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Community structure of Common carp (*Cyprinus carpio* Linnaeus, 1758) gut bacteria in the Cirata Reservoir, West Java Province, Indonesia

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ABSTRACT

Fish are aquatic animals, where they share the same ecosystem with the bacterial community they are associated with. Community structure is a concept that studies the composition of species and their abundance in a community. Information related to bacterial communities associated with their hosts is very important to support fisheries resource management efforts, both through nature conservation and aquaculture. This study of the bacterial community in the digestive tract of fish can be used to support further utilization of potential bacteria that can be used to improve the fish's immune system. This research was conducted to analyze the structure of the bacterial community found in the intestines of Common carp through a metagenomic approach using the NGS (Next-Generation Sequencing) method. The method used is a metagenomic approach, including DNA isolation from Common carp intestines. Then molecular identification was carried out using Illumina's NGS (Next-Generation Sequencing) method using the 16S rRNA gene marker. The results of the analysis using the NGS method showed as many as 19 types of bacterial phyla identified with the top three phyla, namely Fusobacteria (80%), Bacteriodetes (15%), and Firmicutes (3%). The phylum Fusobacteria is dominated by the genera *Fusobacterium, Propionigenium, Ilyobacter, Leptotrichia,* and *Sebaldella*. The phylum Bacteriodetes is dominated by the genera *Porphyromonas, Odoribacter, Bacteroides, Sphingobacterium*, and *Barnesiella*. The phylum Firmicutes is dominated by the genera *Clostridium, Lactococcus, and Sarcina, Bacillus,* and *Lactobacillus*.

***Keywords*:** Next Generation Sequencing, Bacterial community structure, Common carp gut bacteria

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1. INTRODUCTION

Cirata Reservoir is one of the reservoirs used for floating net cage fishing activities. This fishery activity is developing very rapidly, as can be seen from the increase in the number of marine cages scattered in the waters of the reservoir. This rapid increase then encourages an increase in organic and inorganic content in these waters which can further affect the life of various biota in the reservoir, including cultured fish [1].

Fish are aquatic animals, where these animals share the same ecosystem with the bacterial community they are associated with so that the bacterial community that lives in aquatic animals follows the bacterial community in their environment [2]. Community structure is the relative abundance of coexisting species which reflects assembly processes occurring at small scales, and are often available for relatively extensive areas, so could be useful for explaining species distributions [3]. Information related to bacterial communities associated with their hosts is very important to support fisheries resource management efforts, both through nature conservation and aquaculture. This study of the bacterial community in the digestive tract of fish can be used to support further utilization of potential bacteria that can be used to improve the fish's immune system [4].

Research on the bacterial community in fish intestines has been carried out by several researchers. Kashinskaya et.al (2015) [5] conducted an analysis of the intestinal bacteria of the Prussian carp (*Carassius gibelio*), Li et.al (2014) [6] conducted an analysis of the Bighead carp (*Aristichthys nobilis*), and Mulyani (2018) [4] and Fu et.al (2019) [7] conducted an analysis of the gut bacteria of Common carp (*Cyprinus carpio*).

Metagenomics is a molecular-based approach used to study the structure of bacterial communities and their genetic diversity. The sample used in this metagenomic technique is usually isolated from a complex enviromental sample containing diverse microbiota to reveal the true microbial composition of that environment, which is then analyzed by DNA sequencing method without first culturing bacteria in a culture medium [8]. Examples of commonly used sequencing methods include NGS (Next Generation Sequencing) and Sanger Sequencing.

The emergence of NGS technology has triggered rapid progress of studies in characterizing a microbial community, including microbial communities in the fish digestive tract environment [9]. The advantages of the NGS method include: the process is relatively faster, the processing cost is relatively cheaper, the number of DNA samples required for one sequencing process is relatively smaller, and the accuracy of the results provided is relatively higher.

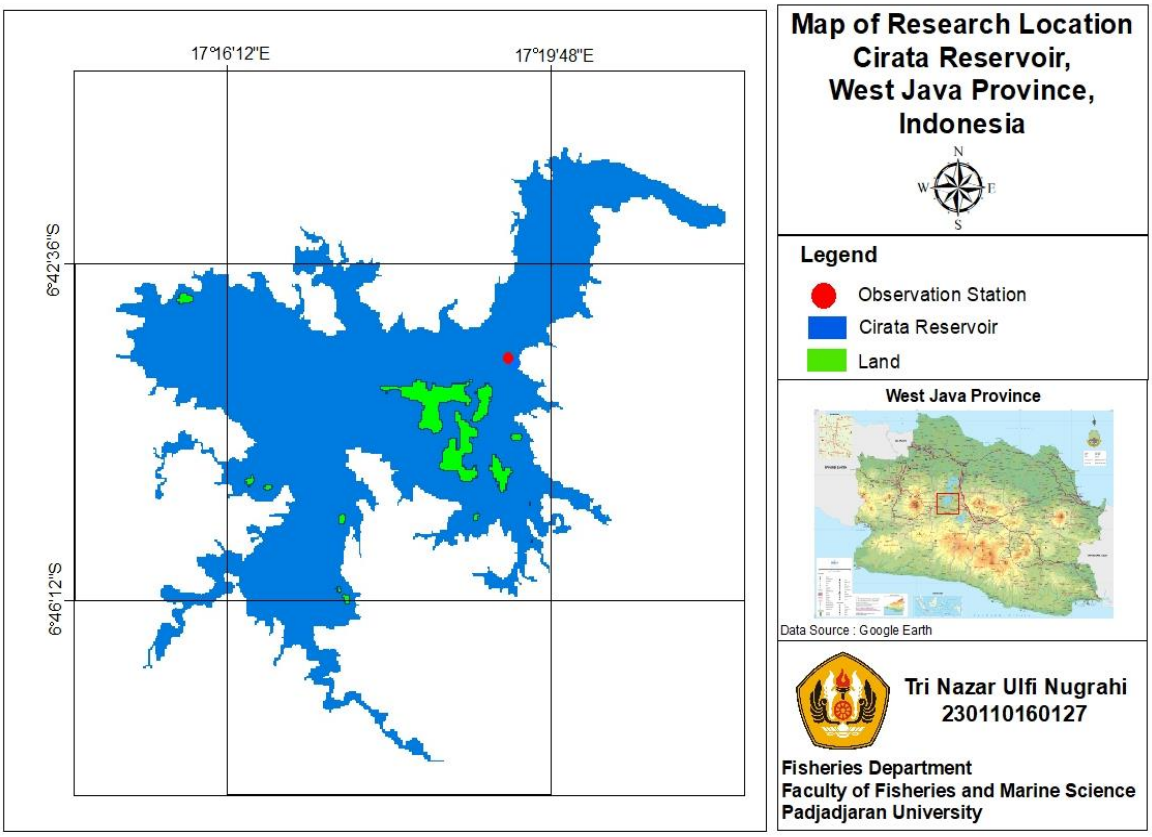
Based on the above background, therefore this research was conducted with the aim of knowing how the structure of the bacterial community that lives in the intestines of Common carp in the Cirata Reservoir is carried out. So it is hoped that this research can provide information related to the structure of the bacterial community found in the intestines of Common carp. The information obtained is also expected to be used as input for the management of the Cirata Reservoir regarding certain potential bacteria.

2. MATERIALS AND METHODS

This research was carried out from March 2019 - June 2020. Fish samples were obtained from Cirata Reservoir, Purwakarta Regency, West Java Province, Indonesia.

2. 1. Research Materials

The material used in this research is carp (*Cyprinus carpio*) obtained from Cirata Reservoir, Purwakarta Regency, West Java Province, Indonesia (Map 1).



Map 1. Map of Cirata Reservoir, West Java Province, Indonesia

2. 2. Research Methods

Data on the structure of the bacterial community in the intestines of Common carp were obtained from processing data sequences based on NGS which were analyzed descriptively.

2. 3. Bacterial Metagenome DNA Isolation

This research uses the metagenomic DNA isolation method to isolate bacteria in the intestines of Common carp. Metagenomic DNA from fish gut bacteria was isolated and extracted according to the procedure using the Quick-DNA™ Fecal/Soil Microbe Miniprep Kit (Zymo Research, catalog no. D6010).

2. 4. Sequencing with Next-Generation Sequencing (NGS) Method

This research uses Illumina's Next-Generation Sequencing (NGS) method which was carried out at one of the NGS service providers in Singapore, namely Novogene. The scope of

work is sequencing the metagenome of the V4 region in the 16s rRNA gene with primers used are primers 515 F and 806 R using the Hiseq Illumina platform.

2. 5. Data Analysis

Sequencing results data that has been obtained from a sequencing service company (Novogene) is in the form of raw data in FASTQ format, then processed using MG-RAST software. The processed products are presented in the form of tables and diagrams, and then analyzed descriptively in terms of bacterial abundance and diversity.

3. RESULT

Based on the research activities that have been carried out, the following results were obtained:

3. 1. DNA isolate

The results of testing the genomic DNA isolates of Common carp gut bacteria in KJA Gandasoli Village, Cirata Reservoir qualitatively by electrophoresis using agarose gel are shown in Figure 1.

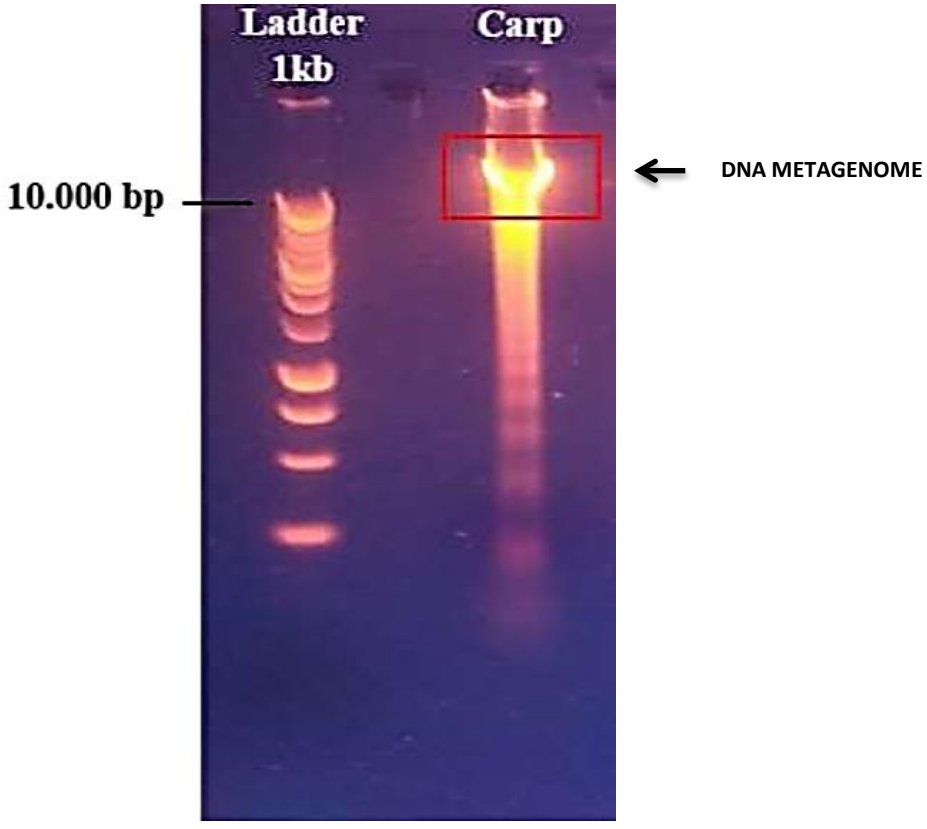


Figure 1. Results of Agarose Gel Analysis showing Metagenomic DNA bands from Common carp gut samples

Based on the electrophoresis results from the isolation of metagenomic DNA of 16s rRNA sequences from Common carp gut bacteria (Figure 1), it appears that the DNA band on the agarose gel is above the DNA marker at a length of 10000 bp. This indicates that the length of genomic DNA that has been isolated is more than 10000 bp. These results indicate that the metagenomic DNA has been well isolated so that it can proceed to the next process.

3. 2. Carp Intestinal Bacteria Community Using Next-Generation Sequencing

After completing the sequencing process at a sequencing service company, raw data is generated in FASTQ format. This data is then reprocessed using MG-RAST software and produces the final data analysis results by first going through the Quality Control (QC) stage.

Table 1. QC Sequence Statistical Data with MG-RAST

Analysis Statistics	Pre QC	Post QC
bp Count	33.589.043	909.411 bp
Sequence Count	81.123	2.151
Mean Sequence Length	414 ±11 bp	423 ±18 bp
Mean GC Percent	49 ±2 %	50 ±4 %

After uploading the raw data into MG-RAST, the results of the QC statistical data are obtained. These results indicate that the sample has an average sequence length of 414 bp before QC (Pre QC) and 423 bp after QC (Post QC) with the number of sequences and total base pairs (bp) are shown in Table 1.

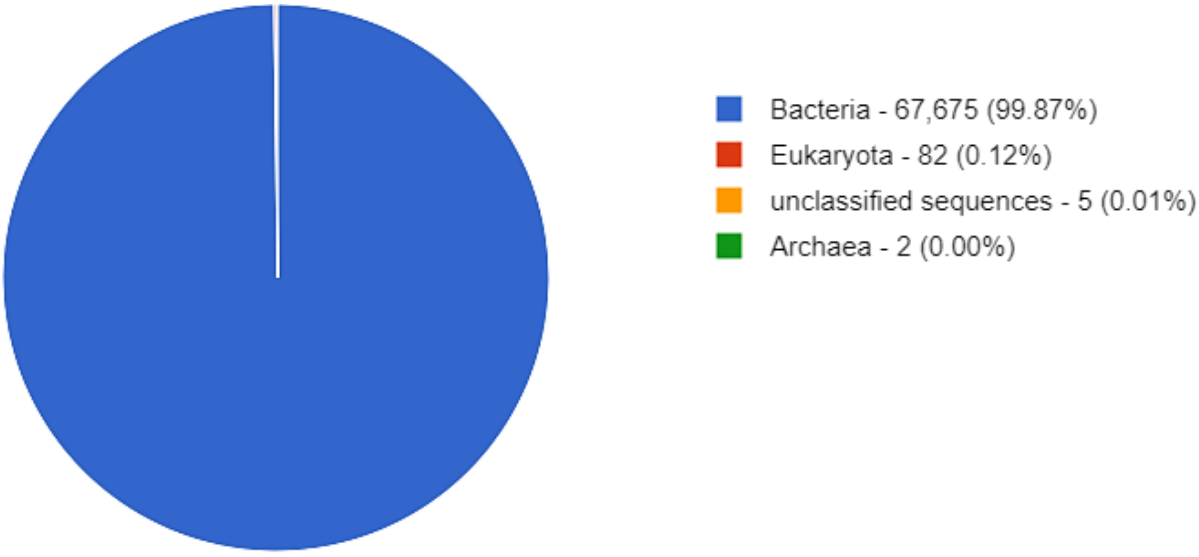


Figure 2. Domain Abundance MG-RAST Analysis Results

Sequences that have gone through the QC stage, can then be analyzed for the structure of the bacterial community using the analysis data from the MG-RAST software. The data analysis resulted in reads data with three types of domains including archaea, bacteria, and eukaryotes and there were unidentified sequences (Figure 2).

– **Identified Bacteria**

Overall, 19 phyla species were identified in the samples of Common carp intestines in the Gandasoli KJA Cirata Reservoir (Table 2).

Table 2. The Abundance of Bacterial Community in Gandasoli KJA Cirata Reservoir

Phylum	**Total *Reads***	**Persentage (%)**
Fusobacteria	117015	80
Bacteroidetes	22506	15
Firmicutes	3945	3
Unclassified (derived from Bacteria)	1744	1
Proteobacteria	586	0.4
Actinobacteria	219	0.1
Tenericutes	119	0.1
Spirochaetes	22	0.02
Verrucomicrobia	21	0.01
Acidobacteria	16	0.01
Cyanobacteria	16	0.01
Chloroflexi	10	0.007
Nitrospirae	3	0.002
Chlamydiae	2	0.001
Synergistetes	3	0.002
Deinococcus-Thermus	2	0.001
Gemmatimonadetes	2	0.001
Chlorobi	1	0.0007
Deferribacteres	1	0.0007
Planctomycetes	0	0.000
Total	146233	100

The phyla identified by the NGS method on the sample placed the phyla Fusobacteria, Bacteriodetes, Firmicutes, Proteobacteria, and Actinobacteria as the top five phyla. This is almost the same as the results of research on intestinal bacteria of Common carp reported by Liu et al. (2016) [10] where of the five types of phyla mentioned above, four of them are dominant phyla except for Actinobacteria. These results indicate that members of the phylum of several types of bacteria are particularly well adapted to conditions in the fish gut. Previous studies on gut bacteria of Common carp also stated that the phyla Proteobacteria, Firmicutes, Fusobacteria, Actinobacteria, and Bacteroidetes were the dominant phyla [4-7, 11-17.

– **Heatmaps**

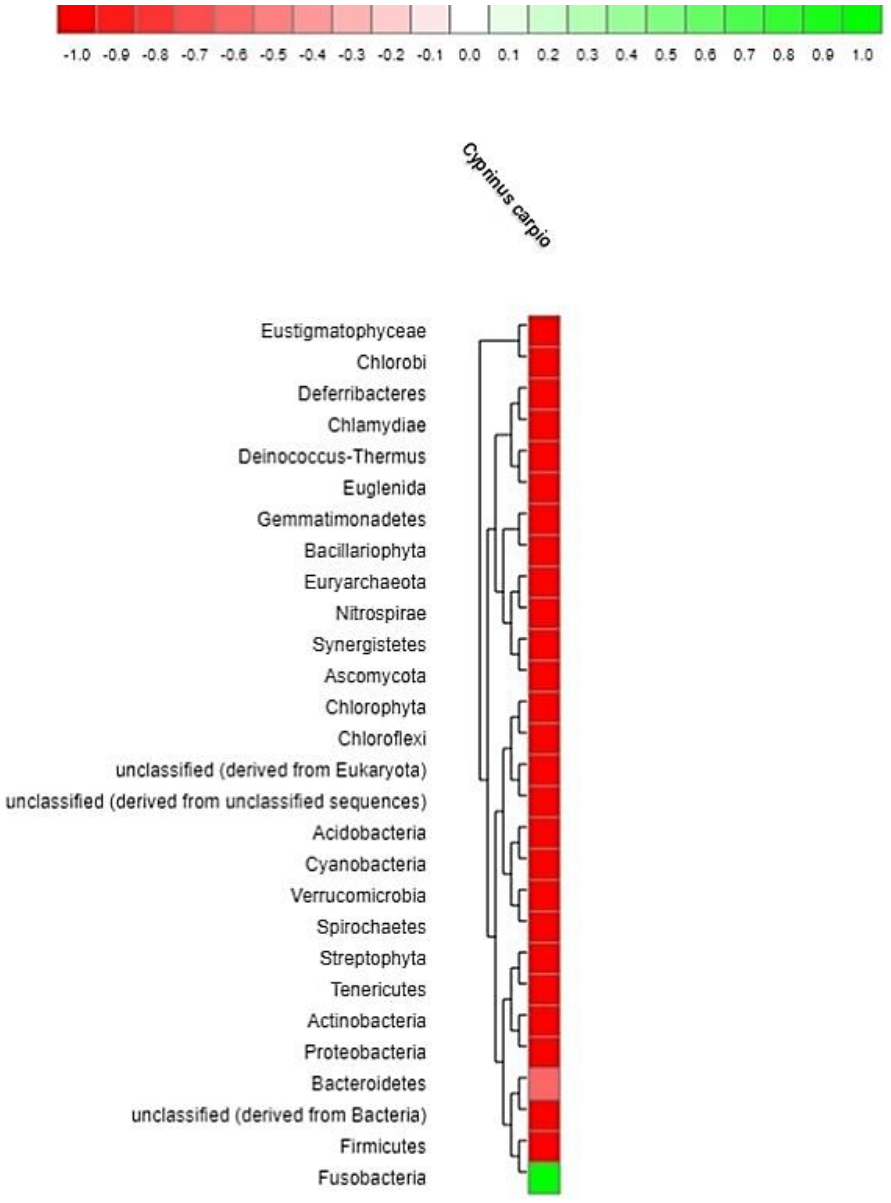


Figure 3. Heatmaps Diagram of Bacterial Abundance (Phylum Level) in Samples with the NGS method using MG-RAST

Analysis of the abundance of bacteria identified by the NGS method can also be visualized using Heatmaps diagrams. Figure 3 shows a diagram of the heatmaps of bacterial abundance in the sample. The diagram displays a color scheme that serves to describe the high and low abundance of bacteria with color indicators [18]. This indicator is seen based on the calculation of the Z score. Z score is the difference between the average abundance value and the standard deviation (SD) value [19]. The green color indicates that the SD value is smaller than the Z score (SD < Z score) which means the abundance of bacteria in the sample is high. Meanwhile, if the abundance of bacteria is low, it will be marked in red with an SD value greater than the Z score (Z score). The white color on the heatmaps diagram indicates that the SD value is close to the Z score. Based on Figure 3, it can be seen that almost all of the boxes are dominated by red color and no other green boxes can be seen besides Phylum Fusobacteria. The abundance of red color indicates the abundance of bacteria in most of the phyla in the sample which has a low abundance [20].

– **Bacteria Abundance**

The three phyla that dominate in this research include the phylum Fusobacteria, Bacteriodetes, Firmicutes. The most dominating phylum is the phylum Fusobacteria. Several studies on gut bacteria of freshwater fish [21-23] showed the same results where the phylum fusobacteria was the predominant phylum. Genus detected from Phylum Fusobacteria, class Fusobacteria, Order Fusobacteriales, and family Fusobacteriaceae include Fusobacterium, Ilyobacter, Leptotrichia, Propionigenium, Sebaldella, Streptobacillus (Table 3). Fusobacteria is anaerobic bacteria and is gram-negative bacteria that produce butyrate, a short-chain fatty acid that is the end product of carbohydrate fermentation [24]. Butyric acid produced by these bacteria can help freshwater fish in inhibiting pathogenic bacteria [25-26].

Table 3. Bacterial Abundance of the Genus Taxa

Phylum	Genus	Reads
Fusobacteria	*Fusobacterium*	111252
	Propionigenium	4162
	Ilyobacter	1379
	Leptotrichia	221
	Sebaldella	1
Bacteriodetes	*Porphyromonas*	9927
	Odoribacter	9645
	Bacteroides	2580
	Sphingobacterium	60
	Barnesiella	52

Phylum	Genus	Reads
Firmicutes	*Clostridium*	2100
	Lactococcus	507
	Sarcina	391
	Bacillus	147
	Lactobacillus	113

Note: displayed are the top five types of genera

The second dominant phylum is Bacteriodetes. This bacterium is a gram-negative rod-shaped bacterium and is widely distributed in soil, sediment, and seawater, as well as in animal skins and insides.

Bacteroidetes are known as one of the bacteria that play an important role in the digestion of food products of plant origin in the intestines of fish [6] [17] [21]. These bacteria function in the metabolism, fermentation, and degradation of oligosaccharides to digest foods of plant origin [14]. Of the 45 genera contained in the phylum Bacteroidetes, the highest abundance was seen in the genus *Porphyromonas* (Table 3).

The third phylum that dominates in both samples is Firmicutes. Bacteria from this phylum are included in the lactic acid bacteria which are a group of gram-positive bacteria, which have the ability to produce lactic acid as the end product of carbohydrate fermentation [27]. Classes detected in this phylum include the Bacilli, Clostridia, Erysipelotrichi, Negativicutes, and Unclassified classes.

Bacilli were the class with the highest abundance in the two samples. Bacilli consist of two orders, namely Bacillales and Lactobacillales. Several types of genera found in the analysis that can be used as probiotics include *Bacillus* from the order Bacillales and *Lactobacillus, Streptococcus, Weissela*, *Enterococcus*, and *Carnobacterium*, from the Order Lactobacillales (Table 2).

This is in accordance with previous research which states that the type of genus is useful, including research conducted by Hoseinifar et al (2018) [28] which stated that bacteria from the genus *Bacillus, Lactobacillus, Enterococcus,* and *Streptococcus* can be used as probiotics in fish. Then research conducted by Mourino et al (2016) [29] regarding bacteria from the genus *Weisella* as probiotics, as well as research conducted by Kim and Austin (2008) [30] regarding bacteria from the genus *Carnobacterium* used as probiotics for freshwater fish species *Oncorhynchus mykiss*.

There are several phyla to a genus that are categorized as Unclassified. This unclassified group of bacteria is caused by the lack of references in the database regarding bacterial samples in a particular environment. As a result, the taxonomic classification of these bacteria cannot be carried out [31].

The unclassified part of microbiological matter is a very valuable material for research, unfortunately, due to the lack of a unified system for their identification it is very difficult to find valuable information about their abundance or characteristics [32-38].

4. CONCLUSIONS

Overall, 19 phyla species were identified in the Common carp gut samples at the Gandasoli KJA Cirata Reservoir. The phyla identified by the NGS method in the sample placed the top three types of phyla, namely Fusobacteria (80%), Bacteriodetes (15%), and Firmicutes (3%). The phylum Fusobacteria is dominated by the genera *Fusobacterium, Propionigenium, Ilyobacter, Leptotrichia*, and *Sebaldella*. The phylum Bacteriodetes is dominated by the genera *Porphyromonas, Odoribacter, Bacteroides, Sphingobacterium*, and *Barnesiella*. The phylum Firmicutes is dominated by the genera *Clostridium, Lactococcus, and Sarcina, Bacillus*, and *Lactobacillus*.

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