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COMMUNITY STRUCTURE OF FISH GUT BACTERIA WHICH HAVE DIFFERENCES OF FEEDING HABITS

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ABSTRACT

This research suggests looking at the community structure in the gut that has different feeding habits using the Next Generation Sequencing (NGS) method. Carp, tilapia, and catfish are fish that live in freshwater with different feeding habits. Samples of carp and tilapia came from the Cirata Reservoir, Purwakarta, West Java and catfish came from the Ciparanje FPIK Unpad Wet Laboratory. This research was conducted in March-August 2019 at the FPIK Unpad Microbiology and Biotechnology Laboratory and subsequently sequenced by the HiSeq NGS in Novogene, Singapore. The results of the study obtained the principal values of coordinates 1 (PC1) and 2 (PC2) obtained were 60.36% and 39.64%. The grouping results made by the bacterial community of carp, tilapia, and catfish form a different group. Highest Abundance of *Cetobacterium, Clostridium sensu stricto 1, Bacteroides, Enterovibrio, Plesiomonas, Lactococcus, Romboutsia, Stenotrophomonas, Turicibacter, Edwardsiella,* and others.

Keywords: Carp, Catfish, Feeding habits, NGS, Tilapia

1. INTRODUCTION

Aquaculture is an activity to produce aquatic biota (organism) in a controlled environment to gain profit (1). The goals of aquaculture include feed production, improvement of natural stocks, fish production for recreation, fish feed production, ornamental fish production, organic material recycling and industrial material production [1]. The need to increase aquaculture production can be supported by increasing growth, fish feed efficiency and maintaining fish health. The bacterial community in the aquaculture environment has an important relationship with the microbiota found in fish. This microbiota can be found in various parts such as the digestive tract [2].

Carp, tilapia, and catfish are fish that live in freshwater with different eating habits. Carp, including omnivores to herbivores, prefer to eat insects or benthic worms [3]. Tilapia including omnivores with elongated body morphology, flat to the side with blackish white color and Catfish including omnivore to carnivores with elongated body shape and smooth skin [4].

At present, much research focuses on microorganisms in the fish gut, but these studies are only concentrated on factors, such as eating habits and host genotypes that can affect microbiota in the fish gut [5,6,7,8,9]. However, research focused on the main commodities of aquaculture in Indonesia is still very limited. Information about the community structure of carp, tilapia, and catfish is important data for the development of the aquaculture industry in Indonesia, so research on the comparative community structure of carp, tilapia, and catfish is needed. The research aims to describe the community structure of bacteria in the gut of carp, tilapia and catfish through the Next Generation Sequencing (NGS) method.

2. MATERIALS AND METHODS

2.1 Sampling of Fish Gut

This research was conducted in March 2019 - August 2019 in the Laboratory of Microbiology and Biotechnology, Faculty of Fisheries and Marine Sciences Unpad. Samples of carp (*Cyprinus carpio*) and tilapia (*Oreochromis niloticus*) came from the Cirata Reservoir, Purwakarta, West Java and catfish (*Clarias gariepinus*) from the Ciparanje FPIK Wet Laboratory. The step used to take a sample of the gut is that the fish is killed by piercing the brain using a sonde needle. Furthermore, fish are weighed using digital scales [10]. After that, the fish is placed on the tray and makes an incision on the belly of the fish until the internal organs are visible. Furthermore, the digestive organs of fish are separated and cut the gut from the stomach to the anus. Then the gut is stored in a sterile petri dish. Intestinal length is measured using a ruler and weighed intestinal mass using a digital scale. The contents of the gut are taken as much as 250 mg by splitting the gut of fish using scissors then scraped off the contents of the gut. The results of scraping the contents of the gut are then stored in a sterile microtube using tweezers to be used as a bacterial DNA isolation metagenome material.

2.2 Isolation of bacterial DNA metagenome

Metagenomic DNA from fish intestinal bacteria was isolated and extracted according to procedures in using the Quick-DNA ™ Fecal / Soil Microbe Miniprep Kit (Zymo Research, catalog no. D6010). The steps taken are the fish gut sample inserted using sterile tweezers into the ZR BashingBead ™ Lysis Tube and then added 750 µl ZR BashingBead ™ Lysis Tube and tightly closed. Furthermore, Microtube is homogeneous using vortex with a maximum speed of 20 minutes. Next, ZR BashingBead ™ Lysis Tube was centrifuged for 1 minute at a speed of 10,000 x g. 400 µl supernatant was transferred into the Zymo-Spin ™ III-F Filter in the Collection Tube and centrifuged at a speed of 8,000 xg for 1 minute. A total of 1,200 µl Genomic Lysis Buffer was added to the filtrate in the Collection Tube. A total of 800 µl of step 5 mixture was transferred to Zymo Spin ™ II C Column in the Collection Tube, then centrifuged 10,000 xg for 1 minute. The liquid from the Collection Tube is poured and done again before. A total of 200 µl of DNA Pre-Wash Buffer was added to the Zymo-Spin ™ II C Column in the new Collection Tube, then centrifuged at 10,000 xg for 1 minute. A total of 500 µl gDNA Wash Buffer was added to the old Zymo Spin II Column. Zymo Spin III Column was transferred to a new 1.5 ml microtube and 100 µl DNA Elution Buffer was added directly to the column, then centrifuged at 10,000 xg for 30 seconds.

Zymo Spin III-HRC Filter is placed into a new Collection tube and 600 µl Prep Solution is added, then centrifuged at 8,000 xg for 3 minutes. DNA elution was transferred to the Zymo-Spin ™ III-HRC filter which had been prepared in a clean 1.5 ml microcentrifuge tube, then centrifuged at 8,000 xg for 3 minutes.

2.3 Visualization and Measurement of Concentrations, Purity of DNA Isolated

Visualization and measurement of concentration, purity of DNA isolation results are needed to determine the quality and quantity of the sample. Visualization of the results of isolation was done by electrophoresis. The first step is to make 1% agarose gel weighed with 1 gram agarose powder and add 100 ml of TAE 1 × to the Erlenmeyer tube. Then the ingredients are heated in the microwave until the ingredients are evenly mixed. Then 10 ml of the red gel was added and the gel was printed. Then 2 µl Bench Top DNA Ladder 1 kb and 2 µl loading dye were put into the first gel well. A total of 4 µl of 2 µl loading dye insulation product was put into the second gel well. The electrophoresis tool is run with an electric current of 80 volts for 55 minutes. After the running process is complete, agarose gel is taken and observed on a UV transilluminator. While the measurement of DNA concentration and purity with a spectrophotometer and absorbance are adjusted at wavelengths (λ) 260 and 280 nm.

2.4 Sequencing with Next Generation Sequencing (NGS) Method and Data Analysis

This research uses the Illumina HiSeq Next Generation Sequencing (NGS) method. DNA samples that have been tested for results by visualizing and measuring the concentration and purity of the DNA are sent to Novogene, Singapore, for sequencing. OTU clustering analysis was performed using the Uparse software.

3. RESULTS AND DISCUSSION

Samples of cyprinus and tilapia were obtained from Cirata Reservoir and catfish samples were obtained from the Ciparanje FPIK Unpad Wet Laboratory. Administratively, Cirata Reservoir covers three districts in the West Java region, namely West Bandung, Purwakarta, and Cianjur Regencies. While the Ciparanje Wet Laboratory FPIK Unpad is located in Cileles, Jatinangor District, Sumedang Regency, West Java.

Table 1. Body Length, Weight, Gut Length and Stadia

No.	Sample	Body Length (cm)	Weight (gram)	Gut length (cm)	Stage
1.	Carp	23	240,34	27	Adult
2.	Tilapia	22	230	25	Adult
3.	Catfish	45	668,33	25	Adult

The results of length measurements in Table 1 found that the length of the gut in carp is longer than its body length. Tilapia have a gut length shorter than the body. Catfish have a shorter gut length than their bodies. By their nature, carp classified as omnivorous tend to be herbivorous [3, 11, 12] tilapia are classified as omnivores [13,14], and catfish that are classified as omnivorous fish tend to be carnivorous [4,15] have shorter bowel length than their body. This is in line with the fact that the herbivorous fish of the digestive tract several times its body length can reach five times its body length, while the intestinal length of carnivorous fish is shorter than the total body length and the intestinal length of omnivorous fish is only slightly longer than the total body [16].

The type of feed eaten is influenced by several factors namely certain types of feed, size, age of fish, season and habitat for life [17]. The type of feed to be eaten by fish depends on the availability of the type of feed in the feed and also the physiological adaptation of the fish such as intestinal length, the nature and physiological conditions of digestion, the shape of teeth and pharyngeal bones, body shape and behavior. While the amount of feed needed by fish depends on eating habits, the abundance of feed, the value of feed conversion and the condition of the fish feed [18].

The type of feed contained in the gut of fish affects the presence of bacteria in it. Also the presence of bacteria in the gut of fish is influenced by other factors such as fish species, fish age, environmental conditions, climate, and other stress factors. Bacteria in the gut among them have the main function to assist the metabolic process in converting feed into components that can be digested and absorbed by the body [19].

The samples obtained were then isolated by a procedure using the Quick-DNA ™ Fecal / Soil Microbe Miniprep Kit (Zymo Research, catalog no. D6010). The next step is to check the quality of the meta-genome DNA isolated using 1% agarose gel electrophoresis. The results of DNA electrophoresis obtained in Figure 1 showed that the DNA bands were isolated from samples of carp, tilapia, and catfish but there were smears. Based on the picture also seen the thickness of the band is a variety of this is caused by different DNA concentrations. Metagenome DNA has averaged over 10,000 bp (10 kb).

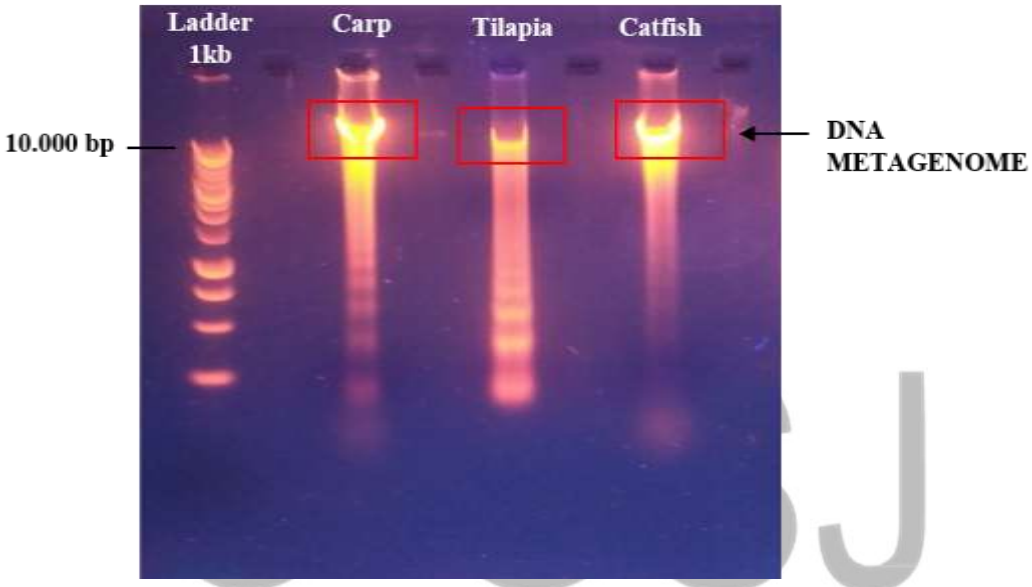

Figure 1. Agarose gel photo showing DNA metagenome bands from carp, tilapia, and catfish

The results obtained from the measurement of DNA purity and concentration through the calculation of the absorbance value of 260 nm divided by the absorbance value of 280 nm (A260 / A280) where the wavelength of UV light at 260 nm can be absorbed by DNA double fragments, while the wavelength of UV light at 280 nm can it is absorbed by protein or phenol contaminants so that by this measurement the level of purity in the genomic DNA can be known [20]. The results of the quantification of purity and concentration can be seen in Table 2.

Table 2. Purity and Concentration DNA Metagenome

No.	Sample	A260	A280	Purity (Ratio A260/A280)	Concentration (ng/µl)
1.	Carp	0,2943	0,1565	1,88	294,3
2.	Tilapia	0,0891	0,0459	1,94	89,1
3.	Catfish	0,0028	0,1049	1,92	201,8

DNA isolation results are pure if the A260 / A280 ratio is between 1.8 to 2.0 [21]. Ratio values lower than 1.8 indicate the presence of protein, salt or solvents, while ratio values above 2.0 indicate the presence of extracted RNA. Ratio values close to 2.90 indicate the presence of a small portion of RNA [22]. Based on Table 1 it was found that the ratio of purity of carp, tilapia, and catfish samples ranged from 1.88 to 1.94.

The concentration values in the three fish are different. The difference in DNA concentration obtained in each sample can be determined by the physical treatment given and the ability of the extraction buffer in breaking down cells. The process of cell destruction physically with perfect grinding can facilitate the extraction buffer in breaking down cells. Besides the extraction buffer used can affect the concentration of DNA produced [23].

The results of DNA concentration and purity measurements showed that the three samples had sufficient quality and quantity to be sequenced with 16S rRNA Next Generation Sequencing in Novogene, Singapore. The requirements for sequencing are minimum degradation, purity A260 / 280 is 1.8 - 2.0, concentrations > 50 ng / µL, and no gDNA and protein contamination [24]. These results indicate that all three samples are eligible to be continued at the next stage.

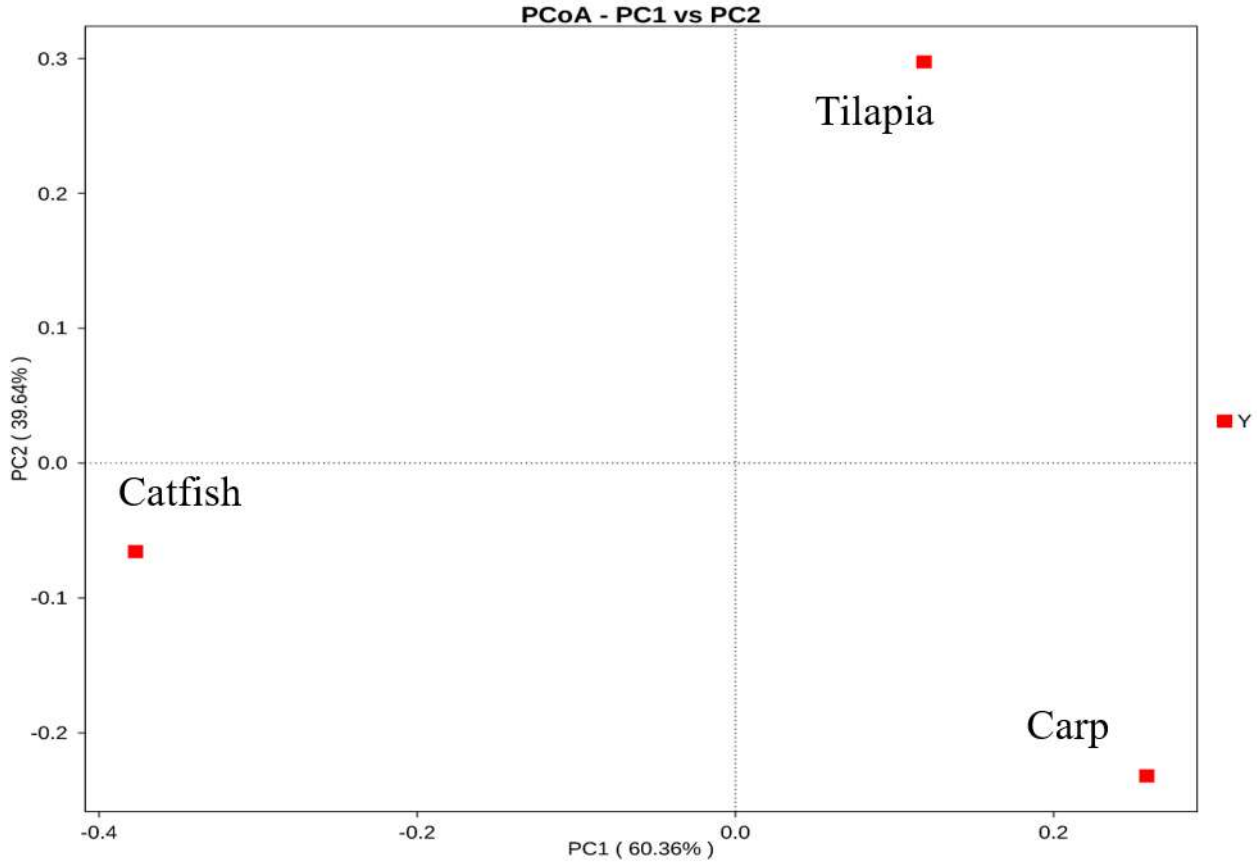

Figure 2. Principal coordinates analysis (PCoA) of bacterial community compositions in fish gut based on the unweighted UniFrac

The principal coordinates 1 (PC1) and 2 (PC2) values obtained from the graphs in Figure 2 are 60.36% and 39.64%, respectively. The grouping results found that the intestinal bacterial community of carp, tilapia, and catfish formed a different group. This is in line with the previous research that explains the PcoA scatter plot between eight fish samples from rivers that are omnivorous, herbivorous, carnivorous and filter feeders showing a clear separation of community composition [25].

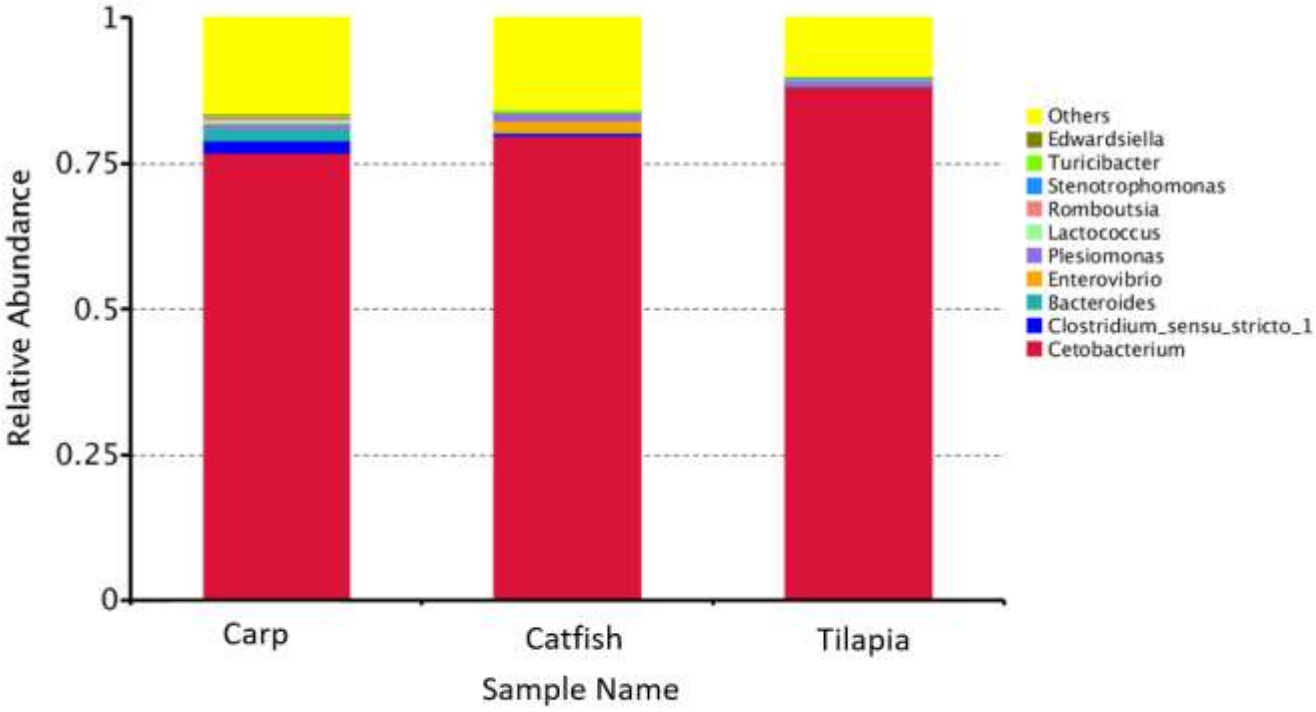

Figure 3. TOP10 Genus of Bacteria in Carp, Tilapia, and Catfish

Based on Figure 3, the highest abundance at the genus level is *Cetobacterium, Clostridium sensu stricto 1, Bacteroides, Enterovibrio, Plesiomonas, Lactococcus, Romboutsia, Stenotrophomonas, Turicibacter, Edwardsiella,* and Others. The abundance of the genus level is dominated by Cetobacterium, fish with relative abundance 0,76 in carp, 0,88 in tilapia, 0,79 in catfish. *Cetobacterium* is known to produce vitamin B12 and is found in plant feed in the gut [26,27,28].

The relative abundance *of Clostridium sensu stricto 1* was found in carp, tilapia, and catfish with relative abundace (0,02; 0,001;0,004). *Clostridium sensu stricto 1* is a genus representing *Clostridium cluster 1* in the 16S rRNA tree, this cluster is defined in phylogenetic terms, and no biochemical, molecular or phenotypic characteristics are known to be unique to the species of this cluster [29]. Clostridium sensu stricto 1 has similarities with *Clostridium thermocellum, Thermoanaerobacter pseudethanolicus, Thermoanaerobacter tengcogensi*s and *Caldicellulosiruptor saccharolyticus* [30].

Bacteria with the genus *Clostridium* are obligate gram-positive anaerobic bacteria with many pathogenic species. This bacterium has been shown to contribute to host nutrition, especially by supplying fatty acids and vitamins [31]. Other bacteria that predominate in all three fish are the genus *Bacteroides* with relative abundances 0,02 in carp, 0,001 in tilapia, and 0,0007 in catfish. This genus functions as carbohydrate fermentation which produces a collection of volatile fatty acids that are reabsorbed through the large gut and utilized by the host as an energy source, providing a significant proportion of the host's daily energy requirements [32]. *Bacteroides* have a function for fermenting carbohydrates which produce a collection of volatile fatty acids that are reabsorbed through the large gut and used as an energy source [33].

Bacteria that abundant only in carp and tilapia is *Enterovibrio* with the relative abundance 0,006 in carp and 0,009 in tilapia. The function of *Enterovibrio* is produced indole acetic acid which can be harmful to lactic acid bacteria in the gut if in excessive amounts [36,37]. Another genus that is high in all three fish is *Plesiomonas* with the relative abundances are 0,009 in carp and tilapia and 0,019 in catfish. Based on the results obtained

sequencing known that the species is P*lesiomonas shigelloides*. *Plesiomonas shigelloides* is a water bacterium and soil sediment that has the ability of proteolytic as well as including pathogenic bacteria which are detrimental to marine organisms [34]. This bacteria is also known to be pathogenic in Silver Carp [35]. Also there is *Lactococcus* the fifth-highest genus in carp and tilapia with an abundance of 0,0064 and 0,001. However, this genus is not found in catfish. This genus is known to be probiotic in tilapia [38].

The genus *Stenotrophomonas* is only identified in catfish, this genus functions as a cellulolytic species, associated with carboxymethyl cellulase (CMCase) or avicelase activity [39]. The genus *Turicibacter* serves to contain butyric acid, an important short-chain fatty acid with anti-microbial properties [40]. *Edwardsiella* is a pathogenic bacterium in aquaculture, more than 20 fish species are affected by this bacterial disease [38]. The high abundance of others at the genus level is due to the lack of databases and poor reads sequencing.

Based on previous research, it was found that *Clostridium, Citrobacter* and *Leptotrichia* are abundant bacteria in carnivorous fish, whereas in herbivorous fish the abundance in the genera Cetobacterium and Halomonas. In abundant bacteria omnivorous fish with the genus *Clostridium, Cetobacterium* and *Halomonas* [25]. Following the results obtained in carp, tilapia, and catfish where all three of these fish are omnivorous and bacterial abundance results obtained by Cetobacterium.

Conclusion

Intestinal bacterial communities of carp, tilapia, and catfish form different groups. The highest abundance of Cetobacterium, Clostridium sensu stricto 1, Bacteroides, Enterovibrio, Plesiomonas, Lactococcus, Romboutsia, Stenotrophomonas, Turicibacter, Edwardsiella, and Others.

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