Assessment of Anaerobic Digestate of Dairy Manure as Biofertilizer: Environmental Risk and Potential in Suppressing Plant Disease

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乳牛ふん尿の嫌気性消化液の肥料としての評価：環境リスク及び土壌病害の抑制可能性

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General Introduction

1. Anaerobic digestion

One of the new trends of dairy farming in Japan is to intensify the animal production, which is always accompanied by the production of large amount of dairy manure. If not properly managed, dairy manure (DM) presents a potential source of various hazards to human life and the environment (Rico et al., 2011; Yamashiro et al., 2013). DM has traditionally been land spread as fertilizer for crops. However, there was concern about the greenhouse gas and odors emissions, and environmental legislation has placed strict constraints on the land application of DM (Xie et al., 2011). Therefore, anaerobic digestion (AD) has been implemented for years for management of dairy manure, which provides several benefits, including the improvement of manure fertilizer quality and reduction of greenhouse gas emissions, odors and pathogens (Micolucci et al., 2016; Qi et al., 2018; Sahlström, 2003). Furthermore, the economical driver of this technology is represented by the production of biogas, composed mainly of methane (CH_4, 60%) and carbon dioxide (CO_2, 40%), which can be converted into electric and thermal energy or purified to obtain biomethane (Holm-Nielsen et al., 2009). Recently, there is increasing worldwide interest in technology producing renewable energy sources, such as biogas, as a result of global warming and the increasing consumption of fossil fuels.

AD is a complex, multi-stage series of biochemical process majorly driven by two groups of microorganisms: Bacteria and Archaea. The bioconversion of organic waste to biogas during AD via the action of enzymes produced by anaerobic microorganisms in four stages: hydrolysis, acidogenesis, acetogenesis, and methanogenesis, which is summarized in Fig. 1.

Phase 1 – Hydrolysis stage: This process can be simply described as the hydrolysis of polymers such as carbohydrates, proteins and fats into soluble monomers (simple sugars, amino acids and long-chain fatty acids). The main enzymes involved in this stage were proteases and lipases, which were released by the hydrolytic or facultative anaerobes (*Clostridia*, *Bacteroides* and *Streptococci*).

Phase 2 – Acidogenesis stage: The monomers from the hydrolysis stage are converted by acid-forming obligatory and facultative anaerobes into short-chain fatty acids, mainly propionic acids, butyric acids, ethanol, carbon dioxide and hydrogen. *Bacillus* and *Pseudomonas* are involved in mainly bacteria in this stage.

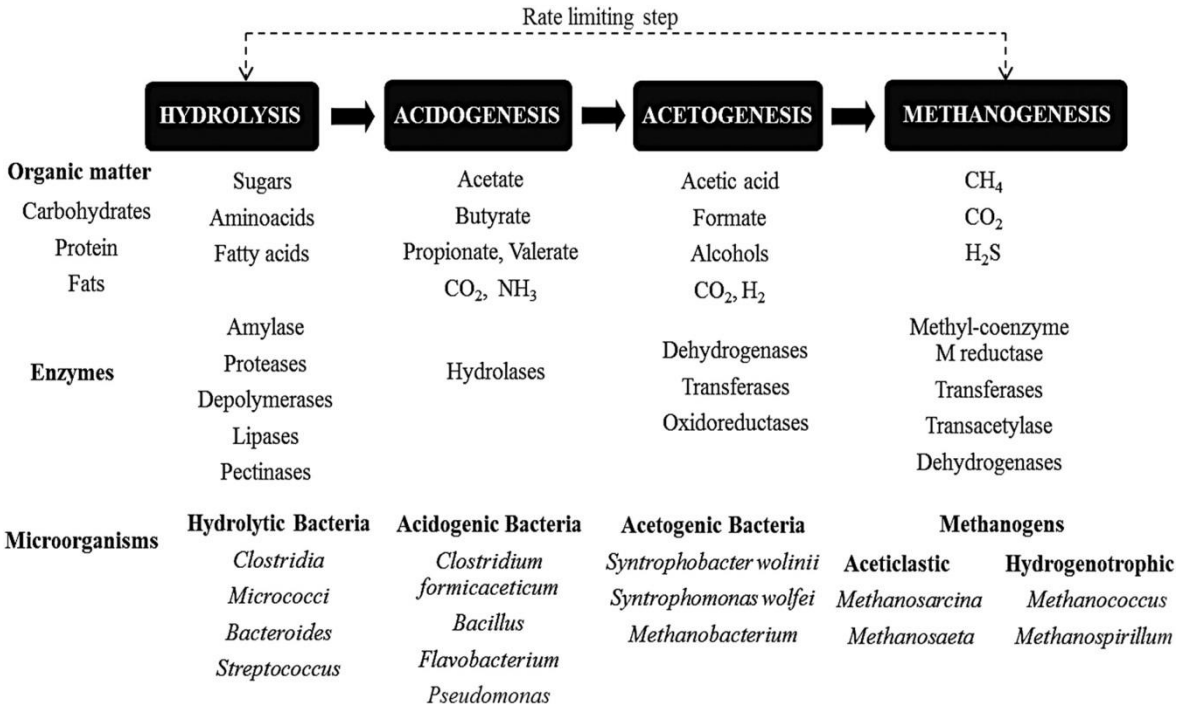


Figure 1: Flow diagram of the degradation of organic matter and production of biogas through anaerobic digestion process. Source: (Arif et al., 2018)

Phase 3 – Acetogenesis stage: This stage comprises of anaerobic oxidation reactions in which short-chain fatty acids are converted into acetic acids. The pre-requisite for this stage is a symbiotic relationship between microorganisms carrying out anaerobic oxidation and methane forming species.

Phase 4 – Methanogenesis stage: In this stage, the methanogens mediate production

of methane from acetate (Aceticlastic methanogenesis), carbon dioxide and hydrogen gas (hydrogenotrophic methanogenesis). Aceticlastic methanogenesis is driven two main genera: *Methanosarcina* and *Methanosaeta*, which contribute to 70% of the methane production, while 30% is produced during hydrogenotrophic methanogenesis by *Methanococcus* and *Methanospirillum*. This is a critical stage as it requires stringent anaerobic conditions and is the slowest biochemical reaction of the AD process.

1.1 Batch or continuous

AD process could be performed as a batch process or a continuous process. In a batch AD process, the influent is added to the reactor at the beginning of the process, and then the reactor is sealed to obtain stringent anaerobic conditions. Biogas production of batch AD process will be formed with a normal distribution pattern over digestion time. As the batch AD process is simple and requires less equipment and lower levels of design work, it is typically used to evaluate the biogas production potential of one influent. In actual biogas plant, more than one batch reactor is necessary for ensuring the constant production of biogas. In continuous AD process, the influent is constantly added to the reactor. Accordingly, the end product, i.e. anaerobic digestate is constantly removed, resulting in constant production of biogas. The typical reactor for continuous AD process is continuous stirred-tank reactors (CSTR) (Yamashiro et al., 2013). This process is usually used to evaluate the stable performance of reactor feeding with single influent or a mixture of influents, which is also called as anaerobic co-digestion.

1.2 Temperature

The temperature is the most important parameter that affects the performance of AD process. The two conventional operational temperature ranges for anaerobic reactors are mesophilic (30 to 38 °C) and thermophilic (49 to 57 °C). Mesophilic digestion still represents the most common technology performed in continuous biogas plants as its lower energy cost and higher process stability. Simultaneously, thermophilic digestion has attracted increasing interest worldwide as it could disposal larger amount of influent with

lower hydraulic retention times (HRT) and is more effective on reducing pathogenic bacteria in influent (Gavala et al., 2003; Min et al., 2016). A comparison of performance of mesophilic and thermophilic digestion is presented in Table 1 (Noike et al., 2009).

Table 1: Performance comparison of mesophilic and thermophilic anaerobic digestion

	Mesophilic digestion	Thermophilic digestion
Temperature	30 - 38°C	49 - 57°C
Gas generation rate	Slow	Fast
Organic loading rate	2.0 ~ 3.0 kg/m^3/day	5.5 ~ 6.5 kg/m^3/day
Hydraulic retention time	20 ~ 30 day	10 ~ 20 day
Sanitization risk	High	Low
Energy consumption	Low	High

2. Anaerobic digestate

The digested residue after AD process, also called as anaerobic digestate, which is a mixture of partially degraded organic matter, microbial biomass and inorganic compounds (Alburquerque et al., 2012). The sustainability of AD systems depends greatly on the appropriate disposal of anaerobic digestate. The growing number of full-scale biogas plants worldwide has resulted in increased amount of anaerobic digestate. Utilization of anaerobic digestate as a biofertilizer in agriculture is considered the most suitable use, as it recycles plant nutrients in digesatate and reduces the consumption of mineral fertilizers (Jiang et al., 2011; Li et al., 2016; Riva et al., 2016). In addition, nutrients are present in inorganic plant-available forms in digestate at a markedly higher level compared to undigested influent, as a large portion of the organic form of nutrients is converted into its inorganic form during

AD process (El-Mashad and Zhang, 2010; Umetsu et al., 2002). For example, the ammonium (NH_4^+-N) concentration is significantly higher in digestate than in the influent (Massé et al., 2007; Riva et al., 2016; Umetsu et al., 2002). However, the fertilizer property of anaerobic digestate is not constant, as it is affected by the influent characteristics and AD process operating conditions (Alfa et al., 2014; Solé-Bundó et al., 2017). In recent years, fertilizer property of digestate from different have been widely investigated focusing on its plant nutrient contents (Abubaker et al., 2012; Alburquerque et al., 2012; Risberg et al., 2017) and ecotoxicity and environmental risk (Tigini et al., 2016). However, there is limited number of researches focusing on the fertilizer property of digestate relating to microorganisms.

It is known that livestock manure is a reservoir of various bacteria, and these bacteria may survive during the AD process and persist in anaerobic digestate. Alfa et al. (2014) and Owamah et al. (2014) have investigated the biofertilizer properties of anaerobic digestate and concluded that it contained many pathogenic and non-pathogenic bacteria, such as *Pseudomonas*, *Klebsiella*, *Salmonella*, *Bacillus*, *Shigella*, *Clostridium*, and other microorganisms. Therefore, utilization of anaerobic digestate as agricultural fertilizer is not completely risk-free as it may increase the risk of pathogenic bacteria spread into environment (Nkoa, 2014). Pathogenic bacteria are thought to be decreased after AD process, and the decay rate is dependent on many factors: digestion temperature, retention time, volatile fatty acids (VFAs) concentration, pH value, reactor configuration, available nutrients, microbial species and chemical interactions, in which temperature represents the most important one (Beneragama et al., 2013b; Sahlström, 2003; Smith et al., 2005). As mentioned in section 1.2, AD process could be performed at mesophilic or thermophilic temperatures. Thermophilic digestion is shown to be more effective on reducing pathogenic bacteria in influent. However, many pathogenic bacteria may still be present in digestate from mesophilic digestion and cause an environmental risk through application. Anaerobic digestate from AD process must be proven hygienically safe before it could be utilized to agricultural lands (Iwasaki et al., 2011; Sahlström, 2003). Therefore, the environmental risk

associated with utilization of digestate as biofertilizer could not be neglected.

3. Antibiotic resistant bacteria

Antibiotics are have been frequently used in livestock industry for therapeutic, prophylactic treatments or growth promotion purposes. Mastitis is one of the most common infectious diseases in dairy cows, which is mainly caused by pathogens such as *Staphylococcus*, *Streptococcus* and *coliform* bacteria (Bradley, 2002). Milk from dairy cows infected with mastitis has a higher somatic cell counts and degraded milk protein. Cefazolin, a β-lactam antibiotic, abbreviated as CEZ, is abundantly used to treat mastitis of dairy cows. Besides CEZ, various types of antibiotics such as neomycin, vancomycin, vancomycin, penicillin, oxtetracycline, ampicillin and streptomycin are widely used antibiotics in livestock industry (Beneragama et al., 2012, 2013b). However, the antibiotics are not completely eliminated in the organism of dairy manure and between 17% and 76% of them are excreted via manure in an unaltered form or as metabolites of parent compounds. It has been indicated that antibiotics in dairy manure had inhibitory effect on biogas production during AD process at a high concentration (Beneragama et al., 2013a). Furthermore, another major concern about the use of antibiotics is the occurrence of antibiotic resistant bacteria (ARB) in dairy manure, as once antibiotics are used ARB are selected and/or evolved (Lateef et al., 2012). Therefore, the dairy manure from dairy cow treated with antibiotics is thought to contain substantial quantities of ARB. AD process has been proven a suitable technology for eliminating ARB, especially at high temperature.

4. Soil-borne plant disease and antagonistic bacteria

Soil-borne plant disease caused by phytopathogens is one of the major factors that cause devastating effects on plant health and production in agricultural fields (Elshahat et al., 2016). Traditionally, pesticides are effective method to control the soil-borne plant diseases, however, frequent use of pesticides may lead to environmental pollution and the development of pesticide resistance in phytopathogens (Mehta et al., 2014). Therefore,

there is growing interest in finding more ecological and economical methods to protect the plant from various phytopathogens.

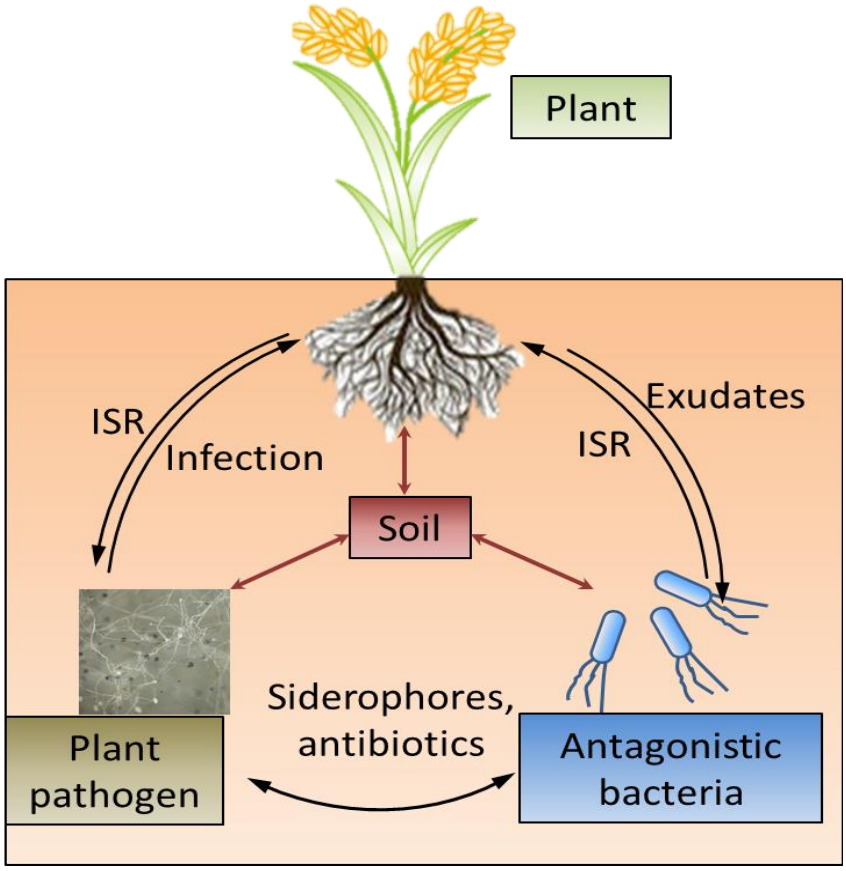


Figure 2: The mechanisms of antagonistic bacteria suppressing phytopathogen

The application of antagonistic bacteria to control plant disease, which is also known as biological control, seems to be the most suitable one. Antagonistic bacteria can protect plants from phytopathogens via multiple mechanisms (Fig. 2), including induction of systemic resistance (ISR), competition for space and nutrients and by secretion of siderophores and antibiotics (Ahemad and Kibret, 2014; Yang et al., 2015).

Bacillus and *Pseudomonas* represent the two major genera of antagonistic bacteria, which are successfully proven to suppress various soil-borne plant diseases (Kumar et al., 2012; Salman, 2010; Yang et al., 2015). *B. subtilis* was demonstrated to be an effective biocontrol agent for chilli anthracnose disease and sugar beet cercospora leaf spot (Ashwini and Srividya, 2014; Collins and Jacobsen, 2003). *B. licheniformis* also showed antagonistic activity against plant diseases by producing antifungal protein and metabolites (Jeong et al., 2017; Wang et al., 2014). Fluorescent pseudomonads also represent an important group of bacteria that have promising antagonistic abilities for used as biological control agents (Elshahat et al., 2016; Salman, 2010).

5. Potential of anaerobic digestate in suppressing plant disease

In recent years, several studies have reported the suppressive effects of organic soil amendment, such as compost on soil-borne plant diseases (Termorshuizen et al., 2006). A comparison of properties of compost and anaerobic digestate is shown in Table 2. Both anaerobic digestate and compost are organic amendments originated from dairy manure that include an available form of plant nutrients (N, P and K), organic carbon, and abundant microbial populations (Alburquerque et al., 2012). However, there is limited information regarding the plant disease suppressive potential of anaerobic digestate.

The plant disease suppressing phenomenon of organic soil amendment consists of various factors, while enzymatic and microbiological parameters, such as antagonistic bacteria, are much more informative for soil-borne disease suppression (Bonanomi et al., 2010). Therefore, population densities of antagonistic bacteria in organic amendment is an important indicator of suppressive activities against soil-borne plant diseases (Boulter et al., 2002), and higher densities of antagonistic bacteria contribute to higher disease suppressive activities (Joshi et al., 2009). Boulter et al. (2002) reported that the dominant antagonistic bacteria in compost were *Pseudomonas* and *Bacillus*. However, the research on the population densities of *Pseudomonas* and *Bacillus* in anaerobic digestate and their antagonistic activities are limited. Therefore, to evaluate the potential of anaerobic digestate

suppressing plant disease, it is important to isolate antagonistic bacteria from digestate and investigate their antagonistic activity.

Table 2: Comparison of properties of compost and anaerobic digestate

Soil amendment	Comparison of properties	
Compost	Organic amendment: Available form of plant nutrients (N, P, K) Abundant microbial populations	Plant disease suppression: Physio-chemical properties Microbiological properties
Anaerobic digestate	Organic amendment: Available form of plant nutrients (N, P, K) Abundant microbial populations	Plant disease suppression: Information is limited

6. Objectives and thesis outlines

Anaerobic digestion is a promising technology treating livestock manure with biogas production and a digested residue, anaerobic digestate. The sustainability of anaerobic digestion systems depends greatly on the appropriate disposal of the anaerobic digestate. Traditionally, anaerobic digestate is spread to agricultural land as biofertilizer. Therefore, the focus of this thesis was on investigating the environmental risk of digestate application relating pathogenic bacteria and antibiotic resistant bacteria. On the other hand, in order to investigate the potential of digestate in suppressing soil-borne plant diseases by detecting the antagonistic bacteria.

The objectives of this PhD thesis were divided into three:

1. To investigate the effect of temperature on survival of pathogenic and antibiotic resistant bacteria during anaerobic digestion of dairy manure in batch reactors.

2. To investigate the survival of pathogenic bacteria as well as antagonistic bacteria during anaerobic digestion of dairy manure in semi-continuous stirred-tank reactors.

3. To investigate the antagonistic activity of antagonistic bacteria (*Bacillus* and *Pseudomonas*) against phytopathogens and soil-borne plant diseases in field experiment.

Chapter 1 was performed with objective of determining the effect of anaerobic digestion temperature on survival of pathogenic and cefazolin resistant bacteria in dairy manure. Lab-scale batch anaerobic digestions were conducted under mesophilic (37 °C), thermophilic (55 °C) and hyper-thermophilic (65 °C) temperatures. Indigenous indicator and pathogenic bacteria (*Escherichia coli, Enterococcus, Salmonella, and Campylobacter*) and one multidrug-resistant bacterium *Acinetobacter* were determined in population densities and their resistance to cefazolin before and after anaerobic digestion.

Chapter 2 was focused on the survival of pathogenic bacteria as well as antagonistic bacteria during anaerobic digestion of dairy manure in semi-continuous stirred-tank reactors. In this chapter, anaerobic digestions of dairy manure were conducted under mesophilic and thermophilic temperatures. Dairy manure and digestates were samples for detecting pathogenic bacteria (*E. coli*, *Salmonella*, *Enterococcus*, and *Campylobacter*) with and without cefazolin resistant and antagonistic bacteria (*Bacillus* and *Pseudomonas*).

In chapter 3, *Bacillus* and *Pseudomonas* isolates from dairy manure, mesophilic and thermophilic digestates were tested for antagonistic activity. The population densities of antagonistic *Pseudomonas* and *Bacillus* against four phytopathogens (*Alternaria Sorani*, *Cercospora beticola*, *Fusarium nivale* f. sp. *graminicola* and *Streptomyces.scabie*) were measured by dual culture method. Furthermore, field experiment was conducted with two selected *Bacillus* isolates from anaerobic digestate to investigate their biocontrol activities against potato late blight (*Phytophthora infestans*).

Chapter 1

Effect of Anaerobic Digestion Temperature on Survival of Pathogenic and Cefazolin Resistant Bacteria in Dairy Manure

1. Introduction

Anaerobic digestion (AD) has been implemented for years for management of dairy manure, which provides several benefits, including the improvement of manure fertilizer quality and reduction of greenhouse gas emissions and odors (Micolucci et al., 2016; Qi et al., 2018; Sahlström, 2003). Furthermore, the economical driver of this technology is represented by the production of biogas, composed mainly of methane (CH_4, 60%) and carbon dioxide (CO_2, 40%), which can be converted into electric and thermal energy or purified to obtain bio-methane (Holm-Nielsen et al., 2009). Recently, there is increasing worldwide interest in technology producing renewable energy sources, such as biogas, as a result of global warming and the increasing consumption of fossil fuels.

Livestock wastes is well known to contain pathogenic bacteria of different species such as *Escherichia coli*, *Enterococcus*, *Salmonella*, *Campylobacter* (Sinton et al., 2007), which are indigenous indicator and pathogenic bacteria in livestock waste causing infections in both people and animals. In recent years, frequently use of antimicrobial drugs in livestock husbandry for both therapeutic and growth promotion purposes is increasing a new public health concerns on presence of antibiotic resistant bacteria (ARB) in environment (Walczak and Xu, 2011). In Japan, monitoring of antimicrobial resistance in *Escherichia coli*, *Enterococcus*, *Salmonella*, *Campylobacter* against common antimicrobials such as cefazolin has been conducted in livestock industry because that it will be potential risk to human and animals health as these indigenous pathogenic bacteria in animal waste appear to resistant to antimicrobial drugs ("Report on the Japanese Veterinary Antimicrobial Resistance Monitoring System -2012 to 2013- National Veterinary Assay Laboratory Ministry of Agriculture , Forestry and Fisheries," 2016). As an ecological consideration, sanitary treatment needs to be implemented to destroy pathogenic and antibiotic resistant bacteria in animal wastes.

Anaerobic digestate, a liquid residue called digestate is produced after anaerobic digestion process. The sustainability of biogas production systems depends greatly on the

appropriate disposal of the anaerobic digestate. The growing number of full-scale biogas plants worldwide has resulted in increased amount of anaerobic digestate. Generally, anaerobic digestate is available to agricultural land as liquid fertilizer. Currently, there is no clearly regulation and standard about biosafety of digestate in all over the world. In particularly in developing countries, treatment of digestate may discharge to agricultural land directly. It leads to increase of risk on dissemination of pathogens. As we have known large quantities of pathogens survive in animal waste, how their fate during anaerobic digestion remains to be unknown. Anaerobic digestate from AD process must be proven hygienically safe before it could be utilized to agricultural lands (Iwasaki et al., 2011; Sahlström, 2003). Therefore, the environmental risk associated with utilization of digestate as biofertilizer could not be neglected. Based on above consideration, the growing interests in researching biosecurity performance of anaerobic digestion attracts more and more attentions from digestate users and consumers. Some literatures have demonstrated that anaerobic digestion was effective process for reducing pathogenic bacteria and antibiotic resistant bacteria in animal waste (Beneragama et al., 2013b; Quessy and Masse, 2006). Generally, the principle factors causing pathogenic bacteria decay or loss during AD process include temperature, retention time, reactor configuration, microbial completion, pH value and chemical interaction (Smith et al., 2005). And temperature is considered the most important one.

In chapter 1, experiments were performed with objective of determining the effect of anaerobic digestion temperature on survival of pathogenic and cefazolin resistant bacteria in dairy manure. Lab-scale batch anaerobic digestions were conducted under mesophilic (37 °C), thermophilic (55 °C) and hyper-thermophilic (65 °C) temperatures. Indigenous indicator and pathogenic bacteria (*Escherichia coli, Enterococcus, Salmonella*, *and Campylobacter*) and one multidrug-resistant bacterium *Acinetobacter* were determined in population densities and their resistance to cefazolin before and after anaerobic digestion using standard dilution plate method.

2. Materials and Methods

2.1 Lab-scale batch anaerobic digestion

Lab-scale batch anaerobic digestions were conducted using 1 L digesters which made from polypropylene with working volume of 600 mL (Andriamanohiarisoamanana et al., 2016; Lateef et al., 2012). Three groups of digesters were kept at mesophilic (37 °C) and thermophilic (55 °C) and hyper-thermophilic (65 °C) conditions in thermostatically controlled water baths. Inoculums were obtained from active thermophilic biogas plants in Hokkaido respectively. Raw manure (RM) was collected from Obihiro University farm and used as feedstock after the total solid (TS) content was adjusted to 10% by diluted water. Before and after digestion process, influent and effluent samples were measured by items of total solid (TS), volatile solid (VS) and pH value. Each experimental group was conducted by triplicate.

2.2 Analysis of parameters

The volume of produced biogas was measured daily with wet gas meter. All gas measurements were expressed at 0 °C and a pressure of one atmosphere. The biogas also was collected and analyzed for gas composition with gas chromatograph (GC) (Shimadzu GC-14A) equipped with a thermal conductivity detector (stainless column and Porapak Qpacking). The operational temperatures of injector port, column and the detector were 220, 150 and 220 °C, respectively. Argon was the carrier gas at a flow rate of 50 mL min^{-1}.

TS and VS contents of samples were determined according to the standard methods (APHA, 1998). The pH before and after digestion was measured using a pH meter (Horiba-D55, Kyoto, Japan).

2.3 Culturing of pathogenic and cefazolin resistant bacteria

Population densities of pathogenic bacteria (*Escherichia coli*, *Enterococcus*, *Salmonella*, *Campylobacter* and *Acinetobacter*) in slurry samples taken before and after

experiment were determined by standard dilution plate method. Samples were diluted 10-fold with phosphate buffered saline (pH 7.4), and 100 µl of diluent was spread on CHROMagar™ ECC (CHROMagar/Paris, France) for *Escherichia coli* detection, Enterococcosel Agar (ECS; Kyokuto Pharmaceuticals Co. Inc., Tokyo, Japan) for *Enterococcus* detection, Deoxycholate Hydrogen Sulfide lactose agar (DHL; Eiken Chemical Co. Ltd., Tokyo, Japan) for *Salmonella* detection, and Cefaperazone Charcol Desoxycholate Agar (CCDA; Kanto Chemical, Tokyo, Japan) for *Campylobacter* detection. Multidrug-resistant *Acinetobacter* were determined with CHROMagar Acinetobacter. The incubation time and temperature were controlled according to the specifications. The appropriate cultures with colonies between 20 and 200 on agar medium were used to estimate the number of bacteria in the samples (Resende et al., 2014). After incubation, typical colonies were counted and calculated as CFU/g dry matter.

In Obihiro University farm, cefazolin (CEZ), a β-lactam antibiotic, which suppresses the growth of bacteria by inhibiting cell wall synthesis, is frequently used to treat the cows with mastitis. Cefazolin was added to plate at a final concentration of 50 mg L^{-1}, the colonies on plates with cefazolin were counted as population densities of cefazolin resistant bacteria (Kobashi et al., 2005).

3. Results and Discussion

3.1 Anaerobic digestion performance

The digestion process was thought completely conducted until cumulative biogas production was not increased for more than 5 days. The operation time of mesophilic, thermophilic and hyper-thermophilic anaerobic digestions were 65, 25 and 30 days respectively. Fig. 3 shows the cumulative amount of biogas generated by batch anaerobic digestion at different temperatures. During the processes of anaerobic digestion, thermophilic digestion showed the fastest biogas generation at the early operation (first 5 days) and got to the stable condition, which is satisfied with high temperature contributes high biogas production rate (Gavala et al., 2003; Micolucci et al., 2016). The maximum

cumulative biogas production was obtained at 20 day. The cumulative biogas production of mesophilic digestion was not increased until day 30, it is thought that methanogens in inoculum from thermophilic biogas plant was not suitable to mesophilic temperature. The maximum biogas production was obtained at day 60. Hyper-thermophilic digestion showed lower biogas production rate and were stable, and maximum biogas production was obtained at day 25.

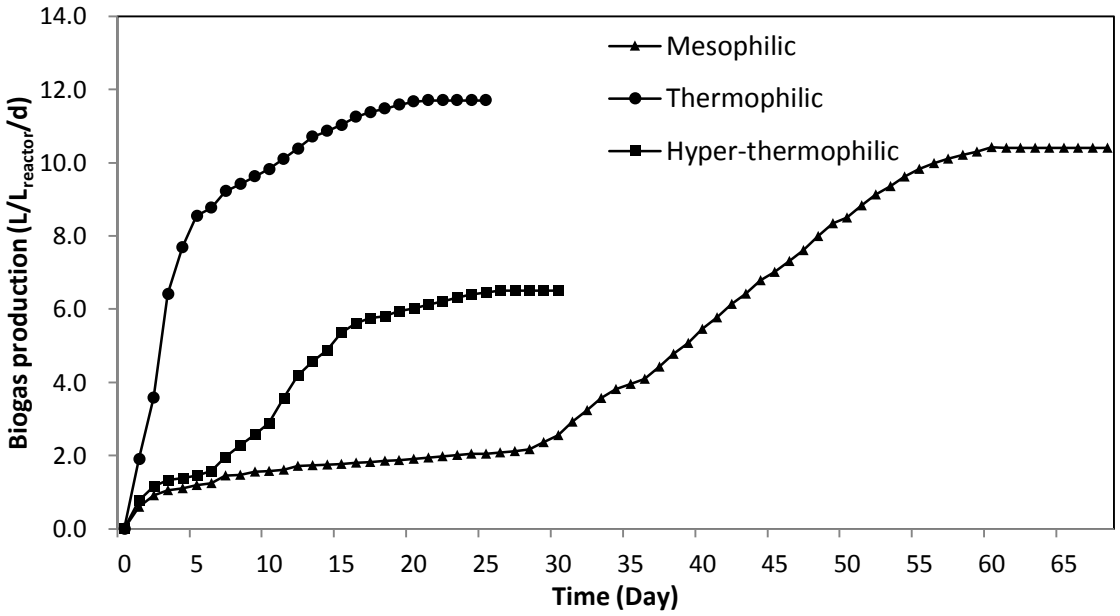


Figure 3: Cumulative biogas production of anaerobic digestion at mesophilic, thermophilic and hyper-thermophilic temperatures

Cumulative biogas and methane production, methane concentration of biogas and methane yield per $gVS_{reduction}$ are presented in Table 3. Thermophilic digestion production highest cumulative biogas and methane production of 11.67 and 6.36 $L/L_{reactor}$, which were little higher than those of mesophilic digestion, which were 10.42 and 5.86 $L/L_{reactor}$.

However, hyper-thermophilic anaerobic digestion produced lowest biogas production of 6.46 $L/L_{reactor}$; it is considered high temperature causes excessive ammonia production which blocked the anaerobic digestion process (Noike et al., 2009). The lowest methane concentration, 48.08% also showed that AD process was not conducted successfully at hyper-thermophilic temperature. The methane concentrations of biogas from mesophilic and thermophilic digestion, 56.22% and 54.66%, were in an acceptable range between 50 and 70%, indicating healthy anaerobic processes (Andriamanohiarisoamanana et al., 2016).

Table 3: Cumulative biogas and methane production, methane concentration of biogas and methane yield of anaerobic digestion at different temperatures

Digestion temperature	Biogas production ($L/L_{reactor}$)	Methane concentration (%)	Methane production ($L/L_{reactor}$)	Methane yield ($L/gVS_{reduction}$)
Mesophilic	10.42 ± 0.04	56.22 ± 1.54	5.86 ± 0.02	0.39 ± 0.01
Thermophilic	11.67 ± 0.51	54.66 ± 3.15	6.36 ± 0.32	0.53 ± 0.03
Hyper-thermophilic	6.46 ± 0.16	48.08 ± 5.58	3.63 ± 0.77	0.36 ± 0.08

The initial and final TS and VS contents and pH value of digestates from anaerobic digestion at different temperatures are presented in Table 4. Initial TS of influents were 8.11%, 7.76% and 7.76% for mesophilic, thermophilic and hyper-thermophilic anaerobic digestion respectively. After experiment period, TS was reduced to 5.05%, 5.3% and 5.6% respectively. Similarly, VS of manures were reduced from 7.04%, 6.69 %and 6.69% to 4.05%, 4.28% and 4.65%, respectively. The pH of digestates from mesophilic and thermophilic digestion were 7.35 and 7.81, respectively. These results also agreed with those of other researchers (Alburquerque et al., 2012; Yamashiro et al., 2013), the pH value of digestate from livestock manure tends towards the alkaline range. All these parameters

show fully conducted and stable processes of anaerobic digestion at mesophilic and thermophilic temperatures. However, the digesate of hyper-thermophilic showed a higher pH value of 8.22, which also indicated the high ammonia concentration.

Table 4: TS, VS contents and pH values of slurry samples before and after anaerobic digestion

Digestion temperature	TS content (%)		VS content (%)		pH value	
	Initial	Final	Initial	Final	Initial	Final
Mesophilic	8.11	5.05	7.04	4.05	7.20	7.35
Thermophilic	7.76	5.30	6.69	4.28	7.15	7.81
Hyper-thermophilic	7.76	5.60	6.69	4.65	7.15	8.22

3.2 Survival of pathogenic bacteria

Through anaerobic digestion, tested pathogenic bacteria were decreased in various degrees, and the results are presented in Fig. 4. Population densities of *Enterococcus, Salmonella,* and *Acinetobacter* reduced to undetectable level after anaerobic digestion. However, *Escherichia coli* and *Campylobacter* still survived after mesophilic anaerobic digestion. It is worth noting that *Campylobacter* showed nearly no reduction, even it can survive during thermophilic anaerobic digestion. Until to hyper-thermophilic anaerobic digestion, *Campylobacter* was reduced to undetectable level. In this experiment, all tested pathogenic bacteria were reduced during anaerobic digestion, and significantly reduced with higher temperature. The temperature has been indicated is the most important factor to reduce pathogens during anaerobic digestion (Sahlström, 2003; Smith et al., 2005). However, *Campylobacter* is tolerant to high temperature, Kearney et al. (1993) also

reported that *Campylobacter* is a resistant bacteria during anaerobic digestion, therefore, decimation reduction time needs longer. Even though hyper-thermophilic anaerobic digestion showed absolutely reduction of pathogenic bacteria, concern on *Campylobacter* in mesophilic and thermophilic digestate still is an environmental risk to agriculture environment.

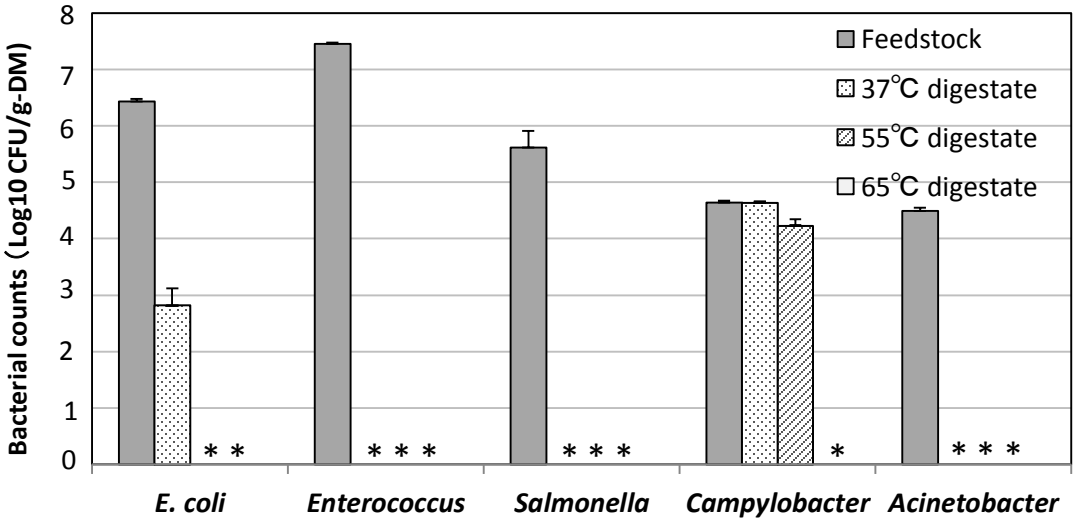


Figure 4: Population densities of bacteria in feedstock and digestates. CFU: colony forming units; DM: dry matter; * means not detected; Values are expressed as the mean ± SE.

3.3 Survival of cefazolin resistant bacteria

The results of CEZ-resistant bacteria are shown in Fig. 5. Except for *Salmonella*, other 4 CEZ-resistant bacteria were detected in various degrees. *Escherichia coli*, *Enterococcus* and *Acinetobacter* were reduced to undetectable level after anaerobic digestion. One side, anaerobic digestion provided an ideal environment to pathogens sterilization. On another side, initial quantity of pathogenic bacteria is related to pathogens

sterilization (Sahlström, 2003; Smith et al., 2005). In this research, all initial CEZ-resistant bacteria in raw manure were detected with small quantities. The largest amount of CEZ-resistant bacteria in among of which were reduced to undetectable levels after anaerobic digestions is *Enterococcus,* it was detected with only amount of 3.51 log_{10}CFU/g-DM. Therefore, main reason for CEZ-resistant bacteria sterilization is considered that initial amount is small. However, *Campylobacter* was detected still surviving after mesophilic anaerobic digestion. Even it was increased lightly compare to initial amount after mesophilic anaerobic digestion.

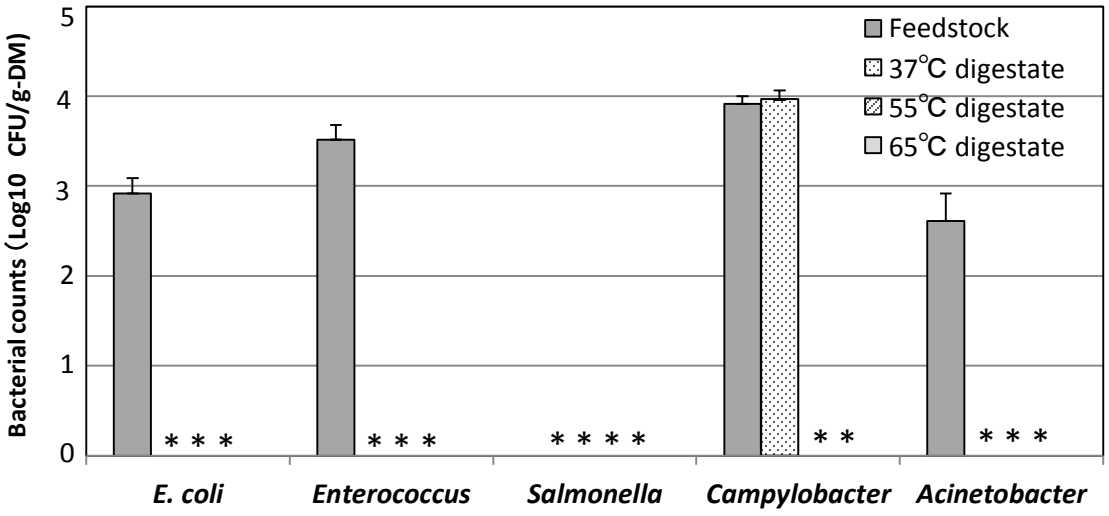


Figure 5: Population densities of CEZ-resistant bacteria in feedstock and digestates. CFU: colony forming units; DM: dry matter; * means not detected; Values are expressed as the mean ± SE.

4. Conclusion

Anaerobic digestion showed effective reduction of pathogenic and CEZ-resistant

bacteria. Pathogenic bacteria can be reduced to undetectable with higher temperature, as temperature is considered important factor to reduce pathogenic bacteria. It worth noting that *Campylobacter* is tolerant to AD treatment, it did not to be reduced significantly during mesophilic and thermophilic anaerobic digestion. Mesophilic digestate utilization needs to be focused on biosecurity because of survival of pathogenic bacteria. Appropriate management practices should be implemented to minimize the sanitary risks of bacterial transmission when applying digestate to agricultural field.

Chapter 2

The Survival of Pathogenic Bacteria and Antagonistic Bacteria during Anaerobic Digestion of Dairy Manure

1. Introduction

Anaerobic digestion (AD) is considered one of the sustainable and ecology friendly processes to dispose organic wastes like animal manure with clean fuel production (Holm-Nielsen et al., 2009). Together with biogas production, AD process also produces a liquid residue called digestate, which is considered a valuable fertilizer consisting of partially degraded organic matter (OM), microbial biomass and inorganic compounds (Alburquerque et al., 2012). Usually, mean of disposal of digestate is directly discharge to agricultural lands, which is currently considered an economical mean for digestate disposal and on the same time, returning their mineral and organic constituents to agricultural systems. It does not only provide a source of available nutrients (nitrogen and phosphorus) to plants but also has positive effects on soil biological properties such as microbial biomass and enzyme activities (Risberg et al., 2017).

In recent years, digestate has been researched as soil amendment on effects on crop growth and improvement of soil properties because of its physico-chemical and biological properties (Alburquerque et al., 2012). On another hand, several previous literatures have reported on various uses of soil amendment to suppress plant diseases caused by soil pathogens and increase soil fertility as well as preserve the environment because of its physio-chemical and biological properties (Bonanomi et al., 2010), such as compost (Termorshuizen et al., 2006). Even though both digestate and compost are organic amendments and are similar in respect to the chemical component including available form of plant nutrients (N, P, K) and organic carbon as well as abundant microbial populations, for research on uses of digestate to suppress soil-borne plant disease is limited. Disease suppressiveness phenomenon of organic soil amendment consists of various factors while enzymatic and microbiological parameters, rather than chemical ones, are much more informative for soil-borne disease suppressiveness (Bonanomi et al., 2010). In among, microbiological parameters usually attract more attentions in biological control of some soil-borne plant diseases such as population density of antagonistic bacteria, which are considered one of indicators in expending potential of organic amendment to antagonize

soil-borne plant disease (Boulter et al., 2002). And higher population of antagonistic bacteria contributes the higher disease reduction (Joshi et al., 2009). Boulter et al. (2002) has investigated dominant bacteria in compost are *Pseudomonas* and *Bacillus.* And these two bacteria are representative antagonistic bacteria which are studied widely in many literatures to control soil-borne plant diseases (Kumar et al., 2012; Salman, 2010; Yang et al., 2015). However, to the best of our knowledges, researches on investigation of population densities of *Pseudomonas* and *Bacillus* in digestate are limited.

On another hand, with application of digestate to agricultural land, concern about the possible persistence and transfer of pathogenic bacteria and antibiotic resistant bacteria (ARB) from digestate is increasing in recent years since pathogens might thus be spread together with the digestate on agricultural soils. And thereby, it may become a public health risk of possible transfer of potential pathogenic bacteria to human and animal (Sahlström et al., 2004). In particularly, widely application of antibiotic drugs in animal husbandry to control animal disease results in the presence and development of unintentional selection of bacteria that are resistant to antibiotics in animal waste, which is becoming one of the most important clinical challenges (Angulo et al., 2004). Even though many literatures have noted anaerobic digestion showed effectiveness on ARB reduction and some studied have been done about the tolerance of resistant bacteria during anaerobic digestion by using multiple antibiotics (Beneragama et al., 2013b), for research on fate of specific indicator bacteria and pathogenic bacteria resistant to antibiotic during anaerobic digestion is limited. *E. coli*, *Enterococcus*, *Salmonella* and *Campylobacter* are indicator bacteria and pathogenic bacteria cause diseases and infection between human and animals, which often are used to detect and estimate contamination level of animal waste and water. Some papers have investigated their persistence in various wastes during anaerobic digestion (Sahlström, 2003; Smith et al., 2005), however, research on fate of them with antibiotic resistance during anaerobic digestion is limited. As an ecological consequence, evaluation of sanitary characteristics of digestate in persistence and prevalence of specific pathogenic and their resistance to antibiotic is necessary prior to its application to agricultural lands.

In chapter 2, laboratory scale continuously fed anaerobic digestions of dairy manure were conducted at mesophilic and thermophilic temperature. Then, two representative antagonistic bacteria *Pseudomonads* and *Bacillus* were determined in population densities and the population densities of specific pathogenic bacteria (*E. coli*, *Salmonella*, *Enterococcus*, and *Campylobacter*) during anaerobic digestion and their antibiotic resistance was also determined.

2. Materials and Methods

2.1 Lab-scale continuous anaerobic digestion

Laboratory scale continuous anaerobic digestions were conducted using two semi-continuous stirred-tank reactors produced with stainless-steel cylindrical digesters with 11.25 L working volume (Yamashiro et al., 2013). Digesters were kept at mesophilic (37 °C) and thermophilic (55 °C) conditions in thermostatically controlled water baths. Digested dairy manures obtained from active mesophilic and thermophilic biogas plants in Hokkaido were used as inoculum. Raw manure (RM) was collected from Obihiro University farm and used as feedstock after the total solid (TS) content was adjusted to 10% by diluted water. Mesophilic and thermophilic digesters were fed with organic loading rates (OLR) of 2.36 gVS/L/day and 4.71 gVS/L/day of feedstock and operated at hydraulic retention times (HRT) of 37.5 and 18.8 days, respectively. Mesophilic anaerobically digestate (MAD) and thermophilic anaerobically digestate (TAD) were simultaneously discharged after raw manure was fed. Raw manure and digestate were sampled to measure total solid (TS), volatile solid (VS) and volatile fatty acids (VFAs) concentration, pH value, and population densities of antagonistic bacteria and pathogenic bacteria with or without antibiotics resistance.

2.2 Analysis of parameters

The volume of produced biogas was measured daily with wet gas meter. All gas measurements were expressed at 0 °C and a pressure of one atmosphere. The biogas also

was collected and analyzed for gas composition with gas chromatograph (GC) (Shimadzu GC-14A) equipped with a thermal conductivity detector (stainless column and Porapak Qpacking). The operational temperatures of injector port, column and the detector were 220, 150 and 220 °C, respectively. Argon was the carrier gas at a flow rate of 50 mL min^{-1}. TS and VS contents of samples were determined according to the standard methods (APHA, 1998). The pH before and after digestion was measured using a pH meter (Horiba-D55, Kyoto, Japan).

The VFAs (acetic acid, propionic acid, and butyric acid) concentrations were determined by high performance liquid chromatography (HPLC, LC-10AD; Shimadzu Co., Kyoto, Japan) with a Shim-Pack SCR-102H column. Sample (3 g) was pre-treated with 6 mL of 10% tungsten acid and 6 mL of 7% sulfuric acid. The mixture was homogenized for 5 min, and then centrifuged at 10,000 g for 20 min. The supernatant of sample was collected and analyzed by HPLC. 5 mM p-toluene sulfonic acid was used as mobile phase at flow rate of 0.8 mL/min at 45 °C. Buffer phase was a mixture of 5 mM p-toluene sulfonic acid, 20 mM Bis-Tris and 100 μM EDTA (Iwasaki et al., 2013).

2.3 Quantitative analysis of pathogenic and cefazolin bacteria

Pathogenic bacteria in RM, MAD and TAD samples were determined standard dilution plate method. Samples were diluted by 10-fold dilutions used phosphate buffered saline (PBS, pH 7.4). The dilution plate method was conducted in triplicate and aliquots of 100 μl of sample were spread on the surface of deoxycholate hydrogen sulfide lactose agar (DHL; Eiken Chemical Co. Ltd., Tokyo, Japan) for *Salmonella* quantification, cefoperazone charcoal deoxycholate agar (CCDA; Kanto Chemical, Tokyo, Japan) for *Campylobacter* quantification, CHROMagar™ ECC (CHROMagar/Paris, France) for *Escherichia coli* quantification and Enterococcosel agar (ECS; Kyokuto Pharmaceuticals Co., Inc., Tokyo, Japan) for *Enterococcus* quantification.

In Obihiro University farm, cefazolin (CEZ), a β-lactam antibiotic, which suppresses the growth of bacteria by inhibiting cell wall synthesis, is frequently used to treat the cows

infected with mastitis. Thus, CEZ was added to selective agar media at a concentration of 50 mg/L to determine relative ARB (Kobashi et al., 2005). Incubation time and temperature were controlled according to the specifications. The appropriate cultures with colonies between 20 and 200 on agar medium were used to estimate the number of bacteria in the samples (Resende et al., 2014). After incubation, typical colonies were counted and calculated as colony forming units per gram of dry matter (CFU/g DM).

2.4 Quantitative analysis of antagonistic bacteria

Antagonistic bacteria in RM, MAD and TAD were determined by standard dilution plate method. Samples were 10-fold diluted using phosphate buffered saline (PBS, pH 7.4) and 100 µl were spread on the surface of BD BBL™ MYP (BD Falcon™, Franklin Lakes, NJ, USA) and Difco™ Cetrimide Agar (Becton, Dickinson and Company, Sparks, MD, USA) in triplicate for the quantification of *Bacillus* and *Pseudomonas*, respectively. The appropriate cultures with colonies between 20 and 200 on agar medium were used to estimate the number of bacteria in the samples (Resende et al., 2014). Incubation time and temperature were controlled according to the manufacturer's specifications. After incubation, typical colonies were counted and calculated as colony forming units per gram of dry matter (CFU/g DM).

3. Results and Discussion

3.1 Anaerobic digestion performance

Anaerobic digestions were performed for 90 days, and their performances were stabilized after the first 10 days. The daily biogas and methane production rates are presented in Fig. 6. In anaerobic digestion process, the temperature is an important factor for biogas production and digestion rate. In general, thermophilic digestion allows higher OLR and reduces hydraulic time, while mesophilic digestion shows more stability (Gavala et al., 2003; Micolucci et al., 2016).

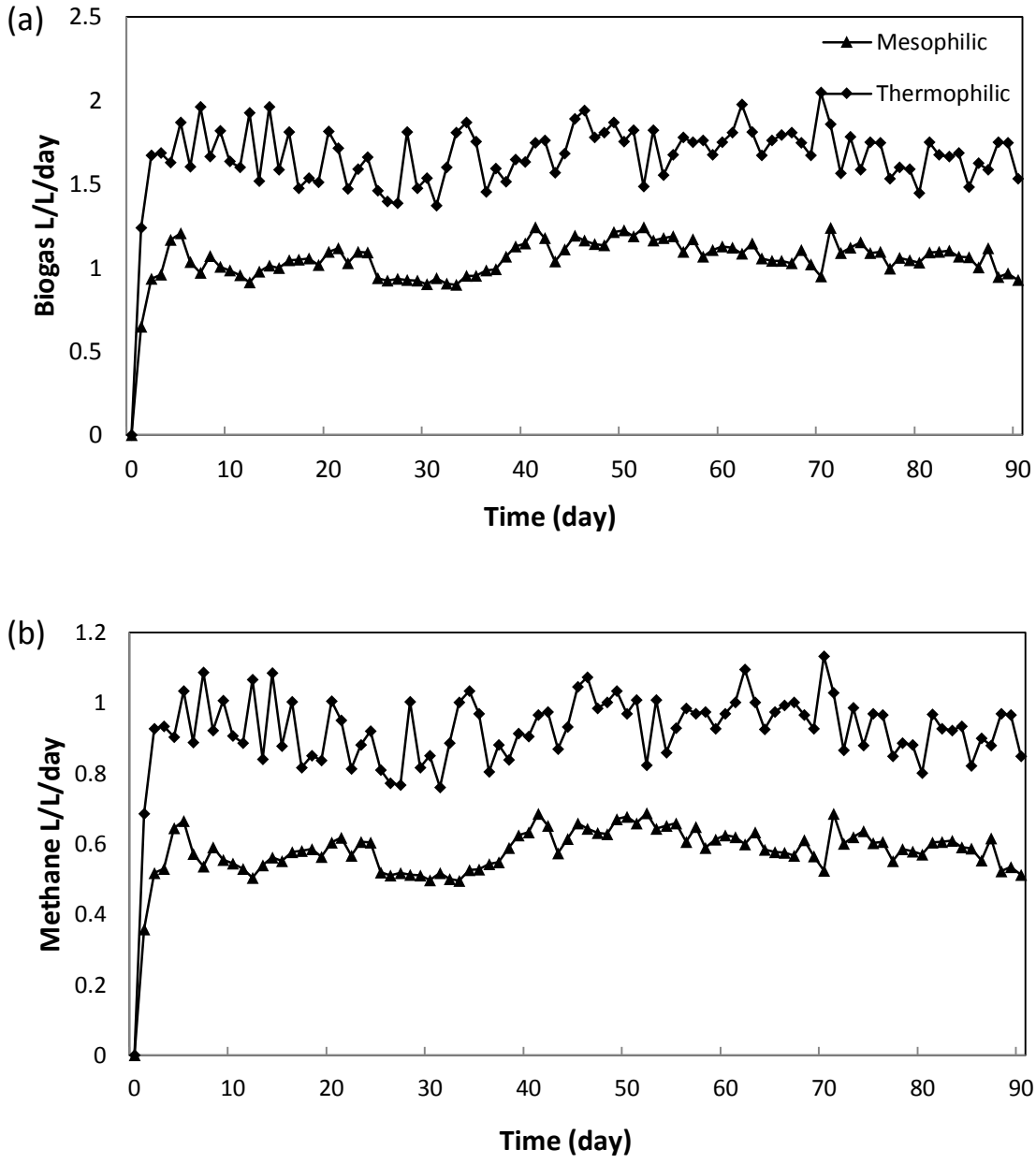


Figure 6: Daily biogas and methane production rates of anaerobic digestion of dairy manure at mesophilic and thermophilic temperatures

In this chapter, thermophilic digestion fed with higher OLR produced higher biogas production than mesophilic condition, however, values arranged from 1.5 to 2 L/L/day while mesophilic digestion showed stable biogas production around 1.0 L/L/day. Regarding with methane production rate, thermophilic digestion produced higher methane production rate ranging from 0.8 to 1.1 L/L/day, while mesophilic digestion showed stable methane production rate around 1.0 L/L/day.

The averages of daily biogas yield, methane concentration and methane yield of mesophilic and thermophilic digestions over the 90 days are shown in Table 5. During the stable period of digestion, mesophilic digestion produced higher biogas and methane yield (0.44 $L/gVS_{loaded}/d$, 0.24 $L/gVS_{loaded}/d$) than thermophilic digestion, which were 0.35 $L/gVS_{loaded}/d$ and 0.21 $L/gVS_{loaded}/d$, respectively. However, the methane yields per $gVS_{reduction}$ were almost the same. The methane concentrations of biogases produced from two digesters of 55.28% and 55.38% were within the reasonable range (50% - 70%), thus indicating successful anaerobic digestions (Andriamanohiarisoamanana et al., 2016).

Table 5: Biogas yield, methane concentration and methane yield of mesophilic and thermophilic anaerobic digestion

	Mesophilic digestion	Thermophilic digestion
Organic loading rate (gVS/L/d)	2.36	4.71
Hydraulic retention time (d)	37.5	18.8
Biogas yield ($L/gVS_{loaded}/d$)	0.44 (± 0.06)	0.35 (± 0.05)
Methane concentration (%)	55.28 (± 2.24)	55.38 (± 1.79)
Methane yield ($L/gVS_{loaded}/d$)	0.24 (± 0.03)	0.21 (± 0.03)
Methane yield ($L/gVS_{reduction}/d$)	0.50 (± 0.05)	0.49 (± 0.05)

TS and VS contents, pH values, and VFAs concentrations are important parameters in anaerobic digestate. Changes of TS and VS contents over anaerobic digestion are presented in Table 6. Significant reduction of TS and VS contents were observed after both mesophilic and thermophilic digestions, which indicated degradation of organic compounds in the feedstock after anaerobic digestion (Orzi et al., 2015). In addition, TS and VS contents in MAD (6.20 and 4.47%) were lower than those in TAD (7.01 and 5.28%), which was likely due to the longer HRT of feedstock in mesophilic digester. The pH value of the raw manure was changed from 6.91 to 7.68 and 7.93 in mesophilic and thermophilic digestates, respectively. The VFAs concentrations of raw manure and digestate are shown in Figure 7. The total VFAs in MAD and TAD decreased sharply from an initial concentration of 4.57 g/L to 0.36 and 0.16 g/L, respectively, which indicated the intensive consumption of VFAs by methanogens during AD process (Riva et al., 2016). Acetic acid was the most abundant acid in both raw manure and digestates, followed by propionic acid. Butyric acid was not detected after digestion.

Table 6: TS and VS contents and pH values of raw manure and mesophilic and thermophilic anaerobic digestates

Parameters	Raw manure	Digestates	
		Mesophilic digestion	Thermophilic digestion
Total solid (%)	10.06 (± 0.37)	6.20 (± 0.20)	7.01 (± 0.68)
Volatile solid (%)	8.80 (± 0.40)	4.47 (± 0.14)	5.28 (± 0.62)
pH	6.91 (± 0.48)	7.68 (± 0.11)	7.93 (± 0.15)

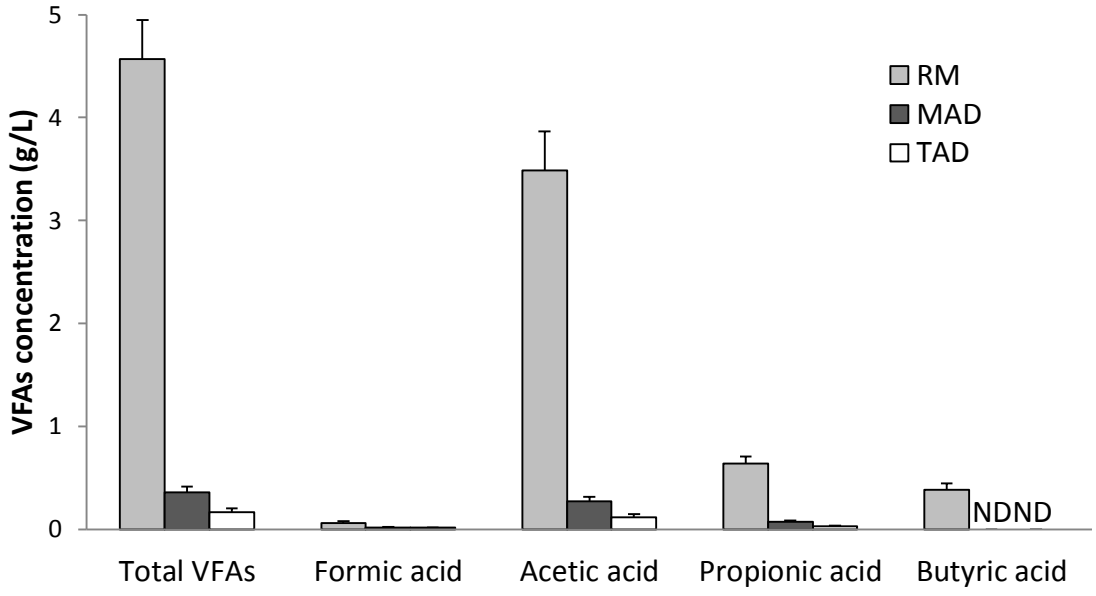


Figure 7: Concentrations of volatile fatty acids (VFAs) in raw manure (RM), mesophilic anaerobic digestate (MAD) and thermophilic anaerobic digestate (TAD). Values are expressed as the mean ± SE. ND: Not detected

3.2 Survival of pathogenic and cefazolin resistant bacteria

The results of quantitative analysis for tested pathogenic bacteria counts are presented in Figure 8. Total *E. coli* count was detected in RM with 6.40 $\log_{10}$CFU/g-DM and it was reduced 86.53% by mesophilic anaerobic digestion and 99.30% by thermophilic anaerobic digestion. Similarly, *Enterococcus* and *Salmonella* also reduced 97.84% and 86.53% in mesophilic anaerobic digestion as well as 100% and 99.73% in thermophilic anaerobic digestion, respectively. From these results, anaerobic digestion showed considerably reduction rate on pathogenic bacteria. Generally, the decay rate of viable bacteria during anaerobic digestion is dependent on several factors, such as bacteria biology, and characteristics of anaerobic digestion including temperature, pH value, retention time, volatile fatty acids, available nutrients and chemical interactions, and among

which, temperature is considered most important factor for pathogenic bacteria reduction (Beneragama et al., 2013b). Because the same feedstock was used and completely digestions were conducted in this study, there was no significant differences in pH value, VFA and chemical characteristics of MAD and TAD. Thermophilic anaerobic digestion showed significantly decay rate of pathogenic bacteria counts. In particularly, *Enterococcus* was reduced completely by thermophilic anaerobic digestion and similarly results also be presented by Iwasaki et al. (2011). In comparison, *Campylobacter* was reduced less with decay rate of 73.19% and 90.86%, respectively in anaerobic digestion. It is because that *Campylobacter* was the most resistant bacteria during anaerobic digestion than *E. coli* and *Salmonella*, therefore, decimation reduction time needs longer (Kearney et al., 1993). Moreover, effect inactivation of pathogenic bacteria also depends on the initial amount of pathogenic bacteria (Sahlström, 2003). Except for *Enterococcus* which was completely inactivated by thermophilic anaerobic digestion, detected amount of another three pathogenic bacteria in MAD and TAD was *E. coli* > *Salmonella* > *Campylobacter*, it is in agreement with the initial amount of these bacteria in the feedstock.

The results of quantitative analysis for CEZ-resistant pathogenic bacteria are also presented in Figure 8. Undetectable levels of CEZ-resistant *E. coli* and *Salmonella* were observed at undetectable levels either in RM or in MAD and TAD. CEZ-resistant *Enterococcus* was detected at 3.86 $\log_{10}$CFU/g-DM in RM and undetectable levels in MAD and TAD. CEZ-resistant *Campylobacter* was detected at count of 3.37 $\log_{10}$CFU/g-DM in RM, and no CEZ-resistant *Campylobacter* was found in MAD and TAD. Compare to researches of pathogenic bacteria, there is limited information on prevalence of antibiotic resistant bacteria during anaerobic digestion. Current study agrees with the result of Beneragama et al. (2013) which found thermophilic anaerobic digestion showing complete reduction on CEZ-resistant bacteria. Temperature is one of important factors on antibiotic resistant bacteria reduction. Add to that, the degree of sensitivity of specific types of pathogenic bacteria is considered to impact upon injury of microorganism during anaerobic digestion. Takemura et al. (2016) found that anaerobic digestion had relative low effect on

CEZ-resistant bacteria reduction and initial amount of resistant bacteria was one reason. In our study, CEZ-resistant *Enterococcus* and *Campylobacter* were reduced to undetectable level both in MAD and TAD. The possible reason for this is the small initial amount.

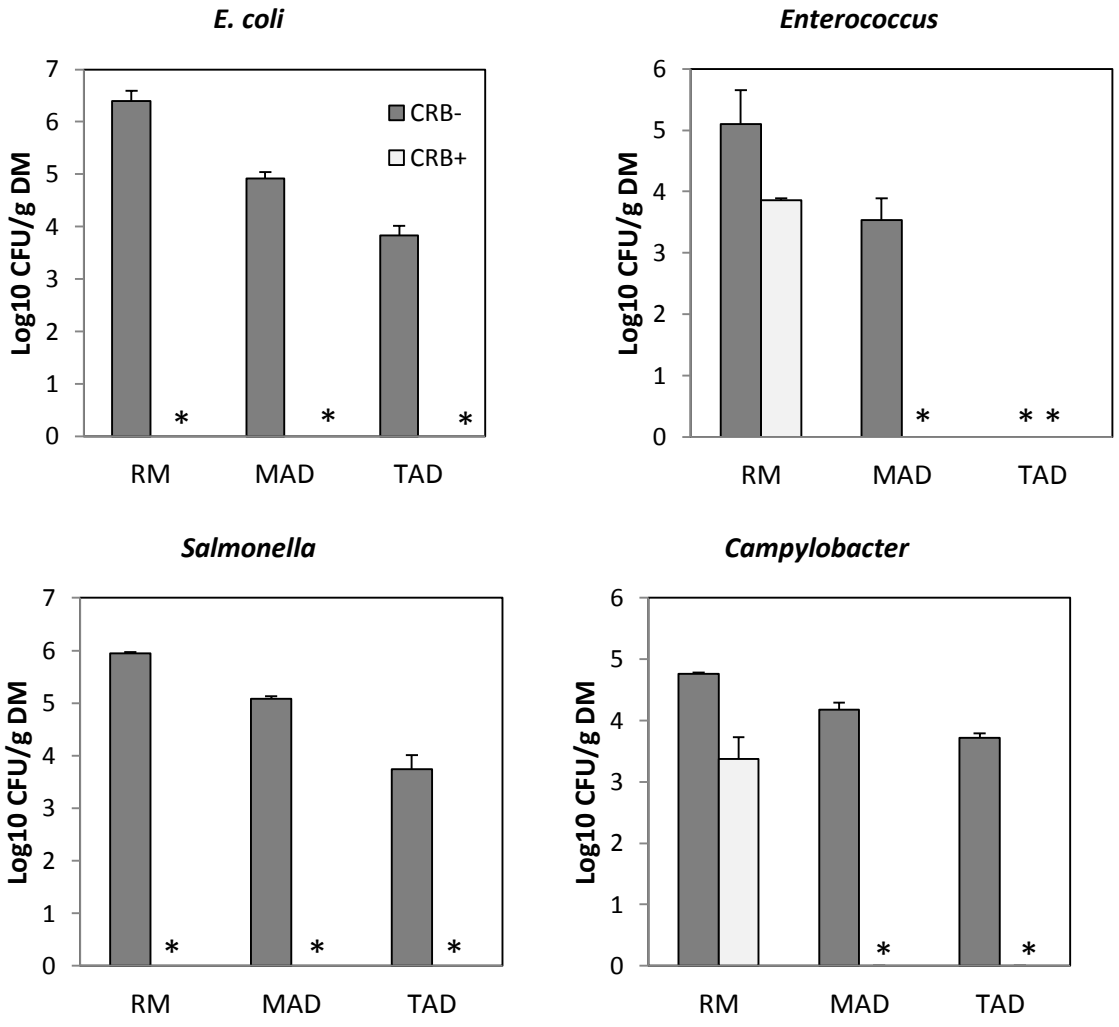


Figure 8: Population densities of pathogenic (CRB-) and CEZ-resistant bacteria (CRB+) in samples. CFU: colony forming units; DM: dry matter; RM: raw manure; MAD: mesophilic anaerobic digestate; TAD: thermophilic anaerobic digestate; * means not detected; Values are expressed as the mean ± SE

3.3 Survival of antagonistic bacteria

The results of quantitative analysis of *Bacillus* and *Pseudomonas* are presented in Figure 2. *Bacillus* population of 0.10×10^6 CFU/g DM in RM was increased to 2.68×10^6 and 0.43×10^6 CFU/g DM in MAD and TAD, respectively. Although the amount of *Bacillus* in TAD was less than that in MAD, anaerobic digestion processes still showed significant benefits for *Bacillus* growth. These results do not correspond with the results from Cao et al. (2013), who reported anaerobic digestion significantly reduced the numbers of *Bacillus*. This might be due to the different operation condtions, such as feedstock TS, HRT and digestate pH values. It is also reported that *Bacillus* is a spore-forming bacterium which produces endospores capable of resisting heat, chemicals, and extreme environments (Nicholson et al., 2000). In this study, it appeared that the suitable temperatures and available nutrients in digesters stimulated the growth of *Bacillus*. The population of *Pseudomonas* was also found to be increased from 0.13×10^4 CFU/g DM to 0.83×10^4 CFU/g DM in MAD and 7.53×10^4 CFU/g DM in TAD. This is likely due to the creation of a favorable condition for *Pseudomonas* growth during acidification phase of anaerobic digestion (Shah et al., 2014). *Bacillus* and *Pseudomonas* are identified as biological control agents that are able to control plant pathogens through competition of nutrients and spaces as well as induction of systemic resistance in the host plant (Hoitink et al., 1997). Many studies demonstrated that organic soil amendment application such as compost can effectively control many soil-borne pathogens, while a microbial component has been identified as a factor for plant disease suppression (Boulter et al., 2002; Hoitink et al., 1997). In this study, the results demonstrated that *Bacillus* and *Pseudomonas* increased both in MAD and in TAD, thus suggesting that digestates have the potential to suppress soil-borne plant diseases.

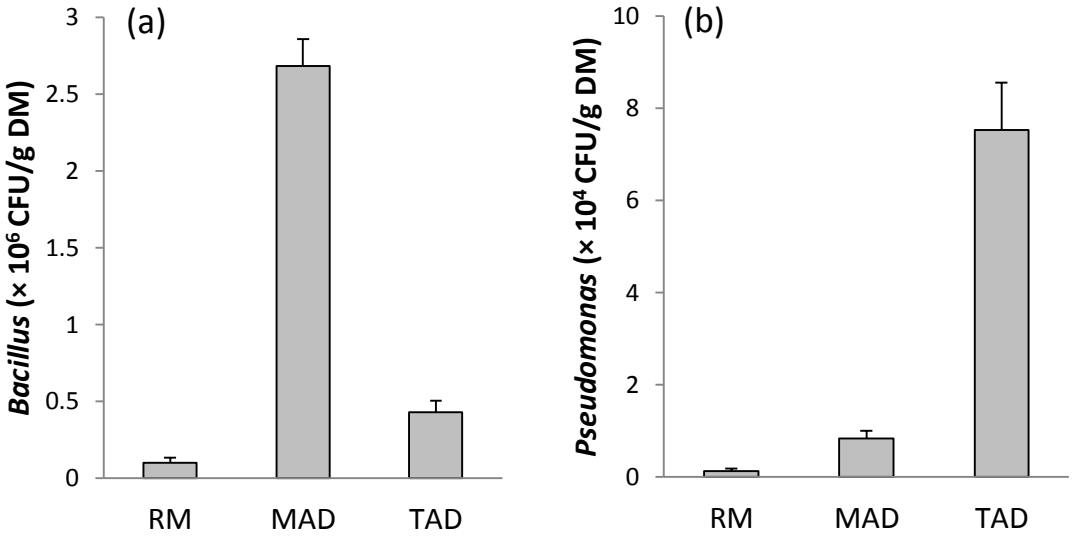


Figure 9: *Bacillus* (a) and *Pseudomonas* (b) counts in raw manure (RM), mesophilic anaerobic digestate (MAD) and thermophilic anaerobic digestate (TAD). CFU: colony forming units; DM: Dry matter; Values are expressed as the mean ± SE

4. Conclusion

The present study showed the impact of mesophilic and thermophilic anaerobic digestion of dairy manure on fate of pathogenic bacteria, CEZ-resistant bacteria and antagonistic bacteria. Results obtained showed that anaerobic digestion especially thermophilic anaerobic digestion was effective in reducing pathogenic and CEZ-resistant bacteria. Moreover, a novel found was that antagonistic bacteria were increased both in mesophilic and thermophilic anaerobic digestion. *Bacillus* spp. may be dominant antagonistic bacteria in digestate to antagonize phytopathogens. These results indicated that digestate was not only sanitary fertilizer but could be potential in plant disease control when using in agricultural land.

Chapter 3

Potential of Anaerobic Digestate of Dairy Manure in Suppressing Soil-borne Plant Disease

1. Introduction

Anaerobic digestion is considered a sustainable and environmentally friendly process that treats organic wastes such as livestock manure. In addition to biogas production, this process also provides a liquid residue called anaerobic digestate (Holm-Nielsen et al., 2009). Anaerobic digestate is considered a valuable biofertilizer because it consists of partially degraded organic matter (OM), microbial biomass and inorganic compounds (Alburquerque et al., 2012). Not only does it provide a source of available nutrients (nitrogen and phosphorus) to plants, but it also has positive effects on soil biological properties, such as microbial biomass and enzyme activities (Risberg et al., 2017).

Fungi infection is one of the major factors that cause plant diseases in agricultural fields. Pesticides are commonly used to control the diseases; however, frequent use of pesticides may lead to environmental pollution and the development of pesticide resistance in fungi (Mehta et al., 2014). In recent years, several studies have reported the suppressive effects of organic soil amendment, such as compost on soil-borne plant diseases (Termorshuizen et al., 2006). Both anaerobic digestate and compost are organic amendments that include an available form of plant nutrients (N, P and K), organic carbon, and abundant microbial populations (Alburquerque et al., 2012); however, there is limited information regarding the plant disease suppressive potential of anaerobic digestate.

The plant disease suppressing phenomenon of organic soil amendment consists of various factors, while enzymatic and microbiological parameters, such as antagonistic bacteria, are much more informative for soil-borne disease suppression (Bonanomi et al., 2010). Generally, the main mechanisms of antagonistic bacteria against plant disease are the production of siderophores, antibiotics, competition with pathogens and induction of systemic resistance (Dimkić et al., 2015). Therefore, population densities of antagonistic bacteria in organic amendment is an important indicator of suppressive activities against soil-borne plant diseases (Boulter et al., 2002), and higher densities of antagonistic bacteria

contribute to higher disease suppressive activities (Joshi et al., 2009). Boulter et al. (2002) reported that the dominant antagonistic bacteria in compost were *Pseudomonas* and *Bacillus*. These two genera are representative antagonistic bacteria, which are studied widely to suppress various soil-borne plant diseases (Kumar et al., 2012; Salman, 2010; Yang et al., 2015). However, the research on the population densities of *Pseudomonas* and *Bacillus* in anaerobic digestate and their antagonistic activities are limited.

Consequently, the objective of this chapter was to investigate the antagonistic activity of *Pseudomonas* and *Bacillus* in anaerobic digestate against phytopathogens and their antagonistic activity against soil-borne plant diseases in field. For this objective, laboratory-scale semi-continuous fed anaerobic digestion of dairy manure was conducted at mesophilic and thermophilic temperatures, and population densities of antagonistic *Pseudomonas* and *Bacillus* against four phytopathogens (*Alternaria Sorani*, *Cercospora beticola*, *Fusarium nivale* f. sp. *graminicola* and *Streptomyces.scabie*) were measured by dual culture method. Furthermore, field experiment was conducted with two selected *Bacillus* isolates from anaerobic digestate to investigate their biocontrol activities against potato late blight (*Phytophthora infestans*).

2. Materials and Methods

2.1 Samples collection and isolation of bacteria

Raw manure and digestate samples were collected from laboratory scale anaerobic digestions kept at mesophilic (37 °C) and thermophilic (55 °C) conditions. The characteristics of samples were shown in chapter 2. Antagonistic bacteria in samples were isolated by standard dilution plate method with BD BBL™ MYP (BD Falcon™, Franklin Lakes, NJ, USA) and Difco™ Cetrimide Agar (Becton, Dickinson and Company, Sparks, MD, USA). Then, one hundred *Bacillus* and *Pseudomonas* isolates were selected randomly from the cultures raw manure, mesophilic and thermophilic digestates and tested for antifungal activity using the dual culture method.

2.2 Screening bacterial antagonistic activities

In this study, three phytopathogenic fungi; *Alternaria solani*, *Cercospora beticola*, *Fusarium nivale* f. sp. *Graminicola* and one phytopathogenic actinomyces, *Streptomyces scabies*, were obtained from the National Institute of Agrobiological Sciences, Japan (NIAS; Tsukuba, Japan), the information of these isolates are presented in Table 7. Spore suspensions of phytopathogenic fungi and actinomyces were atomized over the Potato Dextrose Agar (PDA, Becton, Dickinson and Company, Sparks, MD, USA) plate using a tube atomizer. *Bacillus* and *Pseudomonas* colonies from the culture of RM, MAD and TAD were transferred and point-inoculated on these PDA plates. The plates were inoculated at 25 °C for 7 days. Antagonistic activities were confirmed by the formation of a clear zone around the colonies. After incubation, colonies with a clear zone were counted and calculated as CFU/g DM.

Table 7: Information of phytopathogens used in this chapter

Phytopathogens	Category	Crop diseases	MAFF No.
Alternaria sorani	Fungus	Potato early blight	244033
Cercospora beticola	Fungus	Sugar beet brown spot	241661
Fusarium nivale	Fungus	Wheat pink snow mold	235153
Streptomyces scabies	Actinomycete	Potato common scab	225028

MAFF: Ministry of Agriculture, Forestry and Fisheries

2.3 Identification of antagonistic bacteria

Two bacterial isolates were selected on the basis of their conspicuous clear zone and identified by MALDI-TOF MS (Matrix-assisted laser desorption/ionization- time of flight mass spectrometry) method using Bruker microflex mass spectrometer system

(microflex LT/SH, Bruker Daltonics, Kanagawa, Japan). Two methods, the directx smear method and on-plate extraction method, were used in this study. For the former method, the bacterial colony was directly smeared onto a spot on polished steel MALDI target plates using sterile toothpicks. Thin spots of bacteria were then dried in a safety cabinet and subsequently overlaid with 1 µl of the matrix solution, comprised of a HCCA (α-Cyano-4-hydroxycinnamic acid) matrix (Bruker Daltonik), for 5 min. For the on-plate extraction method, an extraction step by 1 µl of 70% formic acid (Wako Pure Chemical Industries, Osaka, Japan) was introduced before cocrystallization with the matrix. *Escherichia coli* (K-12, laboratory stock) was used as a positive and quality control, and formic acid and the matrix were used as the negative control at each run. The Bacterial Test Standards (Bruker Daltonics) were used for instruments calibration with each run. The samples prepared by each method were subjected to the microflex mass spectrometer, and results were analyzed by MALDI Biotyper 3.0 software (Bruker Daltonics).

2.4 Investigation of bacterial antagonistic activities in field experiment

The field experiment was conducted at the experimental field of Obihiro University. The experimental soil was contaminated with *Phytophthora infestans*, which is the infective agent of potato late blight. Two selected bacterial isolates were applied for field experiment. Half-cut potato tubers were inoculated with bacterial suspensions (1×10^8 CFU/ml) and cured for 3 days before planting. Pesticide treated (RELIABLE Flowable, Bayer Crop Science, Tokyo, Japan) and non-bacteria-treated tubers were planted as positive and negative controls, respectively. Pesticide was applied four times during the outbreak period of late blight. All treatments were conducted in five replications and each replication contained 14 potato seeding tubers. Percent damage of potato leaves caused by insect and percent appearance of potato late blight were calculated according to Eq. (1) and (2):

$$\text{Percent insect damage} = \frac{\text{Number of plants with damaged leaves}}{\text{Number of total plants}} \times 100\% \quad (1)$$

$$\text{Percent disease infection} = \frac{\text{Number of plants with appearance of disease}}{\text{Number of total plants}} \times 100\% \quad (2)$$

2.5 Statistical analysis

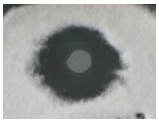
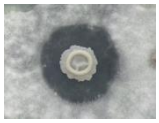
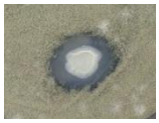
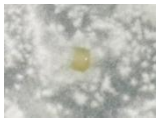
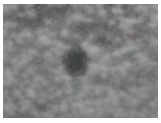
Statistical analyses were performed with SAS Statistical Software version 9.4 (SAS Institute Inc., USA). Values with $p < 0.05$ were considered statistically significant.

3. Results and Discussion

3.1 Bacterial antagonistic activities against phytopathogens

Bacillus and *Pseudomonas* were primarily screened for antagonistic activity against four phytopathogens. The bacterial colonies with inhibition zone were thought with antagonistic activity, which are presented in Table 8.

Table 8: Antagonistic activities of *Bacillus* and *Pseudomonas* against phytopathogens

	Alternaria sorani	*Cercospora beticola*	*Fusarium nivale*	*Streptomyces scabies*
Bacillus colonies with inhibition zone				
Bacillus colonies without inhibition zone				
Pseudomonas colonies without inhibition zone				

The populations of antagonistic *Bacillus* against phytopathogens in RM, MAD and TAD are presented in Fig. 10. The populations of antagonistic *Bacillus* were increased in various degrees after anaerobic digestion, and higher populations were found in MAD than

TAD. Joshi et al. (2009) indicated that a higher population of antagonistic bacteria in soil amendment contributes to a higher disease reduction. Therefore, MAD was expected to have a more effective biological agent than TAD in suppressing soil-borne plant diseases. In particular, the population of antagonistic *Bacillus* against *Cercospora beticola* increased to 2.53 × 10^5 CFU/g DM in MAD, which was much higher compared to *Bacillus* against the other three phytopathogens. Generally, *Bacillus* suppress the growth of *Cercospora beticola* through various mechanisms, such as spore formation, antibiotic production, and glucanalytic and chitinolytic activity (Collins and Jacobsen, 2003).

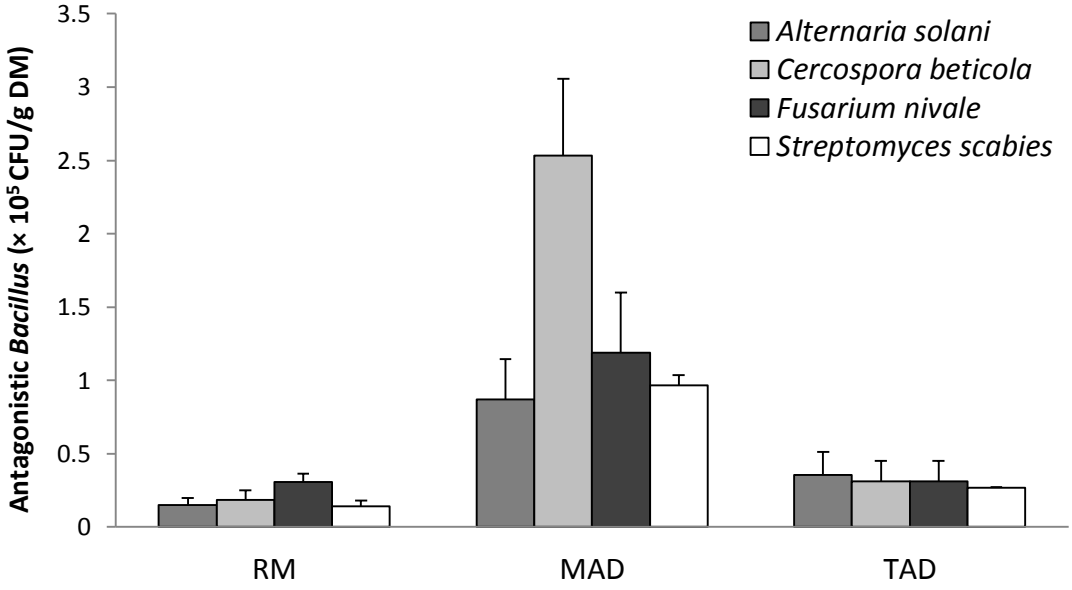


Figure 10: Antagonistic *Bacillus* counts against four phytopathogens in raw manure (RM), mesophilic anaerobic digestate (MAD) and thermophilic anaerobic digestate (TAD). CFU: Colony forming units; DM: Dry matter; Values are expressed as the mean ± SE

The percentages of antagonistic *Bacillus* among total *Bacillus* in two anaerobic digestates are shown in Table 9. Except for the percentage of antagonistic *Bacillus* against

Cercospora beticola, percentages of antagonistic *Bacillus* in TAD, which were approximately 6.25 to 8.33%, were much higher compared to 3.24 to 4.42% in MAD due to the higher population of total *Bacillus* in MAD (Fig. 9).

Table 9: Percentage of antagonistic *Bacillus* among total *Bacillus* in anaerobic digestates

	Mesophilic digestate	Thermophilic digestate
Alternaria solani	3.24%	8.33%
Cercospora beticola	9.43%	7.30%
Fusarium nivale	4.42%	7.30%
Streptomyces scabies	3.60%	6.25%

On the other hand, no *Pseudomonas* colonies that showed antagonistic activity were observed in RM or digestates (MAD and TAD). Among *Pseudomonas* species, fluorescent pseudomonads represent an important group of promising antagonistic potentials in biological control of soil-borne plant diseases (Saber et al., 2015; Salman, 2010). In this study, it was considered that *Pseudomonas* in RM, MAD and TAD were not fluorescent pseudomonads, which is considered one reason that tested *Pseudomonas* had no antagonistic activity. Another possibility is that the four phytopathogens used in this study were resistant to tested *Pseudomonas* colonies. These results suggested that *Bacillus* species may play an important part in suppressing phytopathogens. However, further research is recommended to ascertain the antagonistic activity of *Pseudomonas* in digestates against other phytopathogens.

3.2 Identification of antagonistic bacteria

To confirm the plant disease-suppressive activities of *Bacillus* contained in digestates, two *Bacillus* isolates with superior biocontrol activities were selected and examined in field experiment following the identification by MALDI-TOF MS method. The identification result is shown in Table 4. K-12 (laboratory stock as control) was best match as *Escherichia coli* DH5slpha BRL with score value of 2.333. Two selected bacterial isolates, *Bacillus* B11 and B59, were best matches as *Bacillus subtilis* DSM 10T DSM and *Bacillus licheniformis* CS 54_1 BRB. *Bacillus* has been reported to have antagonistic activity against a wide variety of phytopathogens. *B. subtilis* was demonstrated to be an effective biocontrol agent for chilli anthracnose disease and sugar beet cercospora leaf spot (Ashwini and Srividya, 2014; Collins and Jacobsen, 2003). *B. licheniformis* also showed antagonistic activity against plant diseases by producing antifungal protein and metabolites (Jeong et al., 2017; Wang et al., 2014).

Table 10: Identification of tested *Bacillus* isolates by MALDI-TOF MS method

Analyte ID	Organism (best match)	Score value
K-12	*Escherichia coli* DH5slpha BRL	2.333
B11	*Bacillus subtilis* DSM 10T DSM	2.196
B59	*Bacillus licheniformis* CS 54_1 BRB	2.219

Meaning of score value:
2.300 … 3.000: Highly probable species identification
2.000 … 2.299: Secure genus identification, probable species identification
1.700 … 1.999: Probable genus identification
0.000 … 1.699: Not reliable identification

3.3 Bacterial antagonistic activity in field experiments

In field experiments, damage of potato leaves caused by insects was investigated on day 36 after planting. The results are presented in Fig. 11. Compared with the control group (22.6%), treatment with pesticide, *Bacillus* B11 and B59 reduced percent insect damage to 17.7, 15.2 and 18.7% on day 36 with no significant difference ($p > 0.05$). Insects affect plant health by consumption of plant tissues, and insect damage is considered to facilitate the entry of phytopathogens into plants (Mehta et al., 2014). The reduction in percent insect damage is considered to contribute to a reduction in percent disease infection.

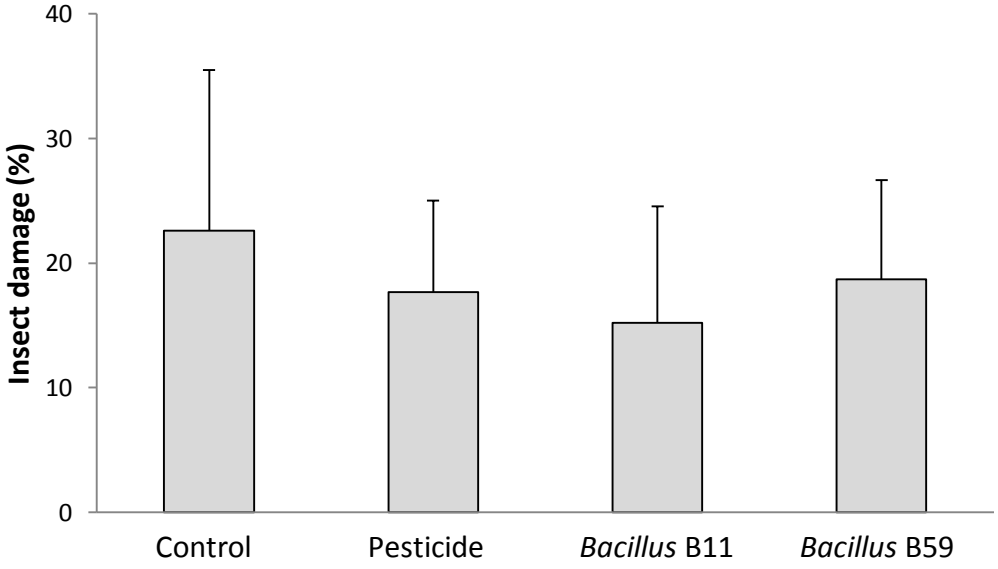


Figure 11: Percent damage of potato leaves by insects in potato plants at day 36

During the experiment period, pesticide was applied four times on days 32, 39, 46 and 53, after which percent disease infection was investigated three days after pesticide application on days 36, 43, 50 and 57. The results are presented in Figure 5. On day 36,

percent disease infection was reduced by treatment with pesticide or *Bacillus* with no significant difference ($p > 0.05$). On day 43, percent disease infection in the control group increased to 43.6%, which was much higher compared to 19.7% in the pesticide-treated group and 27.5% and 27.4% in the *Bacillus*-treated groups ($p < 0.05$). These results showed that potato plants treated with antagonistic bacteria were more resistant to plant disease. However, on day 50, all potato plants were infected except for 81.9% infection in pesticide-treated group, and all treated groups were finally infected on day 57.

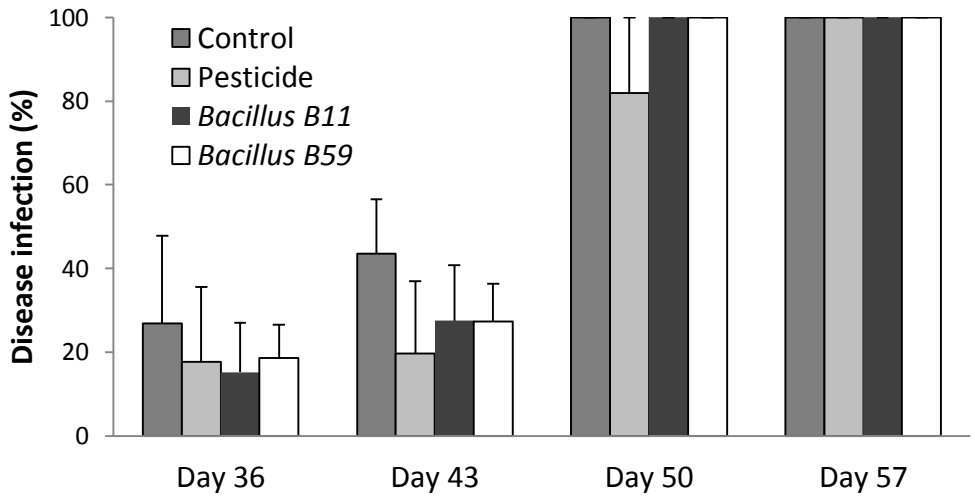


Figure 12: Percent disease infection in potato plants at days 36, 43, 50 and 57

Potato late blight caused by *Phytophthora infestans* is the most destructive disease in potato cultivation worldwide (Ballvora et al., 2002). This disease is very difficult to control, since the pathogen disperses rapidly via splashing rain as well as flowing irrigation and surface water. Thus, potato plants can be destroyed rapidly in 3-5 days under the condition of wet soils above 18 °C and prolonged wet periods with air temperatures ranging from 24 to 29 °C (Jiang et al., 2006). In field experiment, even though all treated groups were

finally infected, *Bacillus* treatment effectively suppressed plant infection when compared to non-treatment group during early outbreak periods (Day 43). These results indicated that inoculation of potato seeding tubers with *Bacillus* suspensions protects potato plants from pathogens in soil; however, it was unable to protect potato plants from airborne asexual sporangia of pathogens during the growing periods. Therefore, a combined treatment method, such as spraying bacterial suspensions directly to plants, may prevent the transmission of airborne asexual sporangia. On days 36 and 43, inoculation with *Bacillus* suspensions showed similar reduction in percent disease infection compared with pesticide-treated group. These results indicated that *Bacillus* species isolated from anaerobic digestate have a credible positive effect on potato late blight suppression, which suggests that digestate can be expected to control soil-borne plant diseases.

4. Conclusion

This chapter provided a new point for the agricultural utilization of anaerobic digestate to suppress soil-borne plant disease. The population densities of antagonistic bacteria (*Pseudomonas* and *Bacillus*) in dairy manure increased by anaerobic digestion suggesting that anaerobic digestion provided environmental benefits for growth of *Bacillus* and *Pseudomonas*. In field experiment, *Bacillus* isolate-treated potato tubers effectively suppressed the appearance of potato late blight. These results suggest that the application of anaerobic digestate could lead to suppression of soil-borne plant diseases caused by antagonistic bacteria. However, further research is needed to ascertain the appropriate application of anaerobic digestate.

General Discussion

Anaerobic digestion (AD) is considered a sustainable and environmentally friendly process that treats organic wastes such as livestock manure with production of biogas, which is a renewable energy source. Recently, there is increasing worldwide interest in AD technology as a result of global warming and the increasing consumption of fossil fuels. In addition to biogas production, AD process also provides a liquid residue called anaerobic digestate. Anaerobic digestate is considered a valuable biofertilizer because it consists of partially degraded organic matter (OM), microbial biomass and inorganic compounds (Alburquerque et al., 2012). Therefore, digestate application on agricultural lands not only provides a source of available nutrients to plants, but it also has effects on soil biological properties, such as microbial biomass and enzyme activities (Risberg et al., 2017). In addition, nutrients are present in inorganic plant-available forms in digestate at a markedly higher level compared to undigested manure, as a large portion of the organic form of nutrients is converted into its inorganic form during AD process (El-Mashad and Zhang, 2010; Umetsu et al., 2002). However, there is also concern about environmental risk with digestate application related with pathogenic bacteria. Furthermore, the widespread use of antibiotics in livestock industry has increased the frequency of antibiotic resistant bacteria (ARB) in livestock wastes, which may still be present in the digestate and cause a health risk for both people and animals.

In addition, the effect of microorganisms in biofertilizer has recently attracted attention, especially for the antagonistic activities of *Bacillus* and *Pseudomonas* species, which have been widely researched. Therefore, the focus of studies presented in this PhD thesis was on the survival of pathogenic bacteria with and without antibiotic resistance during anaerobic digestion at different temperatures and to detect the antagonistic activity of bacteria in digestate.

1. Reduction of pathogenic bacteria during anaerobic digestion

1.1 Effect of temperature

Generally, the principle factors causing pathogenic bacteria reduction during AD

process include temperature, retention time, reactor configuration, microbial completion, pH value and chemical interaction (Smith et al., 2005). And temperature is considered the most important one. In chapter 1, the survival of four pathogenic bacteria (*Escherichia coli, Enterococcus, Salmonella*, *and Campylobacter*) with and without cefazolin resistance, and one multidrug-resistant bacterium *Acinetobacter* were determined in population densities during anaerobic digestion at mesophilic (37 °C), thermophilic (55 °C) and hyper-thermophilic (65 °C) temperatures. *Enterococcus, Salmonella,* and *Acinetobacter* were reduced to undetectable level. However, *Escherichia coli* and *Campylobacter* still survived after mesophilic digestion and *Campylobacter* could be detected after thermophilic digestion. *Campylobacter* was reported as a resistant bacteria during anaerobic digestion (Kearney et al., 1993). Although it was eliminated though hyper-thermophilic digestion, the process stability and high energy input should also be considered. In chapter 2, AD processed were performed at mesophilic (37 °C), thermophilic (55 °C) temperatures, and results showed that anaerobic digestion especially thermophilic anaerobic digestion is effective in reducing pathogenic and cefazolin resistant bacteria. Regarding pathogenic residue in digestate, appropriate management practices should be implemented to minimize the sanitary risks of bacterial transmission when applying digestate to agricultural field.

1.2 Batch or continuous

The results of chapter 1 and 2 indicated the difference in pathogenic bacteria reduction during AD conducted in batch or continuous reactors. Except for *Campylobacter*, which is a resistant bacteria for AD process, only *Escherichia coli* was detected in mesophilic digestate in batch reactors. However, in chapter 2, *Escherichia coli* and *Salmonella* could be detected in mesophilic and thermophilic digestates in continuous reactors. *Enterococcus* was also detected in mesophilic digestate. These results indicated that higher bacteria reduction efficiency of batch reactor than continuous reactor. Similar results of higher reduction of pathogenic bacteria could be achieved through batch digestion have been reported (Kearney et al., 1993; Poudel et al., 2010). This is attributed to that in continuous reactors the retention time may not be long enough to exclude the passage of

not inactivated pathogenic bacteria.

2. Antagonistic bacteria in anaerobic digestate

Many researches indicated the presence of *Bacillus* and *Pseudomonas* in anaerobic digestates from various types of feedstock (Alfa et al., 2014; Owamah et al., 2014). Pathogenic bacteria were reduced after mesophilic or thermophilic digestion. However, antagonistic bacteria (*Bacillus* and *Pseudomonas*) were detected at higher loads in digestates than in feedstock. *Bacillus* loads in mesophilic and thermophilic digestates were 2.68×10^6 and 0.43×10^6 CFU/g DM, and *Pseudomonas* loads were 0.83×10^4 CFU/g DM in MAD and 7.53×10^4 CFU/g DM, respectively. Similarly, Qi et al. (2018) reported a significant increase of *Bacillus* loads in mesophilic and thermophilic digestates than dairy manure. However, the *Pseudomonas* loads were reduced in that study. Another study by Cao et al. (2013) reported anaerobic digestion significantly reduced the numbers of *Bacillus*. These results showed that the fate of *Bacillus* and *Pseudomonas* during AD process was not suitable. Therefore, further research is recommended to ascertain the role of temperature and nutrients on the growth of antagonistic bacteria in the anaerobic digester, which is important for investigating the potential of anaerobic digestate in suppressing phytopathogens.

3. Potential of anaerobic digestate in suppressing plant disease

The plant disease suppressing phenomenon of organic amendment consists of various factors, while enzymatic and microbiological parameters, such as antagonistic bacteria, are much more informative for soil-borne disease suppression (Bonanomi et al., 2010). Therefore, population densities of antagonistic bacteria in organic amendment is an important indicator of suppressive activities against soil-borne plant diseases (Boulter et al., 2002), and higher densities of antagonistic bacteria contribute to higher disease suppressive activities (Joshi et al., 2009). Boulter et al. (2002) reported that the dominant antagonistic bacteria in compost were *Pseudomonas* and *Bacillus*. The results of chapter 2 confirmed the

presence of *Pseudomonas* and *Bacillus* in digestates. In chapter 3, *Bacillus* isolates showed antagonistic activity against four phytopathogens, and higher populations were found in mesophilic digestate than thermophilic digestate. Except for the percentage of antagonistic *Bacillus* against *Cercospora beticola*, percentages of antagonistic *Bacillus* in thermophilic digestate, which were approximately 6.25 to 8.33%, were much higher compared to 3.24 to 4.42% in mesophilic digestate due to the higher population of total *Bacillus* in mesophilic digestate. These results showed that *Bacillus* was an effective antagonistic bacterium in digestate against phytopathogens, and the potential of anaerobic digestate in suppressing plant disease differs widely according to the different phytopathogens.

In field experiment, two selected isolates, B11 (*Bacillus subtilis*) and B59 (*Bacillus licheniformis*), showed significant reduction in percent of damage of potato leaves caused by insect infection of potato late blight (*Phytophthora infestans*). These results indicated the potential of anaerobic digestate in suppressing plant disease. However, further research is needed to ascertain the appropriate application of anaerobic digestate.

General Summary

Anaerobic digestion (AD) has been implemented for years for management of dairy manure, which provides several benefits, including the production of renewable energy source and reduction of greenhouse gas emissions, odors and pathogens. The digested residue, anaerobic digestate could be utilized as a biofertilizer in agriculture to recycle plant nutrients in digestates and reduce the consumption of mineral fertilizers. Therefore, the objectives of this PhD thesis were to investigate the environmental risk relating pathogenic bacteria and potential in suppressing soil-borne plant diseases. In the first two chapters, the study was focused on the survival of pathogenic bacteria with or without antibiotic resistance during AD process at different temperatures and different types of anaerobic reactors, the survival of antagonistic bacteria (*Pseudomonas* and *Bacillus*) was also detected in the second chapter. In the third chapter, the study was focused on the potential of anaerobic digestate in suppressing soil-borne plant disease by investigating the antagonistic activities of bacteria in digestates.

The reduction rate of pathogenic bacteria with or without antibiotic resistance during AD process depends on digestion temperature and also affected by reactor types. In both batch and continuous reactors, thermophilic digestion showed more effective in reducing pathogenic bacteria load in digestates than mesophilic digestion. More bacteria residues were found in continuous reactors than batch reactors at both mesophilic and thermophilic temperatures. However, it worth noting that *Campylobacter* is tolerant to AD treatment, it did not to be reduced significantly during mesophilic and thermophilic anaerobic digestion. Although it was eliminated though hyper-thermophilic digestion in batch reactor, the process stability and high energy input should also be considered. Therefore, appropriate management practices should be implemented to minimize the sanitary risks of bacterial transmission when applying digestate to agricultural field.

A novel found in chapter 2 is that antagonistic bacteria (*Pseudomonas* and *Bacillus*) were increased both in mesophilic and thermophilic anaerobic digestion. Higher population density of *Pseudomonas* was detected in thermophilic digestate, while mesophilic digestate contained higher count of *Bacillus*. Antagonistic activities of bacteria were investigated

against four phytopathogens. *Bacillus* suppressed growth of phytopathogens, while *Pseudomonas* did not show any antagonistic activities. These results showed that *Bacillus* may be dominant antagonistic bacteria in digestate to antagonize phytopathogens. However, the antagonistic activity of *Pseudomonas* could be expected against other phytopathogens and further study is recommended.

In field experiment, *Bacillus* isolate-treated potato tubers effectively suppressed the appearance of potato late blight. These results suggest that the application of anaerobic digestate could lead to suppression of soil-borne plant diseases caused by antagonistic bacteria. However, further research is needed to ascertain the appropriate application of anaerobic digestate. This study provided a new point for the agricultural utilization of anaerobic digestate to suppress soil-borne plant disease.

References

Abubaker, J., Risberg, K., Pell, M., 2012. Biogas residues as fertilisers - Effects on wheat growth and soil microbial activities. Appl. Energy 99, 126–134.

Ahemad, M., Kibret, M., 2014. Mechanisms and applications of plant growth promoting rhizobacteria: Current perspective. J. King Saud Univ. - Sci. 26, 1–20.

Alburquerque, J.A., Fuente, C. de la, Ferrer-Costa, A., Carrasco, L., Cegarra, J., Abad, M., Bernal, M.P., 2012. Assessment of the fertiliser potential of digestates from farm and agroindustrial residues. Biomass and Bioenergy 40, 181–189.

Alfa, M.I., Adie, D.B., Igboro, S.B., Oranusi, U.S., Dahunsi, S.O., Akali, D.M., 2014. Assessment of biofertilizer quality and health implications of anaerobic digestion effluent of cow dung and chicken droppings. Renew. Energy 63, 681–686.

Andriamanohiarisoamanana, F.J., Matsunami, N., Yamashiro, T., Iwasaki, M., Ihara, I., Umetsu, K., 2016. High-solids anaerobic mono-digestion of riverbank grass under thermophilic conditions. J. Environ. Sci. 1–10.

Angulo, F.J., Nunnery, J.A., Bair, H.D., 2004. Antimicrobial resistance in zoonotic enteric pathogens. Rev. Sci. Tech. 23, 485–96.

APHA, 1998. Standard Methods for the Examination of Water and Wastewater, 20 th. ed. American Public Health Association, American Water Works Association and Water Environment Federation, Washington DC, USA.

Arif, S., Liaquat, R., Adil, M., 2018. Applications of materials as additives in anaerobic digestion technology. Renew. Sustain. Energy Rev. 97, 354–366.

Ashwini, N., Srividya, S., 2014. Potentiality of Bacillus subtilis as biocontrol agent for management of anthracnose disease of chilli caused by Colletotrichum gloeosporioides OGC1. 3 Biotech 4, 127–136.

Ballvora, A., Ercolano, M.R., Weiß, J., Meksem, K., Bormann, C.A., Oberhagemann, P., Salamini, F., Gebhardt, C., 2002. The R1 gene for potato resistance to late blight (Phytophthora infestans) belongs to the leucine zipper/NBS/LRR class of plant resistance genes. Plant J. 30, 361–371.

Beneragama, N., Lateef, S.A., Iwasaki, M., Umetsu, K., 2012. Effect of Cefazolin and Waste milk on Multi-drug Resistant Bacterial Reduction during Anaerobic Co-digestion of Dairy Manure and Waste Milk. Annu. Res. J. SLSAL 12, 59–70.

Beneragama, N., Lateef, S.A., Iwasaki, M., Yamashiro, T., Umetsu, K., 2013a. The combined effect of cefazolin and oxytertracycline on biogas production from thermophilic anaerobic digestion of dairy manure. Bioresour. Technol. 133, 23–30.

Beneragama, N., Moriya, Y., Yamashiro, T., Iwasaki, M., Lateef, S.A., Ying, C., Umetsu, K., 2013b. The survival of cefazolin-resistant bacteria in mesophilic co-digestion of dairy manure and waste milk. Waste Manag. Res. 31, 843–848.

Bonanomi, G., Antignani, V., Capodilupo, M., Scala, F., 2010. Identifying the characteristics of organic soil amendments that suppress soilborne plant diseases. Soil Biol. Biochem. 42, 136–144.

Boulter, J.I., Trevors, J.T., Boland, G.J., 2002. Microbial studies of compost: Bacterial identification, and their potential for turfgrass pathogen suppression. World J. Microbiol. Biotechnol. 18, 661–671.

Bradley, A.J., 2002. Bovine mastitis: An evolving disease. Vet. J. 164, 116–128.

Cao, Y., Chang, Z., Wang, J., Ma, Y., Fu, G., 2013. The fate of antagonistic microorganisms and antimicrobial substances during anaerobic digestion of pig and dairy manure. Bioresour. Technol. 136, 664–671.

Collins, D.P., Jacobsen, B.J., 2003. Optimizing a Bacillus subtilis isolate for biological control of sugar beet cercopsora leaf spot. Biol. Control 26, 153–161.

Dimkić, I., Berić, T., Stević, T., Pavlović, S., Šavikin, K., Fira, D., Stanković, S., 2015. Additive and synergistic effects of Bacillus spp. isolates and essential oils on the control of phytopathogenic and saprophytic fungi from medicinal plants and marigold seeds. Biol. Control 87, 6–13.

El-Mashad, H.M., Zhang, R., 2010. Biogas production from co-digestion of dairy manure and food waste. Bioresour. Technol. 101, 4021–4028.

Elshahat, M.R., Ahmed, A.A., Enas, A.H., Fekria, M.S., 2016. Plant growth promoting rhizobacteria and their potential for biocontrol of phytopathogens. African J. Microbiol. Res. 10, 486–504.

Gavala, H.N., Yenal, U., Skiadas, I. V., Westermann, P., Ahring, B.K., 2003. Mesophilic and thermophilic anaerobic digestion of primary and secondary sludge. Effect of pre-treatment at elevated temperature. Water Res. 37, 4561–4572.

Hoitink, H., Stone, A., Han, D., 1997. Suppression of Plant Diseases by Compost. HortScience 32, 184–187.

Holm-Nielsen, J.B., Al Seadi, T., Oleskowicz-Popiel, P., 2009. The future of anaerobic digestion and biogas utilization. Bioresour. Technol. 100, 5478–5484.

Iwasaki, M., Lateef, S.A., Andriamanohiarisoamanana, F.J., Yamashiro, T., Umetsu, K., 2013. Effects of Methane Fermentation on Seed Survival of Broad-Leaved Dock (Rumexobtusifolius L.) with Dairy Manure. J. Agric. Sci. Technol. 3, 561–567.

Iwasaki, M., Yamashiro, T., Beneragama, N., Nishida, T., Kida, K., Ihara, I., Takahashi, J.I., Umetsu, K., 2011. The effect of temperature on survival of pathogenic bacteria in biogas plants. Anim. Sci. J. 82, 707–712.

Jeong, M.H., Lee, Y.S., Cho, J.Y., Ahn, Y.S., Moon, J.H., Hyun, H.N., Cha, G.S., Kim, K.Y., 2017. Isolation and characterization of metabolites from Bacillus licheniformis MH48 with antifungal activity against plant pathogens. Microb. Pathog. 110, 645–653.

Jiang, X., Sommer, S.G., Christensen, K. V., 2011. A review of the biogas industry in China. Energy Policy 39, 6073–6081.

Jiang, Z.Q., Guo, Y.H., Li, S.M., Qi, H.Y., Guo, J.H., 2006. Evaluation of biocontrol efficiency of different Bacillus preparations and field application methods against Phytophthora blight of bell pepper. Biol. Control 36, 216–223.

Joshi, D., Hooda, K.S., Bhatt, J.C., Mina, B.L., Gupta, H.S., 2009. Suppressive effects of composts on soil-borne and foliar diseases of French bean in the field in the western Indian Himalayas. Crop Prot. 28, 608–615.

Kearney, T.E., Larkin, M.J., J.P. Frost, Levett, P.N., 1993. Survival of pathogenic bacteria during mesophilic anaerobic digestion of animal waste. J. Appl. Microbiol. 75, 215–219.

Kobashi, Y., Hasebe, A., Nishio, M., 2005. Antibiotic-resistant Bacteria from Feces of Livestock , Farmyard Manure , and Farmland in Japan — case report —. Microbes Environ. 20, 53–60.

Kumar, P., Dubey, R.C., Maheshwari, D.K., 2012. Bacillus strains isolated from rhizosphere showed plant growth promoting and antagonistic activity against phytopathogens. Microbiol. Res. 167, 493–9.

Lateef, S.A., Beneragama, N., Yamashiro, T., Iwasaki, M., Ying, C., Umetsu, K., 2012. Biohydrogen production from co-digestion of cow manure and waste milk under thermophilic temperature. Bioresour. Technol. 110, 251–257. https://doi.org/10.1016/j.biortech.2012.01.102

Li, X., Guo, J., Dong, R., Ahring, B.K., Zhang, W., 2016. Properties of plant nutrient: Comparison of two nutrient recovery techniques using liquid fraction of digestate from anaerobic digester treating pig manure. Sci. Total Environ. 544, 774–781.

Massé, D.I., Croteau, F., Masse, L., 2007. The fate of crop nutrients during digestion of

swine manure in psychrophilic anaerobic sequencing batch reactors. Bioresour. Technol. 98, 2819–2823.

Mehta, C.M., Palni, U., Franke-Whittle, I.H., Sharma, A.K., 2014. Compost: Its role, mechanism and impact on reducing soil-borne plant diseases. Waste Manag. 34, 607–622.

Micolucci, F., Gottardo, M., Cavinato, C., Pavan, P., Bolzonella, D., 2016. Mesophilic and thermophilic anaerobic digestion of the liquid fraction of pressed biowaste for high energy yields recovery. Waste Manag. 48, 227–235.

Min, H., Hyub, J., Kim, M., Kim, J., Mo, Y., Moon, J., 2016. Effect of increased load of high-strength food wastewater in thermophilic and mesophilic anaerobic co-digestion of waste activated sludge on bacterial community structure. Water Res. 99, 140–148.

Nicholson, W.L., Munakata, N., Horneck, G., Melosh, H.J., Setlow, P., 2000. Resistance of Bacillus endospores to extreme terrestrial and extraterrestrial environments. Microbiol. Mol. Biol. Rev. 64, 548–72.

Nkoa, R., 2014. Agricultural benefits and environmental risks of soil fertilization with anaerobic digestates: A review. Agron. Sustain. Dev. 34, 473–492.

Noike, T., Hidenari, Y., Yuyou, L., Shuichi, O., Syouri, S., 2009. Methane Fermentation, First edit. ed. GIHODO SHUPPAN Co., Ltd., Tokyo.

Orzi, V., Scaglia, B., Lonati, S., Riva, C., Boccasile, G., Alborali, G.L., Adani, F., 2015. The role of biological processes in reducing both odor impact and pathogen content during mesophilic anaerobic digestion. Sci. Total Environ. 526, 116–126.

Owamah, H.I., Dahunsi, S.O., Oranusi, U.S., Alfa, M.I., 2014. Fertilizer and sanitary quality of digestate biofertilizer from the co-digestion of food waste and human excreta. Waste Manag. 34, 747–752.

Poudel, R.C., Joshi, D.R., Dhakal, N.R., Karki, A.B., 2010. Evaluation of Hygienic

Treatment of Biowastes by Anaerobic Digestion in Biogas Plants. Nepal J. Sci. Technol. 10.

Qi, G., Pan, Z., Sugawa, Y., Andriamanohiarisoamanana, F.J., Yamashiro, T., Iwasaki, M., Kawamoto, K., Ihara, I., Umetsu, K., 2018. Comparative fertilizer properties of digestates from mesophilic and thermophilic anaerobic digestion of dairy manure: focusing on plant growth promoting bacteria (PGPB) and environmental risk. J. Mater. Cycles Waste Manag. 20, 1448–1457.

Quessy, S., Masse, D.I., 2006. Reduction of indicator and pathogenic microorganisms by psychrophilic anaerobic digestion in swine slurries 97, 686–691. https://doi.org/10.1016/j.biortech.2005.03.024

Report on the Japanese Veterinary Antimicrobial Resistance Monitoring System -2012 to 2013- National Veterinary Assay Laboratory Ministry of Agriculture , Forestry and Fisheries, 2016.

Resende, J.A., Silva, V.L., de Oliveira, T.L.R., de Oliveira Fortunato, S., da Costa Carneiro, J., Otenio, M.H., Diniz, C.G., 2014. Prevalence and persistence of potentially pathogenic and antibiotic resistant bacteria during anaerobic digestion treatment of cattle manure. Bioresour. Technol. 153, 284–291.

Rico, C., Rico, J.L., Tejero, I., Muñoz, N., Gómez, B., 2011. Anaerobic digestion of the liquid fraction of dairy manure in pilot plant for biogas production: Residual methane yield of digestate. Waste Manag. 31, 2167–2173.

Risberg, K., Cederlund, H., Pell, M., Arthurson, V., Schnürer, A., 2017. Comparative characterization of digestate versus pig slurry and cow manure – Chemical composition and effects on soil microbial activity. Waste Manag. 61, 529–538.

Riva, C., Orzi, V., Carozzi, M., Acutis, M., Boccasile, G., Lonati, S., Tambone, F., D'Imporzano, G., Adani, F., 2016. Short-term experiments in using digestate products as substitutes for mineral (N) fertilizer: Agronomic performance, odours, and ammonia

emission impacts. Sci. Total Environ. 547, 206–214.

Saber, F.M.A., Abdelhafez, A.A., Hassan, E.A., Ramadan, E.M., 2015. Characterization of fluorescent pseudomonads isolates and their efficiency on the growth promotion of tomato plant. Ann. Agric. Sci. 60, 131–140.

Sahlström, L., 2003. A review of survival of pathogenic bacteria in organic waste used in biogas plants. Bioresour. Technol. 87, 161–166.

Sahlström, L., Aspan, A., Bagge, E., Danielsson-Tham, M.L., Albihn, A., 2004. Bacterial pathogen incidences in sludge from Swedish sewage treatment plants. Water Res. 38, 1989–1994.

Salman, M., 2010. Determination of antibiotic activity on plasmids from fluorescent pseudomonads isolates CW2, WB15 and WB52 against pre-emergence damping-off caused by Pythium ultimum and Rhizoctonia solani in cucumber. Biol. Control 53, 161–167.

Shah, F.A., Mahmood, Q., Maroof Shah, M., Pervez, A., Ahmad Asad, S., 2014. Microbial ecology of anaerobic digesters: The key players of anaerobiosis. Sci. World J. 2014, 1–21.

Sinton, L.W., Braithwaite, R.R., Hall, C.H., Mackenzie, M.L., 2007. Survival of indicator and pathogenic bacteria in bovine feces on pasture. Appl. Environ. Microbiol. 73, 7917–7925.

Smith, S.R., Lang, N.L., Cheung, K.H.M., Spanoudaki, K., 2005. Factors controlling pathogen destruction during anaerobic digestion of biowastes. Waste Manag. 25, 417–425.

Solé-Bundó, M., Cucina, M., Folch, M., Tàpias, J., Gigliotti, G., Garfí, M., Ferrer, I., 2017. Assessing the agricultural reuse of the digestate from microalgae anaerobic digestion and co-digestion with sewage sludge. Sci. Total Environ. 586, 1–9.

Takemura, T., Ihara, I., Kitazono, Y., Toyoda, K., Iwasaki, M., Umetsu, K., 2016. Effect of Seed Sludge on Survival of Antibiotic-Resistant Bacteria and Fate of Bacteria Resistant to Different Classes of Antibiotics during Anaerobic Digestion of Dairy Manure. J. Water Environ. Technol. 14, 282–288. https://doi.org/10.2965/jwet.15-070

Termorshuizen, A.J., van Rijn, E., van der Gaag, D.J., Alabouvette, C., Chen, Y., Lagerlöf, J., Malandrakis, A.A., Paplomatas, E.J., Rämert, B., Ryckeboer, J., Steinberg, C., Zmora-Nahum, S., 2006. Suppressiveness of 18 composts against 7 pathosystems: Variability in pathogen response. Soil Biol. Biochem. 38, 2461–2477.

Tigini, V., Franchino, M., Bona, F., Varese, G.C., 2016. Is digestate safe? A study on its ecotoxicity and environmental risk on a pig manure. Sci. Total Environ. 551–552, 127–132.

Umetsu, K., Kondo, R., Tani, M., Hayashi, T., 2002. Fertilizer value of anaerobically co-digested dairy manure and food processing wastes, Greenhouse Gases and Animal Agriculture.

Walczak, J.J., Xu, S., 2011. Manure as a Source of Antibiotic-Resistant Escherichia coli and Enterococci : a Case Study of a Wisconsin, USA Family Dairy Farm 579–589. https://doi.org/10.1007/s11270-010-0729-x

Wang, Z., Wang, Y., Zheng, L., Yang, X., Liu, H., Guo, J., 2014. Isolation and characterization of an antifungal protein from Bacillus licheniformis HS10. Biochem. Biophys. Res. Commun. 454, 48–52.

Xie, S., Lawlor, P.G., Frost, J.P., Hu, Z., Zhan, X., 2011. Effect of pig manure to grass silage ratio on methane production in batch anaerobic co-digestion of concentrated pig manure and grass silage. Bioresour. Technol. 102, 5728–5733.

Yamashiro, T., Lateef, S.A., Ying, C., Beneragama, N., Lukic, M., Masahiro, I., Ihara, I., Nishida, T., Umetsu, K., 2013. Anaerobic co-digestion of dairy cow manure and high concentrated food processing waste. J. Mater. Cycles Waste Manag. 15, 539–547.

Yang, L., Quan, X., Xue, B., Goodwin, P.H., Lu, S., Wang, J., Du, W., Wu, C., 2015. Isolation and identification of bacillus subtilis strain YB-05 and its antifungal substances showing antagonism against gaeumannomyces graminis var. tritici. Biol. Control 85, 52–58.

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Abstract

Recent years, there is increasing worldwide interest in technology producing renewable energy sources as a result of global warming and the increasing consumption of fossil fuels. Livestock wastes, such as manure, present a potential source of various hazards to human life and the environment, and production has increased sharply as the development of livestock industry. Therefore, anaerobic digestion (AD) of livestock manure seems to be a promising method to treat large amount of livestock manure and produce biogas, which is a renewable energy source. Together with biogas production, AD process also produces a liquid residue called digestate, which is considered a valuable fertilizer consisting of partially degraded organic matter, microbial biomass and inorganic compounds. Many studies have been conducted to investigate the fertilizer property of digestate. However, there is concern about the environmental risk of digestate application as livestock manure contains various pathogenic bacteria. Furthermore, the widespread use of antibiotics in livestock has increased the frequency of antibiotic resistant bacteria (ARB) in livestock wastes. On the other hand, some antagonistic bacteria in digestate may extend the utilization of digestate. Therefore, this PhD thesis was focused on two main objectives: to investigate the survival of pathogenic bacteria with and without antibiotic resistance during anaerobic digestion at different temperatures and to detect antagonistic activity of bacteria in digestate.

In chapter 1, the effect of digestion temperature on survival of pathogenic and cefazolin resistant bacteria in dairy manure was investigated in batch reactors. Lab-scale batch anaerobic digestions were conducted under mesophilic (37 °C), thermophilic (55 °C) and hyper-thermophilic (65 °C) temperatures. Results showed that *Enterococcus*, *Salmonella*, and *Acinetobacter* with and without cefazolin resistance were eliminated by AD treatment under each temperature, however, *E.coli* and *Campylobacter* were detected in digestates. Among cefazolin resistant bacteria, only *Campylobacter* was survived under mesophilic temperature. These results indicated that AD process under high temperature could effectively reduce pathogenic and cefazolin resistant bacteria in dairy manure. However, *Campylobacter* was tolerant to AD treatment, appropriate management practices should be implemented to minimize the sanitary risks of bacterial transmission when

applying digestate to agricultural field.

In chapter 2, the survival of pathogenic bacteria with and without cefazolin resistance was investigated in semi-continuous stirred-tank reactors. Two laboratory scale anaerobic reactors digesting dairy manure were conducted at mesophilic and thermophilic temperature respectively. Results showed that pathogenic and cefazolin resistant bacteria were reduced by AD and significantly reduced in thermophilic anaerobic digestion. However, more pathogenic bacteria residues were found in digestates from continuous reactors than those from batch reactors. On the other hand, antagonistic bacteria (*Bacillus* and *Pseudomonas*) were detected at higher loads in digestates than in feedstock. *Bacillus* loads in mesophilic and thermophilic digestates were 2.68×10^6 and 0.43×10^6 CFU/g DM, and *Pseudomonas* loads were 0.83×10^4 CFU/g DM in MAD and 7.53×10^4 CFU/g DM, respectively. These results showed anaerobic digestion is effective on pathogenic bacterial reduction and increased antagonistic bacteria, which may expend biological component potential of digestate to suppress soil-borne plant diseases caused by phytopathogen.

In the last chapter, potential of antagonistic activities of anaerobic digestate against phytopathogens were investigated by detecting the antagonistic activities of *Bacillus* and *Pseudomonas* in anaerobic digestates. *Bacillus* suppressed growth of phytopathogens, while *Pseudomonas* did not show any antagonistic activities. In addition, the populations of antagonistic *Bacillus* were much higher in mesophilic digestate than that in thermophilic digestate, and the highest population was 2.53×10^5 CFU/g DM against *Cercospora beticola*. These results indicated that *Bacillus* was an effective antagonistic bacterium in digestate against phytopathogens. Furthermore, two selected isolates, B11 (*Bacillus subtilis*) and B59 (*Bacillus licheniformis*), were applied in field experiments and showed significant reduction in percent infection of potato late blight (*Phytophthora infestans*). These results demonstrate the benefits of digestate in suppressing soil-borne plant diseases caused by antagonistic bacteria.

The results from this PhD thesis show that (1) higher temperature showed higher

reduction rates, it means temperature of AD process is an important factor that affects the survival of pathogenic bacteria with and without antibiotic resistance. Although hyper-thermophilic digestion showed highest reduction of pathogenic bacteria, high energy input and unstable process stability may be the problem. On another hand, anaerobic batch reactor showed higher bacteria reduction efficiency than continuous reactor under the same temperature, this is attributed to that the retention time may not be long enough to inactivate pathogenic bacteria in continuous digestion condition. Due to some pathogenic bacteria were detected in mesophilic digestate, appropriate management practices, such as sterilization, are recommended to minimize the sanitary risks of bacterial transfer to agricultural land from the application of mesophilic digestate. (2) Anaerobic digestion had increased population densities of *Bacillus* and *Pseudomonas* in digestates compared with dairy manure. Since *Bacillus* and *Pseudomonas* have been reported to be involved in acidogenesis stage of anaerobic digestion process, it was considered that the suitable temperatures and available nutrients in digesters stimulated their growth. As *Bacillus* and *Pseudomonas* have been reported with antagonistic activities against phytopathogens, they are considered biological agents to suppress plant diseases. In current study, digestate with increased *Bacillus* is expected with potential to suppress plant disease when it applies to agricultural land. Due to antagonistic *Pseudomonas* was not detected, further research is recommended to ascertain the antagonistic activity of *Pseudomonas* in digestates against other phytopathogens and the appropriate application of anaerobic digestate.

要旨

近年の地球温暖化と化石燃料の消費の増加の結果、再生可能エネルギーを生産する技術に世界的な関心が集まっている。家畜排せつ物は人間や環境に対し潜在的な汚染源となり、その生産量も畜産業の発展に伴い急激に増加している。家畜ふん尿の嫌気発酵は、大量の家畜ふん尿を処理することができ、再生可能エネルギーであるバイオガスを生産する有望な方法である。嫌気発酵処理後の消化残留物は消化液と呼ばれ、部分的に分解された有機物、微生物バイオマスおよび無機化合物からなる貴重な肥料と考えられている。これまで消化液の肥料特性を調べるために多くの研究が行われてきているが、家畜ふん尿には様々な病原菌が含まれているため、消化液の生物安全性が懸念されている。また、家畜には大量の抗菌性物質が使用されているため、薬剤耐性菌の出現を助長させる危険性もある。しかし一方で、消化液には植物病原菌に対する拮抗性細菌が存在しており、このことは消化液の利用価値を拡大する可能性がある。本博士論文は、異なる温度で嫌気的に消化した乳牛ふん尿に存在する病原菌と薬剤耐性菌の残存量の検討と、消化液中の植物病原菌拮抗細菌の検出の 2 つを目的とした。

第 1 章では、嫌気発酵の温度が乳牛ふん尿中の病原菌やセファゾリン耐性菌の残存に及ぼす影響についてバッチ発酵槽を用いて調べた。実験室スケールで中温（37℃）、高温（55℃）および超高温（65℃）で嫌気性発酵を行った結果、*Enterococcus*、*Salmonella*、および *Acinetobacter* は各温度の嫌気発酵によって減滅したが、*E.coli* と *Campylobacter* の残存が認められた。中温ではセファゾリン耐性 *Campylobacter* の残存も認められた。これらの結果は、高い温度での嫌気発酵はふん尿中の病原菌およびセファゾリン耐性細菌を効果的に減少させることができることを示した。しかし、*Campylobacter* の残存は消化液利用の危害要因となる可能性が考えられたため、より効果的な処理方法が今後の課題である。

第 2 章では、半連続攪拌発酵槽を用いて乳牛ふん尿を嫌気発酵した場合の病原菌やセファゾリン耐性菌の残存を調べた。実験室スケールの嫌気発酵を中温（37℃）と高温（55℃）で行った。その結果、嫌気発酵によって病原菌やセファゾリン耐性菌は減少

し、高温でさらに顕著な減少が認められた。しかしながら、第 1 章の結果と比べ、バッチ発酵槽よりも多くの病原菌の残留が確認された。一方、拮抗性細菌（*Bacillus* および *Pseudomonas*）は嫌気性発酵前のふん尿よりも増加していた。中温消化液及び高温消化液における *Bacillus* 属菌数は 2.68×10^6 CFU/g DM と 0.43×10^6 CFU/g DM、*Pseudomonas* 属菌数は 0.83×10^4 CFU/g DM と 7.53×10^4 CFU/g DM であった。これらの結果は、嫌気発酵は病原菌を減少させる効果があり、さらに拮抗性細菌の増加により消化液は植物病原体による植物病気を抑制する可能性があることを示した。

第 3 章では、乳牛ふん尿を嫌気発酵した消化液の植物病原菌に対する拮抗作用を検討し、さらにその役割を担う拮抗細菌（*Bacillus* および *Pseudomonas*）の量を測定した。その結果、植物病原菌に対し拮抗作用を示す *Bacillus* の量は増加していたが、植物病原菌拮抗性の *Pseudomonas* は検出されなかった。さらに、拮抗作用を示す *Bacillus* 数は中温消化液では高温消化液よりも多く検出され、中でも *Cercospora beticola* に対する拮抗性 *Bacillus* が最も多く、その量は 2.53×10^5 CFU / g DM であった。これらの結果から、消化液では *Bacillus* が植物病原菌に対する有効な拮抗細菌であると考えられた。また、消化液から *Bacillus* 株 B11（*Bacillus subtilis*）と B59（*Bacillus licheniformis*）を分離し、これらを用いた圃場試験を実施したところ、ジャガイモ疫病を有意に低下させることが示された。このことから、消化液は、含まれる植物病原菌拮抗細菌の作用により土壌病害を防除する利点があることが分った。

本博士論文の研究成果をまとめると、（1）病原菌の減少効果は中温発酵よりも高温発酵で顕著であったことから、嫌気発酵の温度が病原菌や薬剤耐性菌の残存に影響を与える主要な因子であると考えられた。超高温処理は病原菌の最大の減少効果を示したが、高いエネルギー投入と発酵プロセスの不安定性が問題になる。また、バッチ発酵槽では連続発酵槽より高い病原菌の減少効果を示したことから、滞留時間も重要な要因であることが分った。しかし、中温消化液には病原菌の残存が認められたため、環境リスクを避けるためには、滅菌などの適切な管理方法が必要である。（2）乳牛糞尿中の *Bacillus* および *Pseudomonas* の量は、嫌気発酵により増加が認められた。*Bacillus* と

Pseudomonas は嫌気発酵の酸生成段階に関与していることが報告されていることから、消化槽中の適切な温度と利用可能な栄養素の存在が増加の要因と考えられた。また、いずれも植物病原菌に対する拮抗作用が報告されており、今回の検討では嫌気発酵によって拮抗性 *Bacillus* の増加が認められ、消化液の生物農薬としての応用が期待される結果であった。拮抗性 *Pseudomonas* は検出されず、他の植物病原体に対する拮抗作用の検討が今後の課題である。