Bacterial diversity in superintensive vanname shrimp aquaculture wastewater treatment plants in Barru and Takalar Regencies

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Abstract. Super intensive wastewater treatment plant (WWTP) is needed to manage wastewater from aquaculture activities before being discharged into the waters. This study aims to determine the diversity of bacterial species in the WWTP super intensive vanname shrimp culture plots located in Barru and Takalar Regencies. The research method was designed by using a survey method in the WWTP super-intensive shrimp cultivation plot in Lajange Village, Mallusetasi District, Barru Regency, and Punanga Village, Takalar Regency. Sampling of DNA from water was carried out every two weeks for one culture cycle by filtering water from the WWTP outlet. The samples obtained were then DNA-extracted using cTAB dTAB DNA extraction kit (iQ2000). Quality of the DNA obtained was measured and analyzed using a qGen machine. Next Generation Sequencing (NGS) analysis was carried out by sending the DNA samples to a commercial company (Genetics Science laboratory) for the analysis. The results showed that the highest relative diversity of samples from Barru was 60% unidentified, followed by vibrio, unidentified Chloroplast, Arcobacter, Pseudoalteromonas, Marinobacterium, Marivita, Thalassotalea, Phaeodactylibacter, and Thalassalituus. Likewise, the relative diversity of bacteria from Takalar originating from Takalar, the highest was also not identified, followed by Pseudoalteromonas, unidentified Chloroplast, Vibrio, Marivita, Thalassotalea, Phaeodactylibacter, Thalassolituus, Arcobacter, and Tenacibaculum. It appears that the types of bacteria from the two sampling locations are still quite diverse and found probiotic bacteria, Pseudoalteromonas, that are commonly used in the cultivation of vanname shrimp in both WWTP plots.

1. Introduction

Super-intensive vanname shrimp culture has been adopted by several farmers in South Sulawesi. This cultivation technology is the hope of future aquaculture systems with the concept of low volume high density [1]. The characteristics of super-intensive vanname shrimp culture are the limited area of ponds range from 1,000 – 3,000 m²; water depth of more than 1.8 m; and high stocking density. Therefore, super-intensive shrimp farming construction should have a slope in the pond bottom of 0.2% towards the outlet; wastewater treatment facilities during the cultivation process should at least 30% available...
from the volume of water used during cultivation so that the waste load can be reduced; and availability of clean water from reservoirs that have been treated before use. The healthy aquatic environment around the pond area which is protected from pond waste pollution and controlled aquaculture with good management of aquaculture waste is expected to become a productive, profitable, and sustainable system of vaname shrimp farming.

Rachman Syah et al., (2014) reported that nutrient load from super-intensive vaname shrimp ponds, is directly proportional to shrimp density [1]. The waste output from aquaculture activities are in the form of uneaten feed, feces, shrimp shells, and metabolic products from shrimp. At a density of 500 shrimp/m² of waste originating from feed which is discharged into the coastal environment reaches 50.12 gTN / kg shrimp; 15.73 gTP / kg shrimp and 126.85 gC / kg shrimp; while at a density of 600 individuals per m², the waste that was disposed was about 43.09 gTN / kg shrimp; 14.21 gTP / kg shrimp and 112.85 gC / kg. Other study shows that, intensive aquaculture technology with a density of 210,000 shrimps / ha, at an area of 5000 m², required input feed of 3.6 tons and produce unwanted output in the form of TSS waste of 1,230 kg during the 120-day cultivation process (Soewardi, 2002 ). [2].

Primavera and Apud (1994) also said that in the intensive cultivation process, about 15% of the feed provided will dissolve in water, while 85% is consumed by aquaculture commodities [3]. Of the total feed consumed, most are returned to the environment in the form of waste. About 17% of the feed given is converted to meat, 45% is wasted in the form of excretion (metabolism, excess nutrients), ecdysis (molting), maintenance (energy) and 20% of the feed given back to the environment in the form of solid waste in the form feces. Estimation of sedimentation rates by Rachmansyah et al., (2006) using sediment traps shows that the amount of sediment in vaname shrimp culture with a density of 50 head / m² increases with the number of paddlewheel used. Further study by Suwoyo et al, (2009) find that the sedimentation rate is 6.89 - 142.71 g / m² / day in shrimp ponds with a density of 50 fish / m² with sedimentation rate ranging from 676.39 - 1262 kg / plot / cycle..

Based on the description above, farmers need to make an aquaculture wastewater treatment plant before waste from shrimp aquaculture being discharged into environments. High organic matter is a source of nutrients for bacteria and can also be a trigger for the dominance of pathogenic bacteria. Advances in science have made it possible to know the bacterial community in waters on a molecular basis. Next generation sequencing is one of the technologies that can be used to improve the sustainability of shrimp farming [4]. This technology has been used to detect the bacterial community in vaname shrimp culture and its role in the environment [5]. The bacterial community in the aquaculture ponds of shrimp, crabs, and shellfish were composed more by bacteria that could cause disease in aquaculture fish than pond water that was not used for aquaculture [6]. Some farmers have constructed WWTP systems, to reduce suspended wastes discharged into the sea environment. It is expected that the existing waste water treatment plant construction can improve the microorganism community, especially the nonpathogenic bacteria in the waste. Therefore, this study aims to determine the diversity and dominance of the bacteria present in the water at the super-intensive pond WWTP outlets in Barru and Takalar Regencies.

2. Materials and Methods

2.1. Sample collection and sampling sites
This study was conducted from August to December 2018. The research was conducted in two places, namely the Experimental Pond Installation of the Brackish Aquaculture Fisheries Research Institute and Fisheries Counseling in Punaga Village, Takalar Regency, South Sulawesi and the super-intensive ponds in Mallawa Village, Mallusetasi District, Barru District. Sampling was done every 2 weeks. A sample of water for observing the density of bacteria was taken as much as 50 ml and then put in a sterile brown bottle. Water samples for molecular bacteriological analysis were taken by filtering 100 liters of water at the WWTP outlet point using a plankton net. The filtered water collected in the holding bottles is then taken as much as 40 ml and then put into a corning steering bottle. All samples are then put into a coolbox containing ice cubes and then taken to the laboratory for further analysis.
Figure 1. Side of sampling, WTTP in super-intensive vaname shrimp culture in Barru (left) and Takalar Regency (right).

2.2. **Bacterial Abundance**

Observation of total heterotrophic bacteria was done by diluting the sample up to $10^{-4}$ and then inoculated into the Tryptic Soy Agar (Difco, United Stated) plate. Whereas observation for pathogenic / vibrio bacteria was carried out by pipetting 100 µL directly onto TCBSA (Difco, United Stated) plate media. Total bacteria were counted manually after the plates were incubated at room temperature for 24 - 48 hours. The bacterial population was calculated using the formula:

$$\frac{\text{Number of colonial bacteria}}{\text{Number of plate}} \times \frac{1}{\text{dilution factor}} \times \frac{1}{\text{volume of sample cultured}}$$

(1)

2.3. **Molecular Analysis of Bacterial Communities**

2.3.1. **DNA sample extraction.** DNA extraction from filtered seawater samples using plankton net was carried out at the Fish and Environmental Health Laboratory, Research Center for Brackish Aquaculture Fisheries and Extension of Maros Fisheries, South Sulawesi. A total of 40 ml samples were centrifuged at 6000 rpm for 15 minutes. The supernatant was discarded and the pellet formed was then weighed 0.03 - 0.05 grams and if only a small amount of pellet was formed, the pellet was immediately transferred to a new microtube. DNA extraction used cTAB dTAB (IQ200) kit following the manufacturer's procedures. Extracted DNA was dissolved in 10 mL TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.5). All DNA samples (collected every 2 weeks) was pooled at the end of the study (Barru 7 samples and Takalar 6 samples). The pooled DNA samples was then electrophoresed using 1% agarose and measured the concentration and purity of the genome using genquant.

2.3.2. **Sequencing Preparation.** DNA samples (raw DNA) were sent to Genetika Science for sequencing analysis using the Next-generation sequencing (NGS) method. Samples were labeled H101SC19042125. There is also a flow of analysis conducted in Novogene on the H101SC19042125 sample is as follows:

a. PCR amplification : 16S rRNA/18SrRNA/ITS genes of distinct regions(16SV4/16SV3/16SV3-V4/16SV4-V5, 18S V4/18S V9, ITS1/ITS2, Arc V4) were amplified used specific primer(e.g. 16S V4: 515F-806R, 18S V4: 528F-706R, 18S V9: 1380F-1510R, et. al ) with the barcode. All PCR reactions were carried out with Phusion® High-Fidelity PCR Master Mix (New England Biolabs).

b. PCR products quantification and qualification/:

   - the mix same volume of 1X loading buffer (contained SYB green) with PCR products and operate electrophoresis on 2% agarose gel for detection. Samples with a bright main strip between 400-450bp were chosen for further experiments.

   - PCR products mixing and purification: The libraries generated with Ion Plus Fragment Library Kit 48 rxns for Thermofisher and quantified via Qubit and Q-PCR, would be sequenced by IonS5™XL(Thermofisher).
2.3.3. Information Analysis

a. Sequencing data processing

Single-end reads were assigned to samples based on their unique barcode and truncated by cutting off the barcode and primer sequence. Quality filtering on the raw reads was performed under specific filtering conditions to obtain the high-quality clean reads according to the Qiime quality-controlled process. The reads were compared with the reference database using UCHIME algorithm to detect chimera sequences. And then the chimera sequences were removed. Then the Effective Reads finally obtained.

b. OUT cluster and species annotation

Sequences analysis was performed by Uparse software (Uparse v7.0.1001) using all the effective reads. Sequences with ≥97% similarity were assigned to the same OTUs [7]. The representative sequence for each OTU was screened for further annotation. For each representative sequence, Mothur software was performed against the SSUrRNA database of the SILVA Database for species annotation at each taxonomic rank (Threshold:0.8~1). (kingdom, phylum, class, order, family, genus, species). To get the phylogenetic relationship of all OTUs representative sequences, the MUSCLE (Version 3.8.31) can compare multiple sequences rapidly. OTUs abundance information was normalized using a standard of sequence number corresponding to the sample with the least sequences. Subsequent analysis of alpha diversity and beta diversity were all performed basing on this output normalized data.

c. Alpha Diversity

Alpha diversity is applied in analyzing the complexity of species diversity for a sample through 6 indices, including Observed-species, Chao1, Shannon, Simpson, ACE, Good-coverage. All these indices in our samples were calculated with QIIME (Version 1.7.0) and displayed with R software (Version 2.15.3).

3. Results and Discussion

3.1. The Abundance of Bacterial Cultivable

The population of cultivable heterotrophic bacteria and total vibrio bacteria observed during the study are presented in Figure 2.

![Figure 2. The Abundance of heterotrophic bacterial (TPC), a total of vibrio (TBV), luminescent of Vibrio, and the ratio of cultivable TBV/TPC of group sample](image-url)

The abundance of heterotrophic bacteria during the study was similar at both locations, 2.106 – 9.225 x 10⁴ cfu/mL. TBV in sample from Takalar (2.4 x 10⁵ cfu/mL) was lower than the samples from Barru (4.948 x 10³ cfu/mL) and vibrio luminescent present only in Barru (3.393 x 10¹ cfu/mL). However, this results was in a higher TBV/TPC ratio (5.136%) compared to...
Takalar (1.293%), but it is generally in good condition (<5%). According to Atmomarsono & Nurbaya [8], increase of TBV/TB ratio by more than 10% may cause some Vibrio pathogens become more pathogenic for aquaculture shrimp.

3.2. DNA concentration and purity

Results of extraction of sediment and shrimp samples from the study site using DNA IQ2000 extraction kits showed that the DNA from these samples was successfully extracted (Figure 5.7). This was indicated by the discovery of DNA bands around more than 2000bp through the electrophoresis process. The single strand of DNA in the upper position (> 23130 bp) is an indicator of the success of DNA isolation with a high degree of purity. Clean DNA genome bands without background indicate a good level of DNA purity. While RNA is indicated by the background or smears along the path of movement, especially at the bottom. The difference is thought to be due to differences in molecular weight between DNA and RNA. DNA and RNA electrophoresis is a technique for separating DNA samples based on size (molecular weight) and the physical structure of the molecule. The quality of DNA or RNA is also very closely related to various contaminants. This can also be seen by running a genome extract obtained using agarose gel (Figure 2).

Figure 3. Visualization of DNA extraction of water samples from super-intensive shrimp ponds in Takalar and Barru South Sulawesi. Line 1 = 100bp Marker; 2 = IPAL outlet water genome from Barru Regency; Line 3 = IPAL outlet genome from Takalar Regency.

Based on these images it can be seen that the DNA band was formed very well and was quite firm, so it could be concluded that the process of isolating DNA from the sediment samples filtered using plankton net was successful. Brown (2016) states that gel electrophoresis will separate DNA molecules according to their size and the larger the DNA molecule, the DNA band produced will be closer to the gel well [9].

DNA purity can be obtained by measuring the absorbance of DNA samples using genquants (Genequant 1300). The results of observations on the level of purity and genome concentration / total DNA from WWTP water outlet sediment samples isolated during the study were then compiled each into one genome / total DNA sample can be seen in Table 1.

Table 1. Concentration and purity level of DNA row isolated from WWTP Outlet water.

| Sample  | DNA concentration (µg/L) | Genom purity(A260/A280) |
|---------|-------------------------|------------------------|
| Barru   | 479                     | 1,939                  |
| Takalar | 10                      | 2.5                    |

DNA purity in water samples from Barru and Pinrang was better (1,735 - 1,939) compared to samples from the Takalar WWTP (2.5). Linacero et al. (1998) state that the purity index values of DNA
and RNA genomes should be in the range of 1.8-2.0 [10]. Ratios which are less than 1.8 indicate that the DNA preparation might have been contaminated, either with protein or with phenol. The reason for the low quality of DNA purity from the Takalar WWTP outlet sample is probably due to the color of the water in the Takalar WWTP which tends to be clearer than other samples so that the deposits formed are less. This perhaps due to the WWTP plot in Takalar has applied several treatment steps; waste goes through the sedimentation stage and then goes to a plot where the waste were filtered by several types of bio filter in the plot, such as fish and green mussels. Water clarity in WWTPs can also be proven by the low concentration of DNA, which is only 10 µg / L. The level of purity of DNA and RNA is influenced by, among others, the extraction methods used, species of organisms, types of tissue extracted, and so on. The results of studies on tiger grouper using a commercial kit for extraction have a DNA purity of 1.7-1.9. Isolation of DNA using conventional methods with a fairly high level of purity of 1.996-2.550 in Oreochromis niloticus gourami tilapia.

3.3. Bacterial Community
The quality of the sample after purification and sequencing using the IonSXTMXL machine can be seen in Table 2.

| Variables     | Sample Name | Takalar | Barru  |
|---------------|-------------|---------|--------|
| Raw Read      |             | 109369  | 103057 |
| Clean reads   |             | 82577   | 80679  |
| Base(nt)      |             | 34844096| 33854425|
| AvgLen(nt)    |             | 421     | 419    |
| Q20           |             | 84.88   | 80.90  |
| GC%           |             | 51.93   | 51.93  |
| Effective %   |             | 75.50   | 78.29  |

Based on these data, the analysis of species diversity in the sample was proceed. The results of species diversity analysis based on OTUs number can be seen in the following figure 4.:

Figure 4. Statistical analysis of the reads and OTUs number (a) and Venn diagram (b) of each sample.

Figure 4 shows that the OTUs Number from the IPru Barru water sample was 643 while from the Takalar WWTP around 542 (Figure 4.a). Between the two samples, 346 species were found to be similar
and specific at the WWTP 297 WWTP site in Takalar 176 (Figure 4.b). High OTUs were also reported to be found in intensive vaname shrimp ponds, 1,312 OTUs per sample [5].

The types of bacteria found in the two WWTPs studied can be seen in the following Figure 5. The classification of bacteria in the two study sites can be seen in this taxonomy tree. The top 10 genera found are Tenacibacilum, Phaeodactylibacter, unidentified_Chloroplast, Marivita, Arcobacter, Thalassotalea, Pseudoalteromonas, Marinobacterium, Thalassolitus, and Vibrio is the most commonly found species. *Tenacibacilum maritum* is a type of bacterial pathogen in Salmon [11]. Phaeodactylibacter is a type of bacteria associated with biofuel-producing algae [12], thus collaboration with Marivita [13]. Marivita also helped arrange floc in fish farming [14]. Thalassolitus opposes the bacterial community in hydrocarbon-contaminated marine waters and is therefore considered a major player in the biological release of petroleum hydrocarbons in the marine environment [15]. Some species of Thalassotalea are bacteria that cause disease in corals [16][17]. Such is the case with Vibrio bacteria, a pathogen in shrimp [5][18].

**Figure 5.** Taxonomy tree in a group of water form WWTP outlets in Barru and Takalar Regency

The top 10 species in the different taxonomic ranks were selected to form the distribution histogram of relative abundance. The distribution in phylum was shown in figure 6.a and genus figure 6b.
Figure 6. Species relative abundance of the sample in phylum (a) and the genus (b).

The relative abundance of bacteria found in the two samples can be seen in the picture above, the left grouped according to the 10 highest phyla, while the right is based on the genus. It appears that the bacteria that dominate in both locations are from proteobacteria, and the dominant genus is Pseudoalteromonas. Pseudoalteromonas is a type of probiotic that is often used in shrimp farming in ponds.

The top 100 genera were selected and the evolutionary tree was drawn using the aligned represent sequences. The relative abundance of each genus was also displayed beside the genus in figure 7.
Figure 7. The evolutionary tree in the genus of water sample form WWTP outlets in Barru and Takalar Regency.

The evolutionary tree in the top one hundred (100) genus of relative abundance in water sample form outlets of WWTP in Barru and Takalar Regency (inside) and the outer bar chart is the highest relative abundance of the genus. The same color in corona is a type of bacteria that comes from the same ancestor. Based on the bar chart outside, the more abundant type of bacteria is Pseudoalteromonas, unidentified chloroplast, Marivita, and Thalassotalea is a type of bacteria whose abundance is higher in Takalar than Barru. Pseudoalteromonas is a type of probiotic used at the pond of shrimp in Takalar. Although one of the characteristics of these bacteria is to inhibit the population of vibrio [19], Vibrio in the Takalar WWTP plot is still found. However, the number was lower compared to the Barru WWTP plot.

Generally speaking, OTUs generated at 97% sequence identity are considered to be homolog in species. Statistical indices of alpha diversity when the clustering threshold is 97% are summarized in Table 3.

Table 3. Alpha diversity indices statistics of water sample form WWTP outlets in Barru and Takalar Regency.

| Variables         | Sample Name |
|-------------------|-------------|
|                   | Takalar     | Barru       |
| Observed species  | 522         | 643         |
| Shannon Index     | 6.042       | 6.564       |
| Simpson Index     | 0.954       | 0.965       |
| Chao1             | 555.548     | 720.027     |
| ACE               | 560.960     | 681.879     |
| Good_coverage     | 0.998       | 0.998       |
| PD_whole_tree     | 52.825      | 67.120      |

These tables showed that, although most of the bacterial communities in the WWTP are pathogenic bacteria, by the ecological index, the ecosystem of bacteria in the water in the study site is still relatively good.

4. Conclusion
We found that 643 homologous OTUs in species in water samples from the Barru WWTP outlet and 522 from Takalar, 346 of which were found in both study sites. The 10 highest genera were found in both locations, namely unidentified, followed by vibrio, unidentified Chloroplast, Arcobacter, Pseudoalteromonas, Marivita, Thalassotalea, Phaeodactylibacter, and Thallassalitus and 2 genera which neither of them possessed, namely Marinobacterium only in the WWTP Barru and the Tenacibaculum only in the WWTP Takalar. Ecologically, the bacterial community to be discharged into the waters is already in good condition, although in the Barru WWTP there is still a colony of luminescent vibrio bacteria.

Ethics statement
This study did not sacrifice any organism, nor introduce viable pathogenic bacteria to the environment. All sampling procedures were already informed and have been agreed upon by shrimp pond owners.

Author contributions
TB, AI, and AH designed the project. TB, AI, UC, and KI collected the samples, conducted the in situ measurement, and completed the statistical analysis. TB prepared the manuscript with input from all co-authors.
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