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Application of a modified membrane-trapping technique to determine fish microflora species from Thai silver BARB (*Barbonymus gonionotus*)

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Abstract

A combined method using both culture-dependent methods (CDM) and culture-independent methods (CIM) was used to investigate the fish microflora of Thai silver BARB fish. The species among microflora communities from different parts of fresh healthy fish, gills, flesh, and digestive tracts as well as fish pond water were investigated. Using a traditional CDM, a total of 316 isolates from the cultivation method were randomly selected based on their morphological, physiological, and biochemical characteristics. Most of the acid-producing Gram-positive fermentative bacteria were identified as genera of lactic acid bacteria, *Enterococcus*, *Pediococcus*, *Streptococcus*, *Lactococcus*, *Aerococcus*, *Tetragenococcus*, and *Thermobacterium*. Moreover, genera of Gram-negative and other Gram-positive bacteria were identified as *Enterobacter* and *Staphylococcus*. In the case of CIM, 18 bands appeared using polymerase chain reaction–denaturing gradient gel electrophoresis (PCR-DGGE). They were identified based on 16S recombinant DNA (rDNA) sequencing analysis as *Weissella confusa, Streptococcus lutetiensis*, *Enterococcus faecium, Enterococcus gallinarum, Lactobacillus fermentum, Pediococcus acidilactici*, and *Enterococcus casselifavus*. Other Gram-positive enteric bacteria species and some yet uncultured bacteria were also identified as *Enterobacter cloacae, Enterobacter ludwigii, Enterobacter mori, Enterobacter roggenkampii, Staphylococcus* sp., *Staphylococcus epidermidis, Bacillus cereus, Oceanobacillus profundus,* and an uncultured bacterium. These results indicate that *Enterococcus*, *Lactobacillus*, *Streptococcus*, and *Pediococcus* are the predominant species of Thai silver BARB microflora.

Keywords: Membrane-trapping technique, Microbiota, Dominant species, PCR-DGGE, CDM, CIM, *Plaa-ta-pian*

1. Introduction

Aquaculture plays a very important role in food security and the Thai economy. Freshwater aquaculture is a crucially important enterprise that largely supplies domestic consumption. In local regions, Thai silver BARB (*Barbonymus gonionotus*) is generally known as *plaa-ta-pian*. It is among the top five freshwater fish species commonly found in Thailand. This species is grown in aquaculture and also used in the aquarium trade. Thai silver BARB cultivation is the third most cultivated species in freshwater aquaculture, accounting for 15 percent of fish produced in this way [1]. Furthermore, many researchers have studied the microflora of fish species in recent years, particularly examining the microbial communities of the digestive tracts and intestines of various aquatic species. Research of this nature has been done using Chinese shrimp [2], gilthead seabream and goldfish [3], as well as yellow catfish [4].

Many researchers have explored the microflora of digestive tracts, especially the gut. The main reason for this is that such approaches reveal crucial factors of essential physiological functions such as food digestion, mucosal system development and the immune system, thus indicating the host's health [2]. Since the microbial components of food webs have important implications in biogeochemical fluxes, it is necessary to investigate their structure and composition. Owing to their short life cycle and high sensitivity to environmental changes, microorganisms are suitable for use as indicators of environmental quality [5]. In addition to these advantages, a

background knowledge of microorganisms is also helpful for research and development of novel fish applications, notably various fermented fish production methods. Previously, some studies have reported on microflora diversity in the intestinal tract of freshwater and sea fish using culture-dependent methods (CDM) and/or culture-independent methods (CIM). In this way, microbiological methods are coupled with molecular approaches [3,4].

CDM is a combination of conventional cultivation techniques that can be used in phenotypic and genotypic identification. It is applied for typing of isolated strains. Generally, microorganisms are enumerated by cultivating them on a properly selected medium [6]. Information about the viable populations obtained from the cultivating medium can further be used to determine viable and culturable (VAC) state cells. Whenever viable cells have been subjected under unhealthy conditions or otherwise stressed, the cells may be viable but non-culturable (VBNC) [7]. Therefore, the current research aimed to minimize stress during the recovery and isolation processes, thereby preventing stress on living cells or VC state cells in the system so that VBNC state cells do not result. Utilization of these strategies showed that fish have an intestinal microbiota that is comprised of aerobic, facultative anaerobes, and obligate anaerobic microbes. The bacterial populations may be shifted, depending on age, species, dietary status, natural conditions, and the complexity of the microbial microflora in a fish stomach [8,9]. Naturally, almost all microbes have a small population density, so it is difficult to cultivate and isolate them on an enriched or selective medium using conventional methods since serial dilution techniques are commonly used. Therefore, CDM gives only a limited understanding of the complexity of the fish intestinal microflora in these biological systems [10]. The current research aimed to create a new approach employing a combination of membrane-trapping techniques with CDM techniques. Molecular or CIM techniques were used to elucidate the microbial diversity in this environmental system and determine the limitations of serial dilution techniques. Moreover, a combination of a membrane-trapping approach with CIM employs amplification of 16S recombinant DNA (rDNA) genes using polymerase chain reaction–denaturing gradient gel electrophoresis (PCR-DGGE) techniques which mainly examines the nucleic acids found in microbes. These methods provide high accuracy to deliver a precise analysis of complex microbial communities [11].

The published literature reveals the background and drawbacks of previously used analytical methods involved in studying fish microbiota. Accordingly, this study proposes to investigate and identify the various fish microflora species recovered and isolated from silver BARB fish using CDM coupled with membrane-trapping techniques. PCR-DGGE techniques were also applied in this work.

2. Materials and methods

2.1 Fish sampling

Thai silver BARB species were supplied by the Department of Fisheries, Faculty of Agriculture, Khon Kaen University. The weight of each fish was approximately 300-400 g. The temperatures and pH values of pond water were in the range of 30.2-32.3 °C and 6.8-7.2, respectively. The samples were collected in April of 2016, which is in the summer in Thailand. All samples were placed in ice upon receipt and kept in this condition prior to the laboratory examinations detailed below. Transport to the research laboratory required a short time (<10 min). This minimized changes in the microbial community.

2.2 Preparation and isolation of fish microflora using a membrane-trapping technique

Fish samples (n = 3 from each fish pond) were used for the recovery and isolation of fish microflora species for each experiment. The gills (G), flesh (F), and digestive tract (D) of each of the three fish, along with a sample of water (W) from each of their ponds were taken and kept separate from other samples. Twenty-five grams of each sample were collected and homogenized in 225 mL of sterile saline peptone water consisting of 0.85 % (w/v) NaCl and 0.1 % (w/v) peptone for 4 min, in a Stomacher 400 Lab Blender (Seward, UK) at 500 rpm. Subsequently, pre-filtration of the homogeneous solution was conducted twice using sterile qualitative filter paper. Fifty milliliters of the homogeneous solution were further recovered and isolated using a membrane-trapping technique through a 0.45 µm cellulose acetate membrane (Filtrex Technologies, India). Each membrane was later put on a modified MRS agar (HiMedia, India) plate supplemented with 1% (w/v) $CaCO_3$ and was incubated at 35 °C for 24 h to distinguish lactic acid bacteria (LAB) from non-lactic acid bacteria (non-LAB).

All membranes having dense colonies were used to recover and isolate the viable and culturable (VC) state of the total bacterial population. Gram-positive bacteria including LAB and Gram-negative enteric bacteria (non-LAB) were cultivated using modified MRS-$CaCO_3$ agar plates incubated at 35 °C for 24 h. The representative strains of LAB presented halo zones surrounding their colonies on modified MRS plates, whereas non-LAB strains did not show these halo zones. Many colonies (>100 points) on the primary membranes were randomly selected and put into fresh MRS medium. Single colonies were purified 2-3 times using a cross-

streaking technique on the same type of agar plates. Pure cultures were preserved in a modified freezing medium with 30% (v/v) glycerol and stored at -20 °C until used in subsequent experiments.

2.3 Physiological and biochemical characterization of fish microflora isolates

Halo zones were presented by 218 isolates from all four microbial sources (i.e., gills, flesh, digestive tract, and pond water) and were classified as LAB. Cell morphologies of all LAB isolates were observed under phase contrast microscopy (Olympus, Japan). The isolates were Gram-stained and catalase positive. Subsequently, both identification and grouping were carried out based on cell morphology and phenotypic properties, such as their utilization of glucose as a carbon source for CO_2 production, hydrolysis of arginine, their growth rate at various temperatures (10, 15 and 45 °C), and at distinctive pH values (3.9 and 9.6), as well as the ability to grow in several concentrations of NaCl [6.5, 10 and 18 % (w/v)] in MRS broth as was done in previously published reports [12].

Accordingly, 98 isolates characterized as non-LAB were Gram stained, tested for oxidase, and subject to IMVIC tests in the presence of indole, methyl red (MR), a Voges-Proskauer (VP) test, and citrate to determine their biochemical characteristics [13]. Verified isolates of Enterobacteriaceae were examined to determine if they were *Escherichia* sp. or *Enterobacter* sp. using previously published methods [13]. Specific identification of both bacteria was carried out on streak plates of McConkey agar with 0.15% (w/v) bile salt (HiMedia, India) incubated at 35 °C for 24 h. Based on the characterization of lactose fermenting strains, the results showed a pink or red zone of acid precipitated bile. The red color was obtained from the generation of acid from lactose and absorption of neutral red. Its color changed when the pH of a medium was lower than 6.8.

2.4 Total genomic DNA (gDNA) extraction

Membranes, containing dense colonies were homogenized in 10 mL of saline peptone water using a stomacher operating at 500 rpm for total gDNA extraction. The extraction was performed using the protocol described in the GF-1 Kit's instructions (Vivantis, Malaysia). The gDNA obtained was suspended in an elution buffer containing sterile TE buffer (10 mM Tris, 1 mM EDTA pH 8.0) and stored at -20 °C until use for 16S rDNA amplification.

2.5 16S rDNA amplification

All primers that were utilized in this work were synthesized and purchased from Ward Medic, Singapore (Table 1). Each gDNA was achieved using a modified protocol [14]. In the first PCR reaction, 16S rDNA was amplified using ~1.4 kb fragments employing the universal primers, 27-F and 1389-R, with a PCR machine (Model is S1000TM Thermal Cycler, Bio-Rad, USA). PCR products were subjected to agarose gel electrophoresis. In the second PCR reaction, the first PCR products were used and the V3 variable region of bacterial 16S rDNA from fish was re-amplified using a touchdown-PCR employing two primers, GC-338-F (with a GC-clamp) and 518-R [16,17,18]. Consequently, PCR products were reexamined using agarose gel electrophoresis and stored at -20 °C for DNA preservation.

Table 1 The sequences of primers used in this study.

Primer	Sequences (5′→ 3′)	References
27-F	AGAGTTTGATCMTGGCTCAG	[15]
1389-R	ACGGGCGGTGTGTACAAG	[15]
GC-338-F	CGCCCGCCGCGCGCGGCGGGCGGGGCGGGGGCACGGGGGGACTCCTAC GGGAGGCAGCAG	[16]
518-R	ATTACCGCGGCTGCTGG	[16]

2.6 Denaturation Gradient Gel Electrophoresis (DGGE) analysis

Details of the methods of the gene amplification methods used in this work have been previously published [14,16-18]. Amplicons in a PCR product size of approximately 200 bp were loaded onto an 8% (w/w) polyacrylamide gel (acrylamide/N, N'-methylene/bis-acrylamide, Vivantis, Malaysia) in 1X TAE buffer. A denatured gradient of urea formamide ranging from 30% to 60% (100% corresponds to 7 M urea and 40% (v/v) formamide) (Vivantis, Malaysia) was used for all electrophoresis experiments at a constant temperature of 60 °C and 20 V for 10 min followed by 80 V for 16 h. The gels were stained with ethidium bromide (Applichem, USA) for 30 min and twice washed in deionized water. They were visualized using UV light and DNA bands on DGGE gel were selected. Various bands were cut from a gel using sterile pipette tips following the scale of a protractor that was used as a maker.

2.7 16S rDNA sequencing analysis

Aliquots of the eluted DNAs were re-amplified using a PCR reaction with both primers, 388-F and 518-R without a GC-clamp, using the PCR reaction mixture and conditions described above. The PCR products (nearly 200 bp) were purified using a Qiagen Universal DNA Purification Kit (Hilden, Germany) according to the manufacturer's protocol. The purified PCR products were sequenced by Ward Medic, Singapore. The homology searches of the 16S rDNA sequences are described in previous work [19]. They were tested against known sequences in the GenBank databases available in the National Center for Biotechnology Information databases (NCBI) (http://www.ncbi.nlm.nih.gov/BLAST/).

3. Results and discussion

3.1 Quantification of fish microflora isolates

According to a preliminary study on the recovery and isolation of microorganisms, the membrane-trapping technique was investigated and improved for the culture-dependent approach in this study. It was successfully conducted to recover all of the viable and cultivable (VC) bacteria on MRS-$CaCO_3$ agar from various parts of silver BARB fish and pond water. The membrane-trapping techniques allowed full recovery of the VC cell states present in any of the samples since the synthetic membrane is made from cellulose acetate (0.45 μm pore size). As a result, the smallest of microbial cells can pass through the membrane easily. Hence, the membrane can efficiently trap all microbial cells larger than this pore size. Table 2 presents the number of isolates found from three parts of the sampled fish and their pond water. A total of 316 isolates were recovered and isolated from three different parts of fish and pond water, D (54 isolates), F (118 isolates), and G (50 isolates), W (94 isolates). The screening of isolates was further divided into two groups according to the generation of halo zones around colonies on the MRS-$CaCO_3$ medium and depending on colony morphology. A total of 218 isolates of acid-producing bacteria (APB) were observed on the agar plates. The appearance of the halo zones was caused by the solubility of calcium carbonate in acidic environments, even in the dilute acid released from the colonies [20,21]. The variation of clear zone size on MRS-$CaCO_3$ agar reflects the amount of acid released from colonies. [21,22]. Alternatively, some bacterial colonies did not exhibit halo zones. They are referred to as non-acidic bacteria (NAB), and accounted for 98 isolates from the four sources describe above. In this study, the pink colonies grown on McConkey agar were used as an indicator of Gram-negative bacteria. This is a selective medium for Gram-negative bacteria and a differential medium to distinguish lactose fermenters from non-lactose fermenting bacteria. It contains crystal violet and bile salts that can inhibit Gram-positive bacteria and promote the growth of Gram-negative bacteria [23]. Based on the results in this study, the MRS medium, a selective and complex medium containing dextrose, yeast extract, meat extract, and peptone that are sources of carbon, nitrogen, and vitamins, was recommended for general cultivation of fastidious strains [24]. The enteric bacterial group could grow even in an enrichment medium that contains only carbon and nitrogen sources from peptone and yeast extract. These nutrients are found in a non-specific medium such as nutrient or plate count broth/agar [13]. For this reason, both APB and NAB groups were isolated. They can grow together on MRS-$CaCO_3$ when obtained from different samples.

Table 2 Number of isolates from various parts of silver BARB fish species using membrane-trapping techniques.

Samples	Number of isolates		Total isolates
	Acid-producing bacteria (APB)	Non-acidic bacteria (NAB)	
Digestive tract (D)	32	22	54
Flesh (F)	68	50	118
Gills (G)	40	10	50
Water (W)	78	16	94
Total	218	98	316

3.2 Physiological and biochemical characteristics of APB and ANB isolates as evaluated by CDM

All of the APB, 218 isolates that presented halo zones around their colonies were subjected to microscopic characterization using a morphological observation technique. It was found that these isolates were Gram-positive, non-spore-forming bacilli and cocci shaped bacteria. The LABs were classified by physiological and biochemical characteristics according to previously published methods [12]. The selected isolates, arranged by genera, showing specific biochemical characteristics from a total of 316 isolates were isolated from four different environments and then subjected to CDM. The results are shown in Table 3. In this study, most LAB strains obtained from the three fish parts and pond water were facultatively homofermentative strains of seven

genera: *Pediococcus* sp., *Enterococcus* sp., *Streptococcus* sp., *Aerococcus* sp., *Tetragenococcus* sp., *Lactococcus* sp., and *Thermobacterium* sp. The most dominant bacterial species were of the genus *Enterococcus* sp. (43%) followed by *Pediococcus* sp. (12%). Both of these species were recovered from other freshwater fish by earlier researchers [11,25]. They reported that bacteria of the most predominant genus, *Enterococcus*, was from the intestines of common carp, freshwater prawns and brown trout. Additionally, *Pediococcus* sp. were also found in freshwater prawns. These are often used in various Thai fermented foods including *plaa-som*, *som-fak* (Thai fermented whole fish and fish mince), and *nham* (Thai fermented pork) [7,25,26]. Such microorganisms are sometimes considered probiotic materials that promote health. One of the necessary properties of a probiotic is high acid-resistance and/or the ability to survive at low pH (≤ 3). For example, *Enterococcus faecium* is often isolated from fermented fish products, [27] and *Pediococcus pentosaceus* can be isolated from fermented vegetables and is detectable in alcoholic fermentations [26,28]. Furthermore, NABs or non-LABs (98 isolates) in this study were evaluated using IMViC tests to characterize enteric bacterial groups as described in previous work [13]. IMViC tests are often used to distinguish *Escherichia coli* from other coliform bacteria. *Escherichia coli* are indole (+), methyl red (+), Voges-Proskauer (-) and citrate test, i.e., (+, +, -, -). *Enterobacter* and *Klebsiella* present (-, -, +, +) results. In the present study, it was found that 31.1% (98 of 316 isolates) of the NAB groups were *Enterobacter* sp. (20.3%) and *Staphylococcus* sp. (10.8 %), as shown in Table 3. There have been several studies that investigated the microflora of the alimentary tracts of freshwater salmonid fish in British Columbia. They found that their predominant microflora consisted of Gram-negative rods within the species *Enterobacter*, *Aeromonas*, and *Acinetobacter* [9,25].

Table 3 Phenotypic characteristics classified by genera from each part of the silver BARB species and their pond water.

Phylogenetic group	Genus	Number of isolates/samples				Total isolates	Total isolates (%)
		D	F	G	W		
Firmicutes							
Lactobacillaceae	*Pediococcus*	1	12	1	24	38	12.0
	Thermobacterium			1		1	0.3
Aerococcaceae	*Aerococcus*				7	7	2.2
Enterococcaceae	*Enterococcus*	20	86	5	25	136	43.0
	Tetragenococcus			1		1	0.3
Streptococcaceae	*Streptococcus*	2			22	24	7.6
	Lactococcus	9		2		11	3.5
Staphylococcaceae	*Staphylococcus*		17	17		34	10.8
γ-Proteobacteria							
Enterobacteriaceae	*Enterobacter*	22	3	23	16	64	20.3
Total						316	100.0

*D, F, G, and W represent digestive tract, flesh, gills, and water, respectively.

3.3 Fish microflora species as evaluated by CIM using PCR-DGGE and 16S rDNA sequencing

The total gDNAs were directly extracted from three different parts of silver BARB fish and their pond water. Then, the 30S regions of 16S rDNA were amplified via two universal primers, 338-F with a GC-clamp and 518-R, before DGGE analysis. Plant chloroplast 16S rDNA co-amplification was previously observed using GC-338-F and 518-R primer sets, which is a specific 30S region [2,14]. The DGGE pattern obtained in the current study revealed a total of 18 discernible bands (Figure 1). The DNA sequences were identified and classified into two phyla, *Firmicutes* and *Proteobacteria*. Other species were identified as luminescent of bacteria (Table 4). Particularly, the *Firmicutes* representatives consisted of six closely related genera with similarities ranging between 86 and 100%, namely *Leuconostocaceae*, *Streptococcaceae*, *Enterococcaceae*, *Lactobacillaceae*, *Staphylococcaceae*, and *Bacillaceae.* In a previous study of the microbial diversity in the intestines of olive flounder (*Paralichthys olivaceus*), it was reported that the *Firmicutes* bacteria retrieved from wild fish were of comprised six genera, *Staphylococcaceae, Streptococcaceae, Bacillaceae, Lactobacillaceae, Enterococcaceae*, and *Leuconostocaceae* [29]. In this study, *Firmicutes* were found to be the most dominant bacteria detected in various parts of the silver BARB fish and their pond water. These fish hosted eight LAB species including *Weissella confusa, Streptococcus lutetiensis, Streptococcus pasteurianus, Enterococcus faecium, Enterococcus casseliflavus, Enterococcus gallinarum, Lactobacillus fermentum,* and *Pediococcus acidilactici.* They presented different GenBank accession numbers (Table 4). It notable that Gram-negative bacteria such as *Escherichia*, *Pseudomonas* and *Proteus* were not detected using CIM methods. Natural variations in water temperature and pH may have been the cause different results from previous studies. Previous studies considered the microbiota of fish intestines, noting bacterial survival at lower pH values and different temperatures ranging from 10 to 45 °C [3,8,9].

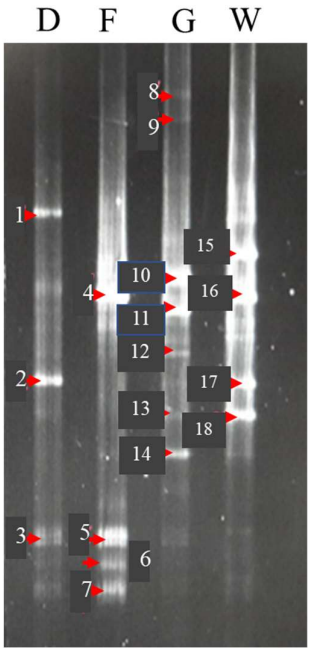

Figure 1 DGGE pattern of 16S rDNA-PCR products of fish microflora extracted from three different parts of silver BARB fish and their pond water. D = digestive tract, F = flesh, G = gills, W = water, and the numerals are DGGE band numbers.

Furthermore, LABs have received considerable attention concerning their advantageous impacts on fish health. Numerous studies have indicated that fish harbor LABs [9,25,28]. Despite the high changeability of fish intestinal microflora, the numbers of LAB from the intestines of freshwater fish are greater than for marine species [3,13]. Various studies found that *Carnobacterium* sp., *Lactobacillus* sp., *Lactococcus* sp., *Aerococcus* sp., *Leuconostoc* sp., *Pediococcus* sp., *Streptococcus* sp., *Vagococcus* sp., and *Weissella* sp. are normally present in the ordinary microflora of healthy freshwater fish [11,25,30]. According to results of 16S rDNA sequencing in the current study, bands 9, 10, 12, and 14 appeared in only fish gills and had the genomic DNA of non-LAB (Gram-positive enteric bacilli) species such as *Oceanobacillus profundus, Staphylococcus epidermidis, Staphylococcus* sp., and *Bacillus cereus*. In the current study, the dominant species of proteobacteria was found in the D and F samples. These phyla consisted of one family, *Enterobacteriaceae*, which was the predominant bacterial genus-group from fish organs. It consisted of *Enterobacter cloacae, Enterobacter ludwigii, Enterobacter mori,* and *Enterobacter roggenkampii* as indicated by bands 3, 5, 6, and 7, respectively (Table 4). In addition to these bacterial groups, other isolates belonging to the phylum *Proteobacteria*, such as *Klebsiella* sp., *Aeromonas* sp., and *Pseudomonas* sp., were found. They can be isolated from water, soil, animals, and humans. Non-LAB (Gram-positive enteric bacilli) species in the family of *Enterobacteriaceae* can be obtained from both CDM and CIM tests. They were not detected because their populations were reduced due to the high-temperature of the water in April and there was no contamination in the area. However, these species were reportedly isolated from the gastrointestinal tracts of freshwater salmonid fish and juvenile olive flounder. These other fish species were determined as another source of both *Klebsiella* sp. and *Aeromonas* sp. [31]. A few of the phylotypes might not be recognized at the class level by the Blast program. They were analyzed by utilizing the Seqmatch program on the RDP website (https://rdp.cme.msu.edu/). Our results showed that there was an assorted group of microorganisms that included *Cetobacterium somerae* from the DGGE band No. 13. These uncultured microbes could not be isolated using CDM. Similarly, a previous study by Cai et al. [14] reported that CDM cannot be used to isolate uncultured bacteria. However, CDM based on membrane-trapping is a very useful technique. Microbes in low numbers, such as *Enterococcus avium*, *Enterococcus gilvus*, and *Staphylococcus epidermidis*, were only found by recovery and isolation using a membrane-trapping technique. This form of CDM has proven more effective than traditional CDM techniques with serial dilutions, which failed to isolate species *in vitro* in low numbers since they are overshadowed by more numerous species [2,6,14,16].

Table 4 Identification of DGGE bands microorganisms from three different parts of silver BARB species and pond water.

Phylogenetic group	Organs	DGGE Band No.	Closest specific species	Similarity (%)	Accession No.
Leuconostocaceae	D	1	Weissella confusa strain 3273	97	MT613585
Streptococcaceae	D	2	*Streptococcus lutetiensis* strain 2709	91	MT611722
	W	17	*Streptococcus pasteurianus* strain NPL630	88	MK908254
Enterococcaceae	F	4	*Enterococcus faecium* strain LC2	100	KT626401
	G	8	*Enterococcus casseliflavus* strain 72D48	91	MT033021
	G	11	*Enterococcus gallinarum* strain 4493	92	MT584665
Lactobacillaceae	W	15	*Lactobacillus fermentum* strain PN8	93	MN559771
	W	16	*Lactobacillus fermentum* strain HBUAS53086	95	MH393160
	W	18	*Pediococcus acidilactici* strain RS1	90	KX611572
Staphylococcaceae	G	10	*Staphylococcus epidermidis* strain RO12	93	MK491020
	G	12	*Staphylococcus* sp. strain 28	94	MG162632
Bacillaceae	G	9	*Oceanobacillus profundus* strain AN-7	86	HQ202873
	G	14	*Bacillus cereus* strain Tom-AES3	100	MF687737
γ-Proteobacteria					
Enterobacteriaceae	D	3	*Enterobacter cloacae* strain ACD1	94	MT613381
	F	5	*Enterobacter ludwigii* strain ED4	98	MT613372
	F	6	*Enterobacter mori* strain RIII-A3	98	MN653289
	F	7	*Enterobacter roggenkampii* strain ED5	98	MT613373
Bacteria	G	13	Uncultured bacterium clone ncd725f05c1	86	HM293524

*D, F, G, and W represent digestive tract, flesh, gills, and water, respectively.

3.4 Summary of fish microflora revealed by both CDM and CIM methods

The results of this study show a comparison of fish microflora dynamics (FMD) found in various fish samples, gills (G), flesh (F), and digestive tract (D) as well as pond water (w) using both CDM and CIM methods. Members of various families, *Enterococcaceae*, *Lactobacillaceae*, *Streptococcaceae*, *Staphylococcaceae*, and *Enterobacteriaceae*, were detected as the predominant taxa using both CDM and CIM. Additionally, *Aerococcaceae, Bacillaceae,* and *Leuconostocaceae*, in the phylum of *Firmicutes*, were also detected with both CDM and CIM methods. These results are shown in Table 5. Previous studies reported a comparison in bacterial abundance between microorganisms not normally found in aquaculture environments and wild olive flounder. These microorganisms were classified into four phyla, *Firmicutes, Proteobacteria, Actinobacteria*, and *Bacteroidetes* [11,29]. Moreover, researchers reported that some isolates showed closer relationships to *Francisella philomiragia*, *Roseovarius crassostreae*, *Shewanella marina*, *Vibrio lentus*, *Algibacter mikhailovii*, and *Tenacibaculum mesophilum.* They might be associated with novel phylogenetic groups also found in the intestinal tracts and mucous membranes of rainbow trout [6,11,25].

Table 5 Comparison of fish microflora dynamics (FMD) found at different parts of fish samples and pond water using CDM and CIM methods.

FMD family	CDM*				CIM			
	D	F	G	W	D	F	G	W
Aerococcaceae				✓				
Bacillaceae							✓	
Enterococcaceae	✓	✓	✓	✓		✓	✓	
Lactobacillaceae	✓	✓	✓	✓				✓
Leuconostocaceae					✓			
Streptococcaceae	✓		✓	✓	✓			✓
Staphylococcaceae		✓	✓				✓	
Enterobacteriaceae	✓	✓	✓	✓	✓	✓		

*Symbols show the association of the FMD families in both methods being studied from different parts of silver BARB fish and pond water. D, F, G, and W represent digestive tract, flesh, gills, and water, respectively.

However, the current study did not consider pathogenic microorganisms that can also be found in the microbial community of silver BARB fish. It can be seen that the results from both CDM and CIM methods showed that LAB are the predominant species in various organs of fish and their environment. The LAB isolated from fish can prevent potential harm from specific species or subspecies of fish pathogens. For instance, it has been reported that *Lactococcus lactis*, *Lactobacillus plantarum*, and *Lactobacillus fermentum* can inhibit the adhesion of several fish pathogens, including *Aeromonas hydrophila*, *Aeromonas salmonicida*, *Yersinia*

ruckeri, and *Vibrio anguillarum*, in the intestinal tract [27-29,32]. These pathogens are often found in sewage and wastewater, which are considered as pathogen sources. Moreover, fish are susceptible to pathogens when they are under stress [8-11]. Particularly, some fish pathogens such as *Photobacterium damselae*, *Vibrio harveyi*, *Vibrio ichthyoenteri*, and *Vibrio scophthalmi*, can be obtained from fish gut infections and have the potential to directly damage their host and/or other aquatic life [31,32]. Control of pathogen populations is critical during fish processing to produce food. In terms of freshwater fish and aquaculture, this is a useful approach to increase the yield and quality from fish farming for commercial fish products.

The current study evaluated the benefits of using membrane-trapping techniques, which can be employed instead of serial dilutions for recovery and isolation of microorganisms from Thai silver BARB. Based on these combined techniques, less numerous microflora species can be recovered in higher density populations and are better isolated to truly reflect microbial diversity. This is provided that they are viable and culturable state cells. The combination of these techniques and culture-independent methods based on PCR-DGGE greatly contribute to the knowledge of biodiversity of fish microflora. Biodiversity data of these BARB strains can be collected and used for the prevention of future disease outbreaks or may be used in the development of vaccines for aquaculture. Additionally, this information is useful for fish processors following FAO recommendations to avoid exposing consumers to *Escherichia coli*. Thus, the results of this study also suggest that LAB are often the dominant species in various organs of *plaa-ta-pian*. It may be interesting to investigate LABs as potential probiotic agents for freshwater fish in terms of promoting the health and well-being of aquatic animals.

4. Conclusion

Both CDM and CIM were employed for investigation of the biodiversity of microbiota in this study. The prominent result of this study was the successful use of a membrane-trapping technique to augment traditional serial dilutions for the recovery and isolation of microorganisms. Using such a technique, microflora species from lower density populations, as well as those from higher density populations, were recovered and isolated, providing that they are in a viable and culturable state. This was done to quantify the high diversity of microorganisms in aquaculture. The combination of this technique and CIM based on PCR-DGGE contributes to our knowledge of biodiversity of fish microbes. Although each method exhibits its specific strengths, the limitations and drawbacks of the approach are revealed. Therefore, this combination of methods is highly recommended to study the biodiversity of various microbial communities.

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