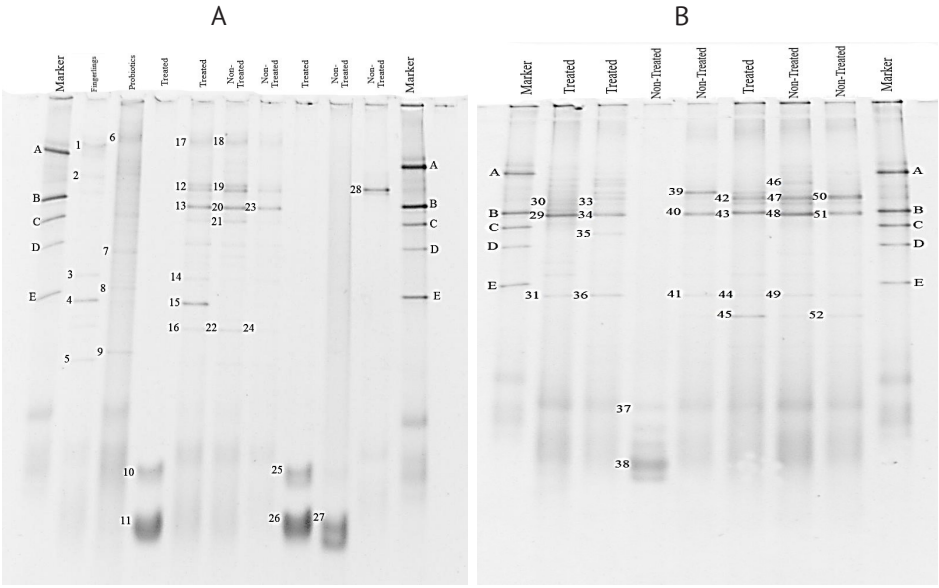

Figure 1. (A) Result of the denaturing gradient gel electrophoresis from different ponds during the 2nd month of collection and the fingerlings and probiotics used in the study. **(B)** Result of the denaturing gradient gel electrophoresis from different ponds during the 4th month of collection.

DGGE analysis shows the presence on the second month of *Synechoccus rubescens* (bands 13, 20, and 23) and *Cyanothece* sp. (bands 12, 16, 18, and 19). None of the fishes, especially those from ponds treated with probiotics, showed the presence of the probiotic strains *Bacillus pumilus* (band 8) and *Chlorobaculum tepidum* (band 9). Strains with closest identities to *Helicobacter* sp. (band 1) and *Clostridium* sp. (band 3) were found in the guts of fingerlings. *Cetobacterium somerae*, corresponding to bands 29, 34, 40, 48, and 51, was present in the 4th month in almost all ponds. The presence of pathogenic organisms related to *Vibrio cholera* (band 35) and *Clostridium ghonii* (band 36) was also noted on the 4th month. The DGGE profile of the probiotics used contained strains of *Bacillus*, *Chlorobium* and *Chlorobaculum*.

A phylogenetic tree (Figure 2) was constructed to determine the relationship of the strains' sequences among each other and with other type strains. The phylogenetic tree identifies seven major clusters to which the gut strains shared closest identity. They are the Actinobacteria-like, Bacillus-like, Chlorobia-like, Clostridia-like, Cyanobacteria-like, Epsilonproteobacteria-like, and Fusobacteria-like clusters. Sequences 7, 33, 35, 36, and 38 did not cluster with a known microorganism and could be an entirely different species.

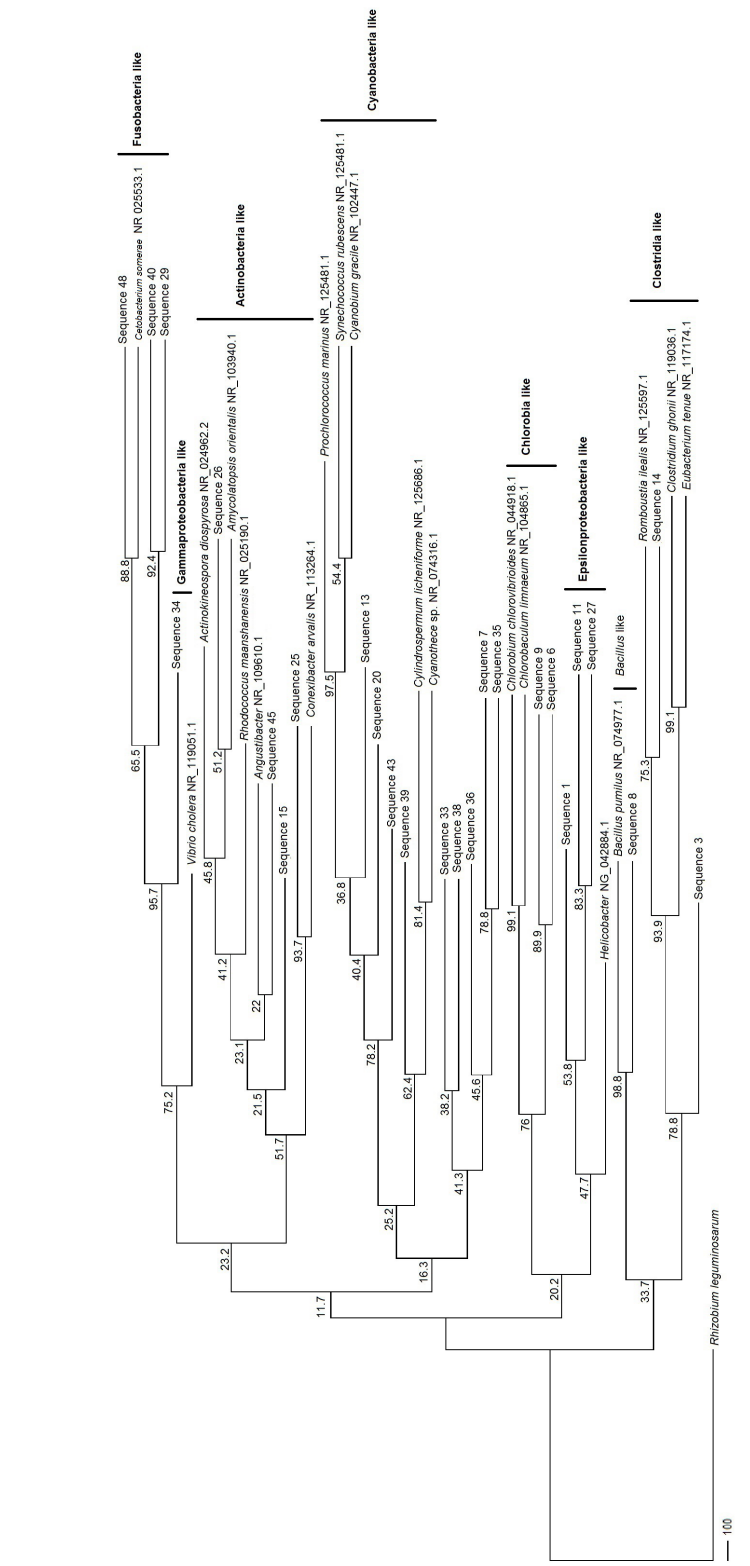

Figure 2. Phylogenetic tree of 16S rRNA sequences from gut of milkfish cultivated in ponds. The sequence length is 140 base pairs. The resultant tree was evaluated by bootstrap analysis based on 1000 replicates to represent the evolutionary history of the taxa analyzed. The percentage of replicate trees in which associated taxa clustered together in the bootstrap test is shown next to the branches. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. *Rhizobium leguminosarum* was the outgroup organism.

DISCUSSION

Impacts of probiotics on water quality

In aquaculture, the common problem in water quality is the increase in organic matter, phosphorus and nitrogen levels due to the feeds and fertilizers being applied in ponds. High concentrations of nitrate can reduce animal growth and survival in aquaculture (Davidson et al. 2014). Studies showed that probiotics improve the quality of water and the pond bottom sediment, thereby creating a stress-free environment for the animals and thus improving their health (Moriarty et al. 2005). Probiotics have proven their effectiveness in improving water quality by enhancing decomposition of organic matter, reducing nitrogen and phosphorus concentrations, and controlling ammonia, nitrite, and hydrogen sulphide (Boyd and Massaut 1999; Ma et al. 2009; Cha et al. 2013). In the study of Mahmud et.al. (2016), ponds treated with probiotics had higher oxygen levels, better water transparency, less ammonium and fewer cyanobacteria. Based on the water quality assessment, probiotic treated ponds had relatively higher DO and lower pH, nitrates, phosphates, and BOD levels, which is beneficial to fish growth. However, ammonia and COD levels were higher in treated ponds. This might be due to the lower frequency of application of probiotics throughout the culture period. It was suggested that maintaining high levels of probiotics in production ponds minimizes the accumulation of dissolved and particulate organic carbon (Balcázar et al. 2006).

Impacts of growth and survival of milkfish

Several studies have found that feeding probiotic bacteria can increase the growth rate, weight gain and feed efficiency (FCR) of several aquaculture species. The increased growth performance produced by the use of probiotics is achieved by enhancing metabolic pathways by contributing vitamins, short-chain fatty acids and enzymes that are either not normally produced by the host or sufficiently included in their diet (Merrifield et al. 2010). Application of probiotics can improve aquatic animal growth rates, feed utility by influencing digestive enzyme processes and survival rates (Hai 2015). This study showed that milkfish grown in probiotic treated ponds have relatively higher average body weight and survival rate and lower FCR. A lower FCR means that it takes less feed to produce 1 kg of fish; hence, production of milkfish is more cost-efficient. The use of probiotics as growth promoters was applied to Nile tilapia (*Oreochromis niloticus*) using the *Streptococcus* strain, significantly increasing crude protein and lipid content in the fish. Also, average weight increased from 0.154 g to 6.164 g in 9 weeks of culture (Lara et al. 2003). The results of the study agreed with related literature that application of probiotics would increase the weight and survival rate of fish as well as lower the FCR.

Microflora of milkfish

The common microflorae of milkfish include *Bacillus* sp., *Bifidobacterium* sp., *Carnoacterium* sp., *Eubacterium* sp., *Lactobacillus* sp., *Lactococcus* sp., *Micrococcus* sp., *Photobacterium* sp., *Pseudomonas* sp., *Shewanella* sp., *Staphylococcus* sp., and *Vibrio* sp. (Prayitno et al. 2015). *Acinetobacter*, *Aeromonas*, *Flavobacterium*, *Lactococcus*, and *Pseudomonas*, obligate anaerobes *Bacteroides*, *Clostridium*, *and Fusobacterium,* and members of family Enterobacteriaceae dominate the guts of freshwater species (Gómez and Balcázar 2008). These microorganisms play important roles in immunity mechanism and provide a positive influence on the health of the fishes. *Lactobacillus fermentum, Lactobacillus gasseri, Lactobacillus delbrueckii,* and *Micrococcus lylae* were potentially used as probiotics in aquaculture (Prayitno et al. 2015). Based on the results obtained, no probiotic strains were found in the guts of the milkfish after the application of probiotics. DGGE analysis was used to analyze the microbial community of milkfish gut. The DGGE profile of the commercial probiotics used in the study contained *Bacillus*, *Chlorobium,* and *Chlorobaculum* species. These probiotics can diminish the growth of pathogens and increase the growth of beneficial bacteria, leading to improved fish quality (Ninawe and Selvin 2009; Chen and Hu 2011). The probiotics species failed to establish in the guts of the milkfish due to the frequency of application. According to Balcázar et al. (2006), probiotic microorganisms are able to colonize gastrointestinal tracts when administered over a long period of time because they have a higher multiplication rate than the rate of expulsion; so, as probiotics are constantly added to fish cultures, they adhere to the fishes' intestinal mucosa, developing and exercising their multiple benefits. DGGE is a useful tool in monitoring spatiotemporal changes in the microbial community structure. It can provide full sequences that can be used for further analysis and can easily compare microbial diversity between samples. However, this technique can only detect dominant species with relative abundance of > 0.1% (Muyzer et al. 1993). DGGE mostly targets abundant taxa present (Jiang et al. 2018). In the study, the dominant taxa might be the species present in the environment and the bacteria present in the probiotics were not that abundant. Another limitation of using DGGE is that the relationship among nucleotide sequence, phylogenetic affiliation and the melting point is not well established and the retardation of the fragment in the gel matrix may not properly indicate phylogenetic relatedness at higher resolution, like species level (Kisand and Wikner 2003).

Strains related to *Cetobacterium, Clostridium, Conexibacter, Cyanobium, Cyanothece Cylindrospermum, Helicobacter, Romboutsia, Synechococcus,* and *Vibrio* were detected in the guts of the milkfish. *Helicobacter* is a major water-borne pathogen but

its occurrence in fishes is still unknown. Several Clostridia inhabit the anoxic environment of the intestinal tract and could cause severe diseases. *Synechococcus* is a phototrophic microbe fundamental in carbon and nutrient cycling. It often constitutes the bulk of the photosynthetic biomass and is responsible for a significant proportion of primary production in oligotrophic water. However, *Synechococcus* blooms may occur, which can produce harmful toxins that could cause fish kill (Seymour et al. 2010). Most Vibrios and related bacteria are found in aquatic environments. These vibrio group bacteria can emit light through the process called bioluminescence (Madigan et al. 2012). *Vibrio parahaemolyticus* and *Vibrio vulnificus* are commonly present in fish gut microbiome and could be transferred to other environmental reservoirs, implicating fish in the persistence and dispersal of potential pathogens. These vibrio species are often found to be the dominant bacteria in and on marine fish and are common members of the gut microflora in both farmed and wild fish (Givens 2012).

Studies have been conducted on determining the adherence and colonization of microorganisms in gastrointestinal tracts. According to Conway (1996), microorganisms can colonize the intestinal tract when they can persist for a long time due to their multiplication rate. Also, another important aspect identified by Nikoskelainen et al. (2001) is that mucosal adhesion is an important criterion for selection of probiotics to be applied for fish. Hence, it is essential to first determine the gut microbiota of milkfish grown in the aquaculture ponds to determine which can colonize longer in the gut. Some yeast strains have a strong adhesion potential to fish guts that could compete with other microorganisms (Li et al. 2018). Also, it was suggested that one approach to evaluate gut microbiome is to follow a 24-hour starvation period (Zhang et al. 2016). Studying the colonization of probiotics in fish guts is very complex due to the different environmental and stochastic factors and high influx of microorganisms in water (Li et al. 2018). According to Li et al. (2018), colonization should be replaced with populate or passive colonization when describing the probiotic content isolated from guts.

CONCLUSIONS AND RECOMMENDATION

Probiotics is a supplemental feed that could improve the growth and survival of fishes as well as the water quality of ponds. The research study focused on determining the impacts of the use of probiotics on fish health and water quality. It also determined the microflora diversity of milkfish guts using the PCR-DGGE method. The results showed that probiotics had a positive impact on water quality. Ponds treated with probiotics had higher DO levels and lower pH, nitrates,

phosphates and BOD levels, which are beneficial for growth and survival. Milkfish grown in probiotic ponds had higher weight and survival rate and better FCR, which is beneficial for production. However, probiotic species (*Bacillus*) failed to establish in the guts of the milkfish due to the frequency of application. In the study, the probiotics contain strains of *Bacillus*, *Chlorobium,* and *Chlorobaculum*, which are beneficial for fishes. Strains related to *Helicobacter, Cetobacterium, Romboutsia, Synechococcus, Vibrio, Cylindrospermum, Cyanothece*, *Conexibacter, Clostridium,* and *Cyanobium* were detected in the guts of the milkfish. The frequency of application of the probiotics might be the reason why the strains were not able to establish. Microbiota data of water and pond sediments should be included to determine how the environment has been altered by the probiotics. This could determine if the probiotic strains used were able to thrive after application. Another recommendation would be mixing the probiotics mixture to the feeds of fishes for establishment in the gut. Other strains of probiotics, such as lactic acid bacteria (*Lactobacillus* sp. and *Carnobacterium* sp.), *Vibrio* sp., and *Pseudomonas* sp., could be tested and utilized as probiotics. Isolating, screening, and culturing of probiotic strains from the river system that can survive in a polluted environment could help in successful introduction to the aquatic organisms.

ACKNOWLEDGMENTS

The authors would like to express their deepest gratitude to the Pure Earth (Blacksmith Institute) Philippines team for its trust, encouragement, and full support in the conduct of this research, and also to University of the Philippines Los Baños – National Institute of Molecular Biology and Biotechnology (UPLB-BIOTECH) for allowing us to conduct the laboratory analysis.

REFERENCES

Balcázar JL, Blas ID, Ruiz-Z I, Cunningham D, Vendrell D, Múzquiz JL. 2006. The role of probiotics in aquaculture. Vet Microbiol. [accessed 2020 Sep 28];114(3-4):173–186. https://doi.org/10.1016/j.vetmic.2006.01.009.

Blacksmith Institute. 2007. Top 10 Most Polluted Places: 2007. New York: Pure Earth. [accessed 2020 Jun 14]. http://worstpolluted.org/.

Boyd CE, Massaut L. 1999. Risks associated with the use of chemicals in pond aquaculture. Aquacult Eng. [accessed 2020 Sep 23];20:113–132. https://doi.org/10.1016/S0144-8609(99)00010-2.

Cha J-H, Rahimnejad S, Yang S-Y, Kim K-W, Lee K-J. 2013. Evaluations of *Bacillus* spp. as dietary additives on growth performance, innate immunity and disease resistance of olive flounder (*Paralichthys olivaceus*) against *Streptococcus iniae* and as water additives. Aquaculture. [accessed 2020 June 13];402–403:50–57. https://doi.org/10.1016/j.aquaculture.2013.03.030.

Chen S, Hu Y. 2011. Use of *Bacillus subtillis* in purification of slightly-polluted water. Acta Sci Cir. [accessed 2020 Jun 14];31(8):1594–1601. https://www.semanticscholar.org/paper/Use-of-Bacillus-subtilis-in-purification-of-water-Yong-you/53052f30d762a849ded1d4ea0418aedf3ab2b32f.

Conway PL. 1996. Pathogenesis and the gastrointestinal tract of growing fish. In: Mackie RI, White BA, Isaacson RE, editors. Gastrointestinal microbiology. New York (NY): Chapman and Hall. p. 3–38.

Cruz P, Ibañez, A, Hermosillo O, Saad H. 2012. Use of probiotics in aquaculture. ISRN Microbiology. [accessed 2020 Sep 23];2012(2):[about 13 p.]. https://doi.org/10.5402/2012/916845.

Davidson J, Good C, Welsh C, Summerfelt ST. 2014. Comparing the effects of high vs. low nitrate on the health, performance, and welfare of juvenile rainbow trout *Oncorhynchus mykiss* within water recirculating aquaculture systems. Aquacult Eng. [accessed 2020 Jun 16];59:30–40. https://doi.org/10.1016/j.aquaeng.2014.01.003.

Decamp O, Moriarty DJW. 2006. Probiotics as alternative to antimicrobials: limitations and potential. World Aquac. [accessed 2020 Jun 10];37(4):60–62. https://doi.org/10.1016/j.jff.2020.104080.

[DENR] Department of Environment and Natural Resources (Philippines). 2016. Water quality guidelines and general effluent standards of 2016. Quezon City, Philippines: DENR.

Givens CE. 2012. A fish tale: comparison of the gut microbiome of 15 fish species and the influence of diet and temperature on its composition [dissertation]. University of South Carolina.

Gómez GD, Balcázar JL. 2008. A review on the interactions between gut microbiota and innate immunity of fish. FEMS Immunol Med Mic. [accessed 2020 Sep 21];52:145–154. doi:10.1111/j.1574-695X.2007.00343.x.

Hai NV. 2015. The use of probiotics in aquaculture. J Appl Microbiol. [accessed 2020 Jun 14];119(4):917–935. DOI: 10.1111/jam.12886.

Jiang H, Huang L, Yang J, Wu G. 2018. Chapter 23 - a microbial analysis primer for biogeochemists. In: De Vivo B, Belkin HE, Lima A, editors. Environmental Geochemistry. Second Edition. Elsevier. p. 599–609.

Kisand V, Wikner J. 2003. Limited resolution of 16S rDNA DGGE caused by melting properties and closely related DNA sequences. J. Microbiol Methods. 54:183–191.

Lara F, Olvera N, Guzmán M, López M. 2003. Use of the bacteria *Streptococcus faecium* and *Lactobacillus acidophilus*, and the yeast *Saccharomyces cerevisiae* as growth promoters in Nile tilapia (*Oreochromis niloticus*). Aquaculture. [accessed 2020 Jun 14];216(1–4):193–201. https://doi.org/10.1016/S0044-8486(02)00277-6.

Leesing R. 2005. Identification and validation of specific markers for traceability of aquaculture fish for import/export [dissertation]. France: University of Montpellier.

Li X, Ringo E, Hoseinifar S, Lauzon H, Birkbeck H, Yang D. 2018. The adherence and colonization of microorganisms in fish gastrointestinal tract. Rev Aquacult. [accessed 2020 Dec 10];11(3):602–618. https://doi.org/10.1111/raq.12248.

Ma CW, Cho YS, Oh KH. 2009. Removal of pathogenic bacteria and nitrogens by *Lactobacillus* spp. JK-8 and JK-11. Aquaculture. [accessed 2020 Jun 14];287:266–270. https://doi.org/10.1016/j.aquaculture.2008.10.061.

Madigan MT, Martinko JM, Bender KS, Buckley DH, Stahl DA. 2012. Brock biology of microorganisms. 13th ed. New York: Pearson Education Inc.

Mahmud S, Ali M, Alam A, Rahman M, Jorgensen N. 2016. Effect of probiotics and sand filtration treatments on water quality and growth of tilapia (*Oreochromis niloticus*) and pangas (*Pangasianodon hypophthalmus*) in earthen ponds of southern Bangladesh. J Apl Aquacult. [accessed 2020 Sept 23];28 (3):199–212. https://doi.org/10.1080/10454438.2016.1188339.

Manzano M, Iacumin L, Giusto C, Cecchini F, Patthey C, Fontanillas R, Comi G. 2012. Utilization of denaturing gradient gel electrophoresis (DGGE) to evaluate the intestinal microbiota of brown trout *Salmo trutta fario*. J Vet Sci Med Diagn. [accessed 2020 Jun 14];1(2). doi:10.4172/2325-9590.1000105.

Merrifield D, Bradley G, Baker R, Davies S. 2010. Probiotic applications for rainbow trout (*Oncorhynchus mykiss* Walbaum) II. Effects of growth performance, feed utilization, intestinal microbiota and related health criteria postantibiotic treatment. Aquacult Nutr. [accessed 2020 Sep 23];16:496-503. https://doi.org/10.1111/j.1365-2095.2009.00688.x.

Moriarty DJW, Decamp O and Lavens, P. 2005. Probiotics in aquaculture. Aquaculture Asia Pacific magazine. September/October 2005 edition. 1:14-16.

Muyzer G, De Waal EC, Uitterlinden AG. 1993. Profiling of complex microbial populations by denaturing gradient gel electrophoresis analysis of polymerase chain reaction-amplified genes coding for 16S rDNA. Appl Environ Microb. [accessed 2020 Jun 14];59:695–700. https://www.ncbi.nlm.nih.gov/pmc/articles/PMC202176/.

Muyzer G, Hottenträger S, Teske A, Wawer C. 1996. Denaturing gradient gel electrophoresis of PCR-amplified 16S rDNA – a new molecular approach to analyse the genetic diversity of mixed microbial communities. Molecular Microbial Ecology Manual. [accessed 2020 Jun 14];34:1–23. http://aem.asm.org/.

Nikoskelainen S, Salminen S, Bylund G, Ouwehand AC. 2001. Characterization of the properties of human- and dairy-derived probiotics for prevention of infectious diseases in fish. Appl Environ Microb. [accessed 2020 Dec 8];67:2430–2435. https://www.ncbi.nlm.nih.gov/pmc/articles/PMC92891/.

Ninawe AS, Selvin J. 2009. Probiotics in shrimp aquaculture: avenues and challenges. Crit Rev Microbiol. [accessed 2020 Sep 30];35(1):43–66. DOI: 10.1080/10408410802667202.

Padmavathi P, Sunitha K, Veeraiah K. 2012. Efficacy of probiotics in improving water quality and bacterial flora in fish ponds. Afr J Microbiol Res. [accessed 2020 Sep 30];6(49):7471–7478. DOI: 10.5897/AJMR12.496.

Prayitno S, Sarwan S. 2015. The diversity of gut bacteria associated with milkfish (*Chanos chanos* Forskal) from Northern Coast of Central Java, Indonesia. Procedia Environ Sci. [accessed 2020 Jun 14];23:375–384. https://doi.org/10.1016/j.proenv.2015.01.054.

Ringø E, Lødemel JB, Myklebust R, Kaino T, Mayhew TM, Olsen RE. 2001. Epithelium-associated bacteria in the gastrointestinal tract of Arctic charr (*Salvelinus alpinus L*.). An electron microscopical study. J Appl Microbiol. [accessed 2020 Jun 14];90:294–300. DOI: 10.1046/j.1365-2672.2001.01246.x.

Sakai M, Yoshida T, Astuta S, Kobayashi M. 1995. Enhancement of resistance to vibriosis in rainbow trout, *Oncorhynchus mykiss* (Walbaum) by oral administration of *Clostridium butyricum* bacteria. J. Fish Dis. [accessed 2020 Jun 14];18:187–190. DOI: 10.1111/j.1365-2761.1995.tb00276.x.

Seymour JR, Ahmed T, Durham WM, Stocker R. 2010. Chemotactic response of marine bacteria to the extracellular products of *Synechococcus* and *Prochlorococcus*. Aquat Microb Ecol. [accessed 2020 Jun 14];59:161–168. https://doi.org/10.3354/ame01400.

Tsuchiya C, Sakata T, Sugita H. 2007. Novel ecological niche of *Cetobacterium somerae*, an anaerobic bacterium in the intestinal tracts of freshwater fish. Letters in Appl Microbiol. [accessed 2020 Jun 14];46(1):43–48. DOI: 10.1111/j.1472-765X.2007.02258.x.

Verschuere L, Rombaut G, Sorgeloos P, Verstraete W, 2000. Probiotic bacteria as biological control agents in aquaculture. Microbiol Mol Biol R. [accessed 2020 Jun 14];64:655–671. doi: 10.1128/mmbr.64.4.655-671.2000.

Zhang M, Sun Y, Liu Y, Qiao F, Chen L, Liu WT et al. 2016. Response of gut microbiota to salinity change in two eurohaline aquatic animals with reverse salinity preference. Aquaculture. [accessed 2020 Dec 8];454:72–80. https://doi.org/10.1016/j.aquaculture.2015.12.014.

Assistant Professor **John Vincent R. Pleto** <jrpleto@up.edu.ph> is a faculty member at the Institute of Biological Sciences, College of Arts and Sciences, University of the Philippines Los Baños. His research interests include aquatic ecology, specifically water quality and bioremediation technology, and environmental impact assessment studies.

Dr. Veronica P. Migo is a Professor at the Department of Chemical Engineering, College of Engineering and Agro-industrial Technology, University of the Philippines Los Baños. Her research interests include wastewater treatment, recycling of agro-industrial wastewater, global inventory of pollutants, validation of analytical methods, design of analytical laboratories for food and feed, soil, plant and fertilizer analysis, and water and wastewater analysis for ISO 17025 accreditation.

Dr. Mark Dondi M. Arboleda is an Associate Professor at the School of Environmental Science and Management, University of the Philippines Los Baños. His research interests include aquatic ecology, microbiology, molecular biology and biotechnology, marine protected areas and sustainable community development.

Dr. Jessica F. Simbahan is an Associate Professor at the Institute of Biology, University of the Philippines Diliman. Her research interests include microbial ecology and biotechnology.