COMPARISON OF BACTERIAL DIVERSITY FROM GOAT FARM AND SEWAGE TREATMENT PLANT EFFLUENT USING METAGENOMIC ANALYSIS

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DEDICATION

This thesis is dedicated to my parents and husband who have supported me in every way for the entire study years. To my parents Santhanasamy and Anthonyachi, for encouraging me through all the times I needed motivation the most. For the encouragement to finish what I started. To my husband Yogarajan, for understanding the time I needed to spend on my studies, adapted to my schedule and overcome the adversity and preserved becoming both physically and emotionally stronger with me.

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ABSTRACT

The need for clean water is challenging when the effectiveness of monitoring the source of pollution is still in question. Water pollution due to fecal contamination has been linked with the entry of sewage from animal husbandry through non-point sources. Due to the inherent limitations of conventional fecal indicator bacteria, microbial source tracking is more appropriate to resolve fecal pollution from non-point sources. Microbial community analysis is the current interest of microbial source tracking. Thus, this project aimed to study the metagenomic microbial community of goat farm effluent to determine its unique microbe for source tracking of goat fecal pollution. Effluents from Osman Goat Farm and sewage treatment plant (STP) in UTM were collected. The STP sample functioned as a reference for comparison. Total DNA from both samples was extracted using the phenol-chloroform method. 16 S rDNA sequencing analysis was done to determine their microbial community. Sequencing results showed 316 *Operational Taxonomic Units* (OTU) represented the goat farm effluent sample, yielding 93 bacterial genera whereas 523 OTUs were in the STP and assigned to 295 bacterial genera. Twenty four genera from the STP effluent sample were associated with human microbes. *Proteobacteria* such as *Alphaproteobacteria* and *Gammaproteobacteria* were observed as dominant in the STP (44.3%) and associated with the human gut. In the goat farm effluent sample, *Bacteroidetes* (28.6%) and *Firmicutes* (49.5%) phyla were dominant. Comparing both samples at the genus level found *Saccharofermentans* uncultured rumen bacterium and *Bacteroidales RF16* uncultured bacterium were genera specific to and differentiate goat farm effluent from the human STP effluent, although both genera were common in other ruminants. In conclusion, 16S rDNA metagenomic sequencing successfully distinguished goat farm effluent from human STP effluent. Suggestions were proposed that the genera be best identified to species level for better association with the goat farm. Their persistence in withstanding artificial external environments must be confirmed before they can be used as goat farm-specific microbial community markers.

ABSTRAK

Keperluan air bersih menjadi cabaran apabila keberkesanan pemantauan sumber pencemaran masih menimbulkan persoalan. Pencemaran air akibat pencemaran tinja telah dikaitkan dengan kemasukan kumbahan daripada penternakan haiwan dari sumber bukan punca. Disebabkan kelemahan hakiki bakteria penanda tinja konvensional, mikrob penjejak sumber adalah lebih sesuai bagi menyelesaikan pencemaran tinja dari sumber tanpa punca. Analisis komuniti mikrob adalah tarikan terkini penjejakan mikrob sumber. Susulan itu, projek ini bertujuan mengkaji metagenomik komuniti mikrob efluen ladang kambing untuk menetukan ahli-ahli unik bagi menjejak pencemaran tinja kambing. Efluen dari Ladang Kambing Osman dan dari loji rawatan kumbahan (STP) di UTM telah diambil. Sampel STP berfungsi sebagai rujukan untuk perbandingan. DNA keseluruhan daripada kedua-dua sampel telah diekstrak menggunakan kaedah fenol-klorofom. Analisis penjujukan 16S rDNA telah dilakukan untuk menentukan komuniti mikrob sampel. Hasil penjujukan mendapati, sampel efluen ladang kambing diwakili 316 *Operational Taxonomic Units* (OTU) yang menghasilkan 93 genera bakteria manakala 523 OTU dijumpai di dalam STP yang disusur kepada 295 genera bakteria. Dua puluh empat genera daripada sampel efluen STP dikaitkan dengan mikrob manusia. *Proteobacteria* seperti *Alphaproteobacteria* dan *Gammaproteobacteria* didapati dominan dalam loji rawatan kumbahan (44.3%) dan dikaitkan dengan usus manusia. Dalam sampel efluen ladang kambing, filum *Bacteroidetes* (28.6%) dan *Firmicutes* (49.5%) adalah dominan. Apabila kedua-dua sampel dibandingkan pada peringkat genus, didapati bakteria rumen tidak terkultur *Saccharofermentans* dan bakteria tidak terkultur *Bacteroidales RF16* adalah genera khusus dan membezakan efluen ladang kambing daripada efluen STP manusia, walaupun kedua-dua genera ini biasa dijumpai di dalam ruminan lain. Kesimpulannya, metagenomik penjujukan 16S rDNA berjaya membezakan efluen ladang kambing daripada efluen STP manusia. Cadangan telah diusulkan adalah lebih berpatutan genera dikenal pasti ke peringkat spesies untuk penyekutuan terbaik dengan ladang kambing. Kebolehan bakteria berterusan tahan dalam persekitaran di luar usus kambing perlu dipastikan sebelum ia boleh digunakan sebagai penanda khusus komuniti mikrob ladang kambing.

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LIST OF ABBREVIATIONS

BOD - Biological Oxygen Demand
DOE - Department of Environment
EPA - Environmental Protection Agency
EQA - Environmental Quality Act 1974
FIB - Fecal Indicator Bacteria
gDNA - Genomic DNA
LDM - Library-independent method
LIM - Library-dependent method
MST - Microbial source tracking
OTU - Operational Taxonomic Unit
PCR - Polymerase Chain Reaction
STP - Sewage Treatment Plant
WHO - World Health Organization

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CHAPTER 1

INTRODUCTION

1.1 Background

The Malaysian Government has targeted 99 percent of the population will have clean water in their homes by the year 2020. For this purpose, Malaysia relies on approximately 150 rivers as natural water reservoirs. But due to climate change globally and the low ranking in the quality of water the river produces, Malaysia is facing water shortage threat (Feng, 2017).

One of the reasons for degraded of water quality is due to the uncontrollable pollution of water bodies (Povera, 2017). 53% of the river's water quality in Malaysia was categorized as slightly polluted or polluted. High levels of pollution of river waters have occurred in states with large numbers of industrial areas and factories, such as in Selangor, Johor, Penang and Perak (Lee, 2020). Following statistics released by the Department of Environment (DoE) in 2018, the main pollution loads were from 5 main sources, namely manufacturing industries, agricultural-based industries, sewage treatment plant, piggery and wet market. A total of 653 tonnes/day of Biochemical Oxygen Demand pollution load, 835 tonnes/day of Suspended Solids Load pollution load and 205.3 tonnes/day of Ammoniacal Nitrogen Load were released in 2018 (Lee, 2020), rendering dilution effect by receiving river not effective.

Fecal indicator bacteria (FIB) such as total coliforms, fecal coliforms, *E. coli*, fecal streptococci, and enterococci have been used to detect sewage contamination in water. Although use of FIB has improved public health protection, the fecal indicator coliform is common to all warm-blooded animals and not specific to host. Thus, they are unable to track source of fecal pollution especially those that enters through non-point sources (Li, 2021). In order to inform total maximum daily load (TMDL) calculations that does not exceed rivers pollution dilution capacity, every source of fecal contamination must be identified. However, unlike sewage from point sources such as sewage treatment plants which are regulated, sewage entry from non-point sources escape regulation. And since, FIBs are designed for the extra-intestinal environment and do not reveal the source(s) of contamination, it could not identify fecal pollution entry from non-point sources such as small farms and animal grazing lands.

Due to this limitation, microbial source tracing (MST) methods were developed to make it possible to identify specific sources of fecal pollution (Tatsuya, 2018). DNA sequencing have been proposed for community-based MST taking inspiration from the increasing usage of next-generation sequencing (NGS) of amplicons from different hypervariable regions of the 16S rRNA gene (i.e., V4, V5, or V6) to define bacterial communities. Therefore, tracking fecal contamination sources by identifying the overlap in community composition between the bacteria in the water and the feces of suspected animal sources. Numerous fecal bacterial species can be classified using OTUs without the necessity for culturing.

Malaysia is encouraging use of plantation lands for animal grazing in order to boost food production (Azhar, 2021). Unfortunately, this caused introduction of untreated fecal contamination from grazing animals through land run-off. Such non-point source fecal pollution remains an unsolved problem in Malaysia (Moriken, 2019). Thus, studying the microbial community using metagenomics analysis of effluent from animal farm representing grazing animals that can contaminate plantation lands and comparing with local sewage effluents would be fundamental towards identifying grazing animal-specific fecal contaminant indicator.

1.2 Statement of the Problem

Fecal pollutants that cause water quality issues can come from both point and non-point sources. Point source pollution refers to fecal pollution events caused by a single identifiable source, such as a waste water treatment plant discharge or a septic tank leak. Non-point source pollution, on the other hand, is caused by diffuse sources such as runoff from residential areas and farmlands (Li et. al, 2021). Husbandry livestock feces has been associated with being one of the sources of water pollution (Lee, 2020) with its entry from run-off or non-point source remain uncontrolled. The current challenge of preventing non-point source fecal pollution is that traditional microbiological methods using the fecal indicator bacteria (FIB) could not identify the source of fecal contamination. This is because, common FIB are found in large quantities in the feces of birds, mammals, and humans. Although this provides a reliable method of detecting fecal pollution, its lack of host specificity prevents determining the specific source of fecal pollution. Additionally, fecal waste from diffuse sources not only act as a cryptic input, but because it can be a mixture of fresh and aged fecal wastes, it is persistent in the environment, thus necessitating more sophisticated detection beyond indication of presence by FIB (Li et. al, 2021).

Due to the inability of traditional FIB to distinguish fecal sources, host-associated fecal microbes have become the subject of extensive research for source-attributable fecal pollution indicator, a method known as microbial source tracking (MST) (Holcomb, 2020). In general, potential sources of fecal bacteria are generally classified into three categories: humans, livestock, and wildlife (Andrew, 2018). Most MST methods are population- or species-based and target microbes that have an ecological or evolutionary relationship with the host animal of interest. Population-based MST methods are frequently library-dependent, requiring prior creation of a library of features or fingerprints associated with a specific source from scratch. Creating a library entails extensive phenotypic or genotypic patterns characterization of a specific population associated with different source of interest. Often, this include labor intensive and costly activities of classifying hundreds to thousands of isolates from each source of interest (Unno et. al, 2018). To use this fingerprint library, indicator bacteria fingerprints from polluted water query are compared to those in the library and finally assigned to the probable source category (Andrew et. al, 2018).

Another possible way to find source-specific fecal bacteria is to compare bacterial community in fecal contamination from different sources or hosts where the fecal originate. This is because, the intestinal environments provide selective pressures to bacteria community which differ from host to host. Subsequently, each host will produce distinct strains of fecal bacteria. Thus, comparison of bacteria community would lead to strains unique to each source. Computational methods for analyzing the large amount of data generated from metagenomic sequencing had helped MST studies and emerged as a standard community-assessment method in microbial ecology. By this, determining the overlap in community composition between bacteria in water versus feces from suspected animal sources have, at least qualitatively, track fecal contamination sources. The concept took advantage of similarities in community composition at the species or operational taxonomic unit (OTU) level for a more discrete assignment of source contamination in waters. Additionally, dual indexing allowed pooling of hundreds of samples and sequenced in a single run, making the generation of fecal taxon libraries (FTLs) from various animal sources relatively simple (Unno et. al, 2018).

1.3 Hypothesis

Fecal indicator bacteria (FIB) is a good fecal indicator but not host-specific. In this case, when sewage enters a river through run-off instead of point sources, FIB could detect the presence of fecal pollution but failed to identify the source. Known is microbial community from goat feces is different from human feces (Kho, 2018 & Ellis, 2013). It is possible that microbial community from untreated effluent from goat farm remained different from human sewage treatment plant effluent, despite both being exposed to the environment.

16S rRNA metagenome sequencing is a powerful tool expected able to identify the complete microbial community and the differences in community of fecal or sewage effluents that's are exposed to the environment. Overall assessment of microbial community, instead of specific bacteria such as FIB in a polluted river sample, gives a big picture pattern able to identify the host or source of fecal pollution. Microbial community analysis of sources of fecal pollutions such as goat farm effluent and human sewage treatment plant effluent gave the expected pattern of host-specific microbial community pattern of sewage pollution that enters the river through run-off.

1.4 Objectives of the Study

Followings are the objectives proposed for this study:

1. To identify bacterial community of goat fecal microbiome and human fecal microbiome from effluents of goat farm and effluents of local sewage using 16S rRNA metagenome sequencing.
2. To compare data from goat fecal microbiome with human fecal microbiome obtained through metagenomics analysis and identify bacterial population acting as fingerprint for each source of fecal pollution.

1.5 Scope of the Study

Current Environmental Quality Act (EQA) only regulates pollution in sewage effluent from point sources. Even so, it only applies for sewage effluent from PE > 150. Smaller human settlements producing < 150 PE is not regulated (Ariffin, 2015). Similarly, effluent from farms due to its high oxygen demand from its organic content, is characterized as non-toxic and polluting and less damaging than industrial chemical effluents, thus excluded from regulation. However, these sewage sources that escaped regulations are significant contributor to ammoniacal nitrogen load that enters the rivers (Roslan, 2019).

For ease of access, this study selected a goat pen located among the community considered as small livestock rearing and its sewage is not regulated under EQA. Study also used effluent from sewage treatment plant to represent effluent of human sewage after treatment. Both are common fecal-related wastewater released into rivers in Malaysia, as allowed by the EQA. Although a higher number of replicates per sites were desired, only two samples were collected from each source for replicates due to time constraint and budgeting limits.

Metagenomics analysis was used to study the genetic materials recovered directly from the effluents of a goat farm and effluents of a sewage treatment plant. Following that, individual bacteria present in the goat farm effluent sample were compared with the bacteria in the effluents of the sewage treatment plant. Both metagenomics results of the effluent samples were compared based on kingdom, phyla, class, order and genus because this is the limit of identification when using 16S rDNA sequencing using the V3-V4 gene region. 16S rDNA sequencing was selected since the desire is for the study to be adaptable to routine water quality activity. 16S rDNA sequencing is more affordable for this intention.

Bacteria identified only in the goat farm effluent sample when compared to the sewage treatment plant effluent were individually analyzed against prior literature on the goat gut microbiome, thus able to identify specific bacterial population that may act as a goat stool indicator.

1.6 Significance of the Study

Water contamination has been linked to livestock husbandry as one of the sources. Human exposure to these pollutants can result in serious illness like enteritis. The effectiveness of monitoring non-point source of pollution is the present problem in preventing water pollution from sewage matter. In this study, metagenomics analysis was used to identify complete genetic materials from effluent samples of a goat farm and sewage treatment plant. Associating bacterial population specific to the goat microbiome is challenging as the bacteria needs to be exclusive and only found in the goat farm effluent. The results of this study would enable the development of a fecal contaminant indicator that could distinguish between sewage from goat farm and human sewage as the sources of contamination.

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