MICROBIAL COMMUNITY DYNAMICS TO UNDERSTAND BACTERIAL INTERACTION FOR BIOREMEDIATION AND BIOENERGY PRODUCTION

バイオレメディエーションとバイオエネルギー生産のための細菌間相互作用を理解するための微生物群集動態解析

Name	**: Nurul Asyifah binti Mustapha**
Student Number	**: 13897019**
Supervisor	**: Dr. Yoshihito Shirai**
Co-supervisors	**: Dr. Toshinari Maeda, Dr. Kenji Sakai, Dr. Tamaki Kato**

Kyushu Institute of Technology

Graduate School of Life Science and Systems Engineering

Department of Biological Functions and Engineering

ABSTRACT

Waste activated sludge (WAS) is a by-product that has been abundantly produced from wastewater treatment plant after secondary biological treatment. The disposal of WAS through landfill or incineration has created more vulnerable problems in term of cost and environmental issues. Many strategies have adapted bioremediation and bioenergy production in order to completely utilize this WAS for sustainable environment and biodiversity conservation. These processes are driven by a complex microorganisms in WAS. Therefore, the successful achievement of both strategies; bioremediation and bioenergy production can be clarified based on the microbial community profile through next-generation sequencing analysis, MiSeq.

Bioremediation strategies utilize specific and powerful exogenous microorganisms to assist in the degradation of various harmful pollutants in WAS. However, this inoculant is unable to retain in WAS for a long period of time to carry out their specific functions in degradation. The interaction between this inoculant and indigenous microbes presence in WAS is suspected to be one of the main reasons. Therefore, through the microbial community analysis in this study, some indigenous bacteria have been identified as bacterial soldiers that are responsible in killing the inoculant bacteria. The enhanced green fluorescence protein (EGFP) expressing *E. coli* was used as the inoculant with the fluorescence marker and some antibiotic-resistant genes. The indigenous bacteria identified as bacterial soldiers were mainly from *Comamonadaceae*, *Myxococcales* and *Sphingobacteriales* communities as later proved by co-culture interaction with inoculant.

Bioenergy production through methane fermentation is another approach that has been taken to utilize WAS. Many studies have been done in laboratory scale to find an efficient methane production approach including application of antibiotic, azithromycin. The effect of antibiotics on microbial diversity during anaerobic digestion stages is important to understand their mechanisms and functions in methane production. Therefore, in this study, different antibiotics that produced different methane profile were used to clarify the microbial interactions and regulatory systems in each stages of anaerobic digestion process. During anaerobic digestion, hydrolysis and fermentation stages were efficiently occurred due to the activation of hydrolytic and fermentative bacterial communities by all antibiotics used in this study. However, some antibiotics have shaped the unfavorable conditions for methanogens in methanogenesis stages. This unbalanced of microbial communities has affected on methane production. Thus, a balanced of microbial communities in all stages of anaerobic digestion is important for efficient methane production as shown by some antibiotics, including azithromycin.

The importance of microbial communities was shown in bioremediation and methane fermentation processes. Another application using by-product taken from refining process of palm oil industry, phospholine gum was done for methane fermentation in WAS. It was found that phospholine gum has inhibited methane production but not affected other anaerobic digestion stages. Therefore, based on these phenomena, phospholine gum has potential to be applied as feed additive for ruminants in mitigating the enteric methane emission which contribute to greenhouse gases.

In conclusion, various reflections and dynamics have been shown by microbial diversity due to the different effects and conditions in the biological processes. This has provided a better and clear understanding on their functions and mechanisms in ensuring a successful bioremediation process and bioenergy production as the excellent approaches for sustainable environment and biodiversity conservation.

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LIST OF ABBREVIATIONS

AZM	Azithromycin
CDM	Clindamycin
CFU	Colony forming unit
CLM	Clarithromycin
CM	Chloramphenicol
CO_2	Carbon dioxide
DGGE	Denaturing gel gradient electrophoresis
EGFP	Enhanced green fluorescent protein
ERM	Erythromycin
FCM	Flow cytometer
FISH	Fluorescent in situ hybridization
GC	Gas chromatography
H_2	Hydrogen
JSM	Josamycin
KAN	Kanamycin
KTM	Kitasamycin
LCM	Lincomycin
OTU	Operational taxonomic units
PANDAseq	PAired-eND Assembler for Illumina sequences
PBS	Phosphate buffered saline
PCoA	Principle coordinates analysis
PCR	Polymerase chain reaction
PFAD	Palm fatty acid distillate
QIIME	Quantitative Insights into Microbial Ecology
RXM	Roxithromycin
SBE	Spent bleaching earth

SBS   Sequencing-by-synthesis

Stp   Standard temperature and pressure

TET   Tetracyline

TLM   Telithromycin

T-RFLP   Terminal restriction fragment length polymorphism

VBNC   Viable but non culturable

WAS   Waste activated sludge

WWTPs   Wastewater Treatment Plants

CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

1.1   Introduction

Waste activated sludge (WAS) production from municipal Wastewater Treatment Plants (WWTPs) had increased sharply in past years. Activated sludge is used during secondary biological treatment to remove organic matters by supplementation of oxygen. This process is carried out by complex microorganisms, particularly aerobic bacteria. After being recycled a few times in the process, the waste activated sludge is normally disposed of by incineration or landfill. However, this situation has contributed to environmental and handling cost issues. Many approaches have been taken to implement the WAS for bioremediation technology or bioenergy production. The successful achievement of both approaches is highly dependent on the microbial communities' function residing in WAS. Therefore, it is important to evaluate the microbial communities of WAS involve during those processes.

In this study, we provide the understanding on the dynamics of microbial or specifically bacterial community involve during bioremediation approach and bioenergy production. In the first part, the dynamics of bacterial community is evaluated by inoculation of exogenous strain to mimic the bioremediation, specifically the bioaugmentation approach. The rest of the study is focused on the dynamics of microbial community during bioenergy production, specifically methane by application of antibiotics and by-product obtained from palm oil industry. The application of

high-throughput sequencing technology has revolutionized the determination of the microbial community in WAS as compared to other conventional methods.

In the first part, the exogenous strain is inoculated in WAS in order to mimic the bioaugmentation process utilized by other studies (Mrozik & Piotrowska-Seget, 2010; Yu et al., 2010; Zhao et al., 2016). The inoculant used in this study is equipped with enhanced green fluorescent protein (EGFP) plasmid with the aim of tracking its survival in WAS. Since many studies reported on the bioaugmentation failures due to non-survival of inoculant (Lladó et al., 2013; Silva et al., 2009; van Veen et al., 1997), an approach using antibiotic is applied to find the possible bacterial soldiers in WAS responsible in killing the inoculant carrying antibiotic resistant gene. The antibiotic addition can retain the inoculant and change the bacterial community in WAS. It is noted that the evaluation of microbial communities during this process is not only important to trace the inoculant but also to find the soldiers.

In the second part of this study also utilized the antibiotics in WAS. The purpose is to evaluate the methane production through anaerobic digestion either increase or decrease, affected by the microbial community dynamics. The study was inspired by the finding from previous research that showed an improvement in the methane production by utilization of azithromycin (Nguyen et al., 2014). However, the details of microbial community were not evaluated. Therefore, in understanding the contribution of microbial community in WAS towards methane production, other antibiotics; chloramphenicol and kanamycin are exploited. Throughout the study, the function of community related with methane production or inhibition is discussed.

As the azithromycin which belongs to macrolide group of antibiotic showed an improvement in methane production, other antibiotic analogs belongs to same group were tested in the third part of this study. The antibiotics exploited included from macrolides and lincosamides which have the same mechanism of action but with different antibacterial spectra. Therefore, the effect of each spectrum of activity by different analogs is evaluated on methane production and other stages in anaerobic digestion. Moreover, the microbial community dynamics and similarity is observed from the selected antibiotic analogs based on the methane production.

Last part of this study is to evaluate the potential of by-product obtained from palm oil industry, namely phospholine gum, as a feed additive for livestock. Livestock, especially cow release a lot of methane to the environment through farting and burping that accounted for about 40% of total greenhouse gas emission from agriculture sector (U.S. EPA. 2014). The WAS is used to mimic the rumen system of cow. Thus, the phospholine gum is added in the WAS and the methane production is evaluated through anaerobic digestion process. The potential of phospholine gum in mitigating the release of methane from WAS is evaluated through the dynamics of microbial community.

Overall, the importance of microbial community in carrying their specific function is essential to understand the successful process involved, in this study the bioremediation and bioenergy production. The information obtained is useful for other studies aiming to improve the bioremediation or bioaugmentation approach and bioenergy production. The factors rising from function of microbial community can be carefully consider to make sure the smooth implementation of WAS.

1.2   Waste activated sludge

WWTPs are the main facility to treat wastewater collected from many sources. Generally, the wastewater coming from domestics, industries, and also agricultural sites are collected in WWTPs for treatment before release back to the environment. Wastewater contains a complex mixture of natural organic and inorganic substances as well as man-made compounds that can cause adverse effects to the human health and environment. Organic substances in wastewater are present as carbohydrates, fats, proteins, amino acids, and volatile acids (Huang et al., 2010) while inorganic constituents include large concentrations of sodium, calcium, potassium, magnesium, chlorine, sulphur, phosphate, bicarbonate, ammonium salts and heavy metals (Abdel-Raouf et al., 2012). Xenobiotics or unnatural synthetic compounds are also commonly found in wastewater (Katsoyiannis & Samara, 2005).

The activated sludge process is by far the most widely used biological treatment for pollutant removal from sewage water. Activated sludge is a heterogeneous mixture of particles, micro-organisms, colloids, organic polymers and cations whose composition depends on the origin of the wastewater (Ye et al., 2016). The main composition in charge for pollutants removal in activated sludge is the bacterial biomass suspension (Gernaey et al., 2004). The clean water is separated from activated sludge in the secondary treatment. The activated sludge is returned to aeration tank for few cycles and finally discarded as waste activated sludge (WAS). In past years, production of WAS from WWTPs had severely increased by bringing many harmful risks to the environment.

1.2.1   Microbial communities in WAS

Various biodegradation processes occur during the treatment involving a complex microbial community in activated sludge.   Basically, WAS consists of a mixed community of microorganism, approximately 95% bacteria and 5% higher organisms such as protozoa, rotifiers and higher forms of invertebrates. Aerobic bacteria are the most predominant population with substantial population of fungi and protozoa. Besides, filamentous bacteria can cause the sludge not to settle properly as low level of filaments is required for solid settling in a healthy activated sludge. This condition is called as bulking. Filamentous sludge bulking is a common problem at small, extended aeration treatment plants which commonly cause a formation of brown viscous sticky foam (Pal et al., 2014).

The microbial community of WAS can be divided into two major groups; decomposer and consumers. Decomposer mainly represented by bacteria, fungi and colorless cyanophyta responsible for biodegradation of pollutant in wastewater. Consumers consist of phagotrophic protozoa and metazoa will utilize bacterial and other microbial cells as substrates. As these consumers presence only 5% in WAS, its role is only marginal (Cloete & Muyima, 1997). The microbial community structure of WAS is not constant but reflects all the effects to which the activated sludge system is exposed during the treatment such as wastewater composition and operational conditions (Wilén et al., 2008).

1.2.2   Functional microbial community

Varieties of treatments are applied using activated sludge carry out by complex microbial population. Each of them carries specific or sometimes redundant functions with other populations. Therefore, these WAS communities can be categorized based on their functions. The degradation of complex organic substances in wastewater is done by oxic organotrophic microorganism such as *Bacillus, Flavobacterium, Moraxella* and *Pseudomonas* by secreting exo- or endoenzymes. Some bacteria that degrade the specific substrates can be accumulated in the sludge after adaptation period to the wastewater (Cloete & Muyima, 1997). The fermentation of organic compounds to volatile fatty acid is carried out by fermentative bacteria such as *Bacteroidetes* and *Spirochaetes* classes (Hernon et al., 2006).

The nitrification and denitrification parallel processes are widely used to treat nitrogenous compounds in wastewater. In nitrification, the ammonium is oxidized to nitrite and then to nitrate by ammonia-oxidizing bacteria such as *Nitrosomonas* and *Nitrosospira* and nitrite-oxidizing bacteria such as *Nitrobacter* and *Nitrococcus*, respectively (Cloete & Muyima, 1997; Shchegolkova et al., 2016). The denitrifier is responsible to reduce the nitrate to nitrous oxide or dinitrogen gas. Common bacteria involve in denitrification is *Proteobacteria* classes and some Gram positive bacteria (Geets et al., 2007).

In addition, the removal of phosphate is carried out by some species from *Acinetobacter*, *Moraxella*, and *Rhodocyclus* (Bond et al., 1995; Sidat et al., 1999). Sulphate is also one of the pollutant presences in wastewater primarily coming from industrial sites. The microbial

communities responsible in removing this pollutant from wastewater is known as sulfate-reducing bacteria include some species of *Thiothrix* and *Beggiatoa* (Williams & Unz, 1985), and recently identified from *Paludibacter*, *Desulfovibrio* and *Desulfuromonas* (Liang et al., 2013). The identification of each community is important in order to understand their function and contribution to many processes.

1.3 Microbial community analysis techniques

It is well known the presence of complex microbial community structure in WAS as it is previously used and functionally contribute in biological treatment process. The increase in knowledge on microbial community involve is important to improve the treatment process and also the quality of end products. There are many techniques available for determining the microbial communities, either culture based or molecular based techniques. Both methods have been used extensively in order to collect the information on microbial communities of WAS as much as possible for a better understanding in their processes.

1.3.1 Culture-based technique

Basically, this is the traditional method to characterize microbial ecology from the environment using commercial growth media such as Luria–Bertani medium, Nutrient Agar, and Tryptic Soy Agar. It is commonly used for cultivation of pure or known strain in the laboratory. Only the culturable microorganisms dependent on the medium and nutrients supplied are able to grow and identify from this technique. Unfortunately, the major limitation is only 0.1 - 1% of total

environmental microorganisms are culturable, while another 99% are unculturable using standard culturing techniques (Hugenholtz, 2002; Ranjard et al., 2000). However, this method is still applicable for some purposes nowadays as it requires less total cost, time and preparation for the whole process.

Some improvements are done to maximize the culturable fraction of microbial community by mimic their natural environment using culture media such as nutrients, pH, oxygen gradient, etc (Rastogi & Sani, 2011). Even so, many of them remain unculturable. These populations are viable under natural environments but cannot grow under laboratory conditions which is categorized as viable but non culturable (VBNC) (Oliver, 2005). In order to have a clear understanding on the contribution of microbial community in WAS including those categorized as VBNC towards related processes, more powerful techniques is required.

1.3.2   Molecular based technique

Currently, the composition of microbial community may be easily evaluated using molecular techniques. In fact, different methodologies have been already developed in order to determine the microbial community structure of various biological systems such as clone library, next generation sequencing, fingerprinting techniques, quantitative real-time PCR (qRT-PCR), etc. However, an investigative study to understand microbial interactions inside a complicate microbial consortium such as WAS by using these methodologies; in particular, by using next generation sequencing technology, is still very scarce (Ng et al., 2015).

To date, many studies have been carried out using less advance methods such as PCR-DGGE (Akyol et al., 2016; Aydin et al., 2015; Lins et al., 2015), T-RFLP (Meng et al., 2015) or FISH (Shimada et al., 2011). Application of DGGE has great value for evaluating environmental microbiology but does have limitations. For example, bands at the same position in DGGE gel is equal to one genome is invalid assumption. This is because bands that has similarity in genetic not necessarily has similar migration in DGGE gel (Jackson et al., 2000). In addition, these conventional methods are unable to detect most of the low-abundance microbes that presence in WAS comparing to next generation sequencing technology (Wang et al., 2012).

Since culture based method is unable to identify the vast majority of microbial communities, molecular based technique is the perfect approach for this purpose. The primary source of information from these communities is their biomolecules such as nucleic acids, lipids and proteins. Whole genomes or selected genes such as 16S and 18S rRNA for prokaryotes (Bacteria and Archaea) and eukaryotes, respectively is achievable by nucleic acid approach (Hugenholtz, 2002). A wide variety of techniques applied this approach which has been progressively developed. In addition, the techniques can be divided into partial community analysis and whole community analysis which applied high-throughput sequencing. Some examples of partial community analysis includes clone-library, genetic fingerprinting methods, real-time PCR, FISH, DNA microarrays, etc. moreover, whole community analysis includes metagenomics, whole genome sequencing, metaproteomics, and metatranscriptomics (Rastogi & Sani, 2011).

Recently, the application of high-throughput sequencing for whole community analysis has been well developed. This technology outperform the Sanger capillary sequencing by a factor of 100-

1000 in daily throughput (Kircher & Kelso, 2010). Some fields use "next-generation sequencing" term to reflect the vast changes made by this kind of technologies. The targets from these advances sequencing is to generate large amount of sequence data rapidly and at a significantly lower cost.

There are many different types of high-throughput sequencer available for microbial community analysis. The first of new high-throughput sequencing was 454 sequencing platform that utilize the pyrosequencing approach. In contrast to Sanger technology, pyrosequencing no longer require electrophoresis as the read out is simultaneously done by signal emitted from the nucleotide in parallel with the sequence extension (Ronaghi et al., 1996). This kind of process is also called as sequencing-by-synthesis (SBS). Another technology that employs same SBS concept is Illumina sequencing platform that utilize the reversible terminator technology (Bentley et al., 2008).

In addition, Life Technologies/Applied Biosystems (ABI) was later sold SOLiD sequencing platform which is the third new high-throughput technology entering the highly competitive sequencing market. This technology applied different concept in which it use sequencing-by-ligation concept combining with a new approach of sequencing library construction and sequence immobilization using rolling circle amplification (Drmanac et al., 2010). Besides, Helicos sequencer known as HeliScope is the first sequencer able to sequence single molecule instead of molecule ensembles obtained from amplification process. The advantages are the sequencing process is not affected by biases or errors during library preparation and also it can be utilized for low input DNA.

However, very limited application of this technology use due to very specific limitations of this platform, the price, and many sequencing users already installed another common high-throughput sequencing technologies (Kircher & Kelso, 2010). Therefore, the important consideration is to select a suitable sequencing platform for specific types of experiments and detail understanding of the available technologies such as source of error, error rate and also total cost of sequencing is required.

1.3.3 Illumina Sequencing platform

Illumina use similar concept to conventional Sanger sequencing by employing the sequencing-by-synthesis but with modified dNTPs containing a terminator. In this concept, the incorporation reaction is blocked by terminator, so only a single base can be added by a polymerase enzyme to each growing DNA copy strand. The base is labeled with fluorescent dyes which can be detected by imaging camera. The sequencing reaction is continued by with incorporation of next base. Since single bases are added to all templates uniformly, the sequencing process produces a set of DNA sequence reads in uniform length. The MiSeq platform is one of the Illumina technologies has provides a way to succeed high-through-put sequencing, which permits greatest coverage of microbial ecology and provide most accurate DNA data for analysis of the microbial community in various environmental samples (Ma et al., 2016).
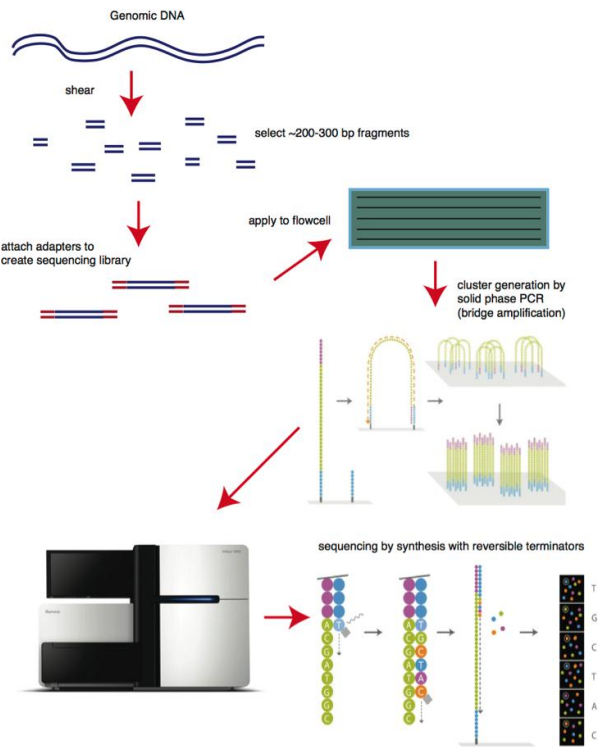
Figure 1.1: Sequencing-by-synthesis through Illumina technology

Briefly, the sequencing process using MiSeq Illumina from sample preparation until sequence reads is shown in Figure 1.1. The DNA sample is prepared into a sequencing library by the fragmentation into 200-300 bases long. The index adapters are added to each end and the library. Next, the sample library is flowed across a flow cell and the template fragments bind to its surface. Consequently, a bridge amplification PCR process (cluster generation) occurs on the flow cell creates approximately one million copies of each template. The fluorescence emitted from the based is captured and identified. The sequencing cycles are repeated to determine the sequence of bases in fragment. Finally, the data from bases are aligned and compared to a reference (Kircher & Kelso, 2010).

1.4　Application of WAS

Although the activated sludge system is widely used for biological wastewater treatment technology, this process finally generates a large amount of WAS as a by-product. The accumulation of WAS from WWTPs has contributed to another associated problems such as environmental issue and cost of handling. This is because the current disposal practice is through the incineration and landfill application. This accounts for about 50-60% of the total operational costs of the overall WWTP operation (Guo et al., 2015). Besides, the environmental regulation on sludge disposal has become more stringent (Lee et al., 2009). Therefore, another approaches has been taken such as bioremediation and bioenergy production in order to remove any remaining pollutant in WAS or efficiently utilize the WAS, respectively.

1.4.1　Bioremediation approach

The application of microorganism or microbial processes to degrade contamination occur in environment is among technologies applied under bioremediation. This technology can be divided into in situ and ex situ treatment technologies. In situ treatment occur in contaminated place whereas ex situ involve the physical removal of contaminated material from the original site (Boopathy, 2000). Importantly, bioremediation depend on the metabolic capabilities of microbial world for transformation of the pollutant to harmless or at least less hazardous compounds.

The original microbial community in contaminated sites might not be able to perform the transformation of pollutant as they do not have the appropriate metabolic potential for degradation. In this situation, the specific competent strain or consortia of microorganism is required to perform the treatment. This is the example of bioaugmentation; one of the bioremediation techniques (El Fantroussi & Agathos, 2005).

1.4.1.1 Bioaugmentation in WAS

Bioaugmentation is an example of in situ bioremediation which apply an introduction of highly active exogenous microorganism into different natural or engineered environments. There are many ways of introducing these exogenous microbes such as using pre-adapted pure bacterial strain or consortia, genetically-engineered bacteria and also biodegradation-related genes (Stenuit et al., 2008). The inoculation of specific microbes in WAS can improve the treatment of WAS. The recalcitrant pollutants in WAS such 3-chloroaniline (Boon et al., 2000) and aromatic hydrocarbons (Dueholm et al., 2015) were degraded via bioaugmentation approach.

Besides, the application of *Exiguobacterium* YS1 as inoculant in WAS has improve the solubility of WAS in anaerobic condition (Lee et al., 2009). Another study used *Brevibacillus sp.* KH3 by inoculation at thermophilic temperature in order to improve the sludge reduction process (Maeda et al., 2011). The inoculation of exogenous microorganism for bioaugmentation either successful or not, will always influence by abiotic or biotic factors. The biotic factors was reported to be more significant (El Fantroussi & Agathos, 2005).

1.4.1.2 Bioaugmentation failures

Some studies that utilize bioaugmentation process explained the factors contributed to this technology failure. The major factor is due to the inability of inoculant to show under natural environment throughout the process, as compared under laboratory scale. For example, a study done by (Xiong et al., 2013) using *Rhodococcus sp*. D310-1 to degrade chlorimuron ethyl showed that the inoculant survival rate was reduced during the treatment. Besides, other factors include the changes in microbial communities has contribute to competition and inhibition (van Veen et al., 1997). The presence on inhibitory compounds towards inoculant in the treatment site or by secretion from other microorganisms such as antibiotics or bacteriocin and presence of bacteriophages or protozoa, and poor biofilm forming ability (Fu et al., 2009) may contribute to unsuccessful bioaugmentation.

In addition, the presence of suitable catabolic gene to degrade the target compound in indigenous community but still utilize the bioaugmentation has led to inconclusive findings and application of same genera with unequally fit for specific target pollutant also caused the bioaugmentation failures (Thompson et al., 2005). Some of these factors shows that in the microbial community of WAS, the bacterial soldiers are presence that closely interact by killing the inoculant. In this study, the bacterial soldiers will be clarified based on the bacterial community analysis.

1.4.2 Bioenergy production

The WAS can be efficiently utilized through bioenergy production. Two main benefits can be obtained from this approach; one is the effective utilization of WAS and the other one is achievement of bioenergy production using zero cost substrate. Moreover, in the next coming years, bioenergy will be one of the most important renewable energy sources, like solar and wind power. Since WAS is made up of complex composition, it is suitable to be use either as substrate or as microbial source for bioenergy production including methane and hydrogen. Many studies have been widely done by different approaches for improving the bioenergy production (Liu et al., 2013; Nguyen et al., 2014; Zhou et al., 2016). It is important to understand the complex composition, especially microbial composition that driven the process for bioenergy production, particularly methane.

Methane gas obtained from biological waste through anaerobic digestion is environmentally and economically ideal to be used. Other benefits are it specifically improve the reduction of pollution by biological waste as the methane production is utilize from these renewable resources, no addition of greenhouse gases production to the atmosphere and its production is independent on oil or natural gas (Chandra et al., 2012). Methane gas achieve through methane fermentation is a versatile technology capable in converting practically all types of polymeric compounds in WAS to methane and carbon dioxide under anaerobic condition.

Methane gas is safe and non-poisonous to be used for internal combustion of engines and power generation, and also as electricity sources for power plant (Tabatabaei et al., 2010). Besides, some researchers found a simple way to convert methane into methyl chloride, which can easily be converted into petrochemicals such as ethylene or propylene, used to make plastics (Podkolzin et al., 2007). As WAS contain complex microbial community, including bacteria and archaea, it is a good source of inoculum for methane production especially as enriched methanogens (Mohd Yasin et al., 2015). Therefore, methane production from WAS or other biological waste offers an effective means of pollution reduction, especially via anaerobic digestion process.

1.4.2.1 Microbial function in anaerobic digestion

The biochemistry and microbiology of various microorganisms are involved during anaerobic breakdown of polymeric substances in WAS to methane production. Generally, four stages involve in anaerobic digestion known as hydrolysis, acidogenesis, acetogenesis and methanogenesis stages. The individual phases are carried out by different groups of micro-organisms. Figure 1.2 shows the flow of anaerobic digestion process and the responsible bacteria in each stage.
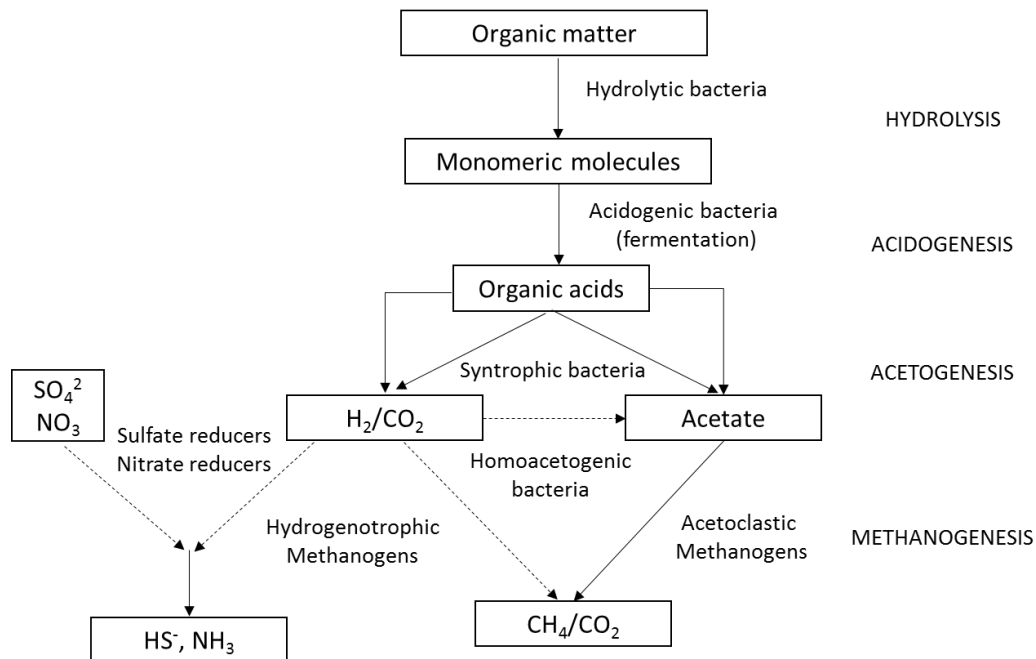
Figure 1.2: Anaerobic digestion stages of methane production including hydrolysis, acidogenesis, acetogenesis and methanogeensis stages. Source: Valdez-Vazquez and Poggi-Varaldo (2009).

In hydrolysis stage, the organic matters like protein, carbohydrates and lipids are breakdown into smaller monomers by their respective hydrolytic enzymes such as protease, amylase and lipase. This process is carried out by hydrolytic bacteria to produce smaller molecules like simple sugars, amino acids and fatty acids (Manyi-Loh et al., 2013). In WAS, the high rate of hydrolysis process commonly related with the presence of *Clostridia* (Guo et al., 2015). The monomeric molecules are later consumed by microbes. Acidogenic bacteria play important roles in second stage. The products from hydrolysis are consumed and degraded into short-chain organic acids such as butyric, propionic, and acetic acids. Besides, those monomers also successively metabolized by syntrophic fermentative bacteria and primarily fermented to acetate, propionate, butyrate, lactate, ethanol, carbon dioxide and hydrogen. Subclasses of *Bacteroidetes* as well as *Clostridia* are prominent as a fermentative bacteria in WAS (Traversi et al., 2012).

Furthermore, in the acetogenic phase, homoacetogenic bacteria or also known as autotrophic acetogens constantly reduce exergonic hydrogen and carbon dioxide to acetic acid. Syntrophic bacteria also generate acetic acid with hydrogen from other short-chain organic acids. Acetogenic bacteria grow in a symbiotic relationship with methane-forming bacteria. During this phase, organic acids and alcohols are also converted into acetate (Chandra et al., 2012). Later, acetate serves as a substrate for methane-forming bacteria. Finally, hydrogenotrophic methanogens consumed hydrogen and organic acids for the production of methane and carbon dioxide. As reported by Guo et al. (2015), the presence of *Methanomicrobia* is associated with the abundant methanogens in WAS. In some anaerobic digestion process, presence of sulfate and nitrate reducer will generate sulfides and ammonia by utilizing hydrogen as electron donor to (Valdez-Vazquez & Poggi-Varaldo, 2009).

Anaerobic digestion is another powerful approach using a biological reaction for the WAS treatment that has been widely applied to achieve an efficient solution to the environmental pollution and energy draining. Methane is considered as a most promising bioenergy generated because organic matters from WAS can be converted into 60-70 vol% of methane in order to meet both requirements of efficient solutions by anaerobic digestion approach (Appels et al., 2008; Nguyen et al., 2014). Four stages of anaerobic digestion involve in biological methane production; hydrolysis, acidogenesis, acetogenesis, and methanogenesis (Bareither et al., 2013; Guo et al., 2015; Mohd Yasin et al., 2015; Nguyen et al., 2014).

All stages in this process were driven by various types of microorganisms. WAS is a substrate in the process of anaerobic digestion for methane production and in the first hydrolysis stage, large

molecules of WAS including proteins or carbohydrates are degraded and converted into smaller molecules such as amino acids or monosaccharides through the stage. Fermentation of these molecules into carboxylic acids (e.g., butyrate or propionate), hydrogen, and carbon dioxide at the acidogenesis stage is combined with the acetogenesis step driven by acetogenic bacteria to produce acetate (Sanz et al 1996). Finally, methane producers, Archaea or methanogens utilize acetate for the methane production at the methanogenesis stage.

1.5   Antibiotics approach

1.5.1   Mechanism of action

Recently, many antibiotics have been developed targeting various type of bacteria. The antibiotics targeted on the bacteria through some mechanism of action. This mechanism can be classified in few groups such as inhibition on protein, nucleic acids and cell wall synthesis, disruption of cell membrane function and act as antimetabolites as shown in Figure 1.3. In this study, only antibiotics targeting on the protein synthesis is used such as chloramphenicol, tetracycline, macrolides and lincosamides.

Application of antibiotics eliminates pathogenic bacteria and sometimes affected on other normal microorganisms. This situation resulted in proliferation and domination of bacteria-carrying genotypes conferring resistance which is called as selective pressure (Colgan & Powers, 2001). Bacteria can receive the antibiotic resistance gene via mobile elements such as plasmid, transposon and integrons (Prescott et al., 2000). Later, the antibiotic resistance gene may be

transferred to other bacteria through conjugation, transformation and transduction (Richmond, 1972). The mechanism of resistance among bacteria include enzymatic modification or inactivation, cell wall or membrane impermeability, efflux pump for expulsion and alteration of target receptors (Levy, 1992).
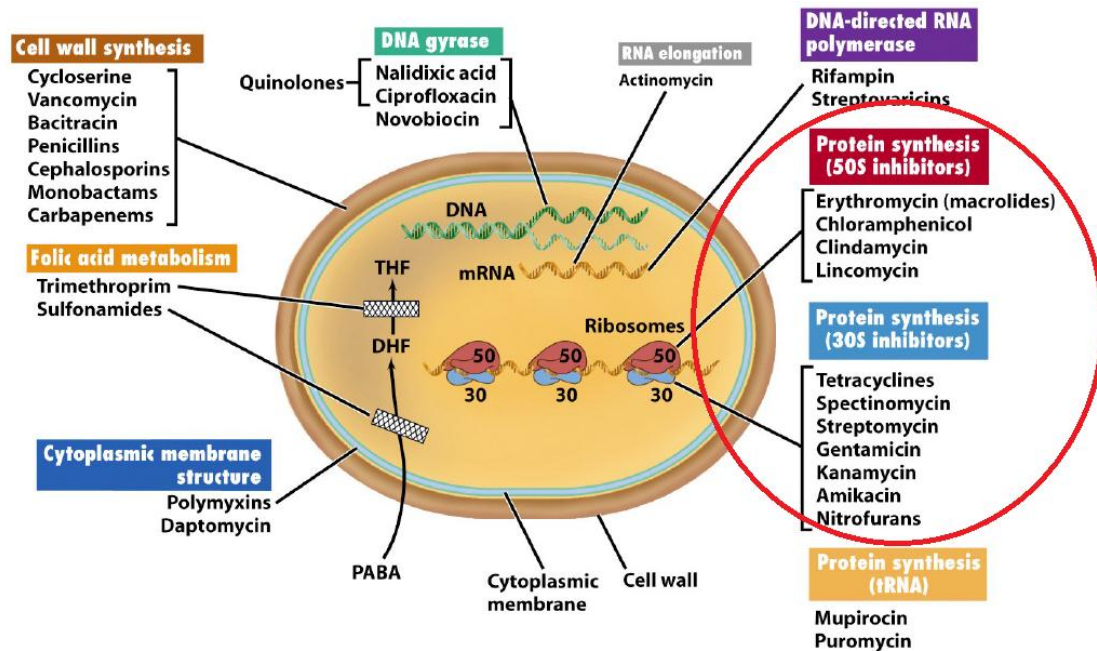
Figure 1.3: Antibiotics mechanism of action. Source: (Madigan et al., 2006)

1.5.2   Antibacterial spectrum

The different effect of antibiotics causing different bacterial interaction is due to the antibacterial spectrum of activity. Antibacterial spectrum for azithromycin is primarily Gram positive bacteria including streptococci and staphylococci, *Enterobacteriaceae* and other rapidly growing pyogenic bacteria (Papich, 2010; Williams, 1991). Besides, chloramphenicol is well-known as

broad range antibacterial spectra including Gram positive, Gram negative, aerobic and anaerobic bacteria (Schwarz et al., 2004). Tetracycline is a broad-spectrum agents, exhibiting activity against a wide range of gram-positive and gram-negative bacteria, atypical organisms such as *chlamydiae*, mycoplasmas, and *rickettsiae*, and protozoan parasites (Chopra & Roberts, 2001). In addition, kanamycin antibacterial spectra activity is Gram negative bacteria and some Gram positive bacteria but has weak activity toward anaerobic bacteria (Papich, 2010). Macrolides and lincosamides basically have the antibacterial activity on Gram positive bacteria (Roberts, 2004). However, some of their analogs may extend on Gram negative bacteria.

Macrolides with a common structure of a large lactone ring were sub-grouped into a few analogs, namely the 14-, 15-, and 16-membered ring and ketolide groups. Erythromycin, the first introduced macrolide (Williams & Sefton, 1993), is a 14-membered ring analog. Other analogs derived from erythromycin are clarithromycin, roxithromycin, and dirithromycin, which are examples of 14-membered ring analogs and azithromycin, which is a 15-membered ring analog. Besides, josamycin, kitasamycin, and tylosin, are some examples of 16-membered ring analogs. Telithromycin was the first of the ketolide group to be derived from erythromycin; it was derived by replacing the L-cladinose in erythromycin with a keto-group (Leclercq, 2002; Roberts, 2004). Lincomycin was the first lincosamide to be discovered and isolated from *Streptomyces lincolnensis* (MacLeod et al., 1964). Later, a new semisynthetic lincosamide, clindamycin, was derived from lincomycin. This structural alteration has led to a modification in the spectrum of activity, dose concentration, and administration of the newer macrolides and lincosamides.

In environmental study, various purposes with positive impact are done by application of antibiotics. This is because the application of antibiotic resistance profile is simple, useful for surveillance and cost-effective (Lewis et al., 2007). One example is improvement in methane production by exploitation of azithromycin (Nguyen et al., 2014). The impact on utilization of antibiotics can be evaluated through changing the bacterial interaction with various antibiotics available. The positive and negative impacts on the bacteria community by the antibiotics provide valuable information for understanding their interactions, functions, activity and process involve.

1.6   Greenhouse gases from livestock

The most important greenhouse gases directly emitted by human's activities include carbon dioxide, methane and nitrous oxide and also other fluorine-containing halogenated substances. Basically some of these gases occur naturally in atmosphere. However, due to human activities, the atmospheric concentration has changed. Greenhouse gases are able to trap heat and make the planet warmer and finally contribute to global warming issues. Methane is reported to be 25 times more effective than carbon dioxide at trapping heat in the atmosphere. Anthropogenic sources of methane include natural gas and petroleum systems, agricultural activities including livestock sector, landfills, wastewater treatment, and certain other industrial processes (Stocker, 2014). Consequently, livestock production accounted for about 25% of total anthropogenic methane in term of enteric fermentation (Lesschen et al., 2011). However, the emission from enteric fermentation contributed to greenhouse gases is dependent on region, agricultural production practice and types of livestock.

1.6.1 Enteric methane production

Anthropogenic methane emission by ruminant animals occurs mainly by microbial anaerobic digestion of feed that take place in reticulo-rumen of stomach and also minor release through anaerobic decomposition of organic matter in manure during storage (Hristov et al., 2013). It is a symbiotic process occurs between ruminant and the microorganisms in rumen. During the anaerobic fermentation of feed digestion, microbes supply energy and protein to ruminant while ruminant provides optimal growth conditions and nutrients for microbes. Therefore, ruminant should efficiently utilize the supplied energy despite of loss it as methane gas through burping or farting.

The process of enteric methane production is similar to other anaerobic digestion process. The energy source from the feed is able to be utilized by ruminant for their maintenance, growth and also milk production (Beauchemin et al., 2008). Briefly, the feed contains carbohydrates and proteins are broken down by hydrolytic bacteria presence in the rumen. This process will later produce volatile fatty acids which is the major source of energy for animals. The proportion of volatile fatty acids such as acetate, butyrate and propionate is dependent on the feed type and composition. Furthermore, bacteria will consume these volatile fatty acids to produce hydrogen and also carbon dioxide. Finally, methanogens will take place for further conversion to produce methane and water.

1.6.2 Mitigation strategy for methane emission

Since ruminants are able to utilize the energy in term of methane for their growth and maintenance, it is important to find a strategy to mitigate the enteric methane emission, which is not only benefits to ruminants but also for the environment. There are many mitigation strategies that has been carried out. For example, the application of vaccine as an antibody for methanogens (Wedlock et al., 2013) and substitutes feed that can improve the concentration which less dependent in fermentation process (Jiao et al., 2014). Besides, the utilization of additives in feed also were done to mitigate the methane emission. Hristov et al. (2013) use 3-nitrooxypropanol as synthetic additive in feed for cow that can block enzyme involve in methane production. The nitrate additive also has been used as additive (Lee & Beauchemin, 2014) but caused a toxicity of nitrate to some cow. Chestnut and valonea extract were used by Wischer et al. (2014) in their study to evaluate its effect on methane release and digestibility by sheep. However, those extracts contribute to high cost of feed. The natural additives from plant-based with low or zero cost and renewable are the most preferable to be investigated for methane inhibition from ruminant animals, especially cow.

1.7   Palm oil refining

Malaysia is currently the world's largest producer and exporter of palm oil (Basiron, 2007). Production of palm oil involves many stages and generates a lots of biomass from the plantation site such as empty fruit bunch, oil palm frond and trunks until processing of oil in plant/factory site. Crude oil must be purified in order to serve for human consumption by producing a refined

oil with light color, bland taste and also to achieve oxidative stability. Specifically, the objective of refining is to remove the minor constituents from the oil without damaging the acylglycerols and other desirable compounds such as vitamins and antioxidants (Gibon et al., 2007). Three major steps in either physical or chemical refining process are degumming, bleaching and deodorization (Manan et al., 2009) as shown in Figure 1.4.
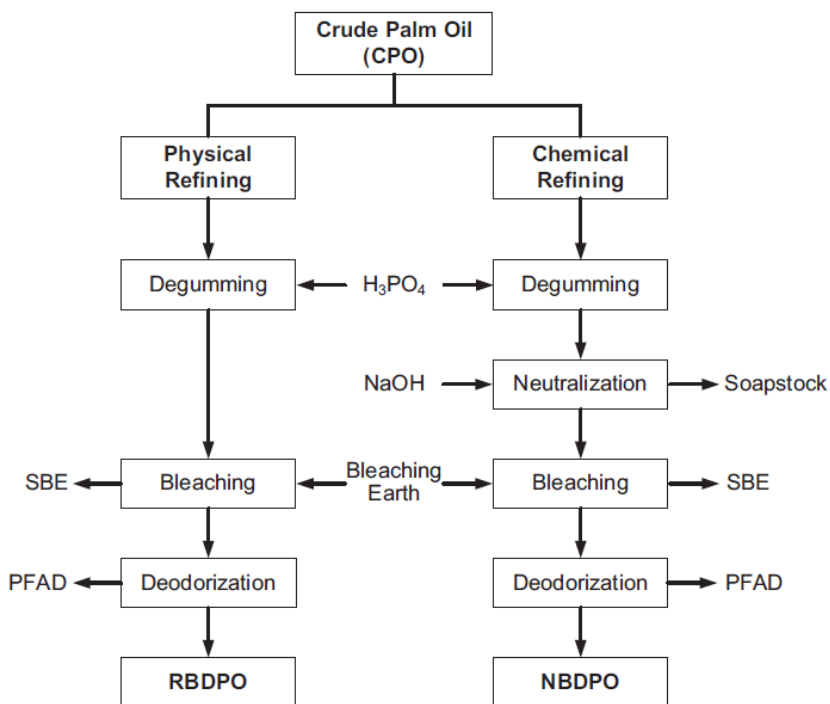
Figure 1.4: Palm oil refining processes. Sources: (Haslenda & Jamaludin, 2011)

One of the constituents in palm oil that need to be removed is phosphatides because the presence of this compound will interfere with further processing of obtaining the refined palm oil. The removal process is achieved through degumming process. There are many degumming approach which depends on the phosphatides content in crude palm oil such as water degumming, wet acid or soft degumming process use for high phosphatides content (Gibon et al., 2007). The main

phosphatide component in palm oil is phosphatidylcholine (Goh et al., 1985). Phosphatide is removed during degumming process which at the end of the process it will produce a by-product called gums. In this study, the term "phospholine gum" is used for the by-product from degumming process of crude palm oil. Besides, instead of phosphatide term, other study also used phospholipids or natural gums (Haslenda & Jamaludin, 2011). Whatever terms used, they are refer to the by-product produce after degumming process of crude palm oil.

There are several other products produced from the refining process of crude palm oil, including spent bleaching earth (SBE), soapstock and palm fatty acid distillate (PFAD) as shown in Figure 1.4. These by-products have the potential to be utilized or sold to other industries. Many other studies have reported on the utilization of SBE, soapstock and PFAD for variety of end uses include as an essential feedstock for animal-feeding industries (Atil, 2004; Dumont & Narine, 2007). However, until now, no report has been published on the production of by-product from degumming process and its utilization from palm oil mill industry in Malaysia. Fortunately, the by-product called phospholine gum is available from some palm oil processing site in Indonesia even though no formal reports has been published. In this study, this phospholine gum is evaluated on its possibility as an additive in cow feeds.

1.8   Objectives

Microbial community function is the key mechanism for various applications of biological WAS treatment such as bioremediation or bioenergy production. The complex microbial community in WAS provide a desirable conditions to be exploited in order to understand the details of each process. Thus, this study provide understanding on the microbial community function in WAS for bioremediation and bioenergy production, specifically methane production and inhibition.

The objectives of this study include:

1. To determine the bacterial soldiers responsible in killing the inoculant during bioremediation process.

2. To investigate the microbial community function on improving or inhibiting methane production by different antibiotics.

3. To study the effect of macrolide- and lincosamide-type antibiotics on microbial community for methane production.

4. To investigate the potential of phospholine gum as ruminant feed additive in inhibiting methane production.

CHAPTER 2

GENERAL MATERIALS AND METHODS

2.1   WAS sampling and preparation

The WAS used in the whole study was taken from the Hiagari Wastewater Treatment Plant in Kitakyushu City, Japan. The fresh raw WAS was initially centrifuged at $8000 \times g$ for 10 minutes at 4°C using TOMY-GRX 250 High Speed Refrigerated Centrifuge and the remaining pellet was resuspended with distilled water by shaking thoroughly. Washing steps is required by washing and resuspending the sludge with distilled water to ensure the consistency of initial sludge conditions. The washing steps were done three times before adjusting to 50% (wet-sludge weight/w) which then adjusted to different final sludge concentration based on experiments by distilled water. Sludge was weekly taken from the plant and freshly washed and prepared for all the experiments.

2.2   Analytical method

2.2.1   Gas measurement

2.2.1.1 Gas chromatograph

Methane gas was analyzed by a GC-3200 gas chromatograph (GL Science, Japan) equipped with a thermal conductive detector and with a column of Molecular Sieve $13 \times 60/80$ mesh column,

SUS 2 m x 3 mm I.D (GL Science, Japan). Helium gas was used as a carrier gas with a flow rate of 40 mL/min. The GC condition was set as follows; current at 100 mA, oven, injector, and detector temperature at 40°C, 50°C, and 65°C, respectively. Hydrogen gas was analyzed by a 6890-N gas chromatograph (Agilent Technologies, Glanstonbury, CT) equipped with the thermal conductive detector. HP-Molesieve 15 m x 0.530 mm id, 25 µm, 19095P-MS5 column (Agilent Technologies Inc.) was used with the column, injector and detector temperature set at 70°C, 100°C and 200°C, respectively. Nitrogen gas was used as a carrier gas with a flow rate of 20 mL/min (Mohd Yusoff et al., 2012).

2.2.1.2 Gas calibration curve

Gas calibration was carried out using pure gases ((Eaton et al., 2005). Pure gas volumes ranging from 10, 20, 30, 40 and 50 µL were injected with a syringe for area measurement. The calibration curve was plotted by the amount of gas against area. Initially, the amount of gas was calculated at standard temperature and pressure to be obtained in µmol. Gas calibration curve equation was constructed based on linear response and regression value more than 0.99. The calibration curve was used to calculate the amount of gas in the samples.

2.2.1.3 Gas calculation at standard temperature and pressure

Standard temperature and pressure (stp) is referred to zero degree Celsius (273 K) and one atmosphere (1 atm) of pressure, respectively. However, commonly used standard was set up at ambient temperature (25°C, 298 K) and absolute pressure (1 atm). Stp is the standard set of

condition for experiments to allow the results to be comparable with other results. Stp can be calculated based on Ideal gas law (Poling et al., 2001);

$$pV = nRT$$

Where,

p is the pressure at 1 atm,

V is the volume of gas in L,

N is number of moles,

R is gas constant (0.0821 mol.L/mol.K), and

T is ambient temperature in Kelvin (298 K).

2.2.2 Organic acids analysis

Organic acid determination was carried out based on method described by Shinagawa et al. (1997). Prior to organic acids measurement, the sample was centrifuged at 13,000 rpm for 7 min to collect the supernatants which were later filtered through a 0.2 µm membrane syringe filter (Minisart, Sartorius Stedim Biotech, Germany). Organic acids in each supernatant were analyzed using high-performance liquid chromatography (Shimadzu LC-10AD, Japan) with conductivity detector CDD-6A. The separation was obtained by two column system, using Shim-packed SCR-102H (8.0 mm ID x 300 mm L) column at 40°C. Buffer (50 µM EDTA.2Na, 10 mM Bis-Tris and 2.5 mM p-Toluene sulphonic acid) and mobile phase (2.5 mM p-Toluene sulphonic acid) were filtered through 0.45 µM pore size filter (Lot 10574.38, Thermo Scientific, Nalgene, Japan). Buffer and mobile phase were used as solvent at flow rate of 0.8 mL/min. Standard curves were constructed for each of organic acid; citric, acetic, butyric, iso-butyric, propionic, succinic,

formic and lactic acids by injecting individually at a range of concentration, and plotted against area. The organic acids from samples were determined based on the retention time.

2.2.3 Total protein

The protein concentration was carried out as described by Lowry et al. (1951). This method is a biochemical test to detect total protein in a sample. Principally, the assay is based on the reaction of peptide bond under alkaline condition with copper ions through the oxidation of protein residues. Lowry A solution (4 g/L NaOH and 20 g/L Na_2CO_3) and Lowry B (5 g/L $CuSO_4.5H_2O$) and Lowry C (10 g/L $KNa.C_4H_4O_6.4H_2O$) were mixed at 1:0.1:0.1 ratios. The mixture was freshly prepared prior to analysis. Then, 1 mL of Lowry A, B and C solution mixture was added to 200 µL sample before standing incubation at 30°C for 10 min. The solution was vortexed prior to addition of 100 µL 1N phenol before standing again at 30°C for 30 min. Finally, the sample was measured at 750 nm absorbance. Distilled water was used as blank instead of sample. Calibration curve was developed using bovine serum albumin (BSA) at a range of concentration.

2.2.4 Total solid

Total solid analysis was carried out as described by Eaton et al. (2005). Total solid is the residue left from a well-mixed sample after evaporation at 103-105°C in drying oven. The increase weight over the empty dish represents the actual total dissolved and total suspended solid in the sample. For determination of total solid, 10 mL of homogenized sample is placed in a pre-weighted crucible prior to overnight drying in 105°C oven. Then, the crucible was cooled in

desiccator before weighing. The process of drying, cooling and weighing were repeated until a constant weight was obtained. The calculation for total solid is:

$$\text{Total solid (g/L)} = \frac{(A - B) \times 1000 \ mL/L}{Sample \ volume, \ mL}$$

Where,

A = weight of crucible and dried residue (g)
B = weight of crucible (g)

2.2.5 Volatile solid

Volatile solid determination was carried out according to Eaton et al. (2005). The residue obtained from total solid determination was ignited to constant weight at 550°C to drive-off volatile solids in the sample. The different in weight before and after ignition represent the fraction of total solid. The suspended solid remained after ignition represents volatile solid value. The process of drying, cooling and weighing were repeated until a constant weight was obtained. The calculation for volatile solid is:

$$\text{Total solid (g/L)} = \frac{(A - B) \times 1000 \ mL/L}{Sample \ volume, \ mL}$$

Where,

A = weight of crucible and residue before ignition (g)
B = weight of crucible and residue after ignition (g)

2.2.6 Protease activity

The protease activity was measured as described previously (Maeda et al., 2011). Initially, 200 μL of sample and 200 μL of 1% w/v casein was mixed and incubated at 37C° for 15 min. Then, 400 μL of 0.44M trichloroacetic acid was added to the mixture and incubate at room temperature for 30 min. Later, mixture was centrifuged to collect 200 μL supernatant before mixing with 200 μL of 2x phenol reagent and 1 mL of 0.4M $NaCO_3$. Again, mixture was incubated at 37C° for 30 min prior to absorbance measurement at 660 nm. One unit of protease activity was calculated as the quantity of tyrosine (μmol) produced from casein by 1 mg of enzyme per min.

2.3   Microbial community analysis

2.3.1 RNA extraction and cDNA synthesis

The microbial community of WAS with or without inoculant was analyzed using RNA based sample. Prior to the RNA extraction, 3 mL of RNA later solution (Ambion, Cat#AM7020) was mixed with 10 mL of sludge sample using an RNAse free falcon tube to avoid the degradation of RNA through the extraction process and then the mixture was centrifuged at 15,000 rpm for 2 min at 4°C to harvest the sludge pellet. The supernatant was discarded and the pellet was immediately resuspended with 1 mL RNA later solution in a screw cap tube and finally centrifuged at 13,000 rpm for 30 s. After removing the supernatant, the remaining sludge pellet was quickly froze in dry ice with ethanol and stored at -70°C until a process of RNA extraction.

Total RNA was extracted using RNeasy kit (Qiagen Inc., Valencia, CA) as described in our previous paper (Mohd Yusoff et al., 2012) and the total concentration of RNA was measured using a Nano drop spectrophotometer (SCRUM Inc., Japan). Prior to analysis, the RNA must be converted to cDNA. The cDNA was synthesized from RNA using PrimeScript RT reagent kit Perfect Real Time (TAKARA Bio Inc., Shiga, Japan). The synthesis was done using 5 µg of total RNA in a mixture of 2 µL of 5 × PrimeScript buffer, 0.5 µL reverse transcriptase enzyme, 0.5 µL oligo dT primer, and 2 µL random oligomers (the total volume of 10 µL along with RNA). The mixture was incubated at 25°C for 10 min, then at 37°C for 30 min and finally the enzyme was deactivated at 85°C for 5 s. The electrophoresis using 1.4% agarose gel was performed to determine the quality and quantity of RNA extracted and cDNA synthesized. The cDNA was used later in high-throughput sequencing for microbial community analysis, specifically bacteria community.

2.3.2 qRT-PCR quantification

The qRT-PCR was performed to quantify the 16S rRNA gene of total Bacteria and Archaea using a TaqMan system. The StepOne Real Time PCR System (Applied Biosystem) was used for amplification and detection of fluorescence by using primers and probes listed in Table 2.1. The total 20 µL of real time PCR mixture was prepared as follows; 10 µL of 2 × Taqman Fast Advance master mix, 0.72 µL of each primer (25 µM), 0.34 µL of Taqman probe (11.8 µM) and 2 µL of cDNA (100 ng). The quantification for each cDNA template was analyzed in triplicate.

The cycling condition was as follows; UNG incubation at 50°C for 2 min, denaturation and polymerase activation at 95°C for 20 min, and 40 cycles of annealing and extension at 95°C for 1 s and 60°C for 20 s, respectively. The standard curve for universal bacteria and archaea were calculated as described in our previous paper (Mohd Yasin et al., 2015). *Escherichia coli*, a rapidly growing aerobic bacteria, was commonly used as the standard for determining the total bacterial numbers because its DNA is consistently detected by RT-PCR (Nadkarni et al., 2002). *Archaeaglobus fulgidus* was used as a representative for Archaeal activities as *Archaeaglobus spp.* are closely related to methanogenic Archaea or methanogens in comparative genomic studies (Gao & Gupta, 2007). The calculation of copy numbers based on the amount of DNA was performed as described by Lee et al. ((Lee et al., 2008).

Table 2.1: Primer and probe sets used in qRT-PCR.

Target	Function	Sequences (5`-3`)	*E. coli* numbering (bp)	Product size (bp)	Reference
Bacteria	Forward	TCCTACGGGAGGCAGCAGT	331-349	466	(Nadkarni et al., 2002)
	Reverse	GGACTACCAGGGTATCTAAT CCTGTT	772-797		
	Probe	CGTATTACCGCGGCTGCTGG CAC	506-528		
Archaea	Forward	ATTAGATACCCSBGTAGTCC	787-806	273	(Lee et al., 2008)
	Reverse	GCCATGCACCWCCTCT	1044-1059		
	Probe	AGGAATTGGCGGGGGAGCAC	915-934		

2.3.3 High-throughput 16S rRNA sequencing

The V4-V5 region of 16S rRNA gene was used as a target of PCR amplification for the high-throughput 16S rRNA sequencing. The primers used were forward primer 515F (5′-GTGCCAGCMGCCGCGG-3′) and reverse primer 907R (5′-CCGTCAATTCMTTTRAGTTT-3′). The PCR mixture (25 µL) consisted of 10 × Taq buffer and Taq polymerase (BioLabs), 20 µM of each primer, 2 mM each dNTPs mix, and 25 mM of $MgSO_4$ (Toyobo) and approximately 50 ng of cDNA template. PCR was carried out under the following conditions: an initial denaturation 94°C for 3 min, followed by 35 cycles of 94°C for 45 s, 50°C for 60 s, and 72°C for 90 s, and a final extension at 72°C for 10 min. The amplified PCR products were purified using QIAquick Gel Extraction Kit (Qiagen, Valencia, CA, USA). Each concentration of purified PCR products was determined using Qubit dsDNA HS Assay Kit (Life Technologies, Oregon, USA).

The PCR products were processed using Nextera XT DNA Library Preparation Kit according to the Illumina manufacturer protocol. Briefly, the input DNA was treated for simultaneous fragmentation and tagmentation using Nextera transposome as well as for the addition of a dual adapter index with a unique barcode sequence through the PCR amplification to differentiate each sample. Then, the purification was performed by AMPure XP beads and normalized to ensure that an equal library was presented in pooled samples. Pooled samples were diluted in hybridization buffer and denatured by heat prior to the sequencing.

A 500-cycle V2 MiSeq reagent cartridge (Illumina) was thawed in water bath and inverted ten times to mix the reagents inside it prior to use. Finally, the pooled samples were loaded in the cartridge and then onto the MiSeq instrument along with the cleaned flow cell. Sequencing was

performed for 251, 8, 8, and 251 cycles for forward Index 1, Index 2, and reverse reads, respectively. The data obtained were demultiplexed and then reads were classified to different taxonomic levels. The raw sequence data were deposited into the NCBI short reads archive database under accession number: SRP072534.

2.3.4   Processing high-throughput data

The raw paired-end reads were assembled using a PAired-eND Assembler for Illumina sequences (PANDAseq) (Masella et al., 2012) to improve the sequencing accuracy of the reads. The resulting sequences were processed using Quantitative Insights into Microbial Ecology (QIIME) v1.9.0 (Caporaso et al., 2010). A low quality sequence (quality score < 20) and any read containing ambiguous bases were discarded from further analysis. The remainders of the high-quality reads were clustered into operational taxonomic units (OTU) at 97% sequence similarity using *de novo* OTU picking pipeline. The UCLUST consensus taxonomy classifier v1.2.22q was used to assigned taxonomy to each representative sequence prior alignment against Greengenes database v13.8 (DeSantis et al., 2006) using PyNAST program (Caporaso et al., 2010; DeSantis et al., 2006).

The rarefied OTU tables were generated as the basis for calculating alpha diversity and rarefaction curves were computed using the Chao1 richness estimator and Shannon index. Beta diversity used to determine the pattern of the bacterial community structure between samples was analyzed by principal coordinate analysis (PCoA) and a cluster analysis using Jackknife beta-diversity and UPGMA of QIIME, respectively. The three-dimensional PCoA plots were visualized using Emperor (Vázquez-Baeza et al., 2013).

CHAPTER 3

BACTERIAL SOLDIERS IN WASTE ACTIVATED SLUDGE – UNDERSTANDING OF THE BACTERIAL NATURAL SELECTION –

3.1   Introduction

The success of bioremediation relies on the transformation of organic pollutants into harmless or at least to less hazardous compounds, depending on the metabolic capabilities by microbes in the community.   Use of a native bacterium is a great interest in bioremediation as it is more applicable to its origin environment. However, bioaugmentation as one of the bioremediation methods is a preferable approach when none of local community representative shows any ability for pollutant remediation. Bioaugmentation can be defined as a pollutant-removal technique by an introduction of specific exogenous competent strains or consortia of microorganisms, ex situ or in situ (El Fantroussi & Agathos, 2005). Although promising, bioaugmentation still far from fully functions for numerous reasons. The utmost significant reason is that the ability of exogenous microorganisms is most often temporary and decreases shortly after the inoculation to the site (Lladó et al., 2013; Silva et al., 2009; van Veen et al., 1997). Consequently, there are biotic and abiotic factors which can be responsible for the unsuccessful bioremediation.

Biotic factors emerging from indigenous population are more often significant (Liang et al., 2011; Yu et al., 2011). Indigenous population becomes attentive for the alien bacteria inoculated in their environment causing some interaction such as predation, mutualism or competition. Thus, these interactions develop a dynamics in their microbial community. For the reason, microbial

community assessment is often taken into account (Lee et al., 2009; Liang et al., 2011; Zhao et al., 2016) to evaluate the effectiveness of bioaugmentation by tracing the inoculants. However, a very scarce study (Zhao et al., 2016) has been done using high-throughput sequencing to assess the inoculant and the overall microbial community of the bioaugmented site.

Bioaugmentation technique is also adopted in Wastewater Treatment Plants (WWTPs) to treat the wastewater obtained from different sources before being able to release to the environment. Many refractory organics compounds were treated using this strategy such as the degradation of 3-chloroaniline (Boon et al., 2000) and aromatic hydrocarbons (Dueholm et al., 2015) in waste activated sludge (WAS), removal or p-nitrophenol and nitrobenzene in mixed wastewater (Hu et al., 2008), drug-based contaminants from pharmaceutical wastewater (Saravanane et al., 2001) and, many more. Even though bioaugmentation strategy seems to have a promising succession to treat wastewaters, failures still occur as the positive effects observed only in short period of time after inoculation (Boon et al., 2000; Patureau et al., 2001; Yu et al., 2010). Hence, the removal efficiency in wastewater treatment system is still not fully successful via bioaugmentation.

Beside the competition between inoculant and indigenous bacteria for nutrients or space during bioaugmentation that cause the failures (van Veen et al., 1997), the direct inhibition and release of inhibitory substances (Fu et al., 2009) revealed that one or more bacterial soldier might exist among indigenous community. To protect their community, the native bacterial will extend their population and kill the inoculant as they recognize it as alien bacteria. Bacteriophages, protists and viruses are known as major contributors in bacterial death at microbial level (Fuhrman &

Noble, 1995). However, information on bacterial soldiers is still scarce and no approach has been done to prove the existence of these soldiers during bioaugmentation.

Antibiotic resistance is one example of natural selection can be a virtuous approach. In this situation, bacteria carrying antibiotic-resistant plasmids would be at extensive advantage compare to bacteria without those plasmids (Bergstrom et al., 2000). Bacteria develop resistant to antibiotics occur rapidly since they can produce several generation within a day. Commonly, the strongest bacteria are last to die or do not cause the death when expose to the antibiotics. The weaker bacteria will die or at least cause reduction in their community abundance (Crump, 2008). This approach has displayed a positive impact in our previous study which the methane production was improved by the application of azithromycin in WAS (Nguyen et al., 2014). Azithromycin targeted on some bacteria caused an affirmative alteration in bacterial interaction. Thus, by using the similar concept in changing the bacterial interaction, other bacteria communities which work competitively against alien bacteria can be found. Based on the natural selection concept, the bacterial soldier responsible in killing the inoculant can be obtained by using inoculant carrying specific antibiotic resistant gene/plasmid. The application of same antibiotic in inoculated polluted sites or samples will allow the inoculant to survive while the possible soldier to die or reduce in population.

Therefore, this study was investigated the existence of possible bacterial soldier in WAS that killed the inoculant by utilizing the antibiotic. Application of antibiotics will develop an antibiotic-resistant community contributing to bacterial natural selection. Enhanced green fluorescent protein (EGFP) plasmid was used as a marker to track the inoculant which carry an

antibiotic resistant gene and tetracycline or chloramphenicol were added for bacterial soldier detection from bacterial community in WAS.

3.2 Materials and Methods

3.2.1 Activated sludge preparation

The WAS was prepared to final concentration of 10% (wet-sludge w/w) with distilled water prior to use in all experiments in this study.

3.2.2 Antibiotics

Antibiotics used in this study were carbenicillin from Nacalai Tesque, tetracycline and chloramphenicol from Wako, Japan. All antibiotics were dissolved into their respective solvent depending on the solubility as a stock solution prior to use in each experiment.

3.2.3 Bacterial strains, plasmid and media

Escherichia coli BW25113 harboring pEGFP plasmid (Clontech) was used as a host bacteria and the strain can express the enhanced green fluorescent protein (EGFP) which has an excitation wavelength of 488 nm as compared to wild type GFP (395 nm) and the emission wavelength of 507 nm. pEGFP has originally the betalactamase gene (ampicillin or carbenicillin resistance).

Therefore, *E. coli* BW25113/pEGFP was grown in Luria Bertani (LB) medium in the presence of carbenicillin (final con. 100 µg/mL) and 1.5% (w/v) agar was added where appropriate.

3.2.4   Cloning of other antibiotic resistant gene in pEGFP

By using the antibiotic resistance, the EGFP-expressing *E. coli* cells can be activated due to killing effect towards another microbial consortium by carbenicillin. However, there was no big difference on with or without carbenicillin.   Therefore, another type of antibiotic-resistance pEGFP plasmid were constructed to evaluate their survival in presence of tetracycline and chloramphenicol antibiotics. Firstly, the pEGFP was extracted from EGFP-expressing *E. coli* using QIAprep Spin Miniprep Kit Protocol (Qiagen) to serve as a cloning vector for antibiotic resistant gene. On the other hand, tetracycline resistant gene was amplified from pBR322 (carrying tet gene) using a designed set of forward (5'-CCGAACATATGCACCTGACGTCTAAGAAAC-3') and reverse (5'-ATGGACATATGCTGCCAAGGGTTGGTTTGC-3') primers. Also, the chloramphenicol resistant gene was amplified from pCA24N (carrying cat gene) using forward primer (5'-AAATACATATGGGTGTCCCTGTTGATACCG-3') and reverse primer (5'-ATTATCATATGTTCAGGCGTAGCACCAGG-3').

Both genes and plasmid were purified using QIAquick Gel Extraction Kit (Qiagen, Valencia, CA, USA).   Later, the purified genes and pEGFP were digested using Nde1 (Biolabs) restriction enzyme and then the digested pEGFP was treated with alkaline phosphatase (Wako) prior to ligation. T4 ligase enzyme (Wako) was used to insert the gene into pEGFP. Ligated product was

transformed into *E. coli* BW25113 competent cell via electroporation followed by short incubation at 37°C for about 1 hour to initiate the growth. Finally, the aliquots of the transformation suspensions were plated on LB agar medium supplemented with either tetracycline or chloramphenicol antibiotic based on their cloned resistant gene, both at 10 µg/mL.

3.2.5   Gene orientation

The insertion of gene direction into the plasmid was determined either in forward or reverse direction. Four single colonies from transformed cell were randomly selected from each selective plate. The cells were re-grown in LB broth with antibiotic for overnight prior to plasmid extraction. The extracted plasmid containing antibiotic resistant gene was cut at two sites; one at the end of the gene and the other one on the plasmid, preferably using the same restriction enzyme. The restriction enzyme used for pEGFP with tet gene was BamHI (Toyobo) and for pEGFP with cat gene was NcoI (Wako). Subsequently, the diagnostic digest was performed using these enzymes to determine the direction of inserted gene. The digested products were analyzed by electrophoresis using 0.8% agar with Marker 6 (Wako) as an indicator of band size. The direction of gene was concluded based on the expected size cut by the restriction enzyme on the gene and plasmid that was confirmed by the size of the band formed on the gel.

3.2.6   Inoculant survival

E. coli harboring the EGFP plasmid was grown on LB agar containing carbenicillin to make sure the selectivity. To make a starter culture, a fresh single colony was picked from the agar plate

and inoculated into a test tube that contained 5 mL LB media in the presence of carbenicillin. The test tube was incubated with 120 rpm of shaking in a waterbath at 37°C for overnight. The grown cells were centrifuged at 13000 rpm for 1 minute. The pellet of bacteria was washed and resuspended in phosphate buffered saline (PBS) solution (0.2 g KH_2PO_4, 0.2 g KCl, 1.15 g Na_2HPO_4, 8 g NaCl per liter) thrice prior to inoculation into WAS.

E. coli BW25113 EGFP cells (5 mL suspension) was inoculated in 100 mL of 5% WAS obtained from initially prepared 50% WAS in a 200 mL Erlenmeyer flask. The mixture was incubated at 37°C and with shaking at 120 rpm. Samples were taken at 0, 8, 24 and 48 hours for the analysis using plating count and fluorescence leaking and also for microbial community. Sterilized WAS was used as a control by treated at 121°C for 40 minutes and either with or without the inoculation of EGFP-expressing *E. coli* cells. WAS without inoculation was used as control. All experimental and control samples were prepared in triplicates.

Same inoculum size, substrate concentration and incubation conditions were utilized for experiments with addition of antibiotics. The antibiotics; carbenicillin, tetracycline or chloramphenicol was added in the WAS at different concentration 5 - 100 μg/mL to observe the resistance of inoculant.

3.2.6.1 Colony forming unit (CFU) count

The number of bacterial cells (CFU) was determined by the colony-counting method. The samples obtained at different hours were sequentially diluted using sterile distilled water in the 1

mL Eppendorf tubes. Later, the 100 µL of diluted sample was spread on LB agar containing 100 µg/mL of carbenicillin as selective plate for counting EGFP-expressing *E. coli* colonies and on LB agar as non-selective plate for counting the total number of bacterial cells. All plates were incubated at 37°C for overnight. Colonies grown on the plates were counted using a plate with 30-300 colonies and calculated as an average of triplicates from an appropriate dilution. For the LB carbenicillin agar plate, only green fluorescent colonies of EGFP-expressing *E. coli* cells were counted by viewing under UV light.

3.2.6.2 Flow cytometer

Flow cytometer (FCM) analysis was done for data comparison with colony counting method. As for detecting the EGFP-expressing *E. coli* in WAS, 1 mL of samples was centrifuged at 1500 rpm for 5 minutes and the supernatants were diluted with PBS solution (0.2 g KH_2PO_4, 0.2 g KCl, 1.15 g Na_2HPO_4, 8 g NaCl per liter) about 100 times (v/v) for FCM accuracy. Pure EGFP-expressing *E. coli* culture was prepared as standard by centrifugation of 1 mL broth at 1000 x g for 10 minutes to discard the supernatant. Pellet was added with 1 mL PBS solution and vortexed for mixing. Second centrifugation to settle down large particles was carried out at 1500 rpm for 5 minutes. Supernatant was collected for dilution with PBS solution to achieve around 10^3-10^4 cells per µL or OD between 0.01-0.05 as a suitable concentration for FCM analysis. All samples were filtered through 40 µm net filter to avoid clogging of the cytometer nozzle prior to analysis.

3.2.6.3 Fluorescent microscope

Fluorescent microscope also was used for data comparison with colony counting method. All samples were diluted to 10 times prior to slide preparation. The diluted sample (10 µL) was carefully pipetted on the center of slide and then 70 µL of 1% agar was pipetted and mixed with the sample. Immediately before the agar solidified, a coverslip was slowly and gently put to cover the sample without having any bubbles. Slides were dried naturally prior to viewing using fluorescent microscope. Slides were viewed under oil immersion with a 100x objective on an Olympus BX51 fluorescence microscope and observed using cooled CCD camera (VB-6010 Keyence, Osaka). EGFP cells were visualized using GFP filter with excitation wavelength 489nm and emission wavelength of 509. The cells were manually counted from 20-30 random areas on the slide.

3.2.7   Fluorescence leaking

The leaking of EGFP obtained from inoculated WAS indicated the dead of inoculant by releasing the fluorescent. Samples obtained from sampling period were centrifuged at 13000 rpm for 1 min to separate WAS particles and cells from the supernatant. Later, the supernatant was filtered through 0.2um filter to remove any remaining cells. All filtered samples were put in 96 well flat bottom plates at 200 µL in triplicates. The fluorescence leaking presence in the filtered supernatant was determined using Varioskan Flash 2.4.5 (Thermo Scientific). The fluorometric measurement was set up to detect the fluorescent from the top with excitation and emission

wavelength at 488 nm and 509 nm, respectively and calculated as Relative Fluorescence Unit (RFU) by subtracting the control.

3.2.8   Isolation of possible bacterial soldier using EGFP-expressing E. coli agar method

Since the EGFP-expressing *E. coli* cannot survive in WAS after inoculation, the indigenous bacteria in WAS was screened using inoculant agar method. The EGFP-expressing *E. coli* was grown in total of 200 mL for overnight prior to 2 times washing with PBS buffer. Final pellet was re-suspended in 50 mL PBS buffer and mixed with 50 mL of 3% (w/v) agar, and immediately poured in big petri dish. After solidified, 500 µL of 1 - 10% (w/v) of WAS was spread on agar. During incubation at 37°C, grown colonies were isolated, re-streaked and proceeded with colony PCR and sequencing to identify the bacteria taxonomy.

3.2.9   Microbial community analysis

The bacteria community was analyzed in the inoculated WAS by comparing with the control WAS, for both experiments; with or without the antibiotics treatment. This was done to evaluate the microbial community dynamics occurred due to the inoculation of exogenous EGFP-expressing *E. coli* and also to find the possible bacterial soldiers that were responsible in killing the inoculant.

3.2.9.1 RNA extraction and cDNA synthesis

RNA extraction was conducted using sludge pellets of fermented sludge. The RNA later solution was used for pellet preparation and details method as described in section 2.3.1. Total RNA was extracted using RNeasy kit (Qiagen Inc., Valencia, CA) as described in our previous paper (Mohd Yusoff et al., 2012). The cDNA was synthesized from RNA using PrimeScript RT reagent kit Perfect Real Time (TAKARA Bio Inc., Shiga, Japan). The cDNA was used later for qRT-PCR to determine bacterial activity and Archaeal activity as well as microbial communities of Bacteria and Archaea population.

3.2.9.2 qRT-PCR quantification

The qRT-PCR was performed to quantify the 16S rRNA gene of total Bacteria and Archaea using a TaqMan system. The StepOne Real Time PCR System (Applied Biosystem) was used for amplification and detection of fluorescence by using primers and probes listed in Table 2.1.

3.2.9.3 High-throughput 16S rRNA sequencing

The V4-V5 region of 16S rRNA gene was used as a target of PCR amplification for the high-throughput 16S rRNA sequencing. Each concentration of purified PCR products was then determined using Qubit dsDNA HS Assay Kit (Life Technologies, Oregon, USA). The PCR products were processed using Nextera XT DNA Library Preparation Kit according to the Illumina manufacturer protocol. The pooled samples were loaded in the 500-cycle V2 MiSeq

reagent cartridge (Illumina) and then onto the MiSeq instrument along with the cleaned flow cell. Sequencing was performed for 251, 8, 8, and 251 cycles for forward Index 1, Index 2, and reverse reads, respectively.

The raw paired-end reads were processed using QIIME v1.9.0 (Caporaso et al., 2010). The high-quality reads were clustered into operational taxonomic units (OTU) at 97% sequence similarity using *de novo* OTU picking pipeline prior to assign taxonomy and alignment against Greengenes database v13.8 (DeSantis et al., 2006) using PyNAST program (Caporaso et al., 2010; DeSantis et al., 2006).

3.2.10 Co-culture interaction between bacterial soldier and inoculant

In order to prove that the bacterial soldiers were functioned in killing the inoculant, the indirect co-culture interaction was carried out using hanging cell culture inserts. The bacterial soldiers were selected from isolated bacteria and also ordered from NITE Biological Resource Center (NBRC) based on bacterial community of MiSeq analysis from antibiotics approach as listed in Table 3.2. All bacteria including EGFP-expressing *E. coli* were grown overnight and prepared to achieve optical density (OD) 1 prior to mixing. The *E. coli* culture was put in the well of 6-well culture plate, while bacterial soldier was put in the hanging insert filter 0.4 μm at 2.5 mL for both cultures. Bacterial soldier culture was replaced with LB medium for control. The sampling was done at 0, 24 and 48 hours using cultures in the well for CFU and fluorescence determination.

3.3 Results and Discussion

3.3.1 *Survival of inoculant and bacterial community dynamics*

The major problem faced by bioaugmentation technology is the non-persistence of inoculant in the polluted sites/environment, thus aborted their ability and functions in remediation. The necessary survival and retention of the inoculating strain are the primary factors for successful bioaugmentation (Boon et al., 2000). This experiment was done to mimic the bioremediation process which utilizes an inoculant carrying specific function. In this study, the inoculant *E. coli* with EGFP plasmid was used as a marker to track their survival in WAS. Although there was some background growth from WAS, the inoculant, EGFP-expressing *E. coli* could specifically be identified by fluorescent colonies viewed under UV light. The inoculant cell number in WAS is shown in Figure 3.1a. During the hours following inoculation, the EGFP-expressing *E. coli* in WAS was increased to 8.1×10^7 at 8 hour of incubation. Thereafter, the number of cell decreased gradually until the end of incubation to 5.5×10^5 CFU/mL from initial inoculant of 7.7×10^7 CFU/mL recorded after being inoculated in WAS. By contrast, cell numbers of EGFP-expressing *E. coli* in sterilized WAS increased at 8 hour from initial incubation of 5.6×10^7 CFU/mL and maintained the growth until 2.5×10^8 CFU/mL at 48 hours.
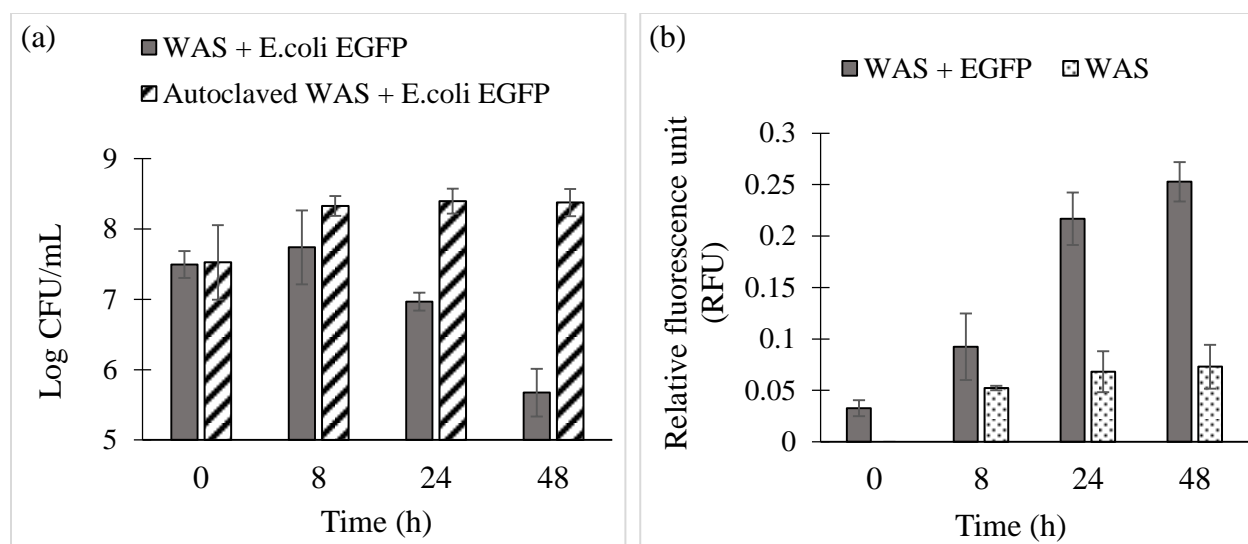
Figure 3.1: Cell numbers of EGFP-expressing *E. coli* in WAS and sterilized WAS (a), and fluorescence leaking of EGFP-expressing *E. coli* in WAS and non-inoculated WAS (b).

In addition, to prove that the inoculant was died in WAS, the fluorescence leaking from *E. coli* was determined from the supernatant of WAS as compared with WAS without inoculation as control. The relative fluorescence obtained is subtracted from the background fluorescence of initial WAS. As shown in Figure 3.1b, the fluorescence in the inoculated WAS was increased compared to WAS without inoculation until 48 h. Some of the dead inoculant cell might release the fluorescence due to cell membrane damaged or cell burst. Therefore, the increased in fluorescence evidenced that the EGFP-expressing *E. coli* was unable to survive in WAS. The bacterial community analysis was carried out to determine the dynamics of indigenous bacteria and possible soldiers among them that killed this inoculant.

Many studies speculated the reasons for unsurvival of inoculant subsequently affecting the bioaugmentation such as strain selection, microbial ecology such as predator, contaminant type, environmental constraints and culture introduction methods (Xiong et al., 2013). Predation by

protozoa is well known in bioaugmentation (Mrozik & Piotrowska-Seget, 2010; Yu et al., 2011) but bacterial predators or soldiers are still not evaluated. In this study, we tried to find the bacterial soldier in WAS contributing to non-survival of EGFP-expressing *E. coli*. *E. coli* was used as inoculant as they are generally common fecal bacteria found in WAS. Thus the abiotic condition of WAS should not affected on their growth. Besides, the incubation was carried out based on the optimal growth condition of *E. coli*. The EGFP plasmid was stably maintained in *E. coli* and had a negligible effect on its growth. However, the inoculant cannot survive in WAS as compared to the sterilized WAS showed that the biotic factors were the main reasons. The sterilized WAS was more hospitable due to relatively low stress in term of competition with indigenous community.
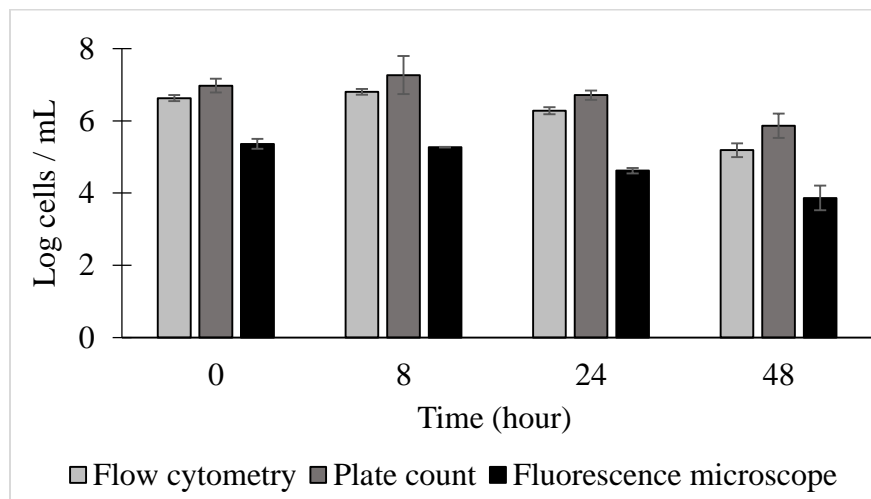
Figure 3.2: Number of EGFP-expressing *E. coli* measured by different approaches.

On the other hand, agar plate method is a conventional method for colony counting. In order to make sure the number of cell is accurate and comparable with other rapid and high sensitivity methods, flow cytometer was used. Also, fluorescence microscope which able to detect the

fluorescence cell using GFP filters was used as comparison with other methods as shown in Figure 3.2. The flow cytometer measurement obtained slightly lower number of EGFP cells compared to colony count but still considerably similar. The lower number might be due to the difficulty or bias occurred during tedious sample preparation prior to flow cytometer analysis. Besides, EGFP cells counted via fluorescence microscope showed highly gapped between the other two methods. This is due to unfair counting of cells from only random areas which not included exact total numbers of EGFP cells. Therefore, further experiments for determination of EGFP-expressing *E. coli* cells were done by colony counting on selective agar plate.

3.3.2   Richness, diversity and dynamics of bacterial communities

Illumina MiSeq analysis was used to determine the effects of inoculation on the dynamics of WAS microbial community. In analyzing the bacterial communities, observed OTUs, Chao1 and, Shannon were clustered at the identity cut-off value of 3%. The observed OTUs number was fluctuated in both WAS and WAS with inoculant ranging from 7580 to 10651, as shown in Table 3.1. The Chao1 values were in accordance with OTUs number. OTUs and Chao1 values are indicators to demonstrate the richness of bacterial community with or without the inoculation of EGFP-expressing *E. coli*. Another indicator, the Shannon index, was used to estimate the microbial population diversity. This diversity index was fluctuated from initial to final day of incubation while in the absent of inoculant the diversity index was similar during the incubation period with a slight increased at 8 h. The details community was evaluated through bacterial community analysis in class level of taxonomy.

Firstly, the present of EGFP-expressing *E. coli* was evaluated from the community. *E. coli* belongs to *Proteobacteria* phylum, specifically *Enterobacteriaceae* in *Gammaproteobacteri*a class. As shown in Figure 3.3, the present of this inoculant was proved by the high abundance percentage of *Gammaproteobacteria* in inoculated WAS, compared to non-inoculated WAS which in agreement with the CFU measurement (Figure 3.1a). During the incubation, the percentage of *Gammaproteobacteria,* specifically *Enterobacteriaceae* was decreased proved that the inoculant cannot survive in WAS. In both samples with or without the inoculant, *Proteobacteria* made up a major percentage of active WAS bacterial community with some other dominant group.

Table 3.1: Diversity statistics of bacterial community in WAS by inoculation.

		Observed OTUs	Chao1	Shannon
Inoculation of EGFP-expressing *E. coli*	WAS_0	8376	33309	12.22
	WAS_8	8612	34136	12.31
	WAS_24	8332	33949	12.21
	WAS_48	8124	31168	12.2
	EGFP_0	7580	27210	11.98
	EGFP_8	10651	69932	12.83
	EGFP_24	8165	29603	12.2
	EGFP_48	8513	31371	12.36
Tetracycline addition	CTRL_0	2861	16316	11
	CTRL_24	2930	15565	11.13
	CTRL_48	2634	11096	10.92
	TET_0	2613	11480	10.79
	TET_24	2470	15691	10.25
	TET_48	2154	11022	9.92
Chloramphenicol addition	CTRL_0	2929	15257	11.15
	CTRL_24	3139	19424	11.31
	CTRL_48	2959	17195	11.15
	CM_0	3148	22443	11.3
	CM_24	2972	20465	11.06
	CM_48	2728	18210	10.67

Our findings demonstrated that the bacterial community composition in the WAS that were not inoculated with EGFP-expressing *E. coli* underwent only minor changes during the 48 hours of incubation. Within phylum *Proteobacteria,* the *Betaproteobacteria, Alphaproteobacteria* and *Gammaproteobacteria* were maintained during incubation with slightly higher *Betaproteobacteria* percentage at 8 hour of incubation while *Deltaproteobacteria* showed a decreasing trend. Furthermore, only *Sphingobacteria* was increased in abundance towards incubation time while other dominant bacteria showed either a minor increased or decreased trends. On the other hand, WAS inoculated with EGFP-expressing *E. coli* showed some changes in the community. Within *Proteobacteria,* the *Deltaproteobacteria* and *Alphaproteobacteria* showed decreased and increased trend, respectively. Some bacteria showed slightly high percentage of abundance including *Alphaproteobacteria, Nitrospira* sp. (*Nitrospira*), *Kouleothrix* sp. (*Chloroflexi*), and *Gemmata* sp. (*Plantomycetia*) when compare with non-inoculated WAS at the end of incubation (Figure 3.3b). These communities were initially considered as soldiers candidates that kill the inoculant in WAS.
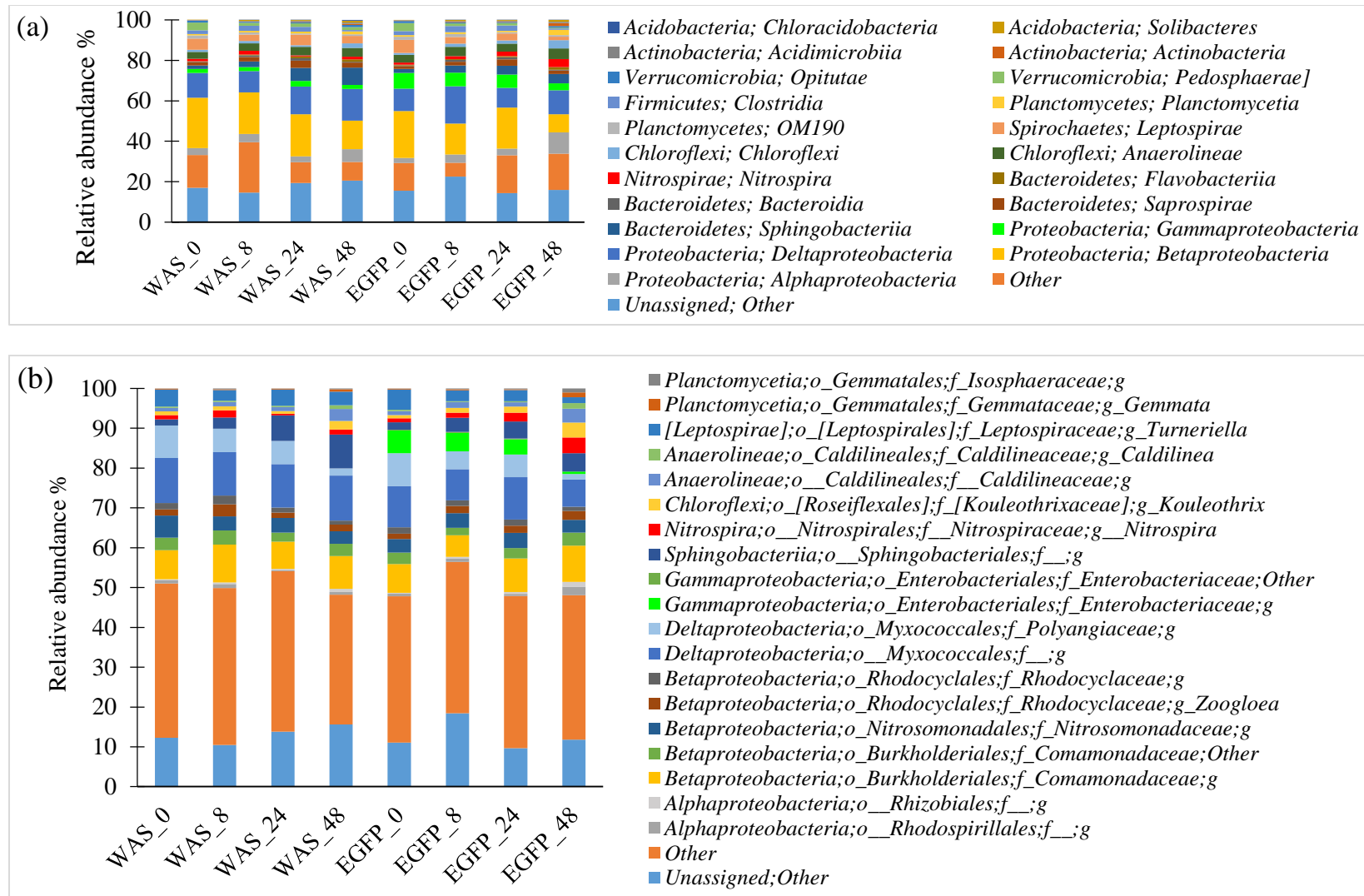
Figure 3.3: Relative abundance of dominant microbial community's dynamics categorized as class (a) and other levels (b) of taxonomy in WAS (WAS_) and WAS with inoculation of EGFP-expressing *E. coli* (EGFP_) from 0 to 48 hours. Results derived from high-throughput 16S rRNA sequencing. Minor classes (> 1%) were summed up in group 'Other'.

The minor dynamics of bacterial community in WAS with or without EGFP-expressing *E. coli* was also proved by the principle coordinate analysis (PCoA). PCoA was done to analyze the similarity of the bacterial community with and without inoculant. Figure 3.4a shows the similarities of both samples were based on the incubation time. Three groups can be observed on their similarity representing in the initial (0 h), middle (8 h and 24 h) and final (48 h) incubation period. This indicate that the major communities of bacteria in WAS were mainly changed according to incubation time, regardless of the inoculation. Overall, the inoculation of EGFP-expressing *E. coli* was not changed the bacterial community of WAS but still affected on some group of indigenous bacterial.

By the inoculation, some indigenous bacterial communities might felt insecure as they recognized the inoculant as alien bacteria. Thus, these communities became a soldier to protect their environment. In order to find the bacterial soldier, one simple assumption is that their abundance will increase as they get the benefits from dead inoculant. Similarly, according to Velicer and Mendes-Soares (2009), the dead cell will creates a `public good` in the form of consumable nutrients for predators or other nearby resistant bacteria for their growth.
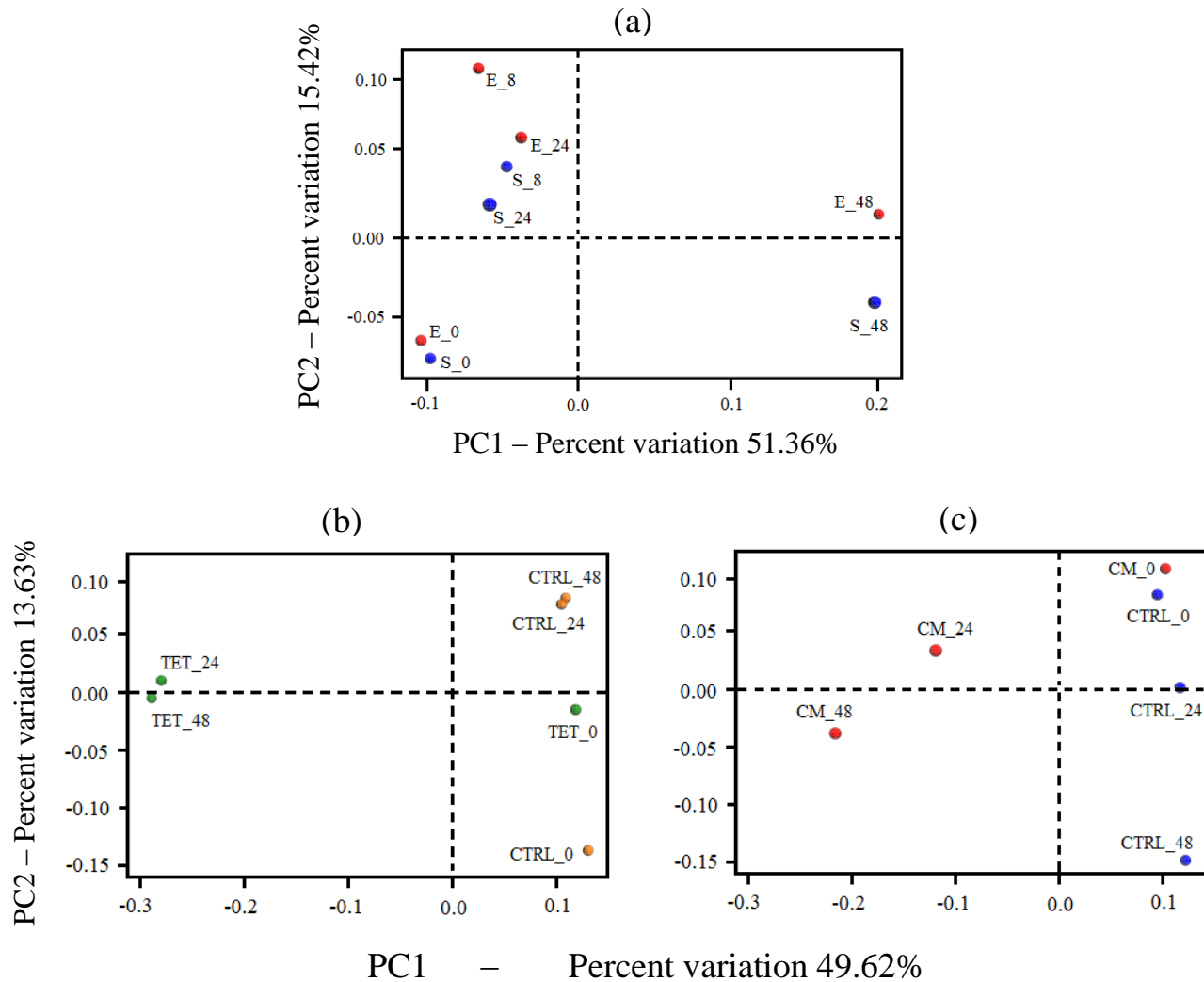
Figure 3.4: Principle coordinate analysis (PCoA) by inoculation of EGFP-expressing *E. coli* in WAS (a) and with or without antibiotic tetracycline (b) and chloramphenicol (c) addition in inoculated WAS. The 2D-plot illustrates the compositional similarity between samples based on weighted UniFrac.

Some classes showed higher abundance percentage including *Betaproteobacteria*, *Alphaproteobacteria, Chloroflexi, Nitrospira* and *Plantomycetia*. However, the different in percentage was not substantial as the increasing trend of these communities were similar with non-inoculated WAS during the incubation. Still, the existence of an alien bacteria in WAS has induced the indigenous community thus altering their diversity. Some studies also suggested that by the bioaugmentation through inoculation process, the microbial community has shifted even though the degradation of target pollutant did not improved (Seshan et al., 2014; Yang et al., 2016). Since some indigenous bacteria were slightly affected, another approach using antibiotic as natural selection was carried out. The antibiotic approach was used with the aim to evaluate the existence of bacterial soldier towards EGFP-expressing *E. coli* as inoculant. Since the inoculant carrying antibiotic resistant gene was maintained or increased by addition of corresponding antibiotic in WAS, the bacterial soldiers can be evaluated by comparing with antibiotic-free inoculated WAS.

3.3.3   *Antibiotics approach affecting survival of inoculant*

We hypothesized that with the presence of antibiotic, the growth of pEGFP-expressing *E. coli* carrying same antibiotic resistant gene will increase or at least maintained the cell numbers during the incubation in WAS while some other indigenous bacterial that not resistant to this antibiotic will die. Therefore, any potential soldier that kill the inoculant is potential to be determined by comparing the community with and without antibiotic in WAS. The pEGFP-expressing *E. coli* carry betalactamase gene was initially used to determine their growth ability in WAS supplied with carbenicillin. A range of carbenicillin concentration was added in the

inoculated WAS to find the most efficient concentration that will support the growth of pEGFP-expressing *E. coli*. However, all concentration (data not shown) showed similar pattern in inoculant cell numbers as 50 µg/mL (Figure 3.5).
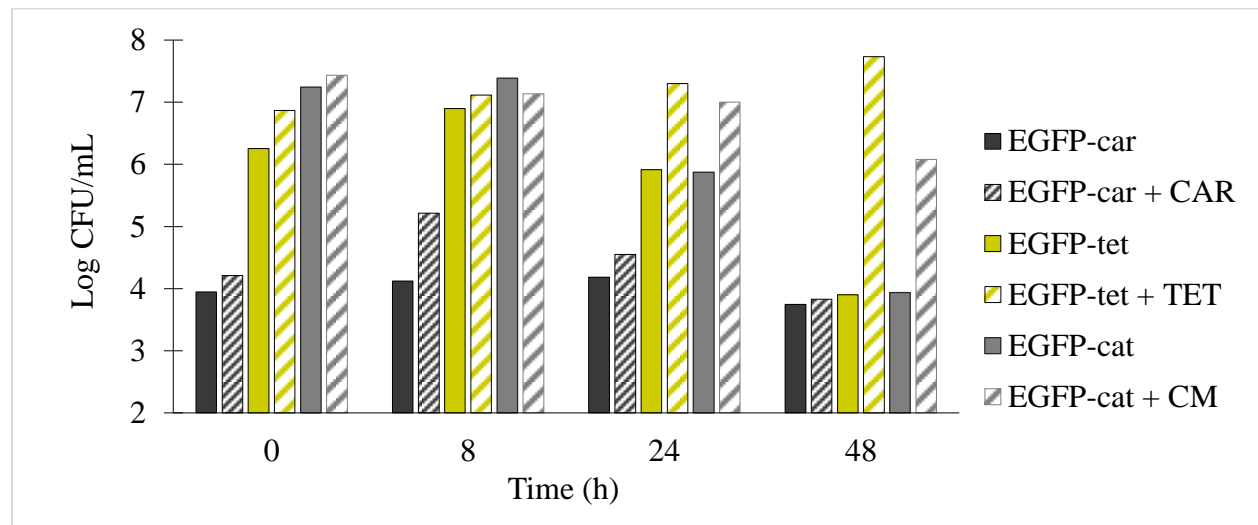
Figure 3.5: Cell numbers of EGFP-expressing *E. coli* with car, tet and, cat resistant gene in WAS without antibiotic (solid) and with addition of their respective antibiotics of carbenicillin, tetracycline and, chloramphenicol (stripe) at 50 µg/mL

The addition of carbenicillin only affective to support the inoculant growth in WAS at 8 hours of incubation and later it was decreased similar to without carbencillin. Due to this situation, another antibiotic resistant gene was constructed in EGFP plasmid and cloned in *E. coli* to observe their growth pattern in WAS by addition of antibiotic. Accordingly, two new pEGFP-expressing *E. coli* were successfully constructed with either tetracycline or chloramphenicol resistant gene. Both strains were used to evaluate their survival in WAS with the presence of corresponding antibiotic. Initially, the addition of 25 µg/mL of tetracycline or chloramphenicol was used in WAS, nonetheless there was no substantial different in inoculant numbers between absent and present of antibiotic (data not shown). Thereafter, 50 µg/mL of antibiotic was added

in inoculated WAS. At this concentration, both inoculant numbers were higher compared to without antibiotic except for chloramphenicol at 8 h (Figure 3.5).

The number of EGFP-expressing *E. coli* with tet gene was gradually increased by tetracycline addition until the end of incubation, while without antibiotic the inoculant only increased at 8 hours and considerably decreased after that. Furthermore, the addition of chloramphenicol also shown an improvement in the cell numbers of EGFP-expressing *E. coli* with cat gene compared to without antibiotic, despite at the end of incubation the cell number was slightly decreased. On top of that, at this antibiotic concentration (50 µg/mL), RNA samples were extracted for further bacterial community analysis.

3.3.4   *Richness and diversity of bacterial communities by antibiotics*

In this antibiotic approach, 2154-3148 OTUs were clustered at the identity cut-off value of 3% for analyzing bacterial communities. As stated in Table 3.1, the number of OTUs varies based on different antibiotic additions and incubation times. OTUs were slightly higher in WAS with inoculation of EGFP-expressing *E. coli* carrying chloramphenicol resistant gene compared to inoculation of EGFP-expressing *E. coli* carrying tetracycline resistant gene, disregard of antibiotic addition for both inoculant type. The changes in Chao1 values were also used to estimate the total number of OTUs. OTUs and Chao1 values are indicators to demonstrate the richness of bacterial community with or without the addition of antibiotics in inoculated WAS. The Shannon index which used to estimate the bacteria population diversity in WAS with or without the addition of antibiotics was ranged from 10.67-11.31 and 9.92-11.13 for

chloramphenicol and tetracycline, respectively. Interestingly, by addition of antibiotic, either chloramphenicol or tetracycline in WAS, the OTU numbers and Shannon index were decreasing accordingly with incubation time. This proved that the antibiotics application has targeting on the non-resistant bacteria communities, thus reducing the richness and diversity of total bacteria communities in WAS. The details of the bacterial diversity changes and the possible soldier exist were evaluated from class level of taxonomic bacterial community.

In order to analyze the similarity of the bacterial community with and without the antibiotic additions, a principle coordinate analysis (PCoA) was conducted using OTUs at a dissimilarity level of 0.03. Figure 3.4b and 3.4c shows the similarity of tetracycline- and chloramphenicol-resistant EGFP-expressing *E. coli*, respectively with and without their corresponding antibiotics. Between inoculated WAS with or without antibiotic, the similarity was only shown during initial incubation. Furthermore, in tetracycline, the similarity can be seen within sample at 24 h and 48 h of the incubation time (Figure 3.4b). The inoculated WAS without tetracycline (CTRL_0-48) was vertically similar and with tetracycline (TET_0-48) was horizontally similar. However, for chloramphenicol, the similarity within sample with or without antibiotic was slightly shown (Figure 3.4c). In addition, inoculated WAS without chloramphenicol (CTRL_0-48) was vertically similar but with chloramphenicol addition (CM_0-48) the community was dissimilar between incubation times but still showed a slightly horizontal similarity. Overall, with or without addition of antibiotics showed the similarity in distance but different in direction as the antibiotics has affected on specific bacteria communities depending on their susceptibility towards antibiotics.

3.3.5   Natural selection by bacterial soldier

Bacteria community analyzed by MiSeq is another proof that showed the ability of pEGFP-expressing *E. coli* to survive in WAS with presence of antibiotic. Without antibiotic, inoculant was dying throughout the incubation as a result of indigenous bacteria recognizing an alien in their environment thus killing by propagating their community. However, addition of antibiotics contributing to natural selection by making the stronger bacteria including inoculant to be resistant but the weaker bacteria including bacterial soldiers to be non-resistant towards the antibiotic and cannot survived. Therefore, declined in the abundance percentage symbolized the possible soldiers. The bacterial communities in WAS with or without addition of antibiotic tetracycline or chloramphenicol was shown in Figure 3.6 and Figure 3.7, respectively with both conditions was inoculated with EGFP-expressing *E. coli* carrying corresponding antibiotic resistant gene.

Gammaproteobacteria in WAS represent the inoculant, EGFP-expressing *E. coli.* From Figure 3.6a, *Gammaproteobacteria* percentage without tetracycline was very low compared with addition of tetracycline which increased drastically at 24 and 48 h. However, when look in details, the *Enterobacteriaceae* percentage (Figure 3.6b) was not in agreement with the CFU numbers (Figure 3.5). Its percentage without tetracycline was higher than with tetracycline addition and even showed a decreasing trend. Still, in the inoculated WAS without tetracycline addition, *Deltaproteobacteria*, *Betaproteobacteria* and *Leptospirae* were the major class showed high percentage of abundance compared to the inoculated WAS with tetracycline addition.
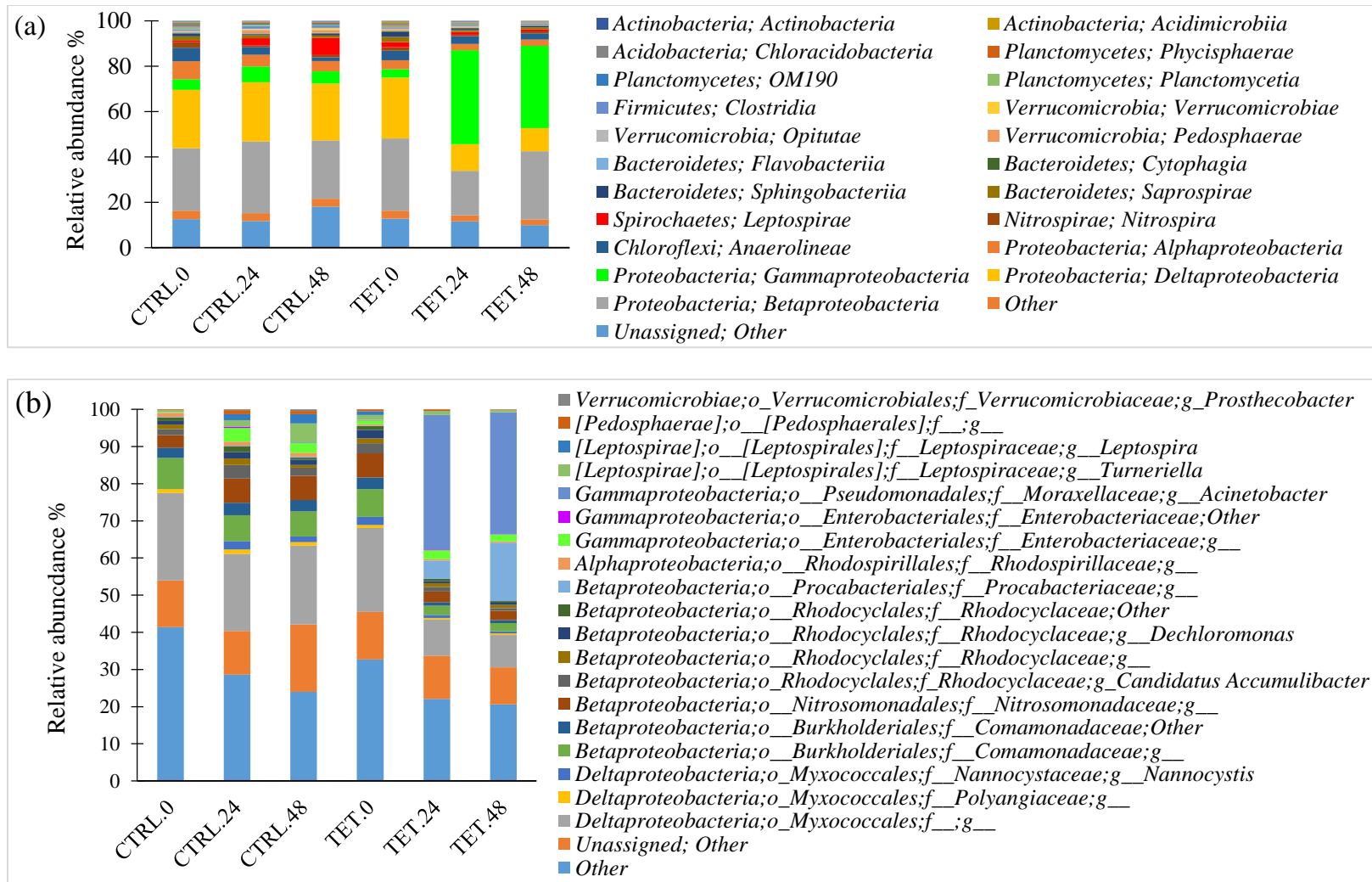
Figure 3.6: Relative abundance of dominant microbial community's dynamics categorized as class (a) and other level (b) of taxonomy in WAS inoculated with EGFP-expressing *E. coli* carrying tet gene without antibiotic (CTRL_) and with tetracycline addition (TET_) from 0 to 48 hours. Results derived from high-throughput 16S rRNA sequencing. Minor classes were summed up in group 'Other'.

From these classes, family or to some extent the genus levels were shown in Fig 3.6b. *Myxococcalles* family belongs to *Deltaproteobacteria,* and *Comamonadaceae* and *Nitrosomonadaceae* family belongs to *Betaproteobacteria* were showed high percentage of abundant without tetracycline. The other communities listed showed minor increment without tetracycline addition, except for *Procabacteriaceae* and *Acinetobacter* that present higher in the addition of tetracycline.

On the other hand, addition of chloramphenicol showed survival of EGFP-expressing *E. coli* in WAS shown in Figure 3.7. The percentage of *Gammaproteobacteria* (Figure 3.7a) was gradually decreased and increased in the absent and present of chloramphenicol, respectively. In addition, the *Enterobacteriaceae* percentage shown in Figure 3.7b that represent the inoculant also showed consistent trend. This result corresponds to the cell numbers of inoculant determined from the agar plate counting. As previously mentioned, the bacterial community with high percentage of abundance is presumably a possible soldier that killed the inoculant. Some major communities showed high abundance percentage without antibiotic compared to with antibiotic addition including *Deltaproteobacteria, Betaproteobacteria, Sphingobacteria, Nitrospira,* and *Planctomycetia*. Specifically, in other levels of taxonomic (Figure 3.7b) were shown the details including *Myxococcalles* (*Deltaproteobacteria*), *Comamonadaceae* (*Betaproteobacteria*) and *Nitrospira* which were major communities that showed higher abundance percentage. Other communities with high percentage without chloramphenicol were also listed.
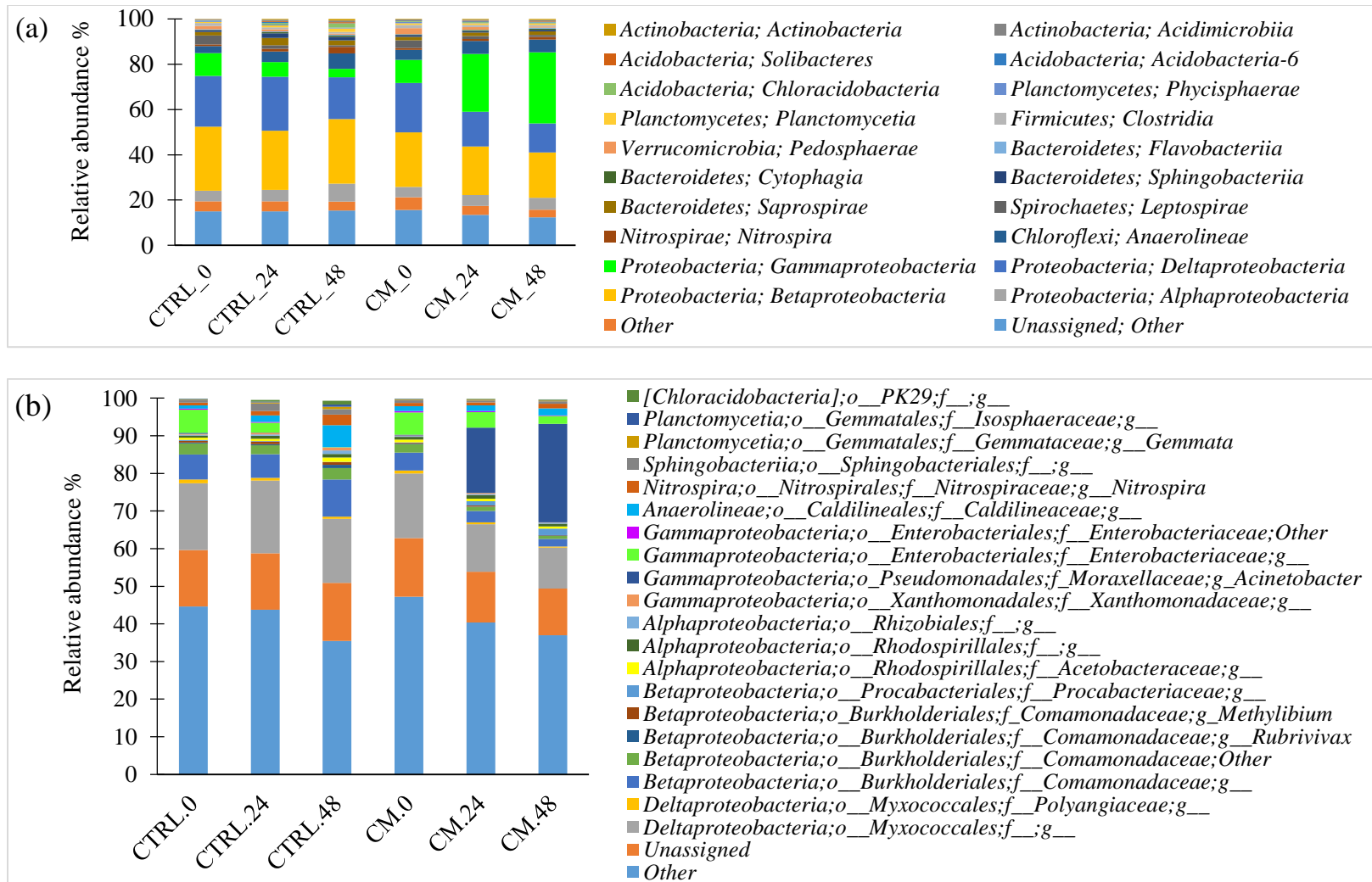
Figure 3.7: Relative abundance of dominant microbial community's dynamics categorized as class (a) and other level (b) of taxonomy in WAS inoculated with EGFP-expressing *E. coli* carrying cat gene without antibiotic (CTRL_) and with chloramphenicol addition (CM_) from 0 to 48 hours. Results derived from high-throughput 16S rRNA sequencing. Minor classes were summed up in group 'Other'.

Similar to tetracycline, *Procabacteriaceae* and *Acinetobacter* were found in higher percentage with the addition of chloramphenicol.

In ecological process, parameters of ecosystem structure and function is affected by predation (Kandel et al., 2014) especially in bioaugmentation process. The antibiotics approach is one of possible method to find the bacterial soldiers. The reason may be that the growth of the dominant species in the sludge was inhibited under antibiotic stress, while more species with low abundance including the inoculant had the opportunity to survive and reproduce. Regardless of inoculant and antibiotic used, *Myxococcalles* and *Comamonadaceae* were expected as bacterial soldier. Besides, other bacterial population listed as possible soldier from both antibiotics used were *Nitrospira*, *Sphingobacteriales*, *Rhodocyclaceae*, *Xanthomonadaceae*, *Caldilineaceae*, and *Leptospiraceae.*

All the listed are a common community's presence during wastewater treatment process and their absence may contribute to malfunctioning or inefficient in treatment. *Myxococcalles* and *Sphingobacteriales* have been categorized as micropredators with gliding motility, performed by lysing other dead or living bacterial (Lueders et al., 2006; Reichenbach, 1999). Besides, *Myxococcalles* was reported to produce toxic secondary metabolite to inhibit *E. coli* RNA polymerase, known as Ripostatin (Reichenbach, 2001). In addition, *Comamonadaceae* was able to kill inoculant as it has been reported to be active in WAS due to high net growth rate (Saunders et al., 2016) and can outcompete other late colonizers (Vuono et al., 2015).

However, *Caldilineaceae* is a filamentous bacteria function as floc-stabilizer and prevent membrane fouling in activated sludge (Yoon et al., 2010). This population is robust and primarily grow on complex polysaccharides and proteins by secreting exoenzymes (Nielsen et al., 2009), presumably not affecting on the inoculant. Hence, the high abundance percentage is due to their robust characteristic and slowly degraded in WAS. Besides, the present of *Nitrospira* might be due to its late colonizing (Gruber-Dorninger et al., 2015) as abundance percentage was increased in WAS with or without chloramphenicol addition. On the other hand, *Leptospira*, motile, pathogenic bacteria was found to be dominant in the inoculated WAS without tetracycline. Barragan et al. (2011) reported that these bacteria may survive in poor-nutrient conditions for long time and also with low population of heterotrophic bacteria. In addition, *Leptospira* has been strongly reported to be susceptible to tetracycline (Johnson, 1982). Therefore, based on researches done by others, not all the listed bacteria can be considered as bacterial soldiers.

In order to prove that other expected bacterial soldiers existed in WAS such as *Myxococcalles*, *Comamonadaceae* or *Sphingomonadales* has an ability to kill the EGFP-expressing *E. coli*, a co-culture interaction between inoculant and bacterial soldiers was done to provide a direct evidence for this assumption.

3.3.6   Direct evidence on bacterial soldiers through co-culture interaction

The ability of possible bacterial soldiers in killing the inoculant was determine by indirect co-culture interaction using 6-well hanging culture insert. Two types of inoculants, EGFP-

expressing *E. coli* resistant to chloramphenicol and tetracycline were used in this experiment for co-culture with all expected bacterial soldiers. The bacteria acted as soldier were selected from isolated bacteria in WAS and NBRC strain (Table 3.2). The NBRC strains selected were based on the bacteria communities obtained from MiSeq analysis using antibiotics approach which belongs to few family level of taxonomy such as *Myxococcaceae*, *Comamonadaceae*, *Sphingomonadaceae*, and *Xanthomonadaceae*.

Table 3.2: List of isolated and expected bacterial soldiers in WAS

	Bacteria	Origin	Taxonomy (Order_Family)
Isolated bacteria	*Acinetobacter sp.*	WAS	*Pseudomonadales_Moraxellaceae*
	Pseudomonas sp.	WAS	*Pseudomonadales_Pseudomonadaceae*
	Klebsiella pneumoniae	WAS	*Enterobacteriales_Enterobacteriaceae*
	Shigella sp.	WAS	*Enterobacteriales_Enterobacteriaceae*
	Shewanella sediminis	WAS	*Alteromonadales_Shewanellaceae*
	Chrysobacterium sp.	WAS	*Flavobacteriales_Flavobacteriaceae*
NBRC strains	*Caldimonas manganoxidans*	Hot spring	*Burkholderiales_Comamonadaceae*
	Diaphorobacter sp.	Manure compost	*Burkholderiales_Comamonadaceae*
	Acidovorax sp.	Toilet flush	*Burkholderiales_Comamonadaceae*
	Alcaligenes faecalis	Soil	*Burkholderiales_Alcaligenaceae*
	Myxococcus virescens	Seaside soil	*Myxococcales_Myxococcaceae*
	Myxococcus xanthus	Granular sludge	*Myxococcales_Myxococcaceae*
	Sphingomonas terrae	Activated sludge	*Sphingomonadales_Sphingomonadaceae*
	Thauera butanivorans	Activated sludge	*Rhodocyclales_Rhodocyclaceae*
	Lysobacter concretionis	Granular sludge	*Xanthomonadales_Xanthomonadaceae*
	Xanthobacter autotropicus	Black pool sludge	*Rhizobiales-Xanthobacteriaceae*
	Rubrivivax gelatinosus	Wastewater food	*Rhodocyclales_Rhodocyclaceae*
	Gemmobacter nectariphilus	Activated sludge	*Rhodobacterales_Rhodobacteraceae*

The isolated bacteria obtained from inoculant agar plate were sequenced and identified as listed in Table 3.2. These isolated bacteria were considered as bacterial soldiers as they were able to grow on agar made up of inoculant as their nutrients. However, all the isolated bacteria were not in the same order or family level of taxonomy as the bacterial soldiers expected from antibiotics

approach through MiSeq analysis. Therefore, the co-culture interaction was carried out for all these isolated bacteria to determine whether they can act as bacterial soldiers in killing the EGFP-expressing *E. coli* used as inoculant in WAS.

Figure 3.8 shows the cell numbers and fluorescence of EGFP-expressing *E. coli* when co-culture with previous isolated bacteria. The cell number of *E. coli* was reduced during the interaction with the isolated bacteria for both *E. coli* resistant to chloramphenicol and tetracycline, Fig. 3.8a and 3.8b, respectively. However, interaction of *E. coli* resistant to tetracycline with *Pseudomonas* sp. had only slight reduced in the cell numbers. This might be due to the initial number of *E. coli* was higher in this samples, which also shown by interaction with *Chrysobacterium* sp. The isolated bacteria which expected as bacterial soldiers in sludge caused more reduction in inoculant number for tetracycline-resistant *E. coli* compare to chloramphenicol due to the initial number of EGFP-expressing *E. coli* used.

In addition, the fluorescent intensity of EGFP-expressing *E. coli* was detected to determine the effect of isolated bacteria on the inoculant cell. Reduction in fluorescent intensity indicates less cell numbers of *E. coli* EGFP presence in the cultures. Fig. 3.8c shows the reduction in fluorescent intensity of chloramphenicol-resistant EGFP-expressing *E. coli* cultures as compared to control, which support the decreased in *E. coli* cell numbers. Besides, the fluorescence of tetracycline-resistant EGFP-expressing *E. coli* also decreased when interacts with expected bacterial soldiers, but showed similar result with the control. However, the reduction of fluorescent intensity in control sample might be due to the *E. coli* ability to retain the fluorescent plasmid during the incubation. Overall, as compared to control culture, all isolated bacteria from

inoculant agar plate were proved to be bacterial soldiers present in WAS which responsible in killing the inoculant, EGFP-expressing *E. coli*.
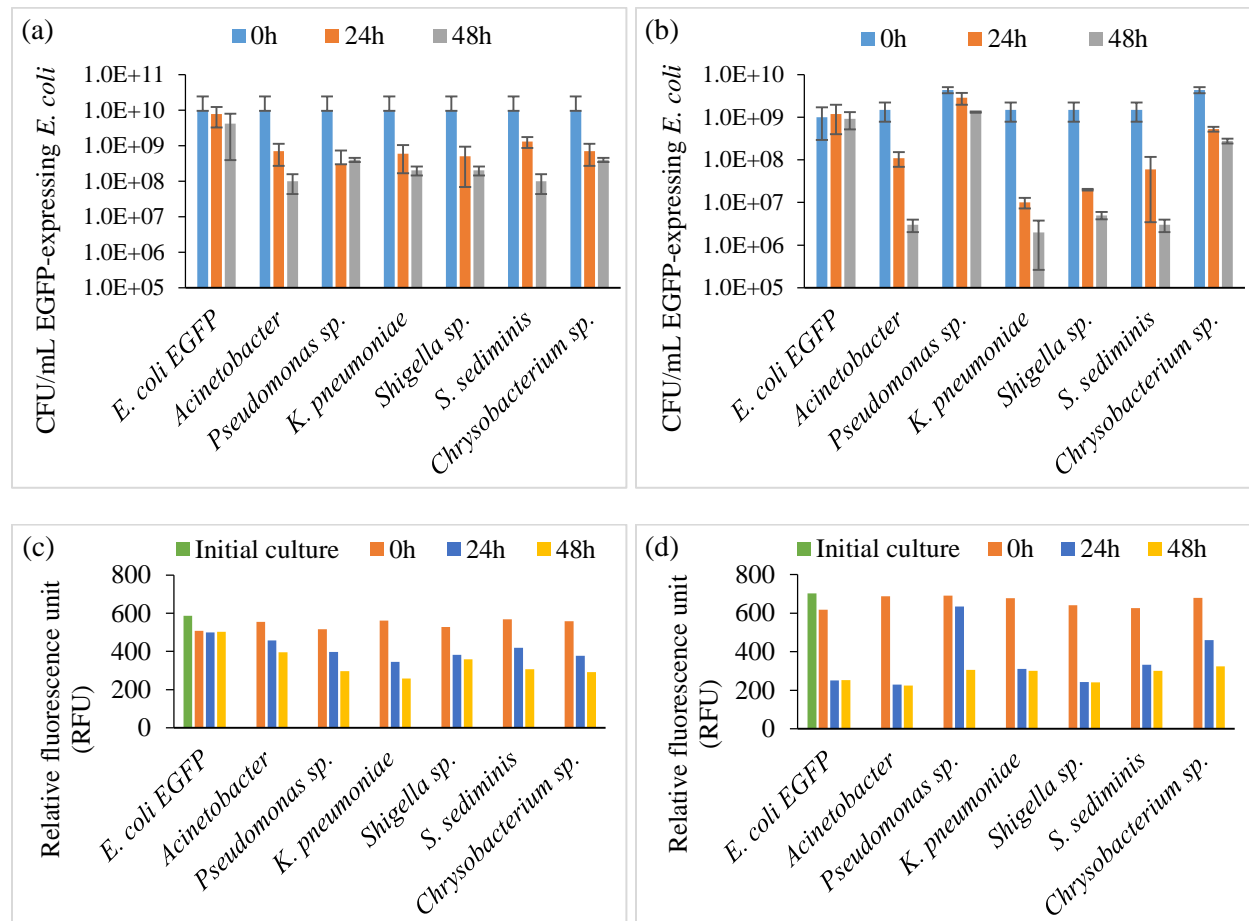
Figure 3.8: Cell numbers and fluorescence value of EGFP-expressing *E. coli* resistant to chloramphenicol (a) and (c) and tetracycline (b) and (d), respectively when co-culture with isolated possible bacteria soldiers.

On the other hand, the co-culture interaction was also done between both antibiotic-resistant *E. coli* with other possible bacterial soldiers obtained based on MiSeq analysis. From the microbial community analysis, *Myxococcaceae*, *Comamonadaceae* and *Sphingomonadaceae* were the most expected bacteria that killed the inoculant in WAS.
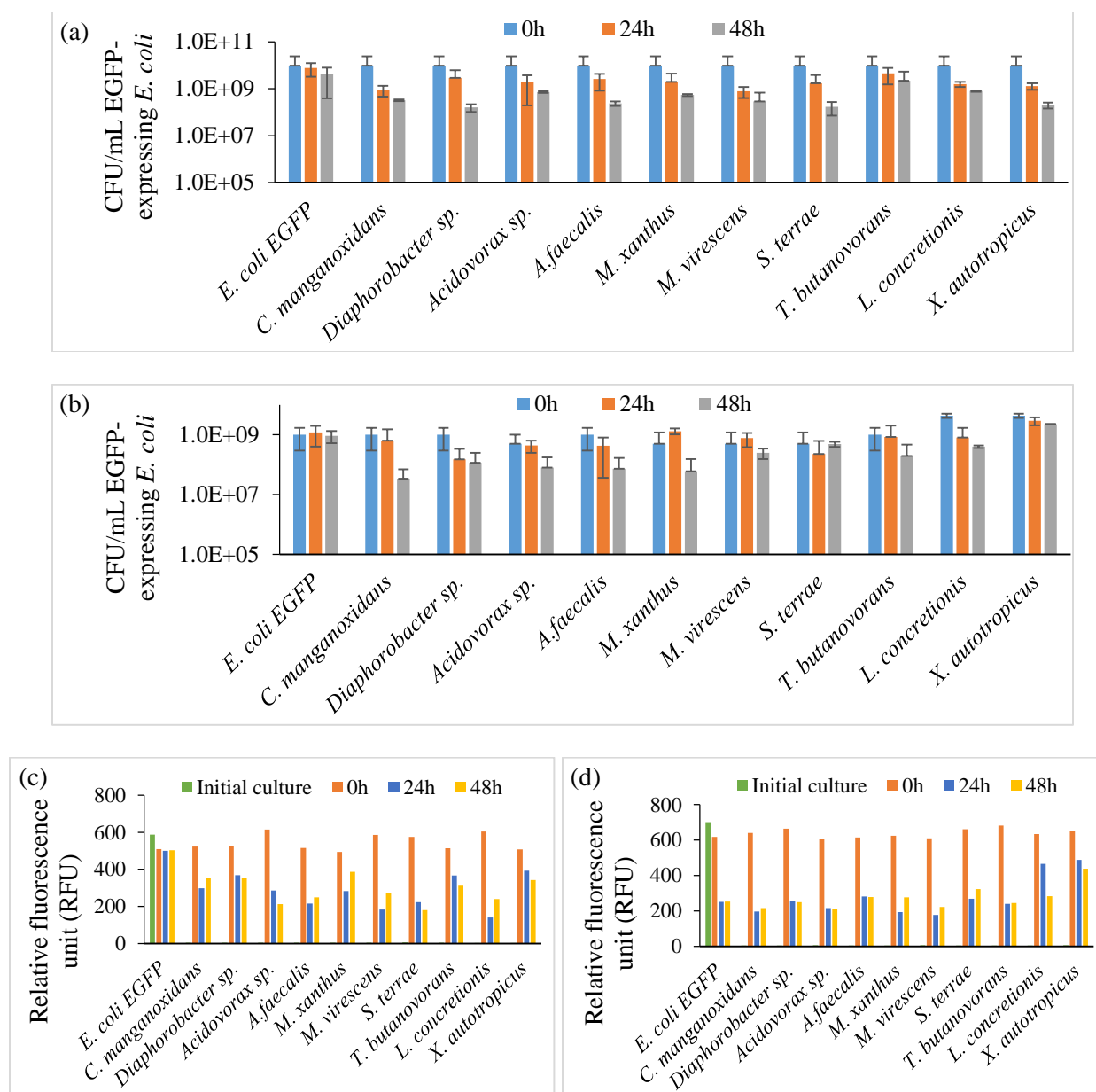
Figure 3.9: Cell numbers and fluorescence value of EGFP-expressing *E. coli* resistant to chloramphenicol (a) and (c) and tetracycline (b) and (d), respectively when co-culture with NBRC strains expected as bacterial soldiers.

Thus, some strains belongs to these families were co-culture with EGFP-expressing *E. coli* to find the evidence of their ability to act as bacterial soldiers. Fig. 3.9 shows the cell number of inoculant and its fluorescent intensity during 48 hour of co-culture interaction. From Fig. 3.9a, *M.*

xanthus and *M. virescens* (*Myxococcaceae*), *C. manganoxidans*, *Diaphorobacter* sp. and *Acidovorax* sp. (*Comamonadaceae*), and *S. terrae* (*Sphingomonadaceae*) as the most expected possible soldiers have reduced the cell numbers of EGFP-expressing *E. coli* with chloramphenicol resistant gene during co-culture. In addition, other selected bacteria soldiers also have reduced this *E. coli* cell numbers as shown in Fig. 3.9a.

Contradictorily, the co-culture interaction of bacterial soldiers with tetracycline-resistant EGFP-expressing *E. coli* did not showed better reduction in their cell numbers as compared to chloramphenicol-resistant *E. coli*, except for bacteria belongs to *Comamonadaceae* family and *A. faecalis*. In addition, selected bacteria belongs to *Myxococcaceae* and *Sphingomonadaceae* showed an increment on inoculant numbers either at 24 hour or 48 hour which not indicated them as bacterial soldiers towards tetracycline-resistant EGFP-expressing *E. coli*. These inoculant cell numbers were supported by the fluorescence detected from the co-culture interaction for both types of antibiotic-resistant inoculants. The tested bacteria based on microbial community analysis with antibiotics approach indicated that *Comamonadaceae* was strongly acted as bacterial soldiers towards inoculant, EGFP-expressing *E. coli* in WAS through indirect co-culture interaction. This is supported by other studies that *Comamonadaceae* has high growth rate and able to compete with other slow-growth bacteria (Saunders et al., 2016; Vuono et al., 2015). Other expected bacterial soldiers might have the ability to kill the inoculant in WAS, but this indirect co-culture method may not suitable to provide the evidence on their ability to act as bacterial soldiers. Also, antibiotics approach may only demonstrated part of bacterial soldiers exist in WAS as the abundance percentage of isolated bacteria detected using MiSeq analysis was not high.

3.4   Conclusion

In conclusion, the determination of microbial community structure of WAS is important to evaluate the presence of communities acting as bacterial soldier affecting on exogenous inoculant during bioaugmentation. Antibiotic approach, an example of natural selection is the best solution to observe bacterial soldiers by exploitation of inoculant carrying EGFP plasmid as tracing marker. All the isolated bacteria from WAS were proved that they can act as bacterial soldiers in killing the inoculant. Some species belongs to *Myxococcales*, *Comamonadaceae* and, *Sphingomonadaceae* also are the potential bacterial soldiers existed in WAS, especially *Comamonadaceae* as evidenced through co-culture interaction with EGFP-expressing *E. coli*. Elimination of these bacterial soldiers may retain the inoculant in WAS thus improving the pollutants degradation efficiency process which finally contribute to bioremediation succession.

CHAPTER 4

IMPACT OF DIFFERENT ANTIBIOTICS ON METHANE PRODUCTION USING WASTE ACTIVATED SLUDGE: MECHANISM AND MICROBIAL COMMUNITY DYNAMICS

4.1   Introduction

Wastewater treatment plants (WWTP) systematically treat the wastewater collected from various sources such as households, industrial sites, and hospitals. The secondary treatment in this system creates an abundant by-product called waste-activated sludge (WAS). The Hiagari Wastewater Treatment Plant in Kitakyushu, Japan, produces approximately 7530 m^3 of WAS daily (Maeda et al., 2011). Normally, further treatment of the generated WAS accounts for about 60% of the total WWTP operating costs (Guo et al., 2015). Several attempts have been made to treat and reduce the amount of WAS that gets generated in WWTPs. An ultrasonic treatment was utilized to solubilize WAS (Bougrier et al., 2005; Dewil et al., 2006), and other treatments, such as thermal and chemical treatments (Bougrier et al., 2006; Valo et al., 2004), have been conducted to utilize WAS in bioenergy production.

Anaerobic digestion is another powerful approach that uses a biological reaction for the treatment of WAS. It has been widely applied as an efficient solution for environmental pollution and energy draining. Methane is considered to be the most promising bioenergy generated, because organic matter from WAS can be converted into 60%−70% volume of methane to meet both of the requirements of an efficient solution (Appels et al., 2008; Nguyen et al., 2014). Four

anaerobic digestion stages are involved in the biological production of methane, namely hydrolysis, acidogenesis, acetogenesis, and methanogenesis (Bareither et al., 2013; Guo et al., 2015; Mohd Yasin et al., 2015; Nguyen et al., 2014). All stages in this process are driven by various types of microorganisms. WAS acts as a substrate in the process of anaerobic digestion for methane production. In the first hydrolysis stage, large WAS molecules, including proteins or carbohydrates, are degraded and converted into smaller molecules such as amino acids or monosaccharides. The fermentation of these molecules into carboxylic acids (e.g., butyrate or propionate), hydrogen, and carbon dioxide during the acidogenesis stage is combined with the acetogenesis stage, which is driven by acetogenic bacteria to produce acetate (Sanz et al. 1996). Finally, methane producers, Archaea or methanogens, utilize acetate to produce methane during the methanogenesis stage.

Thus, complex microbial communities in WAS are indeed essential for the anaerobic digestion process; however, the best conditions for methane fermentation from WAS have still not been found due to the poor investigations regarding microbial interactions and regulatory systems. Our previous study demonstrated the effect of azithromycin (AZM), a macrolide-type antibiotic for enhanced methane production from WAS (Nguyen et al., 2014). The results of a brief microbial community analysis by denaturing gradient gel electrophoresis (DGGE) determined the activation of *Clostridium* species. AZM functions to inhibit the biological protein synthesis through the interaction with the 50S subunit of the bacterial ribosome (Shimada et al., 2008). Chloramphenicol (CM) and kanamycin (KAN) antibiotics have the same inhibitory action as AZM; however, they may have a different antibacterial spectrum. The antibacterial spectrum for AZM is primarily Gram positive bacteria, including streptococci and staphylococci,

Enterobacteriaceae, and other rapidly growing pyogenic bacteria (Papich, 2010; Williams, 1991). Besides, CM is well known as having a broad range of antibacterial spectra, including Gram positive, Gram negative, aerobic, and anaerobic bacteria (Schwarz et al., 2004). In addition, KAN antibacterial spectra activity includes Gram negative bacteria and some Gram positive bacteria, but it has a weak activity toward anaerobic bacteria (Papich, 2010). Sanz et al. (1996) reported that CM inhibited anaerobic digestion systems even at a low concentration, whereas KAN showed a negligible effect.

The different microbial communities under these antibiotics may provide valuable information toward our understanding of microbial interactions and regulatory systems during the anaerobic digestion process of WAS, because quite different microbial activity patterns should be seen in the WAS with or without each antibiotic due to the different antibacterial spectra. Currently, the composition of a microbial community may be easily evaluated using molecular techniques. In fact, different methodologies have already been developed to determine the microbial community structure of various biological systems such as a clone library, next-generation sequencing, fingerprinting techniques, real-time polymerase chain reaction (PCR) and so on. Several investigative studies have been done to understand how microbial interactions are altered by antibiotics inside a complicated microbial consortium such as WAS, using next generation sequencing technology (Ng et al., 2015; Su et al., 2015; Zhang et al., 2011). To date, many studies have been carried out using less advanced methods such as PCR-DGGE (Akyol et al., 2016; Aydin et al., 2015; Lins et al., 2015), terminal restriction fragment length polymorphism (Meng et al., 2015), or fluorescence in situ hybridization (Shimada et al., 2011). The DGGE application has great value for evaluating environmental microbiology but it does have some

limitations. For example, the assumption that bands at the same position in the DGGE gel are equal to one genome is invalid. This is because bands that have a genetic similarity may not necessarily have similar migration patterns in the DGGE gel (Jackson et al., 2000). In addition, these conventional methods are unable to detect most of the low-abundance microbes that are present in WAS compared to next-generation sequencing technology (Wang et al., 2012).

Therefore, in the situation of methane fermentation (anaerobic digestion) using WAS, the effect of several antibiotics (AZM, CM, and KAN) was investigated in this study to understand and detect the important functions for the efficient anaerobic digestion of WAS. In particular, a microbial community analysis using high-throughput sequencing technology (MiSeq Illumina platform) was performed by using the ribonucleic acid (RNA) extracted from WAS in the presence or absence of each antibiotic to evaluate the microbial dynamics of really active microbes during the fermentation from WAS.

4.2   Materials and Methods

4.2.1 Activated sludge

The WAS was prepared to final concentration of 10% (wet-sludge w/w) with distilled water prior to use in all experiments in this study.

4.2.2 Antibiotics

AZM was purchased from LKT Laboratories Inc. (MN, USA) and CM and KAN were purchased from Wako Pure Chemical Industries, Ltd. (Japan). All antibiotics were dissolved in ethanol for AZM and CM stock concentrations of 6 mg/mL and 30 mg/mL, respectively, and into distilled water for KAN (50 mg/mL) as a stock solution. The stock solutions were used to adjust the final concentrations to 15 µg/mL for each antibiotic into the WAS.

4.2.3 Methane assay

The total volume of 30 mL WAS with or without 15 µg/mL of each antibiotic was filled in 66 mL vials. The vials were tightly sealed with butyl rubber stoppers, crimped, and sparged with nitrogen gas for 2 min to provide an anaerobic condition. Then, the vials were incubated at 37°C for 120 rpm. Each experiment was conducted in at least triplicate. Methane was measured by injecting 50 µL of headspace gas in vials into a GC-3200 gas chromatograph (GL Science, Japan).

4.2.4 Analytical methods

WAS samples during the fermentation were used for the following analyses; organic acids, pH, protein concentration, and protease activity. Initially, the fermented WAS samples were centrifuged at 13,000 rpm for 7 min to collect the supernatants which were later filtered through a 0.2-µm membrane syringe filter. Organic acids in each supernatant were analyzed using high-

performance liquid chromatography (Shimadzu LC-10AD) as described previously (Mohd Yusoff et al., 2012). The pH was measured using a compact pH meter (AS ONE, AS-211, Japan). The soluble protein concentration was analyzed by the Lowry method using bovine serum albumin (BSA) as a standard protein (Lowry et al., 1951). The protease activity was measured as described previously (Maeda et al., 2011). One unit of protease activity was calculated as the quantity of tyrosine (μmol) produced from casein by 1 mg of enzyme per min.

4.2.5   RNA extraction and cDNA synthesis

RNA extraction was conducted using sludge pellets of fermented sludge. The RNA later solution was used for pellet preparation and details method as described in section 2.3.1. Total RNA was extracted using RNeasy kit (Qiagen Inc., Valencia, CA) as described in our previous paper (Mohd Yusoff et al., 2012). The cDNA was synthesized from RNA using PrimeScript RT reagent kit Perfect Real Time (TAKARA Bio Inc., Shiga, Japan). The cDNA was used later for quantitative real time polymerase chain reaction (qRT-PCR) to determine bacterial activity and archaeal activity as well as microbial communities of Bacteria and Archaea population.

4.2.6   qRT-PCR quantification

The qRT-PCR was performed to quantify the 16S rRNA gene of total Bacteria and Archaea using a TaqMan system. The StepOne Real Time PCR System (Applied Biosystem) was used for amplification and detection of fluorescence by using primers and probes listed in Table 2.1.

4.2.7 High-throughput 16S rRNA sequencing

The V4-V5 region of 16S rRNA gene was used as a target of PCR amplification for the high-throughput 16S rRNA sequencing. Each concentration of purified PCR products was then determined using Qubit dsDNA HS Assay Kit (Life Technologies, Oregon, USA). The PCR products were processed using Nextera XT DNA Library Preparation Kit according to the Illumina manufacturer protocol. The pooled samples were loaded in the 500-cycle V2 MiSeq reagent cartridge (Illumina) and then onto the MiSeq instrument along with the cleaned flow cell. Sequencing was performed for 251, 8, 8, and 251 cycles for forward Index 1, Index 2, and reverse reads, respectively.

The raw paired-end reads were processed using QIIME v1.9.0 (Caporaso et al., 2010). The high-quality reads were clustered into operational taxonomic units (OTU) at 97% sequence similarity using *de novo* OTU picking pipeline prior to assign taxonomy and alignment against Greengenes database v13.8 (DeSantis et al., 2006) using PyNAST program (Caporaso et al., 2010; DeSantis et al., 2006). The raw sequence data were deposited into the NCBI short reads archive database under accession number: SRP072534.

4.2.8 Statistical analysis

The different antibiotics were compared with control WAS using means from at least triplicate data (n = 3). Comparison was performed using means and standard deviations by the Student's *t*-test (GraphPad software) at a significance level of $p < 0.05$.

4.3 Results and discussion

4.3.1 Effect of each antibiotic in methane fermentation from WAS

This study was investigated how methane fermentation of WAS can influence by using three types antibiotics, AZM, CM, and KAN. They have a certain potential to change the total microbial activity (dynamics) due to the different antibacterial spectra of each antibiotic against the endogenous microbes present in WAS. First, using WAS with 15 μg/mL of AZM, CM, or KAN or antibiotic-free (control), anaerobic fermentation was performed for 10 days to determine the differences of methane production in the presence or absence of each antibiotic. As shown in Fig. 4.1, the addition of AZM showed a 2x higher methane production than the antibiotic-free WAS. The accumulation of methane significantly increased after the second day of fermentation in the WAS in the presence of AZM ($p < 0.05$). By contrast, CM had a significant ($p < 0.05$) inhibitory effect on methane production with the highest production rate of only 684 ± 100 μmol/g VS, and the accumulation of methane was observed after the fifth day of fermentation; there was no methane on the second day.

In addition, methane production from WAS in the presence of KAN was significantly similar to the antibiotic-free WAS. Thus, despite the addition of antibiotics which should change the microbial activity due to their bactericidal effects, three patterns of methane production were observed: 1) a positive impact where methane production was improved in the presence of AZM; 2) a negative impact where CM inhibited methane production; and 3) none or less of an impact with or without KAN. Understanding of the different mechanisms between the three patterns of

methane production may represent an outstanding finding which should be important for the comprehensive understanding of methane fermentation from WAS; in particular regarding microbial interactions (for example, inhibition, repression, acceleration, and dependence). The different effects to methane fermentation should be due to the change of microbial activity in WAS at the hydrolysis, acidogenesis/acetogenesis, and methanogenesis.
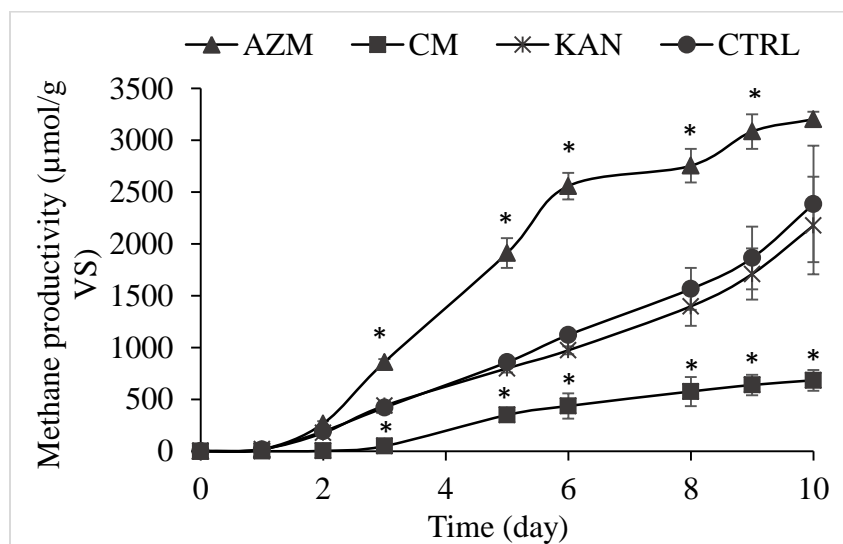
Figure 4.1: Methane production during 10-days of anaerobic digestion using waste-activated sludge with and without any antibiotic as a control. Error bars indicate standard errors (n = 3). *Indicates a significant difference by antibiotic addition.

In this study, AZM promoted the growth of microbes that contributed to improve methane production. However, other types of macrolides used in anaerobic digestion mostly provide a contradictory impact unlike the result in this study. For example, a high concentration of tylosin, a 16-membered ring macrolide showed a certain inhibitory effect on biogas production due to the accumulation of intermediates and pH drop (Shimada et al., 2008). Erythromycin, a parental antibiotic of AZM showed only a slight effect on methanogenic activity but inhibited the

conversion of some volatile fatty acids (Amin et al., 2006), while Du et al. (2015) obtained oppositely. In this study, CM inhibited almost 3-fold of methane production when compared to the control. Also, it may takes longer for some methanogenic bacteria to adapt and acclimatize in this condition since the methane only started to produce after 4 days. Therefore, CM did not affect the microbial community in initial stages of anaerobic digestion as the protease activity and the soluble intermediates (proteins and organic acids) were detected in a high activity and a high concentration from the samples. Additionally, the presence of CM in WAS suppressed the methane production might be due to the inhibition of microbes at only the methanogenesis stage (Jin et al., 2011; Lallai et al., 2002; Sanz et al., 1996) without inhibiting other pathways. However, a previous study by Lins et al. (2015) showed that addition of 100 µg/mL CM did not differ in gas production from the control unlike our results that a low concentration (15 µg/mL) of CM was used. The archaeal population plays an important role in the methanogenesis stage that should be responsible to produce methane from acetate and CO_2/H_2.

4.3.2 *Bacterial activity in the presence of each antibiotic at the hydrolysis*

To determine the differences in the results observed for AZM, CM, and KAN, their bacterial activity at the first hydrolysis step was compared. In the hydrolysis step, complex polymers such as proteins, carbohydrates and lipids are being degraded, resulting in producing readily available organic materials such as amino acids, sugars, and long chain fatty acids, respectively (Appels et al., 2008; Manyi-Loh et al., 2013). This process was primarily contributed by hydrolytic bacteria that secreted extracellular enzymes such as protease, amylase, or lipase to produce substrates for acidogenic microorganisms. Since large molecules of WAS should be converted into small

molecules, degrading products and enzymes are great targets to evaluate the hydrolysis process. Based on the idea that protein is one of the major components in WAS (Maeda et al., 2009), the soluble protein concentration and protease activity were measured and compared in the three antibiotics (Figure 4.2a and 4.2b). The concentration of soluble proteins from WAS during anaerobic digestion in the presence of AZM or CM was higher than that in KAN and the control throughout the incubation period. In the WAS with KAN, the protein concentration had the same trend as the control at an early period (2 days); however, was slightly higher than the control after 7 days.
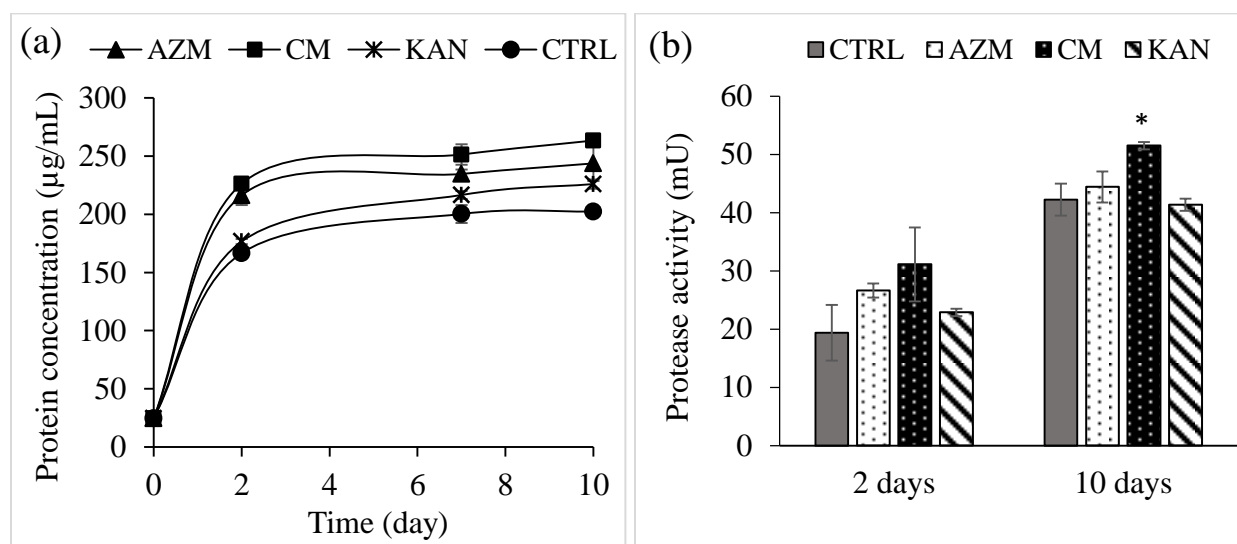
Figure 4.2: Soluble protein concentration (a) accumulated during anaerobic digestion using waste-activated sludge with and without any antibiotic as a control. Protease activity (b) detected in the sample with and without any antibiotic as a control during anaerobic digestion. Error bars indicate standard errors (n = 3). *Indicates a significant difference by antibiotic addition.

The corroborating evidence was obtained from the result that protease activity in the WAS samples with AZM and CM was higher than the control at 2 days (Figure 4.2b). Finally, there was a clear relationship between protein concentration and protease activity. The addition of CM and other antibiotics in WAS did not affect the hydrolytic bacteria that release protease enzymes as the secretion of protease enzyme was almost no difference between antibiotic addition and antibiotic-free WAS. These results demonstrate that the hydrolysis process was not inhibited by the addition of AZM, CM, or KAN.

4.3.3 *Bacterial activity in the presence of each antibiotic at the acidogenesis and acetogenesis*

Anaerobic digestion process continues with the acidogenesis and acetogenesis stages. Figure 4.3 shows the profile of organic acids on days 1, 3, 5, and 10 under anaerobic incubation conditions of WAS with or without the addition of an antibiotic (AZM, CM, or KAN) to evaluate the differences of bacterial activity at the acidogenesis and acetogenesis stages. Acetate and butyrate concentrations were significantly higher ($p < 0.05$) in the presence of AZM compared with those in antibiotic-free WAS. Acetate also accumulated remarkably by day 3 whereas the butyrate concentration was drastically increased by day 5 (Figure 4.3a).
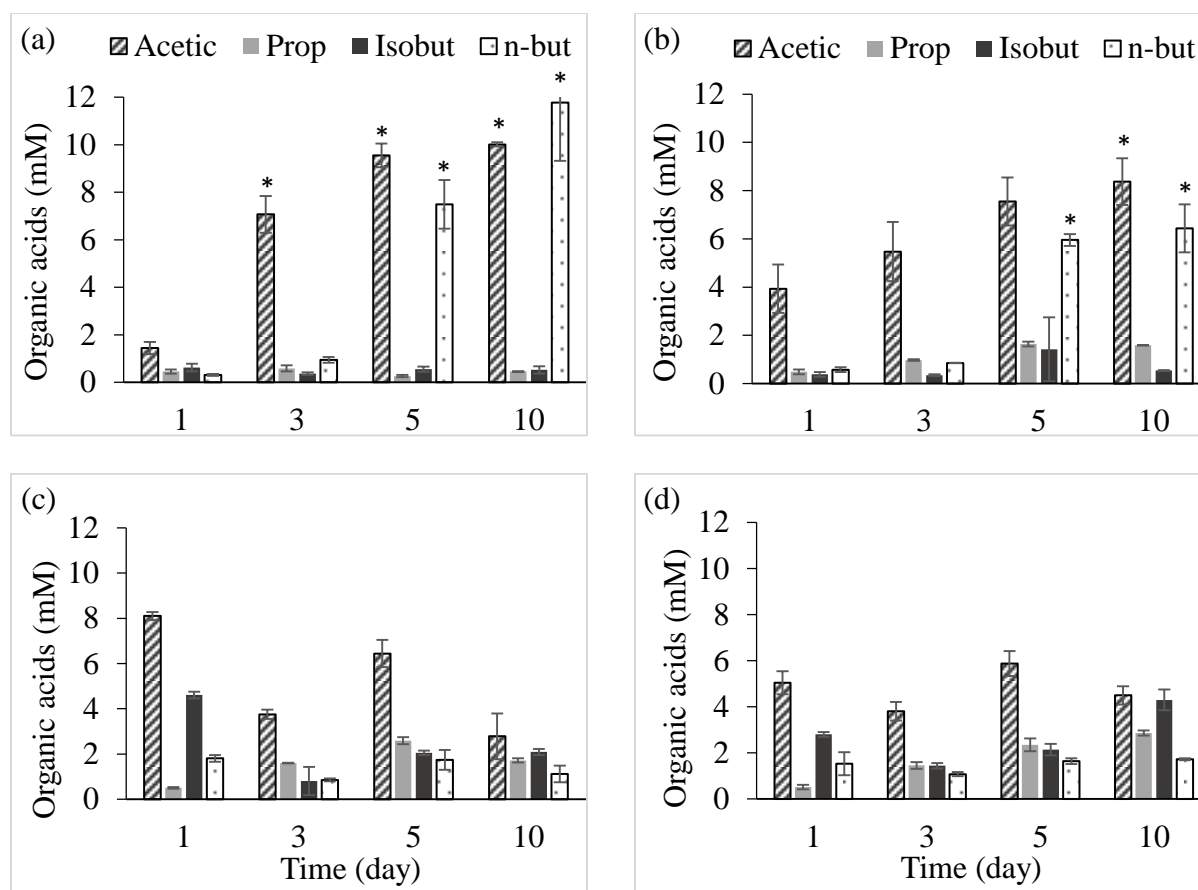
Figure 4.3: Profile of organic acids, acetic acid, propionic acid, isobutyric acid, and butyric acid during the anaerobic digestion of WAS with AZM (a), CM (b), or KAN (c), and without any antibiotic as a control (d). Error bars indicate standard errors (n = 3). *Indicates a significant difference by antibiotic addition.

The addition of CM in WAS had slightly lower intermediates than AZM, especially the butyrate concentration, but still significantly higher than antibiotic-free WAS ($p < 0.05$) at days 5 and 10 (Figure 4.3b). In addition, the production of isobutyrate and propionate were low in both samples with AZM and CM (Figure 4.3a and 4.3b) unlike the WAS samples with or without KAN where isobutyrate and propionate were accumulated throughout the fermentation (Figure 4.3c and 4.3d). In particular, only limited concentration of isobutyrate and propionate were detected in the presence of AZM. Interestingly, despite the presence of CM, which inhibited methane

fermentation, the production of organic acids (acetate and butyrate) was observed. Taken together, the addition of each antibiotic to WAS did not negatively influence the bacterial activity at the acidogenesis/acetogenesis stage, although different patterns of organic acids were observed for each antibiotic.

The profile of soluble metabolites such as acetic, butyric, propionic, and isobutyric fluctuated during the incubation of WAS, demonstrating that they were intermittently produced and consumed by the microbial community in WAS during the acidogenesis and acetogenesis stages. *Clostridia* and *Bacteroidaceae* are the main fermentative bacteria (Guo et al., 2015) that convert dissolved materials into volatile fatty acids, CO_2, and H_2. Later, acetogenic bacteria, known as hydrogen-producing acetogens or homoacetogens, convert these products to acetate. In all WAS in the presence of antibiotics, these bacteria might not be inhibited by antibiotics as the dissolved materials produced in the hydrolysis stage were utilized to produce intermediates. Thus, these intermediates are very important precursors for methanogens to be utilized and converted to methane at the methanogenesis step. Precisely, specific intermediates were used by specific groups of syntrophic bacteria as substrates for methane production. A higher butyric acid accumulation in WAS with AZM might be due to the inhibition of bacteria responsible for the utilization of butyric acid by AZM. This was supported in a previous report by Amin et al. (2006), where butyric acid utilization was inhibited by a macrolide antibiotic at lower concentrations.

Meanwhile, the lower concentration of propionate and isobutyrate were presumably due to the inhibition of these acids producing bacteria. A former study by Mellon et al. (2000) found that

the application of a macrolide antibiotic suppressed the propionate production by affecting some acidogenic anaerobic bacteria. Besides, pH was also measured for all samples at the end of incubation process and the accumulation of organic acids in WAS in the presence of AZM and CM may be responsible for the low pH values (pH 5.1 and 5.3, respectively). On the other hand, addition of KAN did not show a low pH (pH 6.2), and it was similar to the control WAS.

4.3.4 Bacterial and Archaeal activity in the presence of each antibiotic

As shown in Figure 4.1, the addition of each antibiotic (AZM, CM, or KAN) showed different patterns of methane production although there was less of an impact in the hydrolysis, acidogenesis and acetogenesis steps in the presence of antibiotics. Therefore, bacterial and Archaeal activity were determined with qRT-PCR using RNA as a template to evaluate the number of really active bacteria and methanogens in the presence or absence of antibiotics.
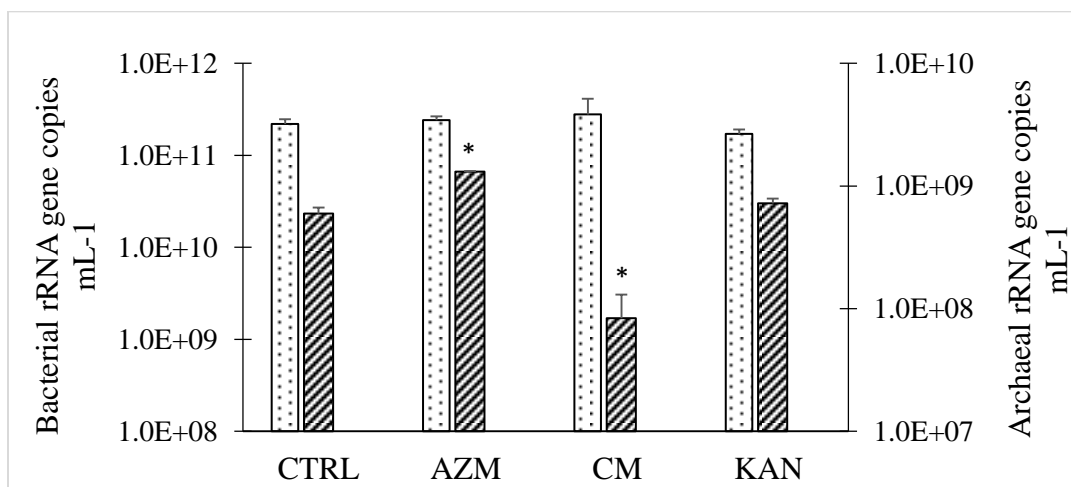
Figure 4.4: Proportion of active bacterial (▦) and Archaeal (▨) populations in waste-activated sludge in the presence of AZM, CM, KAN, and without any antibiotic (CTRL). Error bars indicate standard errors (n = 3). *Indicates a significant difference by antibiotic addition.

Figure 4.4 shows the quantitative activity of bacteria and Archaea in WAS with or without any antibiotic (AZM, CM, KAN, or antibiotic-free). As a result, the activity of active bacteria did not show any change with or without antibiotics, in agreement with the results that bacterial activity at the hydrolysis and acidogenesis/acetogenesis stages was not negatively influenced in the presence of antibiotics. On the other hand, Archaeal activity in the presence of AZM was significantly higher ($p < 0.05$) than that without any antibiotic, as supported by a result that methane production was enhanced under AZM (Figure 4.1). As expected, the addition of CM in WAS showed low Archaeal activity relative to the low methane production in the presence of CM.

In this study, AZM promoted the growth of microbes that contributed to improve methane production. However, other types of macrolides used in anaerobic digestion mostly provide a contradictory impact, unlike the results in this study. For example, a high concentration of tylosin, a 16-membered ring macrolide, showed a certain inhibitory effect on biogas production due to the accumulation of intermediates and a drop in pH (Shimada et al., 2008). Erythromycin, a parental antibiotic of AZM, showed only a slight effect on methanogenic activity but inhibited the conversion of some volatile fatty acids (Amin et al., 2006), while Du et al. (2015) obtained opposite results. In their study, CM inhibited methane production almost three-fold when compared to the control. Also, it may take longer for some methanogenic Archaea to adapt and acclimatize in this condition since they only started to produce methane after 4 days. Therefore, CM did not affect the microbial community in the initial stages of anaerobic digestion as the protease activity and the soluble intermediates (proteins and organic acids) were detected at a high activity and a high concentration in those samples. Additionally, the presence of CM in

WAS suppressed methane production which might be due to the inhibition of microbes only at the methanogenesis stage (Jin et al., 2011; Lallai et al., 2002; Sanz et al., 1996) without inhibiting other pathways. However, Lins et al. (2015) showed that the addition of 100 µg/mL CM showed no difference in gas production compared with the control, unlike our results where a low concentration (15 µg/mL) of CM was used. The Archaeal population plays an important role in the methanogenesis stage that should be responsible for the production of methane from acetate and CO_2/H_2. A low Archaea activity was detected in the WAS with CM (Fig. 4), indicating that methanogens were unable to convert metabolites such as acetate into methane, even though there were enough available substrates for methane production.

Also, bacterial and Archaeal activities in KAN were similar to those in antibiotic-free samples, supported the similarity of methane production in both samples. Despite the addition of KAN which has a certain antibacterial spectrum, methane production with KAN was the same as the control. This indicates that bacteria sensitive to KAN may be not essential to the anaerobic digestion process. However, Lins et al. (2015) found that methane production was slightly inhibited by the addition of 100 µg/mL kanamycin due to the suppression in degradation pathway of intermediates. Thus, a high concentration of antibiotics can provide a different result through the change of microbial community by stronger antibacterial activity.

4.3.5   Richness and diversity of microbial communities

The (Operational Taxonomic Units) OTUs number, diversity index, and taxonomy of the bacterial community were analyzed mainly using Quantitative Insights into Microbial Ecology

(QIIME) software. RNA templates collected at day 2 and 7 of the incubation period were prepared to analyze the active bacterial community responsible for anaerobic digestion. In this part, 8580-9689 OTUs were clustered at the identity cut-off 3% for analyzing bacterial communities. As shown in Table 4.1, only addition of AZM in WAS showed reduction in OTUs, Chao1 and Shannon index, while other samples; CM, KAN and antibiotic-free WAS showed increased in OTUs and indexes by the incubation time. For example, by AZM addition, the Shannon index was dropped from 12.49 to 12.23 at day 2 to day 7, respectively.

Table 4.1: Diversity statistics of the bacterial community in WAS with different antibiotics.

Bacterial Community

Antibiotic	OTUs*	Chao1*	Shannon Index*
AZM 2	9365	45777	12.49
CM 2	9374	45808	12.49
KAN 2	8873	42312	12.247
CTRL 2	9254	43306	12.5
AZM 7	8580	39248	12.23
CM 7	9689	47732	12.61
KAN 7	9210	43487	12.44
CTRL 7	9499	47806	12.48

* Values were defined at a dissimilarity level of 0.03

It is also noted that, by CM addition, the Chao1 and Shannon indexes were increased higher than other samples. Changes in Chao1 values were used to estimate the total number of OTUs. OTUs and Chao1 values were used as indicators to demonstrate the richness of bacterial community changes by the addition of antibiotics in WAS during anaerobic digestion. Another indicator, the Shannon index, was used to estimate the microbial population diversity.

4.3.6　Dynamics of active microbial communities by antibiotics

The similarity of the bacterial community based on different antibiotic additions, a principle coordinate analysis (PCoA) was conducted using OTUs at a dissimilarity level of 0.03. Figure 4.5 shows the PCoA of AZM, CM and KAN and antibiotic-free WAS. The incubation period were differentiating based on color.
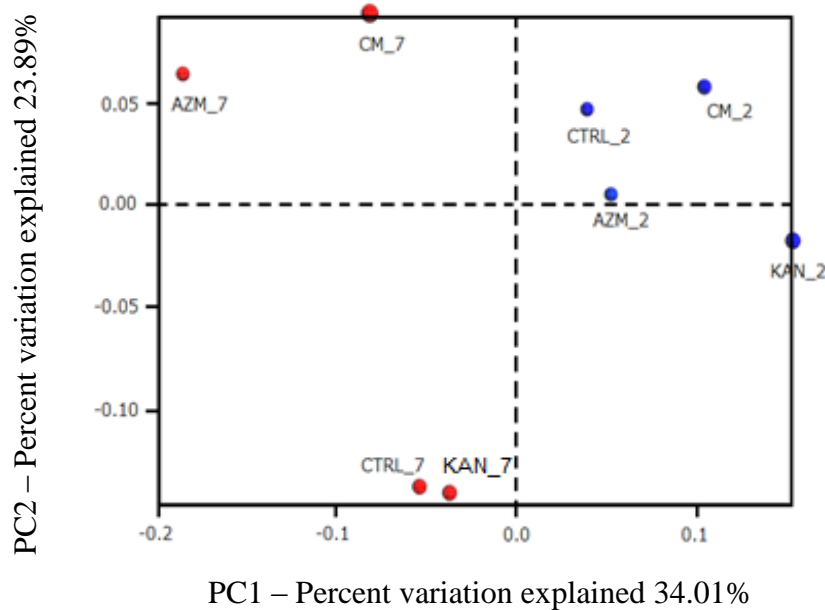
Figure 4.5: Principle coordinate analysis (PCoA) of methane production by different antibiotics addition; AZM, CM, KAN, and antibiotic-free WAS. PCoA was conducted at the 3% cut-off OTU level.

In the initial incubation, the communities were grouped at the same area exhibited that the similar communities were existed in different samples. However, by prolong the incubation until day 7, they were scattered in the graph. The KAN addition and antibiotic-free WAS were grouped together showed that the similar communities were existed. That's the main reason the similar methane was produced from both samples. The AZM and CM samples were existed at

distinct area showed that the microbial communities were dissimilar with each other due to different antibacterial spectrum.

The composition of active microbial communities in WAS with or without antibiotics was evaluated using the MiSeq Illumina analyzer. Figure 4.6 shows the relative abundance of microbial communities present in each sample at day 2 and at day 7 in the presence of AZM, CM, or KAN as well as in the absence of any antibiotic. Understanding the change of microbial community in the presence of each antibiotic allows us to analyze what microorganisms should be important in methane fermentation from WAS. *Proteobacteria* was an important phyla in this system because it has the bacteria responsible for glucose, propionate, butyrate and acetate utilization (Guo et al., 2015). All classes of *Proteobacteria* including *Alpha-*, *Beta-*, *Delta-*, and *Gammaproteobacteria* made up of total percentage in WAS with AZM, CM, KAN and without any antibiotic accounted for 36%, 43%, 40% and 38%, respectively at day 7 (Figure 4.6a). This shows that even though the addition of CM in WAS inhibited methane production, but it did not affected on the important microbes related to the degradative activity from hydrolysis to acetogenesis stages. However, *Flavobacterium* was detected with a relatively high percentage in AZM and CM samples during the fermentation (Figure 4.6b) in agreement with a result that shows a similar pattern of organic acid production, particularly acetic and butyric acids. *Flavobacterium* is one of the acid phase bacteria that involve in anaerobic digestion process (Ali Shah et al., 2014).
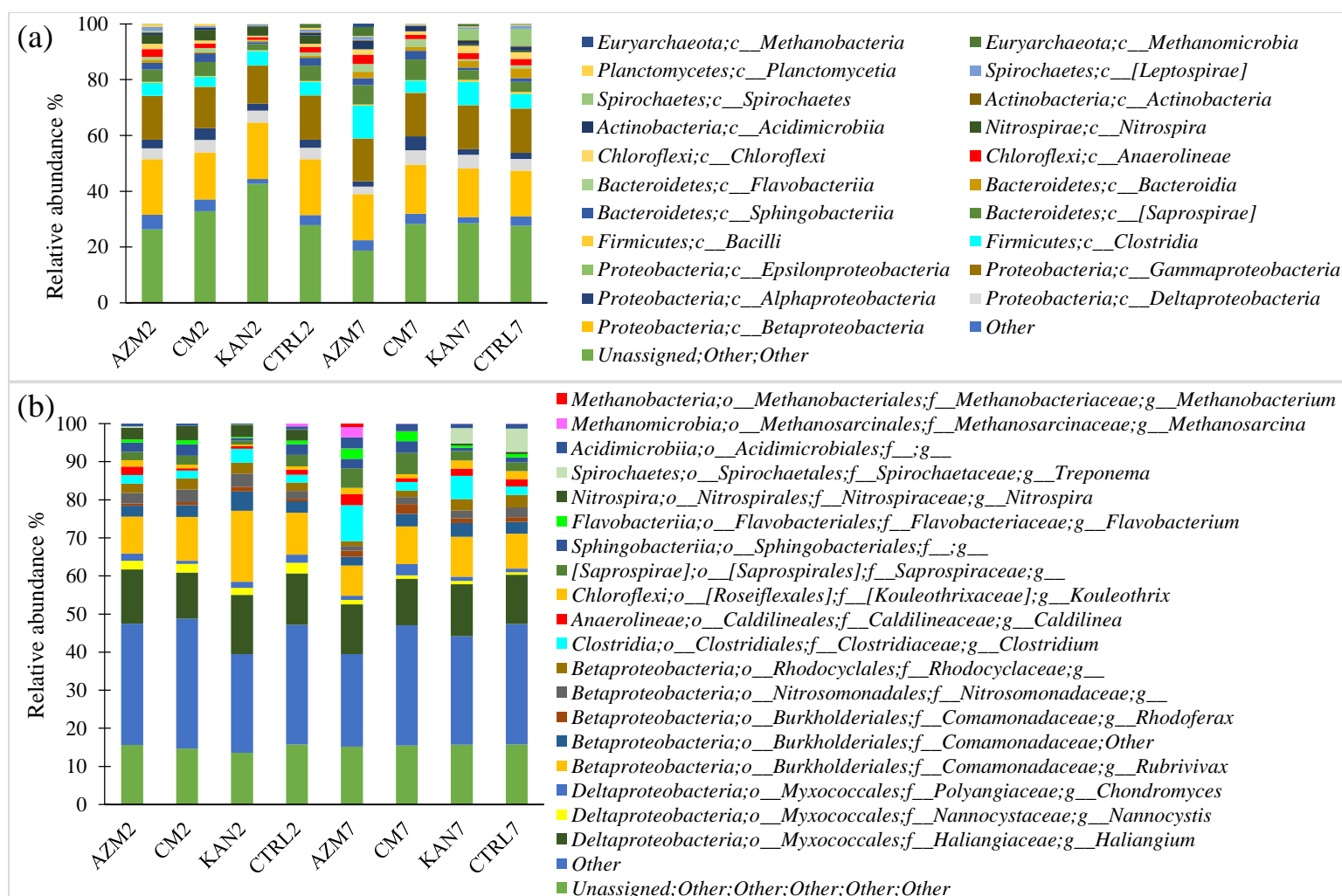
Figure 4.6: Relative abundance of dominant microbial communities categorized as class level (a) and genus level (b) of taxonomy in WAS in the presence of AZM, CM, or KAN and in the absence of any antibiotic (CTRL) at 2 days and 7 days. Results derived from high-throughput 16S rRNA sequencing. Minor classes were summed up in group 'Others.

On the other hand, WAS with AZM contains a high *Clostridium* and *Caldilinea* which may be important to explain a high methane production. The predominance of *Clostridia* contributes to a high rate of hydrolysis and degradation of volatile fatty acid in anaerobic digestion. *Caldilinea* belongs to *Chloroflexi* pylum which is one of the synthrophic bacteria that ubiquitously found in anaerobic digestion system (Narihiro et al., 2015) . Since it is a Gram negative species, AZM might not target these bacteria. Thus, it can be present in a high percentage simultaneously contributing to the improvement of methane production. In addition, WAS with AZM was the only sample that has *Methanomicrobia* and *Methanobacteria which* accounted for about 3% abundance is belonging to Achaea groups. This is also supported by the analysis of genus level that showed the presence of *Methanosarcina*. These methanogens are very well known of their contribution for methane production and the quantitative data from qRT-PCR also shows higher population of Achaea compared to other samples. This situation is presumably due to the resistance of methanogens in WAS towards macrolide antibiotics such as AZM. A former study by Amin et al. (2006) also showed that the presence of erythromycin, a macrolide type antibiotic at a high concentration did not affect the methanogens.

Oppositely, in the WAS with CM that showed lower methane production, the *Clostridia* presence was much lower than with AZM but similar to the control sample (at day 7) which was still able to produce methane. Thus, it proves that the low methane production was not only due to the low *Clostridia* population in the sample but also due to the methanogens as other studies concluded that CM was a powerful inhibitor towards methanogenic Achaea (Lallai et al., 2002; Sanz et al., 1996). In addition, a high percentage of *Chondromyces* was also present in the WAS with CM. The study on these bacteria for anaerobic digestion was very scarce. Its present might

be not contributed to methane production but may involve in the production of organic acid such as acetic acid or butyric acid. *Chondromyces* is belongs to anaerobic Myxobacteria (*Myxococcales* order). According to Veldkamp (1961), anaerobic myxobacteria were able to produce acetic, propionic, and succinic acids from sugar fermentation. Thus, *Chondromyce*s may carry out similar function for organic acids production.

The addition of KAN to WAS was not really affected on dynamics of microbial communities during the anaerobic digestion, but initially supported the growth of *Betaproteobacteria*. Later, no changes occur as shown by similar communities of microbial in WAS. This might be due to the KAN antibiotic did not affect the anaerobes involved in methane production except for slightly improved the *Clostridia*. However, since methane production is a collaboration of complex microbial community, these *Clostridia* alone cannot improve methane production. Importantly, WAS without antibiotics showed a very slight change in their microbial community at initial and final incubation day compared to with antibiotics. This is another evidence that the exploitation of antibiotics has altered the complex microbial communities in WAS which are responsible for improving or inhibiting methane production.

4.4   CONCLUSION

In conclusion, the application of antibiotics with different antibacterial spectrum contributed to better understanding of microbial community involve in bioenergy production. Exploitation of AZM, CM and KAN antibiotics showed different effect on overall anaerobic digestion process through the change of microbial activities and also the simultaneous functions and interactions of diverse bacteria and methanogenic Archaeal. From the finding, it can provide a rough idea on the main methane contributor, inhibitor or even a social cheater exist in microbial community of WAS. Taken together, our results can contribute to the improvement of bioenergy production by controlling/regulating dynamics of microbial community.

CHAPTER 5

EFFECT OF MACROLIDES AND LINCOSAMIDES ON METHANE PRODUCTION USING WASTE ACTIVATED SLUDGE: MECHANISMS AND MICROBIAL COMMUNITY DYNAMICS

5.1   INTRODUCTION

Anaerobic digestion process for bioenergy production has become a globally implemented technology (Weiland, 2009) using waste activated sludge (WAS). Recently, a variety of approaches have been implemented to improve bioenergy production using WAS. One approach studied the exploitation of azithromycin antibiotics in WAS, which has shown to improve sludge degradation and promote methane production (Nguyen et al., 2014). Few other studies have documented the effect of antibiotics on anaerobic treatment processes that occur during wastewater treatment for organic matter degradation, nutrient removal, and methane production. These include studies on the effects of cephalexin in WAS (Lu et al., 2014), tetracycline and sulfamethoxydiazine in livestock wastewater (Shi et al., 2011), erythromycin in pharmaceutical wastewater (Amin et al., 2006), and tylosin in granular sludge (Sanz et al., 1996; Shimada et al., 2008). Studies that evaluate the effect of antibiotics that exhibited a similar mode of action but had various antimicrobial spectra in WAS are scarce.

Macrolides inhibit bacterial protein synthesis by binding to the 50S ribosomal subunit, which inhibits the translocation of peptidyl-tRNA during elongation or inhibits the initial steps of the 50S subunit assembly. Lincosamides are a group of antibiotics that, although distinct from

macrolides, act on the ribosome and share an overlapping binding site with macrolides (Roberts, 2004). Macrolides with a common structure of a large lactone ring were subgrouped into a few analogs, namely the 14-, 15-, and 16-membered ring and ketolide groups. Erythromycin (ERM), the first introduced macrolide (Williams & Sefton, 1993), is a 14-membered ring analog. Other analogs derived from ERM are clarithromycin (CLM) and roxithromycin (RXM) which are 14-membered ring and azithromycin, a 15-membered ring analog. Besides, josamycin (JSM) and kitasamycin (KTM) are 16-membered ring analogs. Telithromycin (TLM) was the first of the ketolide group to be derived from ERM by replacing the L-caldinose with a keto-group (Leclercq, 2002; Roberts, 2004). Lincomycin (LCM) was the first lincosamide to be discovered and isolated from *Streptomyces lincolnensis* (MacLeod et al., 1964). Later, a new semisynthetic lincosamide, clindamycin (CDM), was derived from LCM. This structural alteration has led to a modification in the spectrum of activity, dose concentration, and administration of the newer macrolides and lincosamides.

Based on our previous finding that methane fermentation was improved in the presence of AZM, a macrolide-type antibiotic, we chose to examine other types of macrolide- and lincosamide-type antibiotic analogs, which are considered narrow-spectrum antimicrobials. The newly developed antibiotics, which are derived from their parental structures, are usually aimed at increasing the spectra coverage. These antibiotics may be bacteriostatic or bactericidal based on the concentration used, bacterial sensitivity, and the growth phase of bacteria. Generally, macrolides and lincosamides are effective against Gram-positive aerobic bacteria and Gram-positive and Gram-negative anaerobic bacteria. The specific spectrum activity for each antibiotic is summarized in Table 5.1. As the effectiveness of various antibiotics is different towards different

bacteria, it is worth experimenting the macrolides and lincosamides to determine their effects on microbial communities in WAS that contribute to bioenergy production, particularly methane. In this study, the antibiotics ERM, CLM, RXM, AZM, JSM, KTM, and TLM from the macrolide group and LCM and CDM from the lincosamide group were selected for to evaluate the methane production in prolong time.

5.2 MATERIALS AND METHODS

5.2.1 Activated sludge preparation

The WAS was prepared to final concentration of 10% (wet-sludge w/w) with distilled water prior to use in all experiments in this study.

5.2.2 Antibiotics

Antibiotics used in this study were Erythromycin (ERM) from Nacalai Tesque; Clarithromycin (CLM), Clindamycin (CDM), and Roxithromycin (RXM) from Tokyo Chemical Industry (TCI); Josamycin (JSM) from Funakoshi; Azithromycin (AZM) from LKT Laboratories Inc.; and, Telithromycin (TLM) from Bioaustralis. In addition, Kitasamycin (KTM) and Lincomycin (LCM) are from Wako, Japan. All antibiotics were dissolved in the solvent based on their solubility as a stock solution and the stock solutions were used to adjust the final concentration to be 15 µg/mL of each antibiotic into WAS.

5.2.3 Methane assay

The total volume of 30 mL WAS with or without 15 µg/mL of each antibiotic was filled in 66 mL vials. The vials were tightly sealed with butyl rubber stoppers, crimped, and sparged with nitrogen gas for 2 min to provide an anaerobic condition. Then, the vials were incubated at 37°C for 120 rpm. Each experiment was conducted in at least triplicate. Methane was measured by injecting 50 µL of headspace gas in vials into a GC-3200 gas chromatograph (GL Science, Japan).

5.2.4 Analytical methods

WAS samples during the fermentation were used for the following analyses; organic acids, pH, protein concentration, and protease activity. Initially, the fermented WAS samples were centrifuged at 13,000 rpm for 7 min to collect the supernatants which were later filtered through a 0.2-µm membrane syringe filter. Organic acids in each supernatant were analyzed using high-performance liquid chromatography (Shimadzu LC-10AD) as described previously (Mohd Yusoff et al., 2012). The pH was measured using a compact pH meter (AS ONE, AS-211, Japan). The soluble protein concentration was analyzed by the Lowry method using bovine serum albumin (BSA) as a standard protein (Lowry et al., 1951). The protease activity was measured as described previously (Maeda et al., 2011). One unit of protease activity was calculated as the quantity of tyrosine (µmol) produced from casein by 1 mg of enzyme per min.

5.2.5 *RNA extraction and cDNA synthesis*

RNA extraction was conducted using sludge pellets of fermented sludge. The RNA later solution was used for pellet preparation and details method as described in section 2.3.1. Total RNA was extracted using RNeasy kit (Qiagen Inc., Valencia, CA) as described in our previous paper (Mohd Yusoff et al., 2012). The cDNA was synthesized from RNA using PrimeScript RT reagent kit Perfect Real Time (TAKARA Bio Inc., Shiga, Japan). The cDNA was used later for quantitative real time polymerase chain reaction (qRT-PCR) to determine bacterial activity and archaeal activity as well as microbial communities of Bacteria and Archaea population.

5.2.6 *RT-PCR quantification*

The qRT-PCR was performed to quantify the 16S rRNA gene of total Bacteria and Archaea using a TaqMan system. The StepOne Real Time PCR System (Applied Biosystem) was used for amplification and detection of fluorescence by using primers and probes listed in Table 2.1.

5.2.7 *High-throughput 16S rRNA sequencing*

The V4-V5 region of 16S rRNA gene was used as a target of PCR amplification for the high-throughput 16S rRNA sequencing. Each concentration of purified PCR products was then determined using Qubit dsDNA HS Assay Kit (Life Technologies, Oregon, USA). The PCR products were processed using Nextera XT DNA Library Preparation Kit according to the Illumina manufacturer protocol. The pooled samples were loaded in the 500-cycle V2 MiSeq

reagent cartridge (Illumina) and then onto the MiSeq instrument along with the cleaned flow cell. Sequencing was performed for 251, 8, 8, and 251 cycles for forward Index 1, Index 2, and reverse reads, respectively.

The raw paired-end reads were processed using QIIME v1.9.0 (Caporaso et al., 2010). The high-quality reads were clustered into operational taxonomic units (OTU) at 97% sequence similarity using *de novo* OTU picking pipeline prior to assign taxonomy and alignment against Greengenes database v13.8 (DeSantis et al., 2006) using PyNAST program (Caporaso et al., 2010; DeSantis et al., 2006). The raw sequence data were deposited into the NCBI short reads archive database under accession number: SRP072534.

5.2.8   Statistical analysis

The different antibiotics were compared with control WAS using means from at least triplicate data (n = 3). Comparison was performed using means and standard deviations by the Student's *t*-test (GraphPad software) at a significance level of $p < 0.05$.

5.3 Results and Discsussion

5.3.1 *Effect of macrolides and lincosamides on methane production*

This study investigated how anaerobic digestion for methane production was affected by different antibiotics belonging to the macrolide and lincosamide groups by changing the microbial community in WAS due to the different antibacterial spectra (Table 5.1) but with the same mode of action. Anaerobic fermentation of WAS with or without 15 µg/mL of the antibiotics was performed for 30 d to determine the difference in methane production and the microbial community changes. As shown in Figure 5.1, two patterns of methane production were obtained by comparing antibiotic with antibiotic-free WAS.

Table 5.1: Spectrum activity of macrolides and lincosamides.

Group	Antibiotics	Spectrum of Activity	References
Macrolides	Erythromycin 14-membered ring	Mostly Gram positive and some Gram negative bacteria. Anaerobic bacteria such as *Actinomycetes* and *Arachnia* sp. and also *Chlamydia trachomatis.*	(Amsden, 1996)
	Clarithromycin 14-membered ring	A range of Gram positive and negative organism, atypical pathogens and some anaerobes. Moderately active against *Clostridium* species and it was the most active macrolide tested against *Eubacterium* and *Bacillus* species.	(Peters & Clissold, 1992)
	Roxithromycin 14-membered ring	Same as erythromycin, mainly towards Gram positive cocci and bacilli, and also Gram negative cocci. Inactive against *Enterobacteriaceae*. Reasonably active against anaerobic bacilli such as *Bacteroides.*	(Bryskier, 1998) (Chantot et al., 1986)
	Azithromycin 15-membered ring	Similar spectrum as erythromycin for Gram positive bacteria. Good activity against a variety of anaerobic genera such as *Clostridium* and anaerobic Gram positive cocci. More potent than other macrolides against *Enterobacteriaceae* and related Gram negative bacteria. Has better overall Gram negative activity.	(Williams, 1991)
	Josamycin 16-membered ring	Have the same spectrum of activity as erythromycin. Important for Gram positive bacteria and mycoplasma infection treatment.	(Straneo & Scarpazza, 1990) (Zhao et al., 2014)
	Kitasamycin 16-membered ring	Similar spectrum as erythromycin but weaker antimicrobial activity. Gram positive bacteria and certain *Mycoplasma* strains.	(Doons-Goossens et al., 1990) (Rakhit & Singh, 1974)
	Telithromycin 14-membered ring	Greatest activity against Gram positive aerobes and Gram positive anaerobe compared to clarithromycin and azithromycin. Possesses high activity against Gram negative pathogens *Haemophilus influenzae* and *Moraxella catarrhalis*. Extends to the atypical, intracellular and cell-associated pathogens *Legionella pneumophila, Mycoplasma pneumoniae* and *Chlamydia pneumoniae*.	(Goldstein et al., 2003) (Felmingham, 2001)
Lincosamides	Lincomycin	Has narrow spectrum. Gram positive bacteria and anaerobic bacteria.	(Xu et al., 2014)
	Clindamycin	Improved antibacterial activity and more active *in vivo* and *in vitro* compared to lincomycin. Streptococci, staphylococci and pneumococci. Gram positive aerobes and both Gram positive and Gram negative anaerobes.	(Leigh, 1981) (Smieja, 1998)

Figure 5.1a shows the first pattern of methane production, which drastically increased during the initial phase of incubation due to the addition of certain macrolides; RXM, AZM, JSM, KTM, and TLM. From 2–15 d of incubation, methane production was higher in WAS with these antibiotics although the production of methane was no longer increased after 15 d. It became stable until the end of the incubation period and achieved a lower methane production compared to antibiotic-free WAS (CTRL). The second pattern of methane production is shown in Figure 5.1b. The methane production profiles by the lincosamide group (LCM and CDM) and the macrolide group (ERM and CLM) were similar to antibiotic-free WAS. These samples produced methane much more slowly during initial incubation, but finally achieved a high level of methane production as compared to the first pattern (Figure 5.1a). As a result, some macrolides and lincosamides exhibit distinct patterns in methane production.
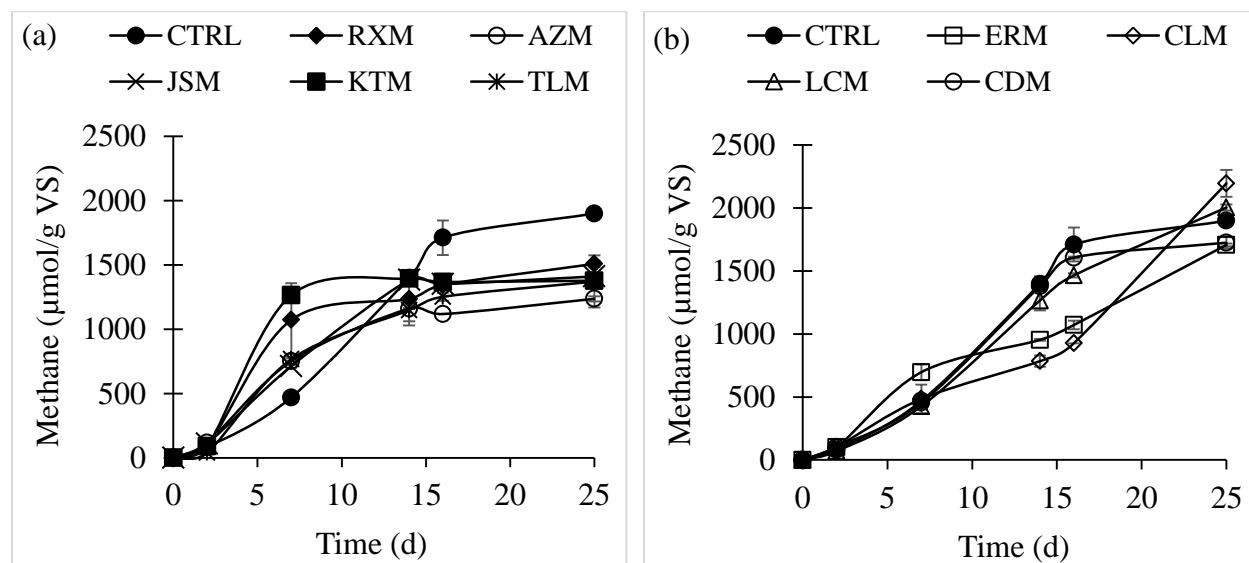
Figure 5.1: Methane production during 25 d of anaerobic digestion using WAS with (a) RXM, AZM, JSM, KTM and TLM, (b) ERM, CLM, LCM, CDM, and without any antibiotics as a control (CTRL) in both figures.

The two distinct patterns of methane production by macrolide and lincosamide analogs shows that they target different microbes involved in methane production. Since the anaerobic digestion stages are driven by a complex microbial community, specific bacteria are important in each stage. In this study, the application of macrolides in WAS for methane production produced results that were contradictory to other studies. For example, methane production was inhibited by the addition of a low ERM concentration in brewery wastewater (Amin et al., 2006), while Cetecioglu et al. (2015) found that biogas and methane production were inhibited by various concentrations of ERM. Tylosin, a macrolide antibiotic, also inhibited methane production (Shimada et al., 2008).

In the macrolides group, some antibiotics such as CLM, ERM, and RXM were more persistent under anaerobic conditions (Schlüsener et al., 2006). These antibiotics may retain their activity in WAS until the end of the incubation period. Therefore, this situation caused the bacteria responsible for methane production to slowly change their community, or perhaps some of the bacteria involved became adapted to this environment. Consequently, they still contributed to high methane production even though the process slowed down (Figure 5.1b). On the other hand, according to (Roberts, 2008), the antibacterial spectrum of lincosamides is more limited than macrolides. Therefore, it is possible that many species of the bacteria involved in methane production were resistant to the addition of lincosamides in WAS and were able to carry out their functions under an anaerobic digestion process. This resulted in better methane production until the end of the incubation period.

5.3.2   Effect of antibiotics at the hydrolysis stage

In order to evaluate the different results based on the varying effects of macrolides and lincosamides, bacterial activity during the hydrolysis stage was compared. Because protein is a major component of WAS (Maeda et al., 2011), determining its degradation product and protease activity can provide information on the efficiency of the degradation process during the hydrolysis stage. For this purpose, only representative macrolides (CLM and KTM) and lincosamide (LCM) were chosen for a 10 d analysis based on their different patterns of methane production. Briefly, the addition of KTM to WAS rapidly produced methane; the addition of CLM resulted in a slower methane production; and LCM showed similar methane production to antibiotic-free WAS. WAS with the addition of antibiotics produced high protein concentration (Figure 5.2a) and high protease activity (Figure 5.2b) compared to antibiotic-free WAS.
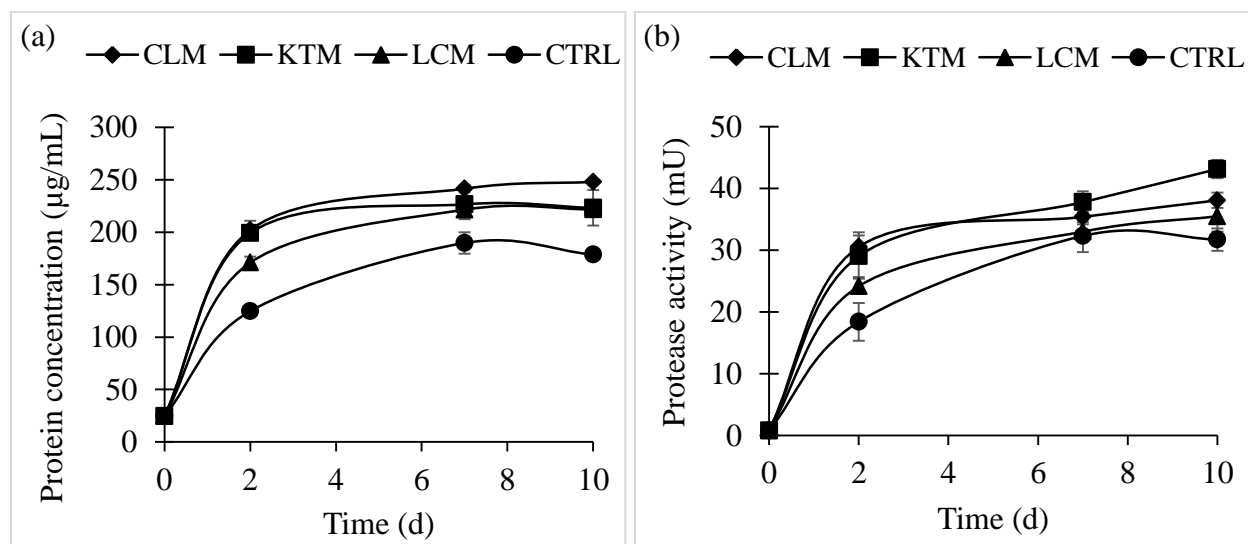
Figure 5.2: Soluble protein concentration accumulated during anaerobic digestion using WAS (a) and protease activity detected in the sample (b) with CLM, KTM, or LCM and without any antibiotics as a control (CTRL).

Both the protein concentration and protease activity increased in increments starting on the first day of incubation and became constant or showed only a very slight increase after 3 d. In contrast, the addition of lincosamide showed a slightly lower protein concentration and protease activity compared to the two macrolides. Despite the CLM addition resulting in a slow increase in methane production, the protein concentration and protease activity were similar to KTM addition in WAS, which rapidly produced methane. The higher converted protein levels and protease activity obtained in the CLM, KTM, and LCM samples demonstrated that the addition of antibiotics, particularly macrolides and lincosamides, stimulated the hydrolytic bacteria in WAS that involved in anaerobic digestion for methane production.

5.3.3   *Effect of antibiotics at the acidogenesis and acetogenesis stages*

Anaerobic digestion process continues with the acidogenesis and acetogenesis stages. The production of organic acids was evaluated in these stages to determine the conversion efficiency from smaller molecules obtained in the hydrolysis process to other soluble intermediates. These intermediates are later required in the methanogenesis stage for conversion to methane. Figure 5.3 shows the profile of organic acids production determined during the middle and final incubation period. Organic acids were determined in order to observe their production/accumulation and consumption/conversion to methane production.

The detection of organic acids was very low in antibiotic-free WAS and WAS with the addition of lincosamide at both 15 d and 30 d. The concentration of organic acids was higher in WAS with the addition of macrolide antibiotics, particularly acetic acid. However, it was noted that the

total production of organic acids was extremely reduced at 30 d for CLM and slightly reduced for ERM; both are 14-membered ring macrolides. On the other hand, the other macrolides, AZM, JSM, KTM, and TLM, showed an incremental increase in the concentration of total organic acids whereas RXM showed similar total concentration with drastically increased of butyric acid up to the final day of incubation. The production of organic acids by the addition of antibiotics was in agreement with methane production, and proved that the organic acids were important soluble intermediates for methane production to some extent.
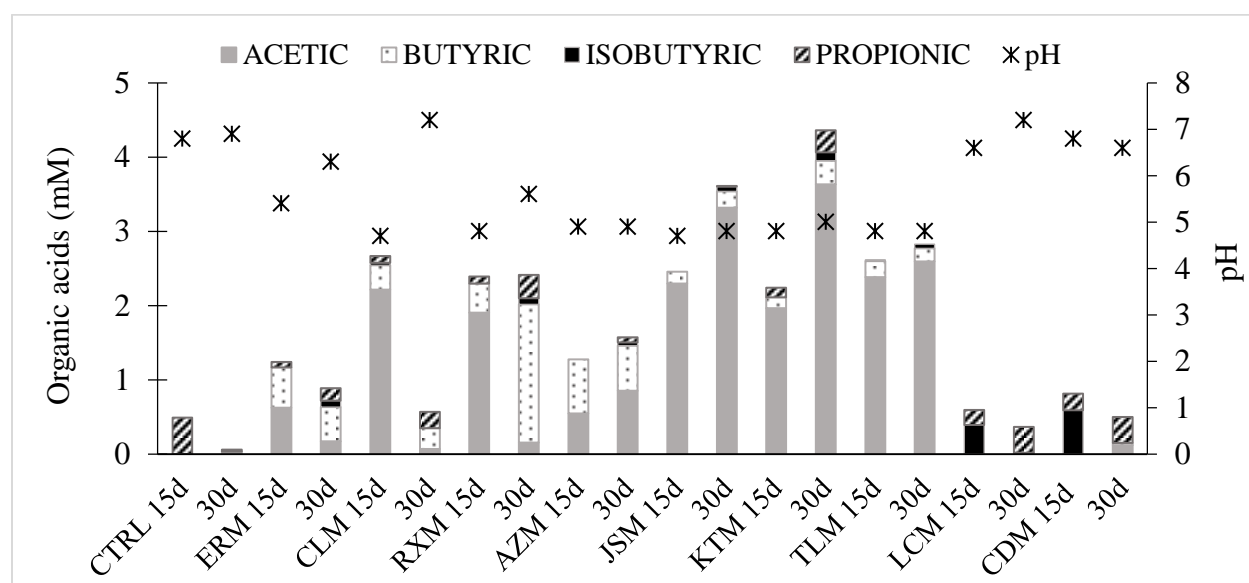
Figure 5.3: Profile of organic acids production; acetic acid, butyric acid, propionic acid, isobutyric acid, and pH value during 15 d and 30 d of anaerobic digestion of WAS with addition of all macrolides and lincosamides and without any antibiotics as a control.

The addition of CLM and ERM antibiotics resulted in an increase in methane production but a decrease in organic acids after 15 d of incubation. This was presumably due to the fact that organic acids had been consumed and converted to other intermediates by acidogenic or acetogenic bacteria, and finally converted to a high methane production. The accumulation of

organic acids by the addition of other macrolides demonstrated that these antibiotics did not inhibited the production of organic acids, but still affected some bacteria, including organic acids utilizing bacteria, homoacetogenic bacteria, or aceticlastic methanogens in WAS. Thus, organic acids cannot be converted to methane production. This situation was proved by the stable production of methane after 15 d from WAS with added macrolides. Another study that used macrolides in anaerobic digestion showed that the antibiotics inhibited homoacetogenic bacteria (Shimada et al., 2008).

Another reason might be due to the higher accumulation of organic acids in long incubation that may be toxic to some bacteria involved in the acidogenesis/acetogenesis stages as a result of imbalance caused by the antibiotic additions. In this case, methanogens are not able to remove the organic acids fast enough. Consequently, the organic acids accumulated in the culture and decreased the pH (Figure 5.3) to suboptimal values that may disturb the growth and functional abilities of methanogens (Huser et al., 1982; Steinhaus et al., 2007). As a result, the inhibition of methane production by the addition of antibiotics was attributed to the decrease in pH. This was also proved by the higher methane production in WAS with the addition of lincosamides and CLM due to the fact that the culture pH was around the optimum value of pH 6.5 to 7.5.

5.3.4   Effect of antibiotics on the total number of bacterial and archaeal populations

Evaluation of the final anaerobic digestion stage was carried out by determining the total number of archaeal and bacterial cells. Figure 5.4 shows the total bacterial and archaeal populations as determined by quantitative real-time PCR using a specific set of primers and probes, which are

shown in Table 2.1. Methanogens belonging to the archaea group are responsible for utilizing soluble intermediates for methane production. Therefore, it is important to obtain the total archaea population present during anaerobic digestion. The total archaea was quite similar between all macrolides and lincosamide analogs, but slightly higher in the antibiotic-free WAS. Interestingly, the total archaea was different in WAS with the addition of lincosamides than in antibiotic-free WAS, even though methane production was similar.

In contrast, the total bacteria population was not significantly different by antibiotic addition (Figure 5.4). Anaerobic digestion process, particularly from hydrolysis to acetogenesis, is normally driven by bacteria. Thus, the addition of antibiotics presumably did not affect the total bacteria populationin WAS that were not inhibited in those stages. However, specific bacteria that are involved in anaerobic digestion process may be affected, as different antibiotic applications will have a different spectrum of activity towards the bacterial community. Hence, the microbial community was evaluated by the ultra-high-throughput microbial community analysis on the Illumina MiSeq sequencers to determine the diversity and dynamics of bacteria in WAS into which antibiotics had been added (Figure 5.6).
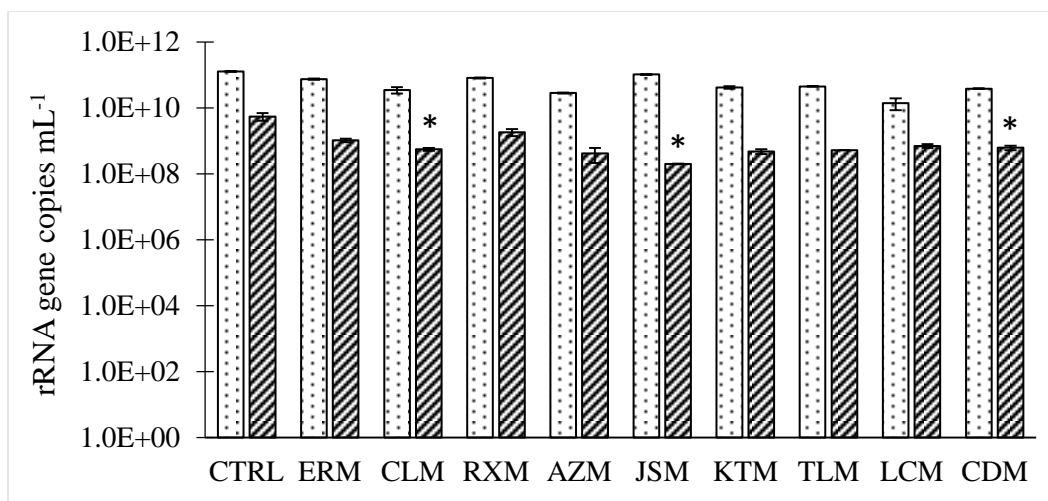
Figure 5.4: Quantity of active bacterial and archaeal populations in WAS in the presence of macrolides (ERM, CLM, RXM, AZM, JSM, KTM, and TLM), lincosamides (LCM and CDM), and without any antibiotics (CTRL). Error bars indicate standard errors (n = 3). * Indicate the significant difference by antibiotics addition

The total archaea population was affected by the addition of antibiotics. According to Shimada et al. (2011), accumulation of volatile fatty acids are attributed to inhibition of methanogens. Antibiotic-free WAS had a very low organic acids, but addition of macrolides and lincosamides increased organic acids production. Some of the accumulated organic acids presumably acetic acid, may indirectly affected the methanogens as the higher levels of butyric acid caused by the addition of RXM did not reduce the archaea population (Fig. 5.4). Therefore, the addition of either macrolides or lincosamide did not affect the total bacteria but slightly affected the total population of archaea in WAS.

5.3.5   Richness and diversity of microbial communities

RNA templates from representative macrolide (CLM, AZM, KTM, and TLM) and lincosamide

(LCM) collected at the end of the incubation period were prepared to analyze the active bacterial

community responsible for anaerobic digestion. WAS with KTM addition at day 5 was selected

as macrolides representative for comparison with WAS, due to irregular methane production

(Figure 5.1a). The OTUs number, diversity index, and taxonomy of the bacterial community

were analyzed mainly using QIIME software. In this study, 6529–8747 Operational Taxonomic

Units (OTUs) were clustered at a dissimilarity level of 0.03 as stated in Table 5.1. OTUs and

Chao1 indicators demonstrate the richness of bacterial community while the Shannon index

estimates the microbial population diversity. All antibiotics were significantly different in OTUs

compared to control WAS, except KTM at 30 day. The Shannon index ranged from 11.37–12.49

with only LCM not significantly different from control WAS. Reduction of diversity within a

sample was shown by control WAS (12.09 to 11.74) and KTM addition (12.49 to 11.77) from 5

day to 30 day. Overall, the richness and diversity of bacterial populations varied based on

antibiotic additions and digestion period.

Table 5.2: Diversity statistics of the bacterial community in WAS with macrolides and lincosamides.

Bacterial Community			
Antibiotic	OTUs*	Chao1*	Shannon Index*
CTRL (day 5)	7683	31489	12.1
KTM (day 5)	8747	39697	12.49
CTRL	7245	30490	11.74
CLM	6529	25345	11.37
AZM	6729	27874	11.43
KTM	7230	30085	11.77
TLM	8055	32987	12.22
LCM	8099	36073	12.13

* Values were defined at a dissimilarity level of 0.03.

Theoretically, the addition of any antibiotic was expected to change the richness or OTUs number of bacteria present in WAS. Differences in the richness and diversity of microbial populations in WAS were dependent on the type of antibiotics. For example, the addition of AZM in WAS showed the lowest OTUs number followed by CLM. This is because AZM is the only macrolide that has an extensive coverage of the antibacterial spectrum and has better activity against Gram-negative bacteria and *Enterobactericeae* (Williams, 1991). Similarly, CLM also covers a range of Gram-negative and Gram-positive bacteria (Table 5.1).

However, the addition of KTM (particularly at 5 d), and also TLM and LCM in WAS obtained higher OTUs numbers compared to antibiotic-free WAS. This showed that the application of antibiotics has an important impact on microbial diversity changes for methane production. Specifically, either increased or reduced community richness was expected to be dependent on

the tolerability of the bacterial community in the presence of antibiotics or the accumulation of organic acids. The small Shannon index range obtained from all samples, with or without the addition of antibiotics, indicated an almost similar diversity because they originally came from same source WAS. Within the same samples at 30 d of fermentation, represented by KTM and CTRL, the diversity of the bacterial community was reduced. The anaerobic conditions presumably affected the initial bacterial communities due to the accumulation of organic acids and pH changes and caused a reduction in the diversity index during the long fermentation process.

5.3.6   Bacterial population dynamics

A principle coordinate analysis (PCoA) was conducted to analyze the similarity of the bacterial community using OTUs based on weighted UniFrac.   Figure 5.5 shows that the antibiotic samples were clustered into three groups. Group I consist of samples collected at day 5 of incubation with KTM addition and antibiotic-free WAS. Group II consists of samples with the addition of macrolides, and Group III consists of samples with lincosamide, specifically LCM addition, and antibiotic-free WAS. The samples collected during the initial incubation period had similar bacterial communities (Group I) and a significant similarity was also demonstrated between macrolide antibiotic additions in WAS samples (Group II), regardless of their methane production. The addition of LCM also showed a similarity with antibiotic-free WAS (Group III), which was in accordance with methane production in Figure 5.1b.
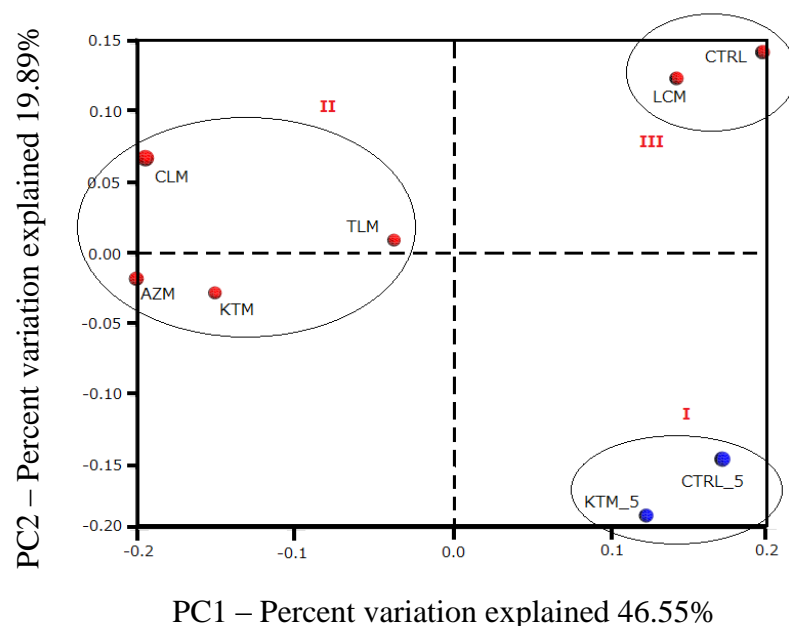
Figure 5.5: Principle coordinate analysis (PCoA) of methane production by different antibiotics macrolides and lincosamides. PCoA was conducted at the 3% cut-off OTU level.

In addition, a considerable horizontal similarity was observed between Group III and CLM, which shared the same pattern of methane production. In the vertical view, Group I and Group III were grouped around the same axis range. This showed that bacterial abundance (Figure 5.6) was also important in contributing to methane production, although the bacterial communities were similar.

It is interesting to note that at the end, the similarity of bacterial communities with the addition of macrolides were still in the same group although the bacteria had undergone various processes during anaerobic digestion.

Figure 5.6 shows the bacterial community based on the class level of taxonomy at day 5, represented by KTM for comparison with antibiotic-free WAS. The other samples were collected at the end of the incubation period and selected from representative 14-, 15-, and 16-membered ring and ketolide macrolide groups and LCM as the representative lincosamide. From the figure, few classes were present at high abundance percentage in KTM 5; these included *Saprospirae, Sphingobacteria, Acidimicrobia, Anaerolineae, Chloroflexi*, and *Clostridia*, while in CTRL 5, only *Spirochaetes* was present in high percentage of relative abundance. At the end of the incubation period, the *Proteobacteria* phylum, particularly *Alphaproteobacteria* and *Betaproteobacteria*, were present at high abundance percentage in CTRL and LCM samples, which both had similar methane production. In addition, *Spirochaetes* was present at high percentage in samples that had a similar pattern of methane production (CTRL, CLM, and LCM) as compared to other samples (Figure 5.1a).

Moreover, *Clostridium* also presents in high percentage with the addition of macrolides and lower with the addition of lincosamide at day 30 of anaerobic digestion. However, in the CTRL sample with no antibiotic additions, the *Clostridium* percentage was very low. Besides, *Acidimicrobia* percentage was higher in WAS with the addition of antibiotics, except for CLM and antibiotic-free WAS whereas *Anaerolineae* percentage was higher in WAS with the addition of antibiotics compared to antibiotic-free WAS, which similar to *Saprospirae*, particularly with KTM and TLM additions. Overall, the *Proteobacteria, Bacteriodetes*, and *Firmicutes* were the predominant phyla in all samples, regardless of the addition of antibiotics.
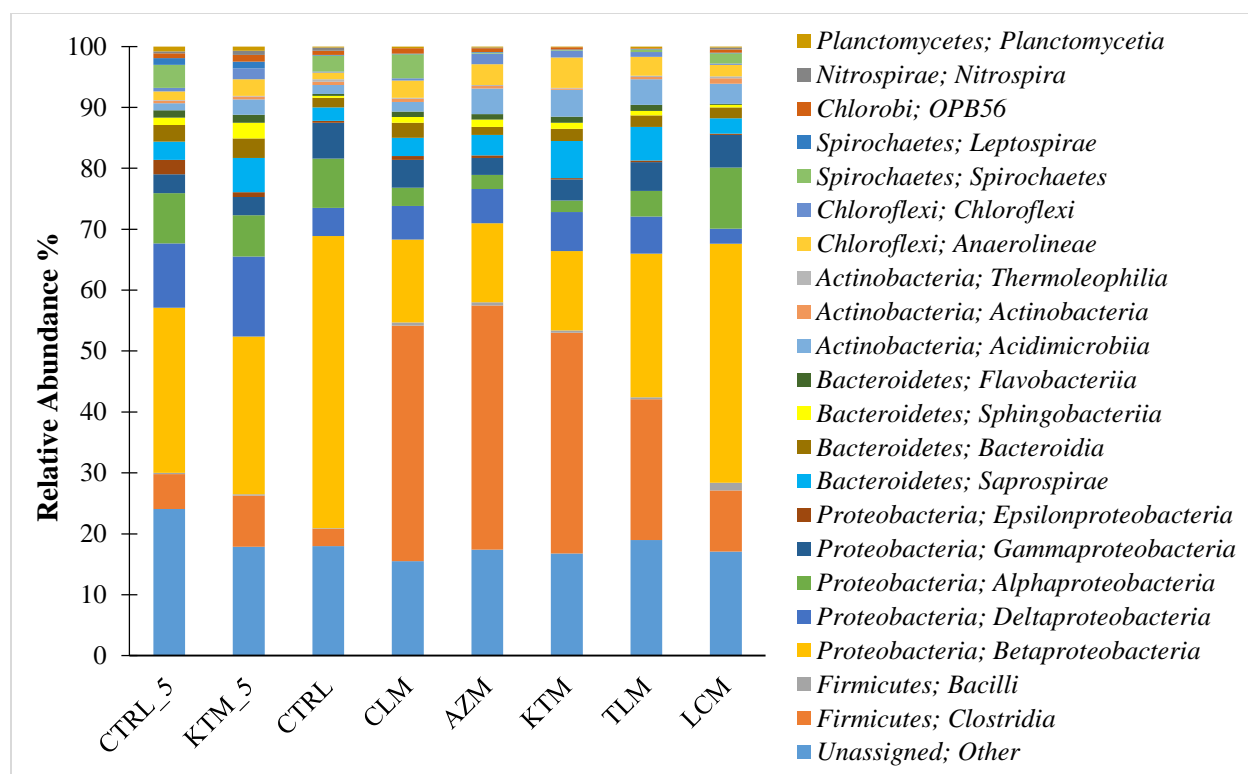
Figure 5.6: Relative abundance of the dominant microbial communities categorized at the taxonomic class level in WAS with KTM (KTM_5) and without antibiotics as a control (CTRL_5) at day 5 and with addition of CLM, AZM, KTM, TLM, and LCM, and in the absence of antibiotics (CTRL) at day 30 of anaerobic digestion. Results were derived from high-throughput 16S rRNA sequencing. Minor classes were summed up in group 'Other'.

The bacteria present at day 5 of incubation in CTRL 5 and KTM 5 samples, such as *Saprospirae, Sphingobacteria, Acidimicrobia, Anaerolineae, Chloroflexi*, and *Clostridia*, are important in anaerobic digestion process. Since their abundances were higher with KTM addition, the hydrolysis and acidogenesis/acetogenesis stages were carried out efficiently to produce a higher methane level compared with antibiotic-free WAS. For example, *Saprospirae* was reported to be a protein degrader (Xia et al., 2008) that provides smaller molecules in the hydrolysis stage, and *Chloroflexi* and *Clostridia* were also associated with a high rate of hydrolysis and organic acids

fermentation (Guo et al., 2015; Shimada et al., 2011). These communities provided sufficient soluble intermediates that could be utilized by methanogens in the methanogenesis stage. *Spirochaetes* was believed to be resistant to lincosamides but susceptible to macrolides and was reported as a fermentative acidogenic bacteria (Ng et al., 2015). Its presence during the whole period of anaerobic digestion might be the reason for the increase in methane from WAS with the addition of lincosamides and antibiotic-free WAS.

Regardless of the antibiotic addition, *Proteobacteria, Bacteriodetes*, and *Firmicutes* have been reported to be common and abundant in anaerobic digestion process (Guo et al., 2015; Ng et al., 2015; Shimada et al., 2011; Yu et al., 2014). These phyla play an important role in producing methanogenic precursors during anaerobic digestion for methanogens. *Clostridia* were assumed to be the most important producer because its high abundance existed with the addition of macrolides. However, as mentioned previously, an accumulation of intermediates, particularly organic acids, may lower the pH value out of the optimum range for methanogens. Thus, these methanogens were unable to convert sufficient intermediates for methane production, which showed as a constant production until end of the incubation period.

5.4   Conclusion

The difference in methane production demonstrated the effect of different antibacterial spectra creating simultaneous interactions and functions in a complex bacterial community during anaerobic digestion. Specifically, the accumulation of organic acids by bacterial community during anaerobic digestion caused the pH to change, which affected on bacterial community for other functions. This is supported by similarity in the bacterial communities during anaerobic digestion was shown by the incubation time and type of antibiotics used. Taken together, the different antibacterial spectra by macrolides and lincosamides stimulated various conditions towards the microbial community in WAS during a prolong period of anaerobic digestion process for methane production.

CHAPTER 6

INHIBITION OF A MIMIC ENTERIC METHANE FERMENTATION USING THE PALM OIL INDUSTRIAL WASTE PHOSPHOLINE GUM: UNDERSTANDING MICROBIAL COMMUNITY DYNAMICS

6.1   Introduction

Methane is an extremely potent greenhouse gas because it can trap heat and make the planet warmer. Natural gas, petroleum, agricultural industries such as livestock production, landfills, wastewater treatment, and other industrial processes are sources of anthropogenic methane. Moreover, livestock production accounts for 25% of the anthropogenic methane released from enteric fermentation, particularly from ruminants (Lesschen et al., 2011). Anaerobic digestion is a natural process wherein bacteria convert organic materials into biogas through hydrolytic, acidogenic, acetogenic, and methanogenic reactions (Hassan et al., 2005). Enteric methane production in the rumen is similar to other anaerobic digestion process (Bayané & Guiot, 2011; Knapp et al., 2014; Yue et al., 2012). Because the gas produced from fermentation is mainly methane, carbon sources can be utilized for the nutrition and milk production of ruminants rather than for methane production. Thus, increases in the quantity and quality of livestock may be achieved if methane production derived from ruminants is inhibited (Beauchemin et al., 2008).

As the ruminants should utilize nutrients for anabolism rather than methane production, it is important to develop a strategy to mitigate enteric methane emission, which benefits ruminants and the environment. To date, many mitigation strategies have been developed such as dietary

manipulation, utilization of additives in feed, and administration of vaccines (Huhtanen et al., 2015). Feed additives have been recently demonstrated to be the simplest approach to mitigate methane emission. Hristov et al. (2013) found that synthetic additives can inhibit enzymes involved in methane production. Moreover, nitrate additives added to the feed of ruminants' exhibit toxicity (Lee & Beauchemin, 2014). The utilization of plant-derived additives in feed is preferable because they are derived from natural sources. However, Wischer et al. (2014) found that chestnut and valonea extracts contribute to the high total cost of the feed. Therefore, it is important to find an alternative natural material that is renewable, abundant, and contributes to low or zero cost of feed production to mitigate methane emission from livestock.

Palm oil production is a major industry in Malaysia and Indonesia. The final stage produces refined palm oil from crude oil through the refining process by removing some undesired constituents. For example, phosphatides or also called phospholipids or phospholine gums, which are constituents of palm oil generated by degumming step, need to be removed. At the end of the degumming process, phosphatides produce a by-product called gums (Haslenda & Jamaludin, 2011). However, until now, no study has described the potential utilization of phospholine gum in any field. However, there are several studies that demonstrate that soy lecithin, a naturally occurring group of phospholipids produced by soy oil refining is used as a commercial product in the food industry as an emulsifier, dispersing agent, or viscosity regulator (Comas et al., 2006; Ozturk & McClements, 2016) and also in the pharmaceutical industry as skin penetration enhancers, moisturizers and anti-irritants (Mehta & Jindal, 2013; Mouri et al., 2015; Valenta et al., 2000). This by-product is a renewable resource that is potentially useful as a feed additive for ruminants.

In this study, methane production during fermentation in the presence of phospholine gum was investigated to evaluate the effects of phospholine gum on the microbial community present in waste activated sludge (WAS), which was used as a substrate, in combination with microbial sources that mimic the rumen of cows.

6.2 Materials and Methods

6.2.1 Phospholine gum

Phospholine gum (a waste produced by crude palm oil refining) is kindly obtained from the Sinarmas Palm Oil Company, Medan, Indonesia. The phospholine gum was directly used for methane fermentation or treated at 550°C for 30 min to remove carbon sources derived from organic compounds in the phospholine gum and used for the experiments.

6.2.2 Activated sludge preparation

The WAS was prepared to final concentration of 10% (wet-sludge w/w) with distilled water prior to use in all experiments in this study.

6.2.3 Methane assay

The anaerobic digestion process was performed using 66-mL vials to understand fundamentally the role of phospholine gum towards methane production and microbial community in WAS.

The total volume of 30 mL WAS with or without phospholine gum was filled in 66 mL vials. The concentrations of phospholine gum used were 5, 10, 25 and 50% w/w. The vials were tightly sealed with butyl rubber stoppers, crimped, and sparged with nitrogen gas for 2 min to provide an anaerobic condition. Then, the vials were incubated at 37°C for 120 rpm. Each experiment was conducted in at least triplicate. Methane was measured by injecting 50 µL of headspace gas in vials into a GC-3200 gas chromatograph (GL Science, Japan).

6.2.4   Analytical methods

WAS samples during the fermentation were used for the following analyses; organic acids, pH, protein concentration, and protease activity.   Initially, the fermented WAS samples were centrifuged at 13,000 rpm for 7 min to collect the supernatants which were later filtered through a 0.2-µm membrane syringe filter. Organic acids in each supernatant were analyzed using high-performance liquid chromatography (Shimadzu LC-10AD) as described previously (Mohd Yusoff et al., 2012). The pH was measured using a compact pH meter (AS ONE, AS-211, Japan). The soluble protein concentration was analyzed by the Lowry method using bovine serum albumin (BSA) as a standard protein (Lowry et al., 1951). The protease activity was measured as described previously (Maeda et al., 2011). One unit of protease activity was calculated as the quantity of tyrosine (µmol) produced from casein by 1 mg of enzyme per min.

6.2.5 RNA extraction and cDNA synthesis

RNA extraction was conducted using sludge pellets of fermented sludge. The RNA later solution was used for pellet preparation and details method as described in section 2.3.1. Total RNA was extracted using RNeasy kit (Qiagen Inc., Valencia, CA) as described in our previous paper (Mohd Yusoff et al., 2012). The cDNA was synthesized from RNA using PrimeScript RT reagent kit Perfect Real Time (TAKARA Bio Inc., Shiga, Japan). The cDNA was used later for quantitative real time polymerase chain reaction (qRT-PCR) to determine bacterial activity and archaeal activity as well as microbial communities of Bacteria and Archaea population.

6.2.6 qRT-PCR quantification

The qRT-PCR was performed to quantify the 16S rRNA gene of total Bacteria and Archaea using a TaqMan system. The StepOne Real Time PCR System (Applied Biosystem) was used for amplification and detection of fluorescence by using primers and probes listed in Table 2.1.

6.2.7 High-throughput 16S rRNA sequencing

The V4-V5 region of 16S rRNA gene was used as a target of PCR amplification for the high-throughput 16S rRNA sequencing. Each concentration of purified PCR products was then determined using Qubit dsDNA HS Assay Kit (Life Technologies, Oregon, USA). The PCR products were processed using Nextera XT DNA Library Preparation Kit according to the Illumina manufacturer protocol. The pooled samples were loaded in the 500-cycle V2 MiSeq

reagent cartridge (Illumina) and then onto the MiSeq instrument along with the cleaned flow cell. Sequencing was performed for 251, 8, 8, and 251 cycles for forward Index 1, Index 2, and reverse reads, respectively.

The raw paired-end reads were processed using QIIME v1.9.0 (Caporaso et al., 2010). The high-quality reads were clustered into operational taxonomic units (OTUs) at 97% sequence similarity using *de novo* OTU picking pipeline prior to assign taxonomy and alignment against Greengenes database v13.8 (DeSantis et al., 2006) using PyNAST program (Caporaso et al., 2010; DeSantis et al., 2006).

6.3   Results and Discussion

6.3.1   *Inhibition of methane by phospholine gum*

In the present study, we used WAS to evaluate the potential of phospholine gum (Fig. 6.1) from palm oil processing to reduce or inhibit methane production. Different concentration of phospholine gum was added in 10% of WAS.

Figure 6.1: Phospholine gum obtained from refining process of crude palm oil.

As shown in Figure 6.2, methane production was remarkbly inhibited compared with control, which contained only WAS. The lowest concentration of phospholine gum was 5% (w/w), at which a 10-fold reduction of methane production was observed compared with the control. The highest concentration of phospholine gum completely inhibited methane production. Further, the addition of 25% (w/w) phospholine gum inhibited methane production to the same level as 50% (w/w) of phospholine gum, which was the highest amount of methane produced after 10 days of fermentation was only 8 ± 4 µmol/g VS. As a result, addition of phospholine gum in WAS has the ability to inhibit the methane production.
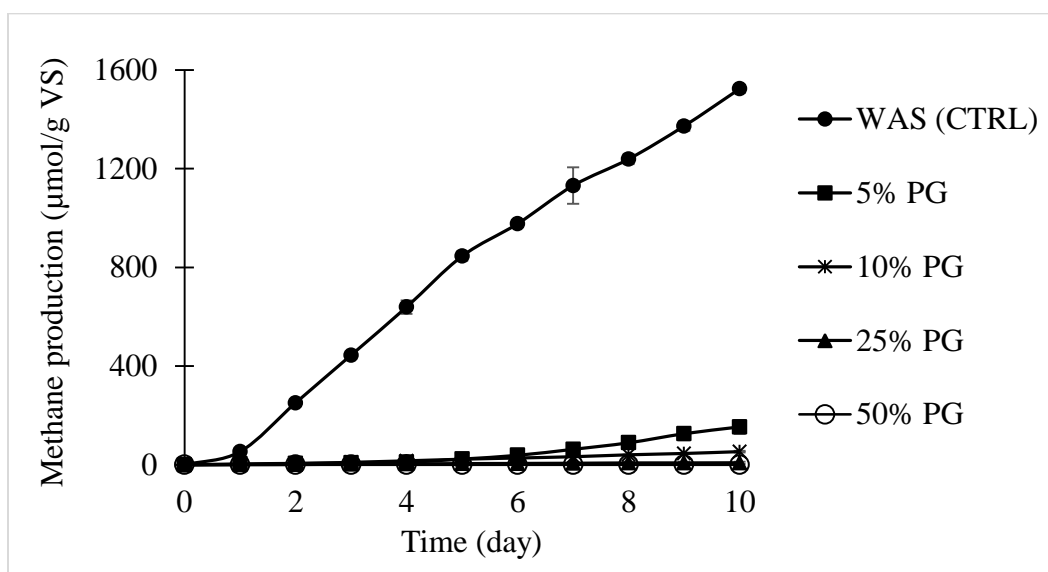
Figure 6.2: Methane production from WAS by addition of different phospholine gum (PG) concentration and WAS only as control (CTRL).

Numerous investigators attempted to mitigate enteric methane production by ruminants. One strategy employs additives (e.g., natural, chemical, or synthetic). Recently, farm producers demand utilization of natural products to alter rumen fermentation. Several investigators used bioactive products extracted from plants, such as essential oil, saponins, and tannins that are

potential inhibitors of methane emission by ruminants (Castro-Montoya et al., 2015; Guglielmelli et al., 2011; Patra et al., 2012), and some of these natural feed additives directly affect methanogens. Moreover, Wanapat et al. (2014) used mangosteen peel as a fruit by-product to significantly reduce methane emissions. Similarly, phospholine gum, which is a by-product of crude palm oil processing, is renewable and inexpensive. As reported by Sinarmas Palm Oil Company, they produce about 1.1 million tons of phospholine gum through degumming process of palm oil every day, which is more than enough to be added as feed additive. In addition, its utilization for ruminants will not increase the cost of feed as phospholine gum is a waste by-product that has not been commercially used in other fields and its component such as phosphorous compounds, which can degrade oil quality, must be removed during the refining process (Goh et al., 1985). Besides, the comparison of methane mitigation strategies that has been summarized by Boadi et al. (2004) showed most of the strategies will increase the cost of feed production. Phospholine gum showed an ability to inhibit methane production using WAS, and therefore has great potential as an additive to ruminants' feed. Furthermore, after treatment process, the phospholine gum could be recovered through manure release and reuse as compost as it is rich with phosphorus and nitrogen contents which are desirable plants nutrients.

6.3.2   *Effect of phospholine gum on bacterial and archaeal population*

To evaluate the effect of phospholine gum on the microbial community of WAS, RNA was extracted from WAS added with the lowest and highest concentrations of phospholine gum and from WAS without phospholine gum on days 2 and 10 of anaerobic fermentation. The total numbers of bacteria and archaea are shown in Fig. 6.3. The total numbers of bacteria were

similar for control WAS or WAS containing (5% w/w) phospholine gum at the initial and final days of fermentation. However, the addition of 50% (w/w) phospholine gum reduced the total number of bacteria on the final day. Further, the total number of Archaea in the control WAS was high on the initial day and slightly increased by the final day of incubation. Besides, addition of 5% (w/w) phospholine gum slightly decreased the total number of Archaea compared with that of the control.
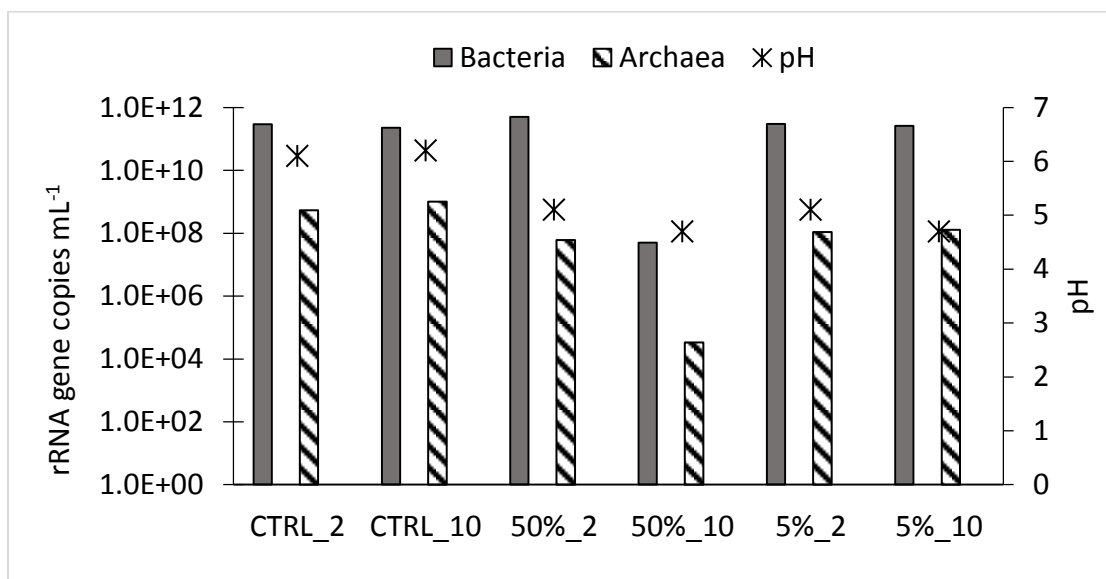
Figure 6.3: Quantity of total active bacterial and archaeal populations in WAS with addition of phospholine gum and pH value.

The addition of 50% (w/w) phospholine gum reduced the total number of archaea on day 2 of fermentation, and a major reduction occurred on the final day of fermentation (6.1×10^7 to 3.3×10^4, respectively). Besides, addition of phospholine gum reduced the pH of WAS from 6 to 5 on day 2 of incubation and then to pH 4.7 on the final day. Certain Archaea, called methanogens are responsible to produce methane. Another study reported similar finding using methanogenic inhibitors that reduced the total number of methanogens to a lesser extent (Zhou et al., 2011).

Addition of a higher concentration of phospholine gum effectively reduced the total population of Archaea, likely by directly inhibiting the methanogens, causing complete inhibition of methane production.

6.3.3   Richness and diversity of microbial community

The sample added with 5% (w/w) phospholine gum on day 2 was not included in the analysis of the microbial community because its methane content was similar compared with that of 50% (w/w) phospholine gum. The RNA templates for other samples were prepared to analyze the active bacterial community in WERE that was affected by the addition of phospholine gum. The OTUs, richness, and diversity indexes are shown in Table 6.1. OTUs and Chao1 values demonstrate the richness of a bacterial community following the addition of phospholine gum to WAS during anaerobic fermentation, while the Shannon index estimates the diversity of the microbial population. A higher index value represents a more homogenous population (Garcia et al., 2011).

Table 6.1: Diversity statistics of the bacterial community in WAS with phospholine gum.

Phospholine gum	Bacterial Community		
	OTUs*	**Chao1***	**Shannon Index***
CTRL 2d	3352	16620	11.30
CTRL 10d	3368	20251	11.18
50% 2d	2994	15652	10.82
50% 10d	3152	16272	10.99
5% 10d	3379	17532	11.30

* Values were defined at a dissimilarity level of 0.03.

The OTU value and Chao1 index of the control WAS was higher compared with that of the WAS with phospholine gum. The Chao1 index was lower for WAS with 50% (w/w) phospholine gum compared with that of 5% (w/w) phospholine gum. As expected, the Shannon index followed the same trend as the Chao1 index. Thus, the 5% (w/w) phospholine gum in WAS had a higher Shannon index compared with that of WAS added with 50% (w/w) phospholine gum. The high Chao1 and Shannon indexes of WAS without phospholine gum and with 5% (w/w) phospholine gum indicated that the richness and diversity of the microbial community was higher compared with that of WAS containing highest concentration of phospholine gum. Phospholine gum affected methane production and the archaeal population as well as the bacterial population, which contributed to the low richness of the microbial community of WAS. In contrast, the addition of phospholine gum to WAS slightly increased diversity during fermentation, which was likely caused by the stimulation of a specific bacterial community or the adaptation of the microbes induced by phospholine gum.

6.3.4   Bacterial population dynamics

To analyze the similarities among bacterial communities in WAS containing phospholine gum to WAS, PCoA was conducted using OTUs at a dissimilarity level of 0.03. Fig. 6.4 shows the PCoAs of WAS and WAS with phospholine gum. The bacterial communities of the control WAS were similar. However, the addition of 5% (w/w) phospholine gum reduced the similarity to the control WAS. The similarity was further reduced by the addition of 50% (w/w) phospholine gum. In addition, the microbial community similarity in the presence of the highest

concentration of phospholine gum was different compared with those during the initial to final days of incubation, but showed a vertical similarity.
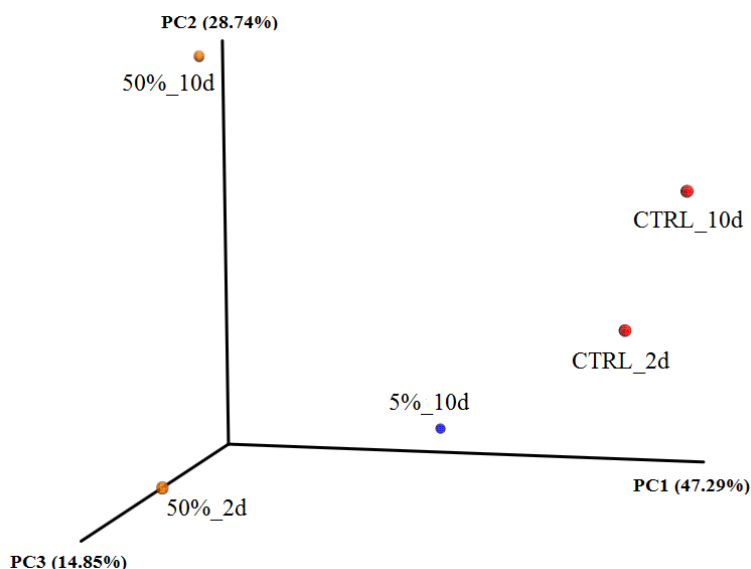
Figure 6.4: Principle coordinate analysis (PCoA) of methane production by phospholine gum addition in WAS. PCoA was conducted at the 3% cut-off OTU level.

MiSeq Illumina analysis was performed to determine the bacterial communities present in WAS with and without addition of phospholine gum. Fig. 6.5a shows the dominant classes present in these samples. The major bacterial community members in control WAS were identified as *Betaproteobacteria* that were present throughout the fermentation. The percentage of *Nitrospira* was high on day 2 and that of *Spirochaetes* was high on day 10. In contrast, the addition of either 5% or 50% (w/w) of phospholine gum to WAS increased the percentage of the *Clostridia*. Further, the percentage of *Bacilli* was higher only in WAS with 50% (w/w) phospholine gum and increased during fermentation. Both *Clostridia* and *Bacilli* classes belongs to *Firmicutes* phyla.
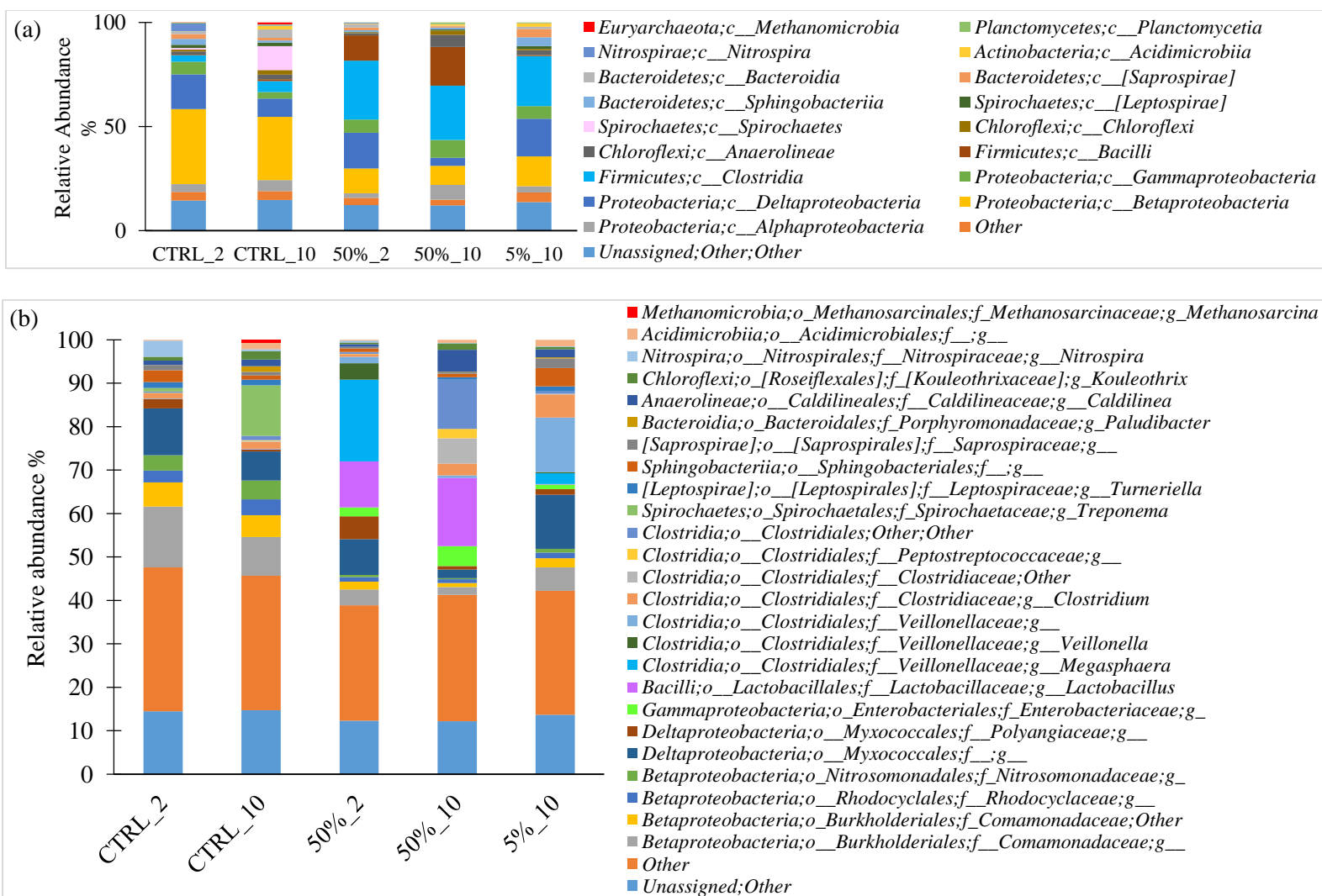
Figure 6.5: Relative abundance of the dominant microbial communities categorized at the taxonomic class level (a) and genus level (b) in WAS with or without addition of phospholine gum. Results derived from high-throughput 16S rRNA sequencing. Minor classes were summed up in group 'Others.

The bacterial community was observed in detail through other taxonomic levels to the extent possible using QIIME (Fig. 6.5b). The highest number of *Betaproteobacteria* was detected in the control WAS, which included *Comamonadaceae, Rhodocyclaceae*, and *Nitrosomonadaceae*. Also, the percentage of *Treponema* species (*Spirochaetes*) was higher percentage at the end of the fermentation. On the other hand, *Lactobacillus* species were detected at an extremely high percentage in WAS with 50% (w/w) phospholine gum compared with other samples. Moreover, the numbers of *Meagsphaera sp*. and *Veillonella sp.* (*Veillonellaceae* of *Clostridia*) were very high during initial incubation, and other families or genera representing *Clostridia* were present at the end of incubation. QIIME analysis detected a small percentage of methanogenic archaea. However, *Methanosarcina* species (*Methanomicrobia*) were only detected in the control WAS. Methanogens were undetectable in WAS added with phospholine gum at any time during fermentation.

Betaproteobacteria and *Spirochaetes* present in control WAS are the common anaerobic bacterial communities involve in the anaerobic fermentation process especially during the hydrolysis and fermentation of volatile fatty acids to soluble intermediates for methane production (Cloete & Muyima, 1997; Cydzik-Kwiatkowska & Zielińska, 2016; Guo et al., 2015). Therefore, all bacteria communities present in control WAS given an overview on the normal communities involved for production of methane from WAS during anaerobic fermentation.

Interestingly, a high percentage of *Lactobacillus* and *Megasphaera* species were detected in the presence of a high amount of phospholine gum probably induced by phospholine gum. Certain *Lactobacillus* species inhibit methanogenesis by producing bacteriocins and other antimicrobial

substances (Soriano et al., 2014). A study by Asa et al. (2010) showed that the utilization of antimicrobial substances secreted by *Lactobacillus plantarum* reduced methane emissions in vitro by affecting methanogenesis in the rumen. *Lactobacillus* species are generally regarded as safe because of their history of safe use and their beneficial effects on animals (Cheng et al., 2014). Moreover, lactobacilli are widely utilized for ruminant feed to improve nutritional value through improvement of aerobic stability during feed conservation. This lactobacillus rapidly produces lactic acid and lower pH values, which improve the productivity of fermentation and reduces nutrient losses during the feed conservation (Caldwell et al., 2013; Comino et al., 2014).

In addition, *Megasphaera* species, which are important ruminal organisms that prevent ruminal lactic acid acidosis (Henning et al., 2010), are beneficial and may be obtained by application of phospholine gum. For example, *M. elsdenii* utilizes lactate in the rumen. Long et al. (2014) constructed an acid-tolerant *Megasphaera* strain to improve their growth in the low pH of rumen environment. The present analysis of a microbial community detected two important bacterial species of ruminants induced by phospholine gum, which may be exploited in the future. In contrast, other *Clostridia* were present at a high percentage, particularly at the end of fermentation. Clostridial species (*Firmicutes*) are common in anaerobic environments and produce fermentation products utilized by methanogens (Bareither et al., 2013). Since the phospholine gum may directly inhibit methanogens, and therefore, the fermentation products produced during anaerobic catabolism cannot be utilized for methane production.

6.3.5   Phospholine gum composition affecting methane production

Further experiments were performed to determine the factors present in phospholine gum that contributed to the inhibition of methane production as well as other stages of anaerobic fermentation. Phospholine gum was incubated at 550°C to remove all the organic compounds before mixing with WAS. Fig. 6.6 shows the methane profile obtained using heat-treated or untreated phospholine gum that was added to WAS.
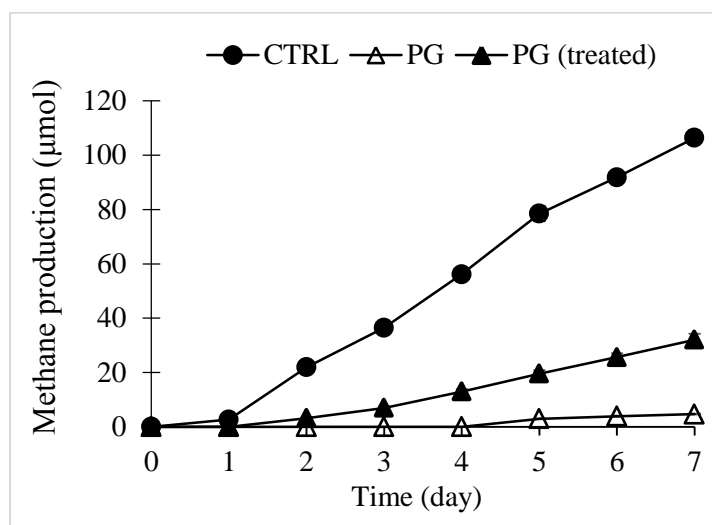
Figure 6.6: Methane production from untreated and treated phospholine gum using heat treatment as compared to WAS only as control.

Similar to previous analyses of methane profiles in the presence of different concentrations of phospholine gum, methane production was inhibited when phospholine gum was added to WAS. However, when heat-treated phospholine gum was added to WAS, the methane production was increasead than untreated phospholine gum (Fig. 6.6). The highest amounts of methane at the final incubation were 32 ± 2 µmol and 4.6 ± 0.1 µmol for treated and untreated phospholine gum, respectively, indicating that the organic compounds present in phospholine gum is one of the

factor contributed to the inhibition of methane production. For example, condensed tannin is an organic substance use in ruminant feed that can directly inhibit methane emissions (Guglielmelli et al., 2011). Moreover, organic compounds that contain sulfur inhibit methane production in landfills (Lee et al., 2015). However, the organic compounds responsible for inhibiting methane production in phospholine gum remain to be identified.

6.3.6 *Effect of phosholine gum in anaerobic fermentation stages*

The addition of phospholine gum to WAS directly inhibited the methanogens involved in methanogenesis. Therefore, further experiments were performed to evaluate the effect of phospholine gum on other stages of anaerobic fermentation such as hydrolysis, acidogenesis, and acetogenesis. During the hydrolysis stage, large molecules such as proteins, carbohydrates, and lipids are converted to smaller molecules such as amino acids, simple sugars, and fatty acids, which are produced by the action of hydrolytic enzymes. Thus, the measurement of protein concentration as one of the major contents in WAS (Maeda et al., 2009) and protease enzyme can provide information on the hydrolysis process of WAS affected by phospholine gum.
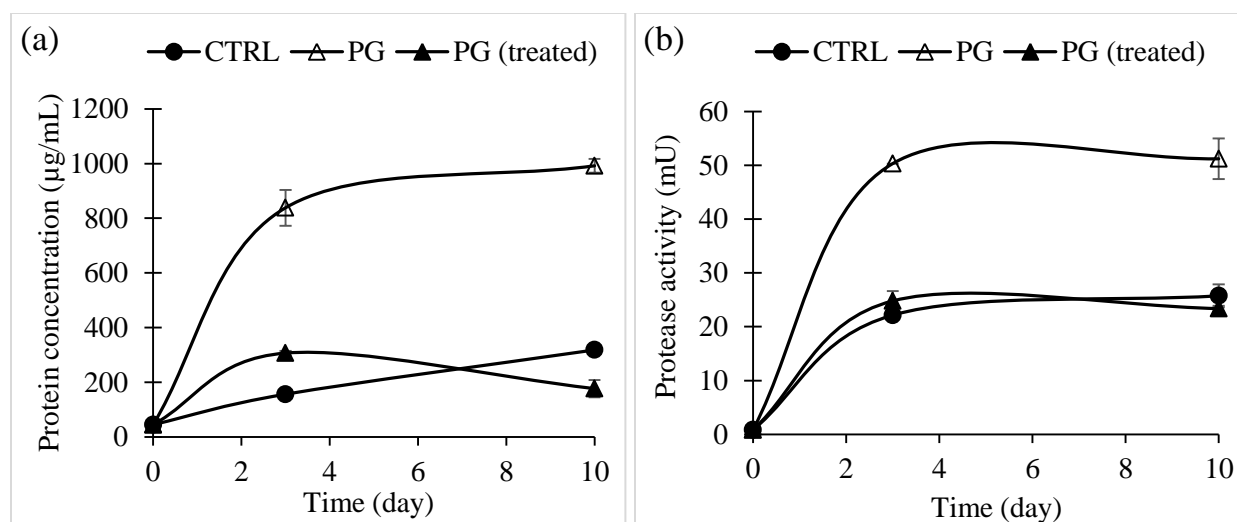
Figure 6.7: Soluble protein concentration (a) and protease activity (b) during anaerobic digestion using WAS with treated and untreated phospholine gum and also without any phospholine gum as a control.

As shown in Figure 6.7a, the protein concentration of WAS added with untreated phospholine gum was very high compared to treated phospholine gum and control WAS. Consequently, the protease activity (Fig. 6.7b) also was much higher in WAS with phospholine gum without heat treatment. On the other hand, treated phospholine gum and control WAS showed similar protease activity which corresponded to their protein concentrations. Therefore, the effect of phospholine gum to WAS was not adversely affected hydrolysis, but have stimulated proteases to hydrolyze proteins, and thus, contributed to rapid hydrolysis. Bacteria in the rumen secrete exoenzymes that degrade soluble proteins to peptides and amino acids (Gresner et al., 2015). Thus, addition of phospholine gum likely will not inhibit the digestion of nutrients required by ruminants.

The next stages of fermentation; acidogenesis, and acetogenesis, were evaluated for the production of organic acids that serve as intermediate substrates for methanogens during methanogenesis. A few organic acids were detected (e.g., isobutyric, acetic, propionic, and butyric acids) on days 3 and 7 of anaerobic fermentation (Fig. 6.8). All organic acids were detected at low levels in control WAS (0.5–3.0 mM) (Fig. 6.8a). In contrast, the addition of phospholine gum to WAS increased the production of organic acids from days 3 to 7, particularly propionic and butyric acids (Fig. 6.8b). Further, compared with control WAS, the level of acetic acid was higher in WAS containing phospholine gum.

The production of organic acids was analyzed using heat-treated phospholine gum (Fig. 6.8c). The production of isobutyric acid was higher than that of other organic acids and was also higher than that of control WAS and untreated phospholine gum. These findings show that the inorganic compounds present in heat-treated phospholine gum contributed to isobutyric acid production, although very low levels of isobutyric acid were produced in the presence of organic compounds (Fig. 6.8b). However, although only isobutyric acid was not produced by the addition of phospholine gum to WAS, other organic acids were produced at impressive levels. Moreover, using phospholine gum in WAS did not inhibit organic acid production, and thus, did not affect the acidogenesis and acetogenesis stages of anaerobic catabolism.
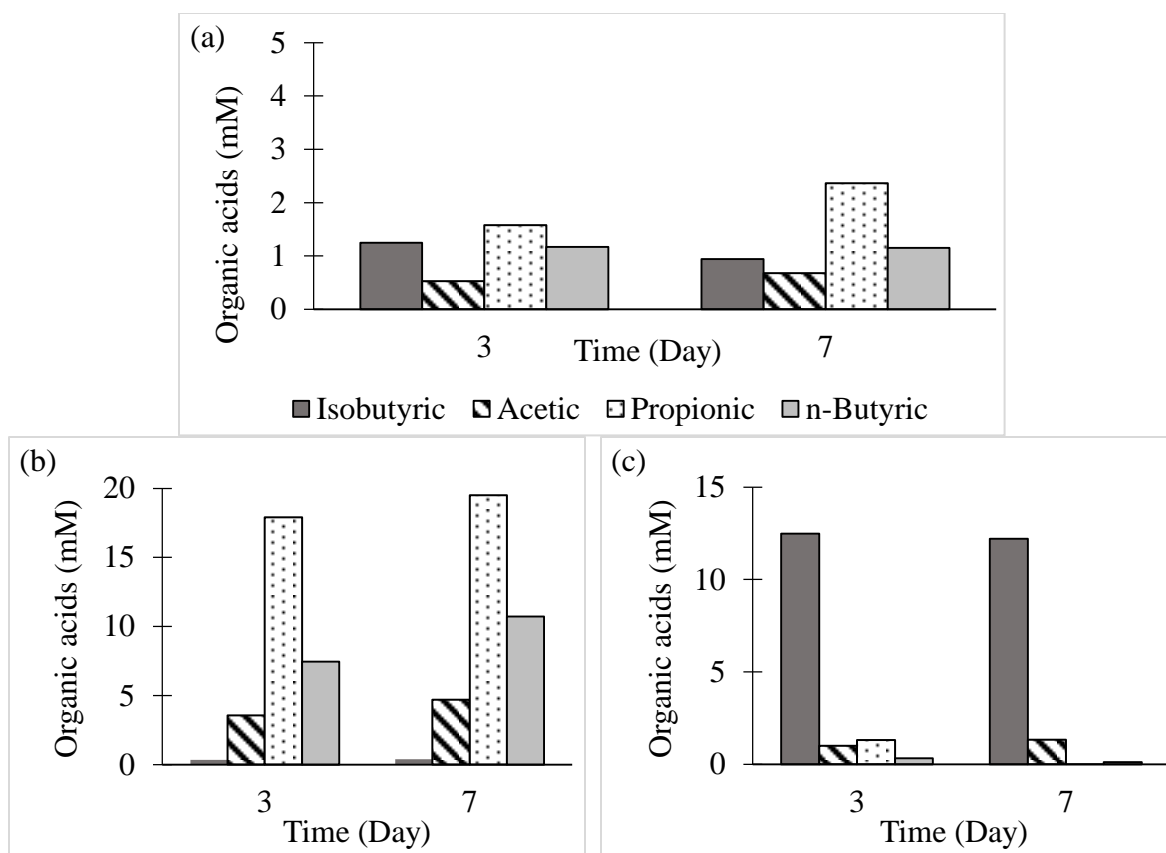
Figure 6.8: Profile of organic acids production at day 3 and 7 of anaerobic fermentation of WAS as control (a) and WAS with addition of untreated (b) and treated (c) phospholine gum.

Butyric and propionic acids are important volatile fatty acids required for the function of the rumen. For example, butyrate is important for ruminant metabolic activity and to maintain the integrity of the ruminal epithelium, whereas propionate is the only glucogenic volatile fatty acid and should be preserved or improved in the rumen (Meissner et al., 2014), which can subsequently improve production efficiency (Leeuw et al., 2016). Besides, according to O'Brien et al. (2013), the accumulation of butyric acid might be explained by the accumulation of hydrogen caused by the direct inhibition of methanogens. Therefore, the application of phospholine gum as a feed additive may provide essential various volatile fatty acids for ruminants and contribute to their growth production and maintenance.

6.4   Conclusion

The ability of phospholine gum to inhibit methane production by WAS provides an overview of its benefits as an additive to ruminant feed. *Lactobacillus sp.* and *Megasphaera sp.* occurrence were induced by phospholine gum are important microorganisms that benefit ruminants and inhibit enteric methane emissions. Phospholine gum directly inhibited methanogens without affecting other functions of the rumen system. The presence of organic compounds in phospholine gum partially contributed to inhibiting methane production. Taken together, phospholine gum shows great potential as an additive to ruminant feed

CHAPTER 7

CONCLUDING REMARKS AND RECOMMENDATIONS FOR FUTURE RESEARCH

The importance of microbial community structure in WAS has been revealed through application of recent high-throughput sequencing technology. The Illumina MiSeq platform deliver a powerful output data and provide a better understanding on the microbial community contributed to successful or unsuccessful bioremediation and bioenergy production by WAS. Furthermore, the exploitation of antibiotics approach has given big impacts on the bacterial interaction thus affecting on the microbial community dynamics. In addition, the potential of industrial by-product has been evaluated on mitigating the greenhouse gases, specifically methane gas. This was also achieved through exploration of microbial community structure.

The first objective of this study is to evaluate the factors contributing to failure of bioremediation through microbial community analysis. The inoculation of exogenous EGFP-expressing *E. coli* in WAS to mimic the bioaugmentation showed that the inoculant was unable to survive shortly after inoculation which expected to be killed by indigenous community. However, the microbial community only changed slightly resulted in difficulty to determine the responsible bacteria. Therefore, by antibiotic approach some indigenous bacterial community in WAS has been identified as bacterial soldier that killed the inoculant. From this study, the bacterial soldiers were captivated benefits from dead inoculant, thus improved in their domination as proved by the co-culture interaction.

Besides, another approach has to be performed to find other microbial communities that contribute to non-survival of inoculant by different interaction. The competition and amensalism are the possible interactions resulting in dead inoculant. Specifically, competition not only cause death to inoculant but also to the other party, while amensalism adversely affecting only the inoculant but not disturbs the growth or death of another community. Competition can be determined through direct or indirect interaction whereas amensalism can be determined by evaluating the inhibitory compounds secreted by those communities to inoculant. The microbial community analysis is the important primary evaluation to determine all these interactions.

In second part of the study, the different types of antibiotics have been used to evaluate the microbial community contribution to methane production. The antibiotics selected were based on the positive impact by improving the methane production, negative impact that inhibited methane production and also no/less impact of antibiotic addition towards methane production. These three different patterns of methane production allowed to a better understanding on the microbial community functions. For further improvement of methane production, the microbial community responsible to produce methane primarily detected using AZM from each anaerobic digestion stage should be retain in the WAS by isolation, enrichment and directly apply again in WAS for rapid methane production. Besides, the addition of CM directly inhibited methanogens but not inhibited other communities. These communities can be exploited for another production of intermediates substrates such as organic acids for bioplastic production or bio-alcohols as a source for biofuel production.

Continuing the inspiration from the improvement of methane production by addition of AZM in second objective, the other antibiotic analogs belong to macrolide and lincosamide has been selected to evaluate the methane production in third part of this study. Macrolides and lincosamides have same mechanism of action by targeting the protein synthesis but with different antibacterial spectrum of activity. Evaluation of microbial community structure and stages of anaerobic digestion has found out that some antibiotics produced high organic acids which indirectly inhibited methanogens. Therefore, further study can exploit these antibiotics for intermediate substrates production like organic acids or bio-alcohols. Besides, in term of methane production, enriched methanogens can be used simultaneously with these antibiotics to improve the methane production from WAS. On the other hand, other various antibiotics with different mechanism of action and spectrum of activity shall be evaluated to explore their potential in methane production based on microbial community dynamics of WAS.

Last part of this study showed the potentials of by-product from palm oil refining process to mitigate the methane emissions which further can be applied as feed additive for ruminants. Since this is the preliminary study to understand the mechanisms affected on anaerobic digestion and the responsible microbial community, more details study is required. Simple suggestion is by utilizing the rumen feces as a source of substrates and microorganisms instead of WAS for a more specific evaluation on methane inhibition from ruminants. Also, the experiment should be done in longer period of time to see if the microbial communities become adapted to phospholine gum addition as reported by other study using plant-based compound that the important rumen bacteria can improved in longer period.

On the other hand, this study has found out that two important ruminal bacteria, *Lactobacillus sp.* and *Megasphaera sp*. originated from phospholine gum has the ability to inhibit methane emissions. Therefore, further study can be done by isolation and enrichment of these cultures then use it as direct feed for ruminants. In addition, organic compounds of phospholine gum have been confirmed to inhibit methane production from this study. Thus, the determination of exact compositions or extracts that responsible to inhibit methane production should be done in future. Finally, the potential of phospholine gum as feed additive must be tested in vivo or in real application for ruminant, specifically on cow. This is important to evaluate not only the methane inhibition but also the feed intake, fiber degradability, animal productivity and rumen methanogens adaptability with the phospholine gum.

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PUBLICATION AND CONFERENCES ATTENDED

Publications

1. **Nurul Asyifah MUSTAPHA**, Kenji SAKAI, Yoshihito SHIRAI, Toshinari MAEDA. Impact of different antibiotics on methane production using waste-activated sludge: mechanisms and microbial community dynamics. Published in **Applied Microbiology and Biotechnology**. IF: 3.337

2. **Nurul Asyifah MUSTAPHA**, Siti Suhailah SHARUDDIN, Norhayati RAMLI, Yoshihito SHIRAI, Toshinari MAEDA. Effect of macrolides and lincosamides on methane production using waste activated sludge: mechanisms and microbial community dynamics. Submitted to **Microbiological Research**. IF: 2.561

3. **Nurul Asyifah MUSTAPHA**, Keita KUBO, Siti Suhailah SHARUDDIN, Norhayati RAMLI, Yoshihito SHIRAI, Toshinari MAEDA. Potential application of phospholine gum as feed additive in ruminant to mitigate methane emission. In revision Journal **of Cleaner Production**. IF: 3.59

4. **Nurul Asyifah MUSTAPHA**, Siti Suhailah SHARUDDIN, Norhayati RAMLI, Yoshihito SHIRAI, Toshinari MAEDA. Bacterial soldiers in waste activated sludge – Understanding of the bacterial natural selection. To be submitted to **Nature Microbiology**.

Conferences

1. Nurul Asyifah MUSTAPHA, Toshinari MAEDA, Yoshihito SHIRAI. Effect of Macrolide-, Lincosamide- and Ketolide-type Antibiotics on Methane Production from Waste Activated Sludge. The 50th Annual Conference of Japan Society on Water Environment, Tokushima, Japan. (March 16-18, 2016).

2. Nurul Asyifah MUSTAPHA, Toshinari MAEDA, Kenji SAKAI, Yoshihito SHIRAI. Microbial community analysis to understand a positive/negative impact of antibiotics on methane production from waste activated sludge. The 67th Annual Meeting of the Society for Biotechnology, Japan. (October 26-28, 2015).

3. Nurul Asyifah MUSTAPHA, Toshinari MAEDA, Yoshihito SHIRAI. Microbial Diversity Changes in Waste Activated Sludge by the Inoculation of an Alien Bacterial Organism. The 1st International Symposium for Women Researchers on Advanced Science and Technology conjugated with the Seminar for Young Researchers. Yamaguchi, Japan. (July 10-14, 2015).

4. Nurul Asyifah MUSTAPHA, Toshinari MAEDA, Yoshihito SHIRAI. Inhibition of Methane Production from Waste Activated Sludge by a Palm Oil Industrial Waste. The First Asian Symposium on Chemistry-based Biotechnology, Kitakyushu, Japan. (June 11-12, 2015).

5. Nurul Asyifah MUSTAPHA, Natsumi ISHIDA, Toshinari MAEDA, Yukihiro TASHIRO, Kenji SAKAI, Yoshihito SHIRAI. Flow Cytometry Quantification of Green Fluorescent Protein-expressing Escherichia coli Cells Inoculated into Sewage Sludge. UPM-Kyutech Symposium on Applied Engineering and Sciences, Kyutech Tobata Campus. (December 20-21, 2014).

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