Evaluation of hygienic properties of residues after anaerobic digestion (digestate) of dairy cow manure estimated from biological analysis

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生物学的分析に基づく乳牛ふん尿嫌気発酵消化液の衛生評価

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Abstract

To determine the effects of anaerobic digestion treatment on the survival of weed seeds in dairy cow manure, weed seeds (*Rumex obtusifolius* L.) were mixed into dairy cow manure, and the seed germination rates and percentage of dormant seeds after mesophilic (35 °C) and thermophilic (55 °C) anaerobic digestion processes were investigated in Chapter 1. The survival rates of *Rumex obtusifolius* L. seeds heat-treated at 35 °C and 55 °C were 75.5% and 0%, respectively, compared to 81.6% and 0% in anaerobic digestion processes at the same temperatures. The survival rates at 35 °C treatment were similar between the anaerobic digestion and heat treatments, but the percentage of seeds in primary and secondary dormancy was higher in the anaerobic digestion treatment compared to heat treatment. Although digestate have a high value as liquid fertilizer, mesophilic digestate should be treated with caution owing to the presence of dormant weed seeds.

In Chapter 2, the elimination of pathogenic bacteria in livestock manure during anaerobic diegstion in a full-scale biogas plant was investigated using livestock manure as a raw material. Manure was collected from reception pits, digester tanks, and digestate storage tanks of four biogas plants with mesophilic (38 °C) and thermophilic (55 °C) fermenters currently in operation in Hokkaido, Japan. *Coli-aerogenes* and *Enterococcus* in manure were cultured to analyze the survival rates after the digestion process. As a result, no *Coli-aerogenes* and *Enterococcus* were detected in either manure in the fermenter or digestate after anaerobic digestion in the thermophilic biogas plant. In the mesophilic biogas plant, although the number of *Coli-aerogenes* and *Enterococcus* decreased with digestion, *Coli-aerogenes* and *Enterococcus* in manure were found in the fermenter and digestate after anaerobic digestion. The mean decimation reduction time (T_{90}) of *Coli-aerogenes* and *Enterococcus* in mesophilic digestion were 13.3 and 16.7 days, respectively.

Large quantities of antimicrobial agents have been used in dairy farming to treat and prevent livestock diseases. There has been a global problem of the resulting emergence of antimicrobial-resistant bacteria in livestock manure. Cefazolin (CEZ) is the antibiotic most commonly used in treating dairy mastitis, and CEZ-resistant (CEZ-R) bacteria are detected in manure of dairy cows administered with the antimicrobial. The study in Chapter 3, the effects of thermophilic anaerobic digestion on the survival of CEZ-R and CEZ-susceptible (CEZ-S) bacteria and substrate-specific extended-spectrum β-lactamase (ESBL)-producing bacteria (*E. coli*, *Klebsiella*/*Enterobacter*/*Citrobacter*, *Proteus*, *Pseudomonas*, and *Acinetobacter*) in dairy cow manure was examined. As a result, the number of CEZ-R bacteria was reduced to 0.93% by thermophilic anaerobic digestion, including a decrease in the number of *E. coli*, other coliforms, and other bacteria to below the limit of detection, 0.27%, and 1.05%, respectively. In contrast, there was a slight increase in ESBL-producing *Pseudomonas*. These results suggested that the risk of spreading cephazolin-resistant bacteria was reduced in the thermophilic anaerobic digestion process of livestock manure,

but some antimicrobial-resistant bacteria remained.

The study in Chapter 4 also focused on the analysis of CEZ-R *Pseudomonas* spp. because there was a slight increase in ESBL-producing *Pseudomonas* spp. during thermophilic anaerobic digestion. Due to the mixture of pathogenic and plant growth-promoting species in *Pseudomonas* spp., *P. aeruginosa*, *P. fluorescens*, and other pseudomonads were classified and quantitatively analyzed using commercial PASA medium and newly prepared CAP medium (commercial Cetrimide medium to which L-arginine and phenol red were added to classify species according to differences in color development).

The evidence for these classifications was performed by PCR confirmation. *Pseudomonas* spp. decreased after both mesophilic and thermophilic anaerobic digestion, indicating that anaerobic digestion at thermophilic was more effective than at mesophilic during the anaerobic digestion process. *P. aeruginosa* was not detected in either manure collected or digestate after the mesophilic and thermophilic anaerobic digestion process. In contrast, CEZ-R *Pseudomonas* in mesophilic digestate increased relative to manure before the digestion process. *P. fluorescens*, which was present in manure and all CEZ-R, was no longer detected after mesophilic or thermophilic anaerobic digestion. Fluorescent pseudomonads, other than *P. aeruginosa* and *P. fluorescens*, increased after the anaerobic digestion process, and most of them were CEZ-S. In addition, an increase in the mesophilic anaerobic digestion process was observed in CEZ-R *Pseudomonas* spp. other than fluorescent pseudomonads. The percentage of bacterial counts suggested that most CEZ-R *Pseudomonas* spp. increased by the mesophilic anaerobic digestion process were other than fluorescent pseudomonads. Thus, due to the increase of CEZ-R *Pseudomonas* spp. other than fluorescent pseudomonads in mesophilic anaerobic digestion, the risk of spread of resistant bacteria to the environment should be considered when using digestate as liquid fertilizer.

General Introduction

One of the major problems for dairy farmers is that the volume of dairy cow manure produced per dairy unit has been increasing yearly with the decreasing number of dairy cows and the growing scale of operations. The average processing volume per dairy farmer in Japan in 2020 (1,514 t/year/farm) increased approximately three times compared with that in 1989 (493 t/year/farm) (Fig. 1). In addition to the challenges of manure processing, increased environmental consciousness in recent years has given rise to an effort to use livestock manure along with an increased interest in biogas plants as an option.

A biogas plant is a processing facility wherein organic waste (biomass) is decomposed and decreased in volume through anaerobic digestion (methane fermentation). The main products are methane gas and digestate (fermentation residue). Methane, derived from biomass materials, including livestock manure, has been recognized as a renewable energy and has garnered attention as an energy source with low environmental impact. In many biogas plants, various organic wastes are processed together with livestock manure for anaerobic co-digestion to increase the methane production. The use of such additional materials has been considered for anaerobic co-digestion, including different types of organic waste, such as kitchen garbage from homes and businesses, food waste, food processing residues, marine processing residues, weeds, and sewage sludge, as well as their effectiveness in various ratios for increasing methane gas production. Moreover, Germany and some other European countries are growing crops (energy crops) that are used in biogas plants to increase biogas production.

Since the Kyoto Protocol was adopted in 1997, many biogas plants have been constructed in Japan, and the cumulative number of biogas plants built reached 131 units by 2004 when the "Act on the Proper Management and Promotion of Use of Livestock Manure (Manure Law)" was enforced in earnest, with the largest number in Hokkaido at 41 units. The change in the number of livestock biogas plants installed in Hokkaido by year is shown in Fig. 2.

No facilities were built between 2008 and 2010 because many challenges occurred after the initial installation and the cost of electricity was low under the Renewable Portfolio Standard (RPS) law. Meanwhile, there has been an increase in the number of constructions starts for anaerobic digestion and processing facilities equipped with power since the energy crisis that followed the Great East Japan Earthquake in March 2011 and the feed-in tariff (FIT) scheme in July 2012. Between 1995 and 2020, 112 units were built. That includes 17 units that were stopped or removed, which were mostly installed as part of experimental projects that were terminated.

As for the scale of facilities, the greatest number were in facilities with 500–999 cows, at 21 units (22.1%); the next highest was 20 units (21.1%) with 200–299 cows. Recently, there has been a trend toward centralized and on-farm (stand-alone) large-scale facilities. The facilities of a small scale with less than 100 cows (11.6%) are mostly research and experimental facilities, such as public testing

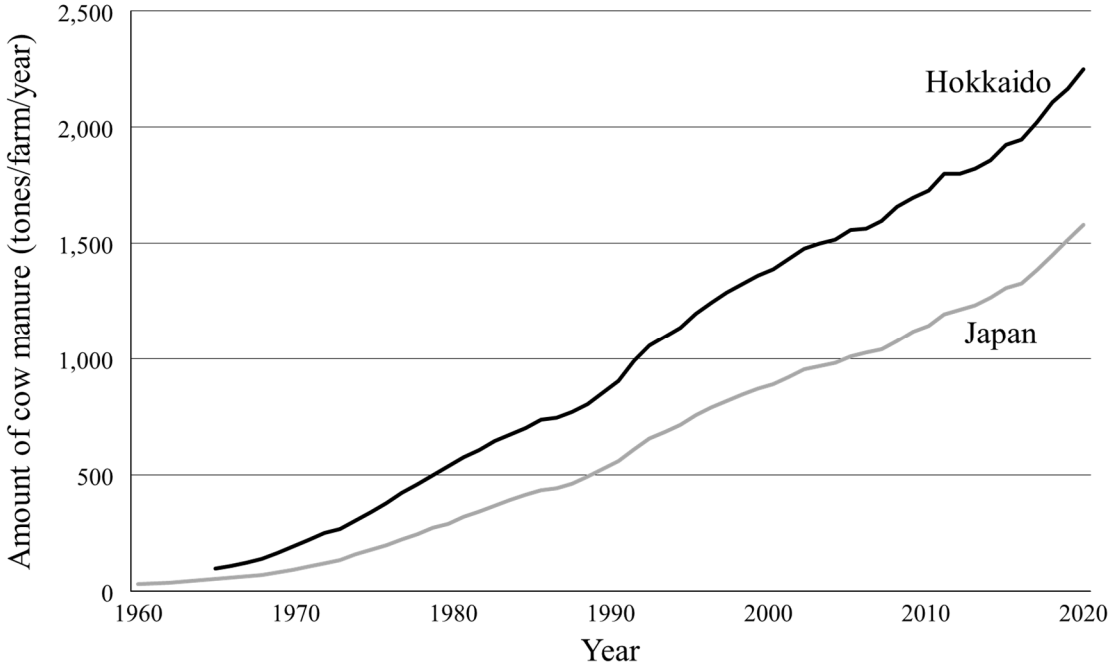

Fig. 1 Excreated amount of dairy cow manure per farm in Japan and Hokkaido region

Data source: The 95th Statistical Yearbook of Ministry of Agriculture, Forestry and Fisheries, Japan

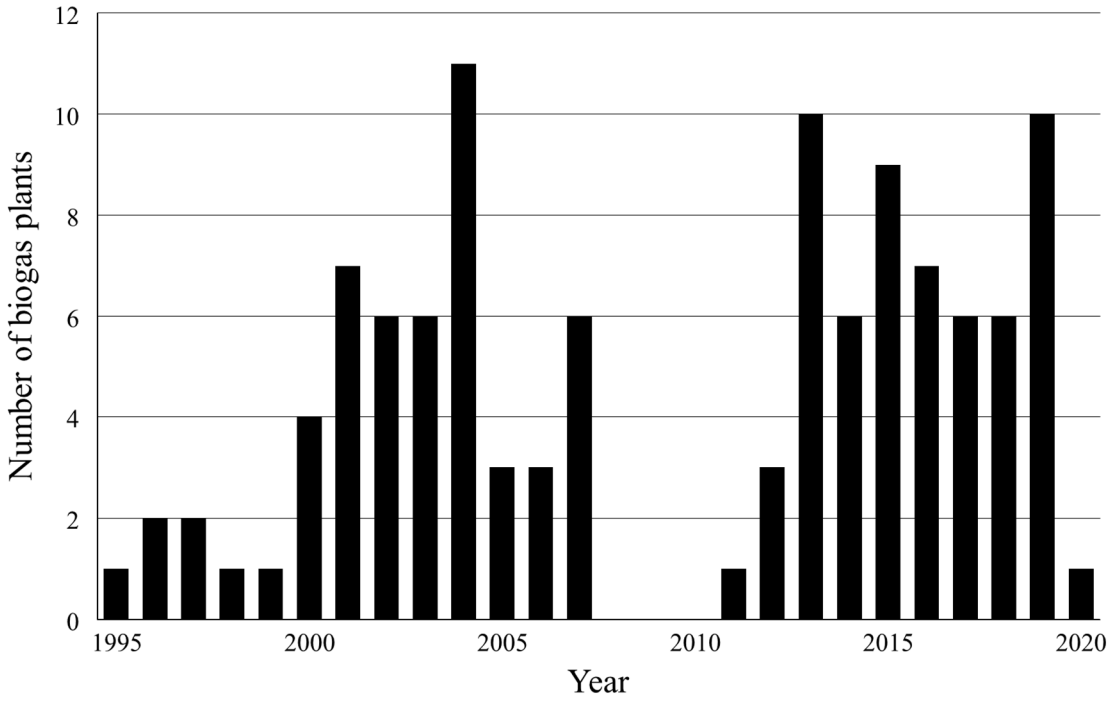

Fig. 2 Number of installed biogas plants in Hokkaido

Data source: Biomass Research Co. Ltd.

laboratories, universities, and agricultural high schools.

Changes in the volume of cow manure processed per day in biogas plants in Hokkaido are indicated in Fig. 3. Although the construction of centralized facilities has been increasing since 2015, the volume of manure processed has been surging proportionally with the larger scale of the facilities. Changes in the power output of biogas plants in dairy farms in Hokkaido are indicated in Fig. 4. Before the implementation of the FIT scheme, the installation of power generation was not conducive to biogas plant because the biogas desulfurization technology and the maintenance systems were underdeveloped and low economic benefit because of the price of electricity was low under the Renewables Portfolio Standard (RPS) law. Triggered by the introduction of the FIT scheme, there was an increase in power generation output from biogas plants in Hokkaido, especially from large-scale centralized biogas plants, with total power output of 15,180-kW by 2020, increasing 8.45 times compared with the total power output of 1,797-kW before 2011, prior to the implementation of the FIT scheme.

It is increasingly crucial that renewable energy sources are introduced and expanded in the run-up to “carbon neutral 2050.” For this purpose, a new FIP (Feed-in Premium) system will be introduced and a full-scale effort to achieve zero greenhouse gas emissions will start in 2022. Building a decarbonized society is a long-term goal in Japan, and the biomass energy market is expected to grow in the future. However, presently, because the increase in the volume of manure processed by biogas plants will be equal to the increase in the volume of digestate, there is an issue of insufficient farm land area for using the digestate as liquid fertilizer, causing a barrier to the promotion of biogas plants.

Knowledge accumulated regarding the fertilizing effect on crops, odor control, incidence of disease, and economic benefits of using digestate as an organic fertilizer. Additionally, a broad range of uses has been studied aside from use as a replacement for chemical fertilizer, as indicated in Table 1 (Baştabak and Koçar 2020).

The quality of digestate, including nutrient content, physical and chemical properties, microbial species, and the presence or absence of harmful substances and impurities, is important for its effective and safe use. The specific factors possibly indicating the quality of digestate are listed in Table 2. When used, as most frequently, as liquid fertilizer, analysis of the contents of digestate is important, including major nutrients (N, P, and K) and trace elements (Na, Mg, and Ca, among others). However, the residual amount of toxic substances and odor effects on human, livestock, and animal health during field spraying should also be considered.

Each factor that expresses the quality of digestate is largely affected by the properties of livestock manure used as raw material and the type and mixing ratio of organic wastes when mixed and fermented. Attention should be paid to contamination of heavy metals and toxic organic matter when treating urban waste or sewage sludge from factories. Regulations of countries such as the

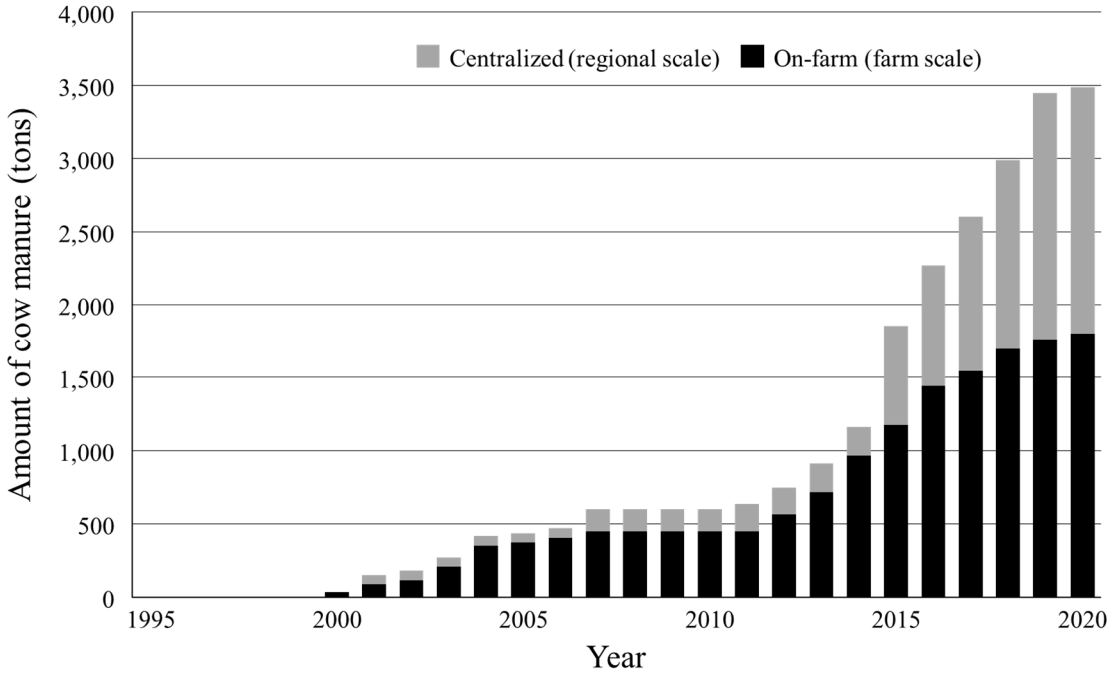

Fig. 3 Utilized amount of dairy cow manure for biogas plants in Hokkaido

Data source: Biomass Research Co. Ltd.

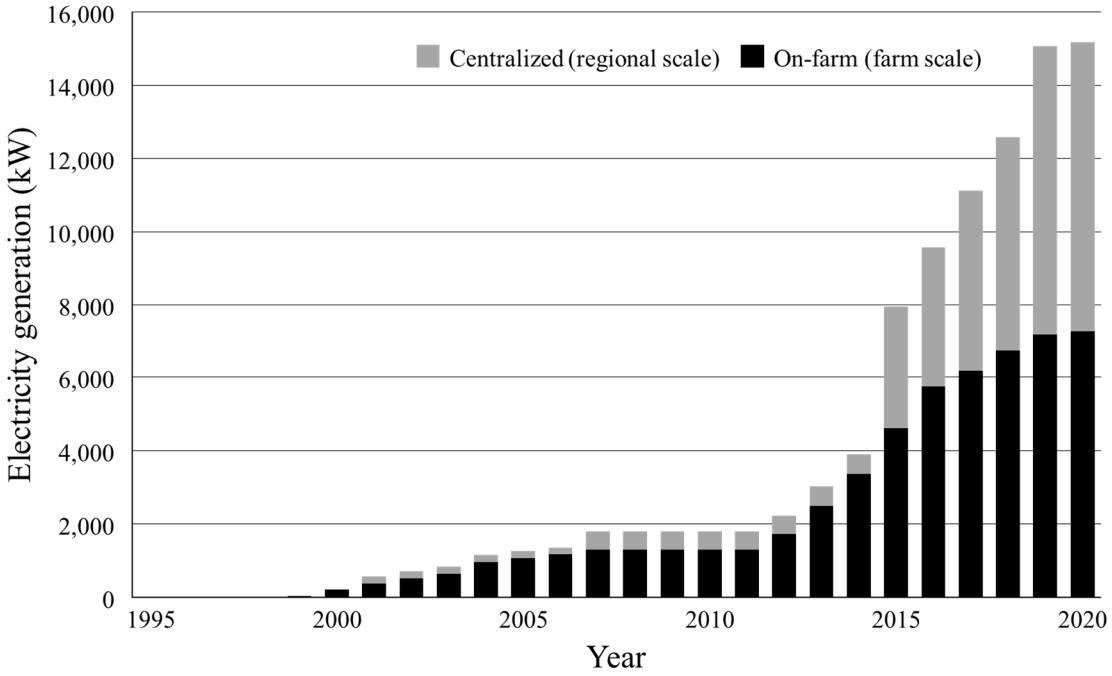

Fig. 4 Electricity generation in biogas plants in Hokkaido

Data source: Biomass Research Co. Ltd.

Table 1 Various uses of digestate

Use	Feature	References
Organic fertilizer, Biofertilizer	It is used to grow agricultural or feed crops as well as hydroponics and the growth of aquatic plants, such as seaweeds and algae, because it contains the three major nutrients in fertilizers (nitrogen, phosphate, and potassium) and various trace elements.	Nasir, et al., 2012
Soil improver	It reportedly increases productivity when used to improve soil aggregate formation (affecting water retention, permeability, cohesion, plasticity, and aeration) and improving pH.	Logan and Visvanathan, 2019
Animal feed	In some cases, it is given to pigs, chickens, shrimp, and fish as a feed additive in some countries, including China, but there is no social consensus due to legal restrictions.	Logan and Visvanathan, 2019
Irrigation water	In some cases, the water in the digestate is separated and distilled for use as irrigation water in plants equipped with large-scale digestate concentrators.	Guilayn, et al., 2020
Biopesticide	*Bacillus thuringiensis* and *Pseudomonas fluorescens*, etc. contained in manure, inhibit the growth of pathogenic bacteria and promote plant growth.	Pan, et al., 2018
Seed pretreatment	Promotes seed germination and growth by penetration and absorption of nutrients contained in digestate.	Feng, et al., 2011; Zhao, et al., 2014
Phosphorus (phosphate) recovery	The MAP method (method of crystallization as magnesium ammonium phosphate) can recover and recycle phosphorus at a low cost.	Barampouti, et al., 2020
Carbon source for industrial products	There has been a technique reported for producing electrode material for capacitors from carbon compounds obtained via pyrolysis of digestate.	Enock, et al., 2017; Enock, et al., 2018

Table 2 Quality indicators of digestate

Classification	Factors
Nutrients	Major nutrients and trace elements
Toxic substances	Chemicals, inorganic and organic compounds, heavy metals, antimicrobial-resistant genes
Pathogenic and harmful microbes	Pathogenic bacteria, plant pathogens, antimicrobial-resistant bacteria, viruses, parasites
Useful microbes and products	Enzymes, plant growth factors, plant-pathogen growth inhibitors (biopesticides), etc.
Residual compounds	Antibiotics, herbicides (clopyralid)
Odor	Hydrogen sulfide, ammonia, methyl mercaptan, etc.
Others	Weed seeds, prions, etc.

Netherlands, Switzerland, Austria, and others allow the use of digestate from sewage sludge in field land. The quality of digestate is variable, even when using the same raw materials, depending on the fermentation temperatures (psychrophilic, mesophilic, or thermophilic), fermentation method (wet or dry, one-tank, or two-tank), shape of the digester tank, how water is added (return digestate to digester tank, etc.), pH, organic loading rate and hydraulic retention time (HRT), and mixing method. Therefore, the operating conditions of the biogas plant should be examined to get digestate appropriate for use.

Even if digestate released from a biogas plant has a constant quality, its quality may change owing to the subsequent storage conditions, especially temperature and length of storage. This was primarily because of the decrease, volatilization, oxidation/reduction in the contents, or generation and decomposition by residual microbes. Therefore, quality control should be paid attention to during storage.

All quality indicators listed in Table 2 need to be checked for the safe use of digestate from biogas plants. Microbiological factors (a) affect the hygiene status and disease incidence of livestock manure and (b) affect crop yields if returned to the field. Although the causes of disease incidence are mainly viruses and pathogens, the presence of antimicrobial-resistant bacteria has been an important issue recently. Owing to the fact that livestock use more antimicrobial agents than humans, their manure is a reservoir for antimicrobial-resistant bacteria and resistant genes. This may cause uncontrolled clustering of diseases if antimicrobial-resistant pathogens emerge spontaneously or through the transmission of resistance genes.

Anaerobic digestion of organic waste derived mainly from livestock manure is also expected to increase more and more in the future from the perspective of resource recycling and energy self-sufficiency. Therefore, multidimensional analysis, including fertilizer components, must be conducted as it is important in the quality control of digestate. To utilize digestate, risk factors must be evaluated, and steps should be taken to identify a safety risk.

In this study, the experiments to analyze the effects of digestion temperature and HRT in the biogas plant on the number of survived weed seeds, bacteria, and antimicrobial-resistant bacteria in digestate were conducted by focusing on risk factors regarding digestate derived from dairy cow manure. In Chapter 1, I examined the effects of anaerobic digestion treatment on the viability of weed seeds released in dairy cow manure. In Chapter 2, the correlation between the digestion temperature and the survival rate of pathogenic bacteria, mainly hygienic indicator bacteria, was also examined. Furthermore, in Chapter 3, because there is often an issue of antimicrobial-resistant bacteria emerging in livestock manure, this study examined their survival rate of antimicrobial-resistant bacteria against cefazolin, which is often used in dairy farming as a treatment for dairy cow mastitis, in dairy cow manure with anaerobic digestion treatment. In Chapter 4, *Pseudomonas* spp. that have little reduction by anaerobic digestion treatment among CEZ-resistant bacteria were thoroughly analyzed.

Chapter 1

Effects of Anaerobic Digestion on Seed Survival of Broad-leaved Dock (*Rumex obtusifolius* L.)

ABSTRACT

To clarify the effect of exposure to anaerobic digestion on the survival of seeds of *Rumex obtusifolius* L. contained in dairy manure, the percentage of seed germination was observed after the mesophilic (35 °C) and thermophilic (55 °C) anaerobic digestion. The number of survival seed was 0% at 55 °C, 81.6% at 35 °C from anaerobic digestion, and 0% at 55 °C, 75.5% at 35 °C after heat treatment. The survival rate of the seeds in anaerobic digestion was similar to heat treatment at 35°C. However, in the investigation of seed status, the number of primary and secondary dormant seeds was higher than after heat treatment. This result suggests that since *Rumex obtusifolius* L. seeds survive in dormant state in mesophilic anaerobic digestion, the usage of manure as fertilizer need to be considered.

The research in this chapter was reported in Journal of Agricultural Science and Technology A in 2013 by Iwasaki et al. (Iwasaki, et al., 2013).

1.1 INTRODUCTION

Dairy manure is an important organic fertilizer providing valuable nutrients for crops. However, they have some issues to be considered for suitable management; qualities, characters, malodor, produced gas like hydrogen sulfide or existence of pathogenic bacteria (Kearney et al.,1993) and the presence of survival weed seeds. Survival of weed seeds in manure is a serious problem in using dairy manure for crop land. It would cause dispersing of weed seeds on the field. Some weeds have seeds that are hard to digest because of the presence of hard testa which keeps them uninjured during the passage in the digestive tracts of dairy cattle, therefore, they returned to field with manure. Generally, cow does not eat weeds as they are not palatable. But the amount of weed seeds which turns to the land with manure is very high because the feed was contaminated depending on the situation. Usually, it is essential work for farmers to control weeds by using herbicides, and remove grown weeds by tilling after fertilization of dairy manure.

The most common treatment of dairy manure to be used as fertilizer is composting. There are several reports related to the survival of weed seeds in compost (Larney and Blacks, 2003). Before using dairy manure as fertilizer, it usually needs to be composted for a period of time. High temperature composting (or aerobic fermentation) of dairy manure can damage some weed seeds. However, some are still survived and have high germination rate even after composting that often leads to the dispersal

of seeds on crop lands (Barberi, 2002).

In addition to composting, digestate from biogas plants is also an important fertilizer source. Biogas plant is anaerobic digestion (methane fermentation) system which leads degradation of complex organics and wastes including dairy manure by anaerobic microorganisms in the absence of oxygen (Angelidaki and Ellegaard, 2003). Anaerobic digestion is also an energy producing method from some organic materials. The produced methane from organics is used for electric energy production by using rotary electric generator. After digestion, the digestate can be used as fertilizer because it is rich in nutrients. Therefore, biogas plants became popular and used widely in recent times. A lot of biogas plants have been constructed in Japan. Hokkaido is the northern land and major region of dairy farming in Japan where many biogas plants have been constructed. It is presumed that about 218 thousand tons per year of cow manures are treated in biogas plants in Hokkaido. Generally, HRT for anaerobic digestion operated in mesophilic condition (37 °C) is longer than that of thermophilic condition (55 °C) due to the faster digestion of organics.

Anaerobic digestion also eliminates weed seeds from dairy manure which is thought to be able to solve this dispersal problem of weed seeds. Several researchers have contributed to examine the relationship between anaerobic digestion and deletion of weed seeds (Kimura, et al., 1994). Jeyanayagam and Collins (1984) reported the deletion rate of Johnson grass (*Sorghum halepense*) and Fall Panicum (*Panicum dichotomoflora*) seeds in anaerobic digestion, but there are limited numbers of information about other weeds.

Among many weeds, *Rumex obtusifolius* L. is the most popular weed and sometime annoying for farming in Hokkaido region. *Rumex obtusifolius* L. is a perennial plant and native to Europe (Martinkova, et al., 2009). They are gregarious plants and have the position fortify tactics; exclude other plants and substantially expand its territory (Gilgen, et al., 2010). Today, they are found everywhere in Japan. One of the possible reasons for expansion of this weed is through the contamination of its seeds in dairy manure. In this study, the effects of temperature on seed of *Rumex obtusifolius* L. in anaerobic digestion with mesophilic system (35 °C) and thermophilic system (55 °C) were investigated.

1.2 MATERIALS AND METHODS

Materials collection and preparation

Broad-leaved Dock (*Rumex obtusifolius* L.) seeds were obtained from the university farm of Obihiro University of Agricultural and Veterinary Medicine, Obihiro, Hokkaido, Japan. Prior to use, the seeds were separated from perianth manually and sorted by size, shape and color. The initial weight was 2.88 ± 0.20 g per 1,000 seeds. Cow manure was obtained from concrete floor of an enclosed dairy barn at Obihiro University. The collection was carefully done to prevent inclusion of sand and other

inert materials. Five samples were randomly taken for analysis of total solids (TS) and volatile solids (VS). The manure was later stored at -18 °C until further use.

Description of digester

Schematic diagram of the digester used in the experiments is presented in Fig. 1. The digester was made from 2 L glass jar (working volume of 1.5 L) with three outlets. Biogas produced during digestion was collected in gas storage bag. The produced gas was passed through a column that contained $Fe(OH)_3$ (to remove H_2S) before being collected in a gas storage bag. Manure was introduced and effluent was collected from the digester through an outlet with the aid of a syringe. The three outlets were tightly fitted with rubber stoppers.

Anaerobic digestion experiments were conducted at both mesophilic (35 °C) and thermophilic temperature (55 °C). The digesters were kept in water baths maintained at 35 and 55°C. They were operated in continuous mode with HRT of 25 and 15 days for mesophilic and thermophilic temperatures, respectively. Prior to use for digestion, manure was slowly thawed at room temperature and the TS content was adjusted to 9% with distilled water. This TS concentration was maintained for all the influent slurries. Manure was fed into the digesters once every day at the loading rates of 3.6 g/L/day at 35 °C and 6.0 g/L/day at 55 °C. Biogas production was monitored daily and effluent samples were analyzed for TS, VS and Volatile fatty acids (VFAs) concentration.

For anaerobic digestion experiment, seeds were packed in 5 bags (100 pieces of seed per bag) made from 18 mesh nylon bags. The mesh bags were placed into a larger mesh bag and the bag was submerged in manure for period similar to the HRT applied in each digester. Subsequently, the viabilities of the seeds were evaluated.

Heat Treatment of weed seeds

To determine the effect of temperature on seed viability, the seeds were incubated before germination tests, under anaerobic condition. One hundred seeds were put into a 50 mL polypropylene tube filled with distilled water. Ten tubes were prepared in this manner and placed in water bath maintained at 35 °C for 25 days and 55 °C for 15 days.

Seed germination test

The standard germination test was carried out to measure the number of viable seeds without dormancy. Petri dishes (diameter 7cm x height 2.5 cm) were lined with layers of filter paper and moistened with distilled water. The seeds were added to petri dishes after sterilized with 70% v/v ethanol solution and subsequently rinsed with distilled water, then incubated at 25 °C under light intensity of 2,000 lux for seven days to separate dormant seeds from non-dormant seeds (Noronha, et al., 1997). After that, the petri dishes were transferred to a dark room at 4 °C for another 30 days and

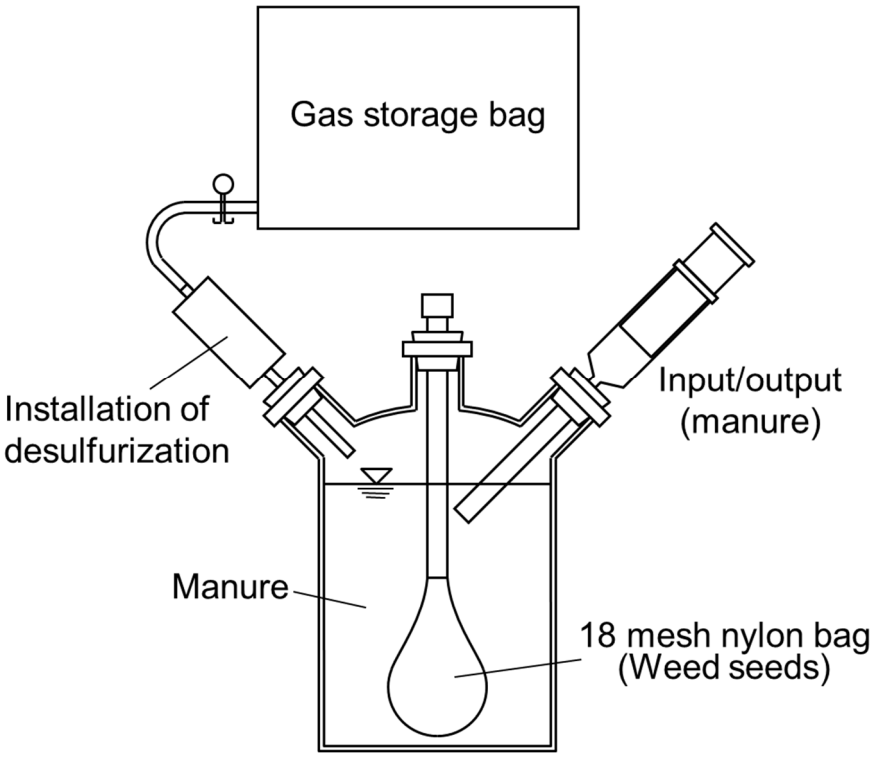

Fig. 1 Installation for anaerobic digestion

carried out the standard germination test to separate primary dormant seeds from others. Finally, Tetrazolium test was performed to identify secondary dormant seeds and non-viable seeds (dead seeds) (Moore, 1985). This germination test was applied to non-treated (initial) seeds, heat-treated seeds and digested seeds.

Analyses of components in biogas and digestate

Methane concentration in the produced gas was measured by gas chromatography (Shimadzu Co., GC-4C, Japan) with 0.3 cm x 200 cm silica gel column (80/100 mesh). Helium gas was used as carrier gas with a flow rate of 28 mL/min at 80 and 120 °C. The pH of raw and digestate was measured every day by a pH meter (HORIBA Ltd., D-52, Japan). Concentrations of TS, VS and VFAs (formic, acetic, propionic and butyric acids) in influents and effluents were measured every time the seeds were applied and removed from the digesters. Total solid and VS were measured by standard methods (American Public Health Association, 1985). Volatile fatty acids were measured using high performance liquid chromatography (HPLC, Shimadzu Co., LC-10AD, Japan) with Shim-pack SCR-102H column (Shimadzu Co., Japan). Five 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. For sample preparation, 6 mL of 10% tungsten acid and 6 mL of 7% sulfuric acid were added to 3 g sample. The mixture was homogenized for 5 min, and centrifuged at 10,000 x g for 20 min at room temperature. The supernatant was collected and analyzed using HPLC.

1.3 RESULTS AND DISCUSSION

Seed viability potential

The results of potential viability of *Rumex obtusifolius* L. seeds before experimental treatments are shown in Table 1. The results show that 98.2% of the total seeds examined were viable before digestion or heat treatment, 0.5% were primary dormant, and no secondary dormancy seeds were found in these samples.

Shimizu and Tajima (1974a, b) had reported that the growth of *Rumex obtusifolius* L. in nature was hastened by mild temperature for 30-40 days, and 2 weeks after blooming, the seeds obtained the abilities to germinate. Another 2 weeks later, the seeds establish dormancy which correlated with a decrease of seed moisture content during air drying period. Indeed, the rate of dormant seeds used in this experiment was far lower than the results reported by Hongo (1986). The probable reasons for failure in establishment of dormancy were the change of harvest time coincided with grass harvesting or the period of carrying out the experiment which was during in winter, because cold stratification is effective in breaking dormancy (Hidayati, et al., 2001). The results of initial germination test were very high, proving the seeds had high germination potential.

Table 1 Results of initial germination tests

Seed Status		
Not dormancy	(%)	98.2 ± 1.4
Primary dormancy	(%)	0.5 ± 0.7
Secondary dormancy	(%)	0.0 ± 0.0
Dead seed	(%)	1.3 ± 1.1
Total	(%)	100

Values are expressed as mean ± SD ($p < 0.05$)

Table 2 Results of germination tests after heat treatment

Digester temperature		(ºC)	35	55
Retention time		(day)	25	15
Seed status	Not dormancy	(%)	55.3 ± 7.1	0.0 ± 0.0
	Primary dormancy	(%)	11.2 ± 3.1	0.0 ± 0.0
	Secondary dormancy	(%)	9.0 ± 2.5	0.0 ± 0.0
	Dead seed	(%)	24.5 ± 5.3	100.0 ± 0.0

Values are expressed as mean ± SD ($p < 0.05$)

The result of investigation of seeds viability after heat treatment is shown in Table 2. At 35 °C, the rate of non-dormancy and dead seeds was 55.3 ± 7.1% and 24.5 ± 5.3%, respectively. In contrast, the rate of the dead seeds at 55 °C reached 100% on day 15. These results showed that the death of seeds was caused, especially by anaerobic circumstance at 35 °C and effect of heat stress at 55 °C. Thompson et al. (1997) had reported about the effect of heat treatment on germination rate of weed seeds. They demonstrated that *Rumex obtusifolius* seeds treated at 56 °C for 8-16 days kept high germination rate (> 80%). The differences between the results of this experiment and the latter may be due to differences in anaerobic conditions or imbibition of seeds (Bloemhard, et al., 1992; van Assche and Vanlerberghe, 1989). They determined germination rate using the soil which was kept moist in aerobic conditions. There were no significant differences in seed viability in both studies investigated at 35 °C (about 80%).

Some regulatory factors related to dormancy of weed seeds were reported. The seeds with broken primary dormancy will moderately lead to secondary dormancy. This process was developed more hot circumstances and dormancy was stopped by high temperature (Brandel, 2004). Secondary dormancy is developed by hypoxia (Benech-Arnold, et al., 2000). Taylorson (1979) also reported that the weed seeds which were treated at 50 °C for 7 days showed remarkable increase in absorption of oxygen and release of carbon dioxide, which correlate with breaking of dormancy. The results in this study suggested that the circumstances in anaerobic conditions in water and high temperatures at 35 °C led the seeds to dormancy; furthermore, prolonged period of these conditions led the seeds to secondary dormancy or death from primary dormancy. Thus, it is clear that temperature is also an important factor for viability of *Rumex obtusifolius* L.

Digester Performance

The parameters of the digester performance are shown in Table 3. After stable working conditions, digester produced biogas at a fairly constant amount (0.89-1.51 L/L/d). Methane concentration was similar or slightly lower than the reported values by Amon et al. (2007) and Gallert and Winter (1997), while pH was slightly higher than these reports. Probably, the lower amounts of biogas production and methane concentration per effective volume of digestion jars depends on the lower concentration of organic matters in substrates and the lower amount of acetic acid, butyric acid and total volatile organic acid result from digestion of organic matters.

Table 4 shows the compositions of influents and effluents. The VS/TS ratio of influents was 84.7%. In influent, the concentrations of acetic acid and butyric acid were little higher, while propionic acid was little lower than ordinary reports (1,000 – 3,500 mg/L, 100 – 1,000 mg/L and 1500 – 2,000 mg/L, respectively) (Paul and Beauchamp, 1989; Illmer and Gstraunthaler, 2009). The amount of propionic acid, one of the important indicators of digestion condition, was detected in low level (216 mg/L) on day 25, which indicates successful digestion without major problems (Bjornsson, et al.,

Table 3 Dairy-fed anaerobic digester performance

Digester temperature	(ºC)	35	55
Retention time	(day)	25	15
VS† loading rate	(g/L/day)	3.6	6.0
Biogas production per digester working volume	(L/L/day)	0.89 ± 0.10	1.51 ± 0.21
Methane concentration	(%)	56.35 ± 3.98	55.20 ± 3.44
pH		7.63 ± 0.08	7.80 ± 0.07

† VS = volatile solids; Values are expressed as mean ± SD ($p < 0.05$)

Table 4 Composition of influent and effluent (mean value)

			Effluent	
Digester temperature	(ºC)	Influent	35	55
Retention time	(day)		25	15
Total solids	(%)	9.00	6.50	6.49
Volatile solids	(%)	7.62	5.10	5.08
Formic acid	(mg/L)	14	2	0
Acetic acid	(mg/L)	6,983	1,063	1,002
Propionic acid	(mg/L)	1,610	216	209
Butyric acid	(mg/L)	1,030	9	3
Total volatile acids	(mg/L)	9,637	1,290	1,214

1997).

Viability of seeds in anaerobic digestion process

The results of germination test of the seeds after exposition of anaerobic digestion are shown in Table 5. At 35 °C digestion, it was found that the rate of seeds in not dormancy was decreased, while the rate of primary and secondary dormancy seeds was increased dramatically (31.0% and 22.0%, respectively). Jeyanayagam and Collins (1984) had reported the effect of anaerobic digestion on seed viability of Sorgham halepense (L.) pers. and Panicum dichotomiflorum michx. They found that the seed viability decreased faster for 20-days than 15-days of HRT at 35 °C. In this study, the rate of dead seeds in anaerobic digestion (18.4%) did not so differ from heat treatment (24.5%) at 35°C.

Comparing seed viability at 35 °C and 55 °C in exposure to anaerobic digestion, the viabilities were low at both temperatures. Especially at 55 °C, seeds in the state of not dormancy and dormancy were not recognized on day 15; they were all dead seeds. Similar to the result of heat treatment, the decreased number of viable seeds at 55 °C in anaerobic digestion was thought to be read by heat stress. Considering the results of heat treatment and anaerobic digestion together, the main factor effect on seed viability is thought high temperature. However, the viable seeds less decreased than heat treatment at 35 °C and increase primary and secondary dormant seeds than heat treatment only, means that anaerobic digestion help for dormancy rather than add damage to death on *Rumex obtusifolius* seeds. The seed was thought to be affected to digest by methanogens in anaerobic digestion process because seed is an organic matter. However, it was thought that the nutrients (nitrogen, phosphoric acid and potassium) and other components like fatty acids, amino acids and saccharides which are made from fats, carbohydrates and proteins in digestate reduced the damage of the seeds. Engeli et al. (1993) examined the survival of *Rumex obtusifolius* seeds in two stage pilot plant which consisted of hydrolysis step and methanogenesis step at 35 °C. They found that hydrolytic conditions and a low pH of 5.6 to 7.6 were mainly responsible for the killing of weed seeds. In addition, the survival of pathogenic bacteria in organic waste used in biogas plants is also affected by high concentration of VFAs (formic, acetic, propionic and butyric acids) and lower pH (Sahlstrom, 2003). As shown in Table 3, pH data in this experiment were not low as to 7.6 (35 °C) and 7.8 (55 °C) which is generally in anaerobic digestion. Therefore, incomplete hydrolysis, low concentration of VFAs and moderate pH may be the reasons for the small number of dead seeds.

These results suggest that the condition of anaerobic digestion might effective on viability of weed seeds through the temperature stress and components in digestate. Therefore, to reduce the seeds of *Rumex obtusifolius* L. in digestate, it will be better to work the biogas plant at thermophilic temperature. This approach will be applicable to other weed seeds.

Table 5 Results of germination tests after treatment of anaerobic digestion

Digester temperature		(ºC)	35	55
Retention time		(day)	25	15
Seed status	Not dormancy	(%)	28.6 ± 4.7	0.0 ± 0.0
	Primary dormancy	(%)	31.0 ± 0.7	0.0 ± 0.0
	Secondary dormancy	(%)	22.0 ± 5.6	0.0 ± 0.0
	Dead seed	(%)	18.4 ± 8.4	100.0 ± 0.0

Values are expressed as mean ± SD ($p < 0.05$)

1.4 CONCLUSIONS

Digestate from anaerobic digestion can be used as organic source to crop land; however, weed seeds in manure make issues for the land. The survival rate of weed seeds from anaerobic digestion depends on the digestion temperature. Under mesophilic condition, the survival rate was higher than that in thermophilic condition, which was 0%. Thus, for complete reduction of weed seeds, anaerobic digestion should be carried out in thermophilic condition.

Chapter 2

The Effect of Temperature on Survival of Pathogenic Bacteria in Biogas Plants

ABSTRACT

The paper deals with the hygienic advantages of sanitation to treat dairy manure in full-scale biogas plants. The slurry samples were collected from two thermophilic biogas plants (55 °C) and two mesophilic biogas plants (38 °C) in Hokkaido Japan. A detectable number of *Coli-aerogenes* group and *Enterococcus* in the digestate could not be found in either thermophilic biogas plants. However, in both mesophilic biogas plants the viable numbers of *Coli-aerogenes* group and *Enterococcus* were detected in the slurries even after anaerobic digestion. The mean decimation reduction time (T_{90}) values of the *Coli-aerogenes* group and *Enterococcus* in the slurries during mesophilic digestion were 13.3 days and 16.7 days, respectively.

The research in this chapter was reported in Animal Science Journal in 2011 by Iwasaki et al. (Iwasaki, et al., 2011).

2.1 INTRODUCTION

Anaerobic digestion in biogas plants is an alternative way to handle animal manure and reduce greenhouse gas emission. The digestate may be used as a fertilizer on agricultural land. Animal manure is known to contain pathogenic bacteria that may be a health risk for both human and animals. The digestate must be proven hygienically safe in order to be recycled (Sahlstrom, 2003). However, there is no regulation concerning the hygienic standard of the biogas plants residue in Japan. The growing interest in biogas plants in Japan makes it important to consider biosecurity aspects of recycling digestate. Umetsu et al. (2002) studied the survival of coliform bacteria during mesophilic anaerobic digestion of dairy manure. Minato et al. (2003) also reported the monitoring of *Coli-aerogenes* group and *Enterococcus* in practical mesophilic biogas plants. But there is no data on hygienic requirements in full-scale thermophilic biogas plants in Hokkaido Japan. In thermophilic process, the temperature was known to be beneficial for the conversion rate, but was originally feared to be more difficult to be controlled. Umetsu et al. (2005) and Aoki et al. (2005) demonstrated the practicality of thermophilic temperature of biogas plants in the cold regions. The objective of the present paper is to clarify the survival of *Coli-aerogenes* group and *Enterococcus* in full-scall mesophilic and thermophilic biogas plants in Hokkaido Japan.

2.2 MATERIALS AND METHODS

Sampling

The material organisms were added into all investigated plants mainly consisted of manure and waste water mixed with waste milk. The slurry samples were collected from reception pit, digester and storage tank in two thermophilic (55 ˚C) and two mesophilic biogas plants (38 ˚C) on August 5, 2005. Specification of these biogas plants and operating conditions of this study were presented in Table 1. Ambient temperature of the day was around 30 ˚C.

Analytical method

Total solids (TS) were determined by drying in a fan-assisted oven at 105 ˚C for 24 hours. Volatile solids (VS) were determined by combusting the oven dried material at 550 ˚C for 4 hours. The pH values of the slurry samples were measured with pH meter (TOA Inc, Tokyo, Japan). Total volatile acids (TVA) were determined with a Shimadzu HPLC (LC-10A), using a Shim-pack SGR-102H. The concentration of lactic, formamide, acetic, propionic and butyric acids was measured. The detail of the analytical procedure for TVA was described in a previous paper (Kimura, et al., 1994).

Indicator organisms

Bacteria used as the most common indicator for public health monitoring is faecal *E. coli*. Enterococci can validate the hygienic treatment of biowaste in biogas plants (Larsen, et al., 1994). In this study, indicator organisms, *Coli-aerogenes* group and *Enterococcus* were detected. *Coli-aerogenes* group and *Enterococcus* from the slurry samples were isolated on Desoxycholate *E. coli* selective agar plates incubated at 35 ˚C for 20h, and M-*Enterococcus* selective agar plates incubated at 35 ˚C for 48h, respectivery. Desoxycholate *E. coli* selective agar plates contain 10 g/L peptone, 5 g/L sodium chloride, 2 g/L dipotassium hydrogenphosphate, 10 g/L lactose, 2 g/L ferric ammonium citrate and 15 g/L agar. M-*Enterococcus* selective agar plates contain 20 g/L tryptose, 5 g/L yeast extract, 2 g/L glucose, 4 g/L dipotassium hydrogenphosphate, 0.4 g/L sodium azide and 15 g/L agar. All tests were carried out in quintuplicate or sextuplicate.

Statistical analysis of bacterial reduction

The time required to achieve 90% reduction of bacteria (T_{90}) is given by $-1/\alpha$ values when deletion curve of elapsed time versus logarithmic bacterial cell numbers has approximate slope α (Gibbs, et al., 1995). Therefore, T_{90} can be calculated by the following equation.

$$T_{90} = (t2 - t1) / (\log N1 - \log N2)$$

where,

Table 1 Specification and operating conditions of the biogas plants of this study

Parameter		Plant A	Plant B	Plant C	Plant D
Digester temperature	˚C	55	55	38	38
Digester volume	m^3	60	540	424	671
Amount of feeding per day	m^3/day	4	45	16	18
Hydraulic retention time (HRT)					
Reception pit	day	7.5	3	3.1	4.4
Digester	day	15	12	26.5	37.3

N1 = the number of bacterial cells at time t1

N2 = the number of bacterial cells at time t2

In this study, T_{90} values of digester can be calculated by knowing

t2 – t1 = HRT of each digester

logN1 – logN2 = (bacterial cell concentration in reception pit)
– (bacterial cell concentration in digester)

and applying these to above equation.

2.3 RESULTS AND DISCUSSION

Anaerobic digestion can be performed either mesophilic at 30-38 ˚C or thermophilic at 50-55 ˚C. The advantages of thermophilic temperatures include increased loading rates which lead to less capital costs as a result of smaller digester size; increased efficiency of organic matter decomposition. Disadvantages of thermophilic system include higher energy requirements to heat influent substrate and maintain digester temperature.

Figure 1 shows the changes in TS and VS in the slurries of the thermophilic (A, B) and mesophilic (C, D) biogas plants. The TS contents of the slurries taken from the reception pits ranged from 3.97 to 11.95% and the VS contents remained relatively constant averaging 83.0% of the TS. The slurries taken from A plant had higher water content because of the dilution by milking parlor wastewater. On the contrary, the TS content of the slurries taken from the C plant, was over 12% higher because of no dilution. Figure 2 shows the changes in TVA in the slurries of the thermophilic (A, B) and mesophilic (C, D) biogas plants. The main volatile acids present in the slurries in the reception pits were acetic acid, propionic acid and butyric acid. The TVA content of the slurries taken from the reception pits was similar to those of TS or VS content as shown in Fig. 1. During anaerobic digestion, the concentration of TVA decreased sharply with the decomposition performed in each biogas plant. Figure 3 shows the changes in pH value of the slurries for each biogas plant. The pH value is one of the important clues for understanding methane bacteria activity. In general animal manure has sufficient alkalinity to maintain the pH in an anaerobic digester for a relatively stable process of methane production. However, as a result of overloading, excessive concentration of volatile acids and ammonia nitrogen is considered to hinder methane production because of the drastic changes in pH values. But in this observation, no special differences in pH were found in each of the biogas plants. From the data of composition of the digestate, the authors have concluded that each biogas plant was

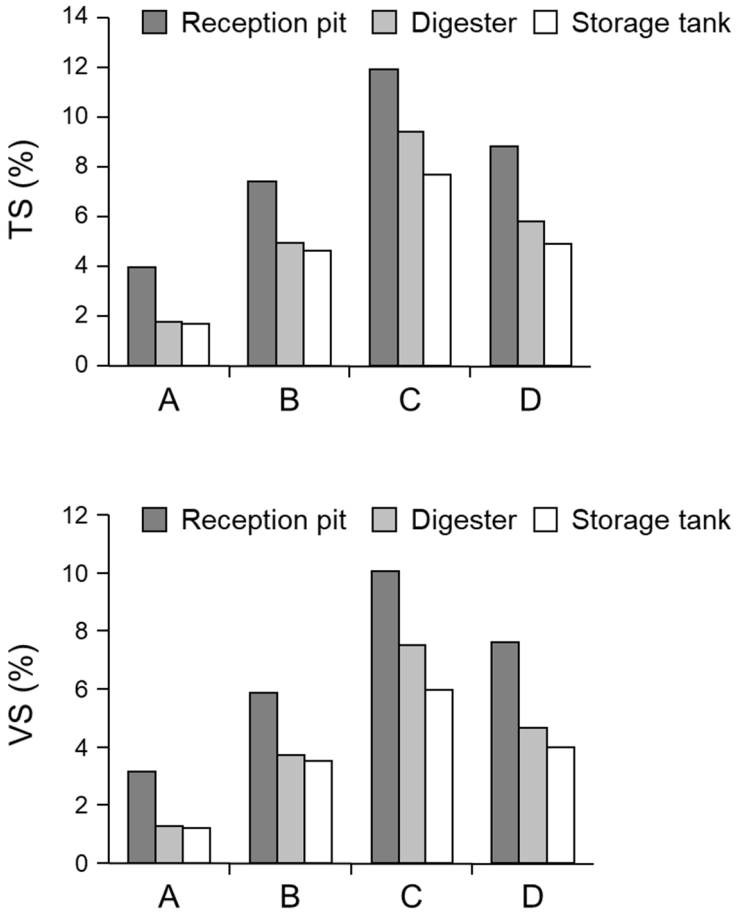

Fig. 1 Changes in total solids (TS) and volatile solids (VS) in the slurries of the thermophilic (A, B) and mesophilic (C, D) biogas plants

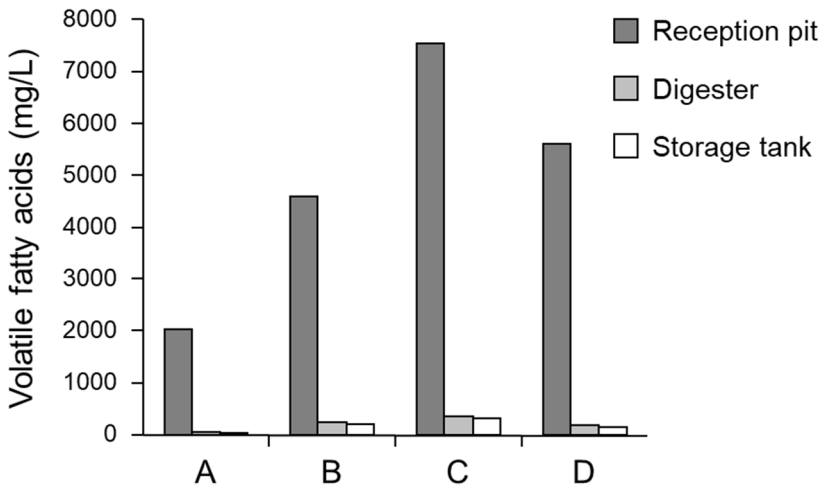

Fig. 2 Changes in total volatile acids (TVA) in the slurries of the thermophilic (A, B) and mesophilic (C, D) biogas plants

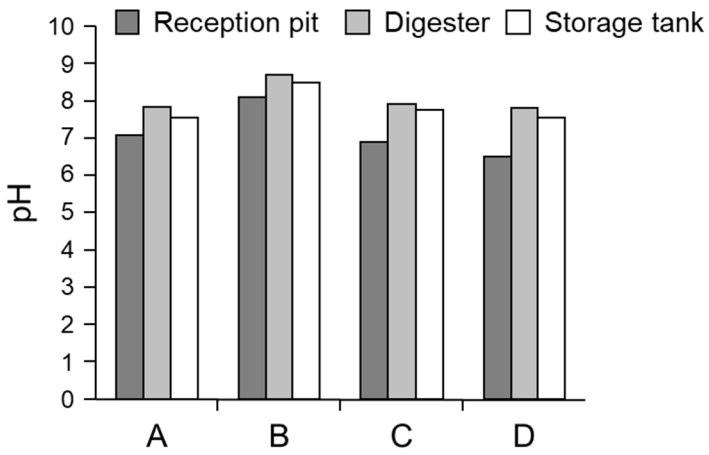

Fig. 3 Changes in pH value of the slurries in the thermophilic (A, B) and mesophilic (C, D) biogas plants

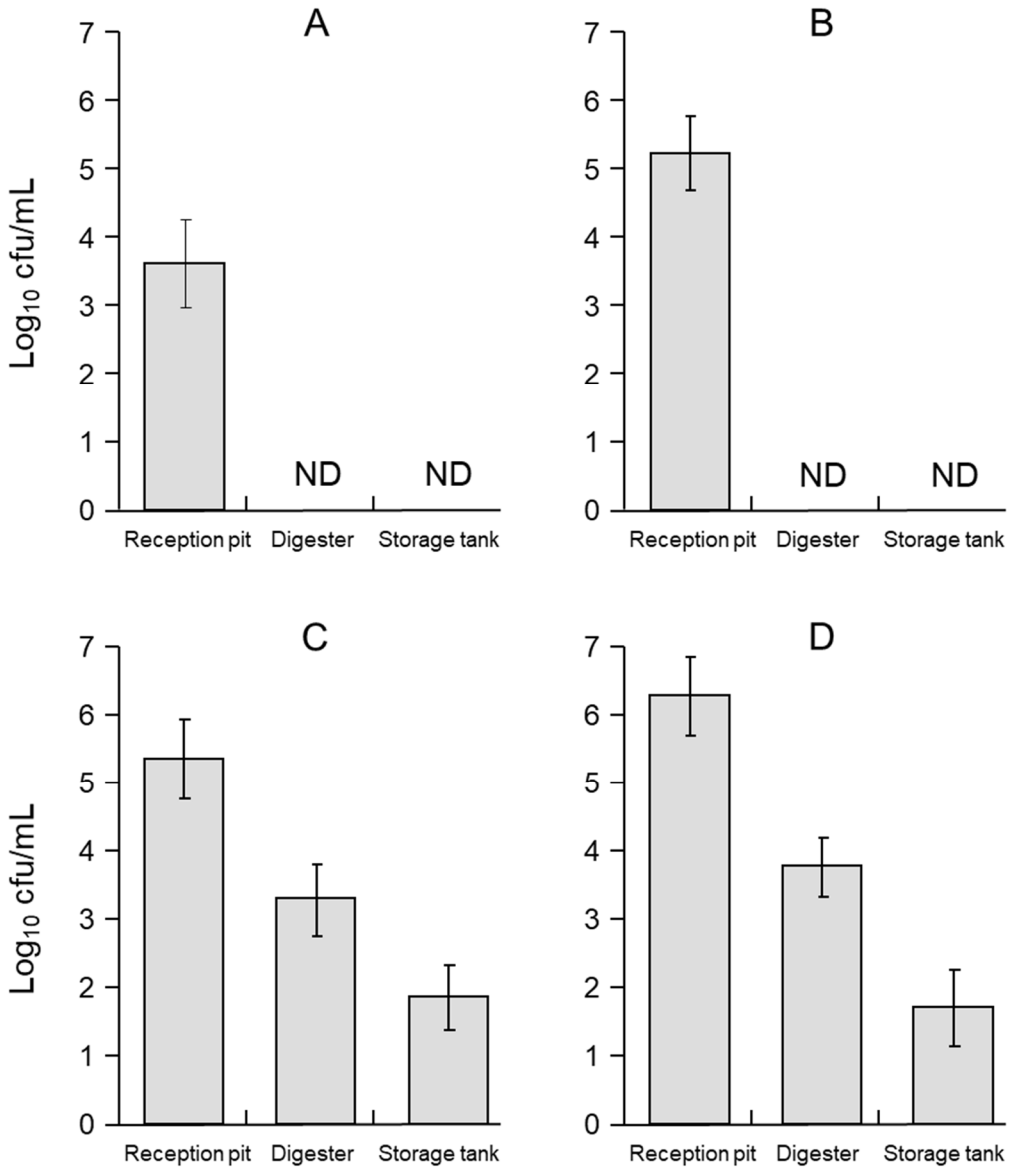

Fig. 4 Arithmetic mean viable counts of *Coli-aerogenes* group in the thermophilic (A, B) and mesophilic (C, D) biogas plants

ND, not detected. Data are mean ± SD.

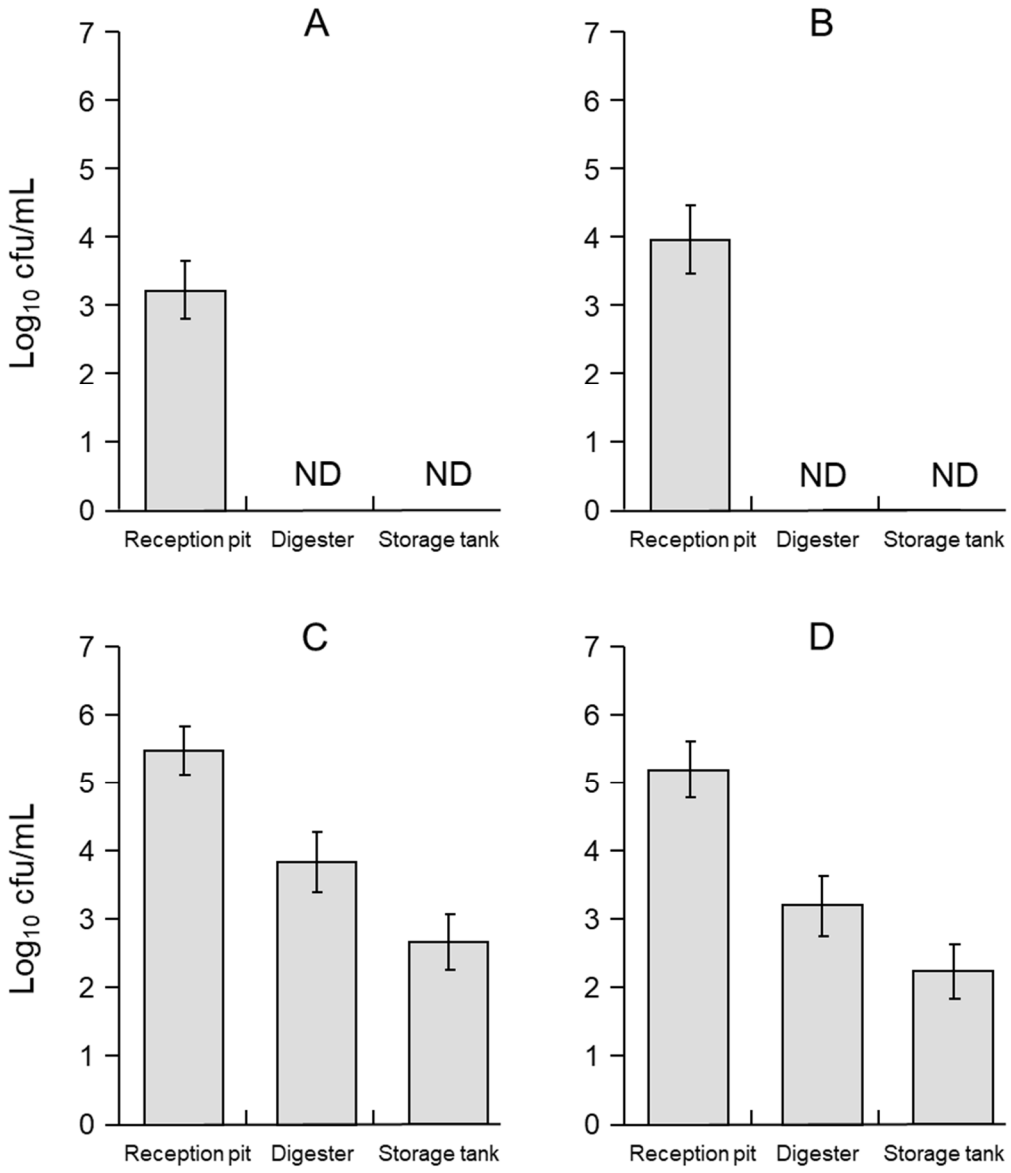

Fig. 5 Arithmetic mean viable counts of *Enterococcus* in the thermophilic (A, B) and mesophilic (C, D) biogas plants

ND, not detected. Data are mean ± SD.

running favorably.

The survival patterns of *Coli-aerogenes* group and *Enterococcus* in the thermophilic (A, B) and mesophilic biogas plants (C, D) were shown in Figs. 4 and 5, respectivery. The viable number of *Coli-aerogenes* and *Enterococcus* couldn't be found in the slurries after anaerobic digestion of both thermophilic biogas plants. In both mesophilic biogas plants, the viable numbers of *Coli-aerogenes* and *Enterococcus* were detected in the slurries, though the number of viable bacteria declined to low level after anaerobic digestion. The decay rate of viable bacteria has been reported to depend on temperature, retention time, pH, volatile acids, bacterial species and available nutrients (Farrah and Bitton, 1983; Kearney, et al., 1993). Among these factors, the temperature is the most important factor concerning survival of pathogenic bacteria during anaerobic digestion (Kearney, et al., 1993; Dumontet, et al., 1999). Bacterial inactivation due to temperature is related to time (Olsen and Larsen, 1987).

Each plant HRT of reception pit was several days. Therefore, it may be considered that the number of bacterial cells occurred within reception pit was low. On the other hand, the number of bacterial cells in storage tank in thermophilic plants was less than that of in digester suggesting reduced bacterial growth in storage tank. The time required for a 90% reduction of viable counts of a population of microorganisms or a decrease by one logarithmic unit (log 10) is called the decimation reduction time (T_{90}) (Schlundt, 1984). The T_{90} value indicates the differences of inactivation of bacterial pathogens in anaerobic digestion. T_{90} for many bacteria can be counted in hours in thermophilic digestion and in days in mesophilic digestion (Gibbs, et al., 1995). The mean T_{90} values of *Coli-aerogenes* and *Enterococcus* for each biogas plants are shown in Table 2. In the present investigation, *Coli-aerogenes* and *Enterococcus* in the digestate of thermophilic biogas plants (A, B) were not detected. The mean T_{90} values of both *Coli-aerogenes* and *Enterococcus* in mesophilic biogas plants (C, D) were 13.3 d and 16.7 d, respectively. These findings agreed with the experimental results obtained by Olsen and Larsen (1987). *Salmonella* and *M. paratuberculosis* are inactivated within 24 h in thermophilic anaerobic digestion compared to weeks and even months in mesophilic anaerobic digestion (Plym-Forshell, 1995; Olsen, et al., 1985).

2.4 CONCLUSIONS

In Japan, many farmers spread raw or untreated manure straight to land. However, it is important that appropriate management practices are implemented to minimize the risks of pathogen transfer to the food chain from the management of animal manures. The present investigation was undertaken to study the hygiene and sanitation in full scale biogas plants treating dairy manure. This paper reported the survival of *Coli-aerogenes* and *Enterococcus* in full scalle mesophilic and thermophilic biogas plants in Hokkaido Japan. *Coli-aerogenes* and *Enterococcus* in thermophilic

biogas plants were not detected. In both mesophilic biogas plants, the viable numbers of *Coli-aerogenes* and *Enterococcus* in the slurries declined to low level after anaerobic digestion. In this study, digester high temperature would have a suppressing effect on the population of *Coli-aerogenes* and *Enterococcus* in the slurries during anaerobic digestion. Finding of this study suggest that biogas plants offer benefit towards net reduction of pathogenic bacterial numbers in dairy manure.

Table 2 The mean T_{90} values of *Coli-aerogenes* and *Enterococcus* for each biogas plant

Plant		*n*	T_{90} for *Coli-aerogenes*	T_{90} for *Enterococcus*
A	Reception pit	6		
	Digester	5	–	–
	Storage tank	5		
B	Reception pit	6		
	Digester	6	–	–
	Storage tank	6		
C	Reception pit	5		
	Digester	6	14.5	18.3
	Storage tank	5		
D	Reception pit	6		
	Digester	6	12.1	15.0
	Storage tank	6		

n, number of samples

Chapter 3

Thermophilic Anaerobic Digestion Is an Effective Treatment for Reducing Cefazolin-resistant Bacteria and ESBL-producers in Dairy Manure

ABSTRACT

The application of thermophilic anaerobic digestion treatments to dairy manure in a biogas plant was evaluated to investigate whether the prominent countermeasure was sufficient for the dissemination of antimicrobial-resistant bacteria in the dairy industry. To determine the changes in the number of antimicrobial-resistant bacteria in dairy manure after thermophilic anaerobic digestion, cefazolin-resistant (CEZ-R) and ampicillin-resistant (AMP-R) bacteria in dairy manure and digestate were quantified by plate spread methods performed with combinations of antimicrobials added to agar plates and selective and differential agar plates. In addition, extended-spectrum β-lactamase (ESBL)-producing bacteria were also quantified to evaluate the effect of thermophilic anaerobic digestion on plasmid-borne resistant bacteria. CEZ-R and AMP-R bacteria were widely reduced after thermophilic anaerobic digestion compared with the susceptible bacteria against these antimicrobials. The classification into *E. coli*, other coliforms, and non-coliform bacteria revealed that CEZ-R and AMP-R coliform bacteria were more significantly reduced than non-coliform bacteria by thermophilic anaerobic digestion. Moreover, ESBL-producing bacteria were reduced significantly among CEZ-R bacteria. From these results, thermophilic anaerobic digestion appears to be a useful treatment to counter the dissemination of antimicrobial-resistant bacteria in dairy manure by the preferential elimination of antimicrobial-resistant bacteria.

The research in this chapter was reported in Journal of Material Cycles and Waste Management in 2019 by Iwasaki et al. (Iwasaki, et al., 2019a).

3.1 INTRODUCTION

The appearance of antimicrobial-resistant bacteria is a growing global issue. Today, the amount of antimicrobial-resistant bacteria is increasing, and the social and economic impacts of antimicrobial-resistant bacteria are becoming critical (Roca, et al., 2015). Most of the discussions and efforts to establish countermeasures against antimicrobial-resistant bacteria have been limited to human medicine for a long time. In the veterinary sector, large quantities of veterinary antimicrobial agents (antibiotics and synthetic chemotherapeutics) are used in livestock to treat infectious diseases, for prophylactic control, and for growth promotion via food additives. The number of antimicrobial agents used for livestock is much higher than that used in humans, and the excessive use of

antimicrobial agents results in promoting antimicrobial-resistance in microorganisms (Asai, et al., 2005; Furuya and Franklin, 2006). Because some antimicrobial-resistant bacterial infections can spread from animals to humans via livestock and livestock products, it is thought that countermeasures that are limited to human medicine are unable to control antimicrobial-resistant bacteria.

To address these critical situations, a new concept called “One Health” has been proposed worldwide. The One Health concept is based on three sectors, human, animal, and ecosystem, and argues for the necessity to foster a collaborative multidisciplinary approach among them for reducing the risk of zoonosis and antimicrobial-resistant bacteria. In Japan, surveillance of antimicrobial-resistance in indicator bacteria (*E. coli*, *Enterococcus*, *Salmonella*, and *Campylobacter*) in dairy products and against several frequently administering antimicrobials such as cefazolin (CEZ), fluoroquinolone, ceftiofur and cefotaxime has been conducted to investigate the progress of antimicrobial-resistant bacteria in the livestock industry (The National Veterinary Assay Laboratory, Ministry of Agriculture, Forestry, and Fisheries, 2013). However, the countermeasures to increasing antimicrobial-resistant bacteria in animal and ecosystem sectors are behind those being taken in the human sector. In 2016, The National Action Plan on Antimicrobial Resistance in Japan was formulated, and a structure based on the One Health concept was organized. Following this plan, the first Conference on Antimicrobial Resistant One-Health Surveillance was held in February of 2017 with cooperation with the Ministry of Health, Labour and Welfare (MHLW), the Ministry of Agriculture, Forestry and Fisheries (MAFF) and the Ministry of the Environment (MOE).

Livestock waste is considered a pollution source of antimicrobial-resistant bacteria in the livestock industry. Composting and anaerobic digestion are two major treatments and recycling processes for livestock wastes. Since anaerobic digestion process has an additional advantage by producing biogas which can be used for power and heat generation, 79 biogas plants have been constructed in Japan on September of 2016, and these are expected to reduce the emission of greenhouse gases. Most of these biogas plants are operated at mesophilic or thermophilic temperature. In terms of accumulated biogas yield and methane concentration, there is no difference between thermophilic and mesophilic anaerobic digestion. However, thermophilic digestion has higher stability at high organic loading rate than mesophilic digestion for the treatment of livestock wastes. Moreover, because approximately 15 days of digestion periods in usual thermophilic biogas plants, which is shorter than 30 days in mesophilic biogas plants, adequate volume of digester tank in thermophilic plants is smaller than that in mesophilic plants when treating the same amount of biomass. Therefore, the construction cost of thermophilic biogas plant is smaller than that of mesophilic plant. Many researchers also have described the benefit of these processes in reducing pathogens in livestock wastes. In Chapter 2, it was revealed that *Coli-aerogenes* and *Enterococcus* in dairy manure are more significantly reduced in thermophilic biogas plant than in mesophilic plant (Iwasaki, et al., 2011). Therefore, similar to these effects, anaerobic digestion is expected to reduce antimicrobial-resistant

bacteria. However, it is still poorly understood because of the rarity of studies.

In this study, I focused on the reduction effect of thermophilic anaerobic digestion treatment in a biogas plant on antimicrobial-resistant bacteria in livestock wastes. Dairy manure and its digestate were sampled from a full-scale biogas plant, which was fed the manure of CEZ-treated dairy cows, and the quantities of CEZ-resistant (CEZ-R) bacteria were investigated. In addition, detected bacteria were classified into several groups by culturing with selective and differential medium, and the effect of anaerobic digestion on each bacterial group was observed. Extended-spectrum β-lactamase (ESBL) is considered the main factor inducing resistance against β-lactams (Brinas, et al., 2003). Since ESBL genes, which constitute some gene families (TEM, SHV, CTX-M family, etc.) are intrinsically plasmid-borne (Jacoby, 2006), ESBL genes could further spread into other non-CEZ-R host bacteria, making them resistant to β-lactams. Since digestate is generally applied to crop fields as an organic fertilizer, there are fears that resistance will expand if the digestate contained the plasmid-borne ESBL gene. In fact, recently, several studies have reported that ESBL-producers have been increasing in *Salmonella* and *E. coli* in the dairy industry (Batchelor, et al., 2005; Carattoli, 2008). Therefore, ESBL-producing bacteria need to be promptly eliminated. In this study, the effects of thermophilic anaerobic digestion on ESBL-producers were investigated. If thermophilic anaerobic digestion treatment could eliminate ESBL-producers, thermophilic anaerobic digestion could be assessed to have crucial value because it implies the possibility to eliminate the source of antimicrobial-resistance.

3.2 MATERIALS AND METHODS

Examined Samples

Dairy manure and digestate were sampled from the biogas plant working at a thermophilic temperature (55 °C) at the farm of the Field Science Center at Obihiro University. Fifty cows were bred, and the therapeutic antimicrobial agents for cows used at the farm were CEZ, cefuroxime sodium and penicillin for mastitis, penicillin and streptomycin for pneumonia and bronchitis, and neomycin (fradiomycin trisulfate) for bacterial gastroenteritis. However, ampicillin (AMP) was not used to treat the cows. Approximately 4 t/day of manure was excreted and almost the whole manure was used as feedstock for the biogas plant and processed as follow. Collected manure is kept in a reception pit of the biogas plant for approximately 1 day and is added to diluent water to avoid digestion disturbance caused by a high organic loading rate. Diluted dairy manure is transported to a continuously fed digester tank where it is digested at 55 °C for approximately 15 days as the HRT. After digestion, the digestate is transported to a slurry storage tank. Dairy manure and digestate were sampled from a reception pit and slurry storage tank of the biogas plant, respectively.

Quantification of classified CEZ-R bacteria and ESBL-producers

CEZ-R and ESBL-producing bacteria in samples were classified and quantified by the spread plate technique using three kinds of agar plates: peptone, tryptone yeast extract and glucose (PTYG) agar plate (Asuming-Brempong and Aferi, 2014), CHROMagar *E. coli* & Coliform Chromogenic (ECC), and CHROMagar ESBL plate (CHROMagar, Paris, France) (Fig. 1). In addition, quantification of AMP-resistant (AMP-R) bacteria was also conducted to compare with CEZ-R bacteria. Dairy manure was appropriately diluted in 1X phosphate-buffered saline (1X PBS), plated on three PTYG agar plates and incubated at 30 °C for 48 h. After counting the colony numbers on each plate, 300 colonies were randomly selected and plated onto new PTYG agar plates with and without 50 μg/ml CEZ (Sigma-Aldrich Co. LLC, St Louis, MO) and numbered (Fig. 1a). After incubation at 30 °C for 24 h, the susceptibility of each colony to CEZ was determined from PTYG + CEZ plate cultures. All colonies on the PTYG agar plates without CEZ were transferred to CHROMagar ECC plates (Fig. 1b), and CEZ-R colonies were simultaneously transferred to CHROMagar ESBL plates (Fig. 1c) and incubated for 24 h at 30 °C and 37 °C, respectively. Colonies on the CHROMagar ECC plates were classified into three groups by diverse color developments: *E. coli* (blue), other (fecal) coliform bacteria (mauve), and non-coliform bacteria (colorless or growth inhibition). Colonies on CHROMagar ESBL plates were classified into six groups: ESBL-producing *E. coli* (*E. coli*-ESBL), *Klebsiella*/*Enterobacter*/*Citrobacter*-ESBL, *Proteus*-ESBL, *Pseudomonas*-ESBL, *Acinetobacter*-ESBL, and others (other genera and/or non-ESBL-producers) by diverse color developments referring to the manufacturer's instructions. Total bacteria numbers in samples were averaged across three PTYG agar plates, and the numbers of each group (*E. coli*, other coliforms, non-coliform bacteria, and ESBL-producers) were calculated by multiplication of total bacteria numbers by the percentage of each group observed on CHROMagar ECC plates and CHROMagar ESBL plates. The numbers of CEZ-R bacteria of *E. coli*, other coliforms, and non-coliform bacteria were calculated by multiplication of the numbers of each group and the percentages found by culturing with PTYG + CEZ plates. For detection of AMP-R bacteria, diluted samples were plated onto PTYG plates with 50 μg/ml AMP (Sigma-Aldrich Co. LLC, St Louis, MO), and experiments were conducted with the same method except for transferring to CHROMagar ESBL plates. The applied concentrations of antibiotics were referred to the web database of antibiotic books in Japan (http://www.antibiotic-books.jp).

Analysis of the moisture contents of samples

Diluent water was added into the dairy manure to avoid higher substrate loading and make anaerobic digestion satisfactory in the biogas plants. Therefore, the amount of bacteria was calculated on the basis of the dry matter (DM) weight and expressed as cfu/g-DM to allow correct competition of bacterial quantities of dairy manure and digestate. The moisture contents of the samples were measured using a Moisture Analyzer (MX-50, A&D Co. Ltd, Japan). The samples were automatically

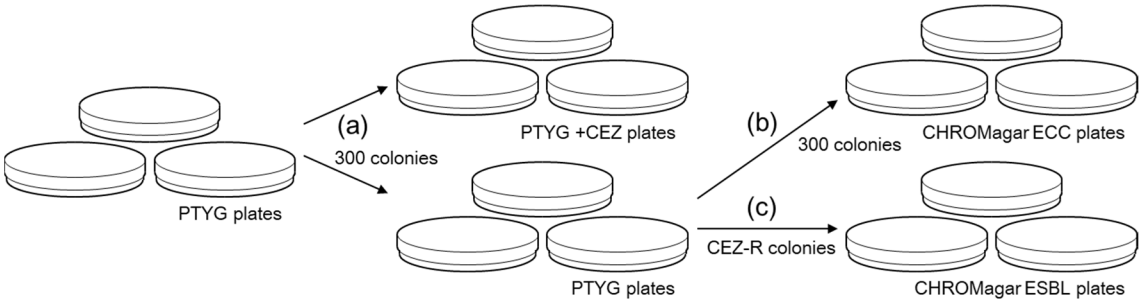

Fig. 1 Schematic diagram of culturing steps to identify and quantify bacteria

heat treated at 160 °C - 200 °C for 30 - 60 min. The moisture contents were calculated on the basis of the difference between wet and dry weights. Statistical analysis of bacterial quantities was performed with Microsoft Excel 2010 (Microsoft Corporation, Seattle, WA, USA).

3.3 RESULTS AND DISCUSSION

The effect of thermophilic anaerobic digestion on CEZ-R bacteria

The effect of thermophilic anaerobic digestion on CEZ-R bacteria was confirmed by comparing the number of *E. coli*, other coliforms and non-coliform bacteria in dairy manure and digestate. These numbers were calculated from colony numbers on CEZ-added and non-added PTYG plates and CHROMagar ECC plates. Figure 2 shows the comparison of bacterial numbers in dairy manure and digestate. The number of CEZ-R bacteria in dairy manure was 4.41% of the total bacteria (7.19×10^6 cfu/g-DM in 1.63×10^8 cfu/g-DM). After thermophilic anaerobic digestion, the average numbers of CEZ-R and CEZ-susceptible (CEZ-S) bacteria were 6.65×10^4 cfu/g-DM and 1.63×10^7 cfu/g-DM in digestate. The survival rates after thermophilic anaerobic digestion were 0.93% in CEZ-R bacteria and 10.5% in CEZ-S bacteria. As a result, the number of CEZ-R bacteria significantly decreased to 0.41% (6.65×10^4 cfu/g-DM) of total bacteria (1.64×10^7 cfu/g-DM) in digestate (Fig. 2a). Comparing the survival rates of CEZ-R and CEZ-S bacteria, thermophilic anaerobic digestion preferentially reduced CEZ-R bacteria rather than CEZ-S bacteria.

The rates of *E. coli* and other coliforms in total bacteria in dairy manure were 23.2% (3.77×10^7 cfu/g-DM) and 2.8% (4.56×10^6 cfu/g-DM), respectively; coliform bacteria (i.e., aggregation of *E. coli* and other coliforms) amounted to greater than 1/4 of the total bacteria. On the other hand, the number of coliform bacteria in digestate was <0.02% (3.01×10^3 cfu/g-DM) (Fig. 2b). This result indicated the preferential reduction effect of thermophilic anaerobic digestion on coliform bacteria.

The numbers of *E. coli*, other coliforms and non-coliform bacteria in CEZ-R bacteria are shown in Fig. 2c. The amount of *E. coli* was 0.34% (2.43×10^4 cfu/g-DM), and other coliforms was a relatively high percentage 15.5% (1.12×10^6 cfu/g-DM) in dairy manure. The percent content of coliform bacteria, 15.8% in dairy manure, was decreased significantly to 4.52% in digestate, and 95.5% of residual CEZ-R bacteria in digestate was found to be non-coliform bacteria. While most residues were non-coliform bacteria, the survival rate of these bacteria was very small at 1.1%. These results clarified that thermophilic anaerobic digestion could reduce all CEZ-R *E. coli*, other coliforms, and non-coliform bacteria.

On the other hand, though the reduction rate was smaller than that of CEZ-R, CEZ-S bacteria also decreased to 10.5% after thermophilic anaerobic digestion (to 1.63×10^7 cfu/g-DM from 1.56×10^8 cfu/g-DM). In contrast to CEZ-R bacteria, in dairy manure, CEZ-S bacteria contained relatively large number of *E. coli*. *E. coli* and other coliforms could not be detected in digestate, and all CEZ-S

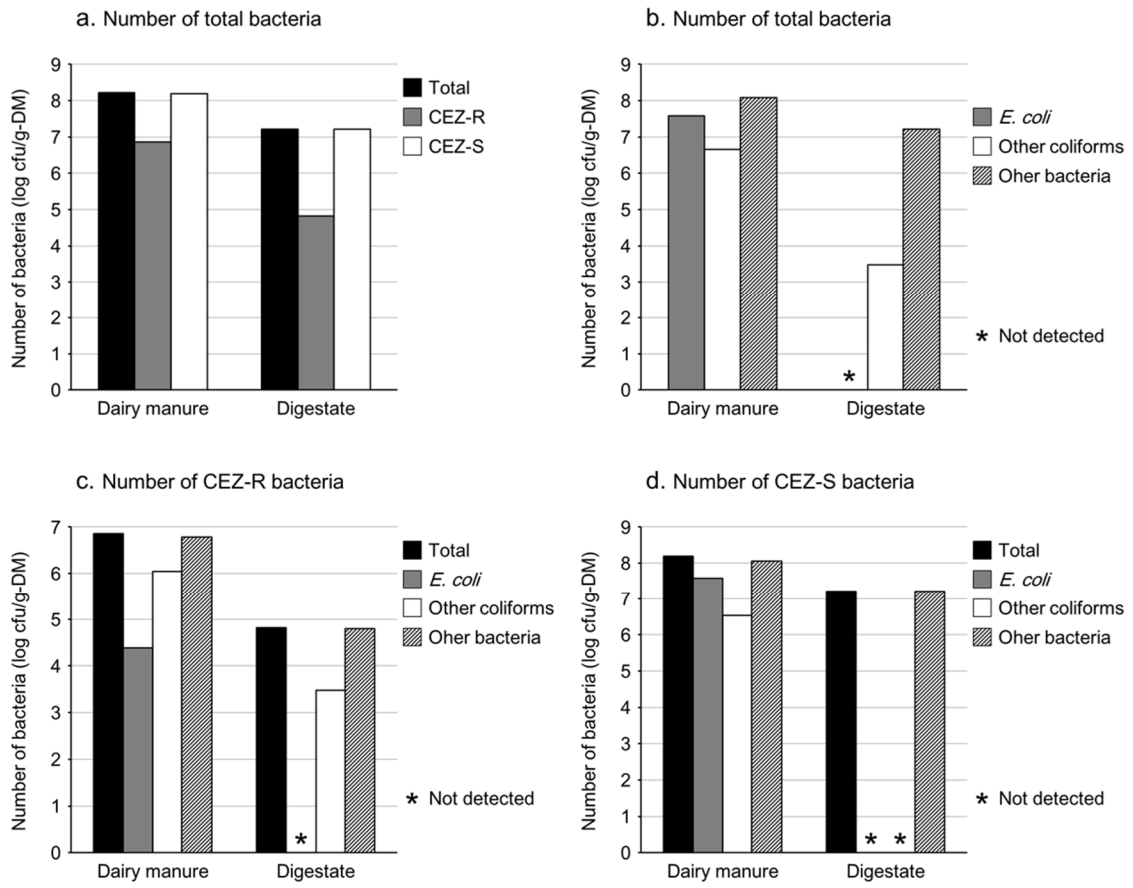

Fig. 2 Numbers of bacteria classified as susceptible to CEZ in dairy manure and digestate

bacteria that survived in digestate were non-coliform bacteria (Fig. 2d).

In dairy manure, total coliform bacteria made up a huge proportion (approximately 1/3) of the total bacteria. The number of coliform bacteria was reduced from 4.23×10^7 cfu/g-DM to 3.01×10^3 cfu/g-DM by thermophilic anaerobic digestion, which was a 0.01% survival rate. These large reductions were observed in both CEZ-R and CEZ-S coliform bacteria (Table 1). Because coliform bacteria made up 15.9% of the total CEZ-R bacteria (1.14×10^6 cfu/g-DM in 7.19×10^6 cfu/g-DM), more than 1/6 of the CEZ-R bacteria were eliminated along with the elimination of coliform bacteria. On the other hand, approximately 5/6 of the CEZ-R bacteria were composed of non-coliform bacteria. The survival rate of non-coliform bacteria was 13.6%, which was higher than that of coliform bacteria. However, the survival rates of the non-coliform bacteria were significantly different between CEZ-R and CEZ-S (1.05% and 14.3%). From these results, it was clear that elimination of CEZ-R bacteria by thermophilic anaerobic digestion was the combined result of the elimination of total coliform bacteria, which made up 1/6 of the CEZ-R bacteria, and the preferential elimination of CEZ-R among non-coliform bacteria.

The effect of thermophilic anaerobic digestion on ESBL-producing bacteria

To confirm the effect of thermophilic anaerobic digestion on ESBL-producers, the quantities of ESBL-producers in samples were measured. Table 2 shows a summary of the number of ESBL-producers classified by culturing with CHROMagar ESBL plates. In this study, CEZ-R bacterial colonies were classified into *E. coli*, other coliforms, and non-coliform bacteria with CHROMagar ECC plates and were further distributed into groups of ESBL-producers and non-ESBL-producers by culturing with CHROMagar ESBL plates. Note that this method could not distinguish between ESBL-producing bacteria except for the indicated genus groups in Table 2 and non-ESBL producers. These groups were expressed as other coliform-(ESBLs + non-ESBLs) in other coliform groups and other-(ESBLs + non-ESBLs) in the non-coliform bacteria group. All CEZ-R *E. coli* in dairy manure were identified as *E. coli*-ESBL and completely eliminated by thermophilic anaerobic digestion. *Klebsiella*/*Enterobacter*/*Citrobacter*-ESBL and *Proteus*-ESBL, which amounted to approximately 67% of CEZ-R of other coliforms in dairy manure, were significantly decreased by thermophilic anaerobic digestion, with 0% and 0.07% survival rates, respectively. Similarly, ESBL-producing non-coliform bacteria except for *Pseudomonas*-ESBL also decreased remarkably.

Organic acids such as volatile acids and higher fatty acids, which were produced during anaerobic digestion, were reported as the possible factors eliminating the bacteria (Lowe, et al., 1993; Sahlstrom, 2003; Desbois and Smith, 2010). In addition, other factors, such as nutrient starvation among competitive microflora or free radical production in bacteria due to sublethal stress, were also possibly contributed to the decrease of sanitary bacteria in anaerobic digestion (Aldsworth, et al., 1999). However, the temperature and the length of treatment are the most important factors affecting

Table 1 Numbers of CEZ-R and CEZ-S bacteria and their survival rates

	Number of bacteria (average ± S.D.) (cfu/g-DM)		Survival rate (%)
	Dairy manure	Digestate	
E. coli			
CEZ-R	$2.43 \times 10^4 \pm 1.08 \times 10^3$	0	0
CEZ-S	$3.77 \times 10^7 \pm 1.07 \times 10^7$	0	0
Total	$3.77 \times 10^7 \pm 1.07 \times 10^7$	0	0
Other coliforms			
CEZ-R	$1.11 \times 10^6 \pm 4.96 \times 10^4$	$3.01 \times 10^3 \pm 2.85 \times 10^2$	0.27
CEZ-S	$3.44 \times 10^6 \pm 9.74 \times 10^5$	0	0
Total	$4.56 \times 10^6 \pm 1.02 \times 10^6$	$3.01 \times 10^3 \pm 2.85 \times 10^2$	0.07
Non-coliform bacteria			
CEZ-R	$6.05 \times 10^6 \pm 2.68 \times 10^5$	$6.35 \times 10^4 \pm 6.01 \times 10^3$	1.05
CEZ-S	$1.15 \times 10^8 \pm 3.26 \times 10^7$	$1.63 \times 10^7 \pm 2.34 \times 10^6$	14.3
Total	$1.21 \times 10^8 \pm 3.28 \times 10^7$	$1.64 \times 10^7 \pm 2.37 \times 10^6$	13.6

Table 2 Numbers of ESBL-producers among CEZ-R bacteria in dairy manure and digestate

	Numbers of bacteria (average ± S.D.) (cfu/g-DM)		Survival rate (%)
	Dairy manure	Digestate	
Total CEZ-R bacteria	$7.19 \times 10^6 \pm 3.19 \times 10^5$	$6.65 \times 10^4 \pm 6.30 \times 10^3$	0.93
E. coli	$2.43 \times 10^4 \pm 1.08 \times 10^3$	0	0
E. coli-ESBL	$2.43 \times 10^4 \pm 1.08 \times 10^3$	0	0
E. coli-non-ESBL	0	0	-
Other coliforms	$1.12 \times 10^6 \pm 4.96 \times 10^4$	$3.01 \times 10^3 \pm 2.85 \times 10^2$	0.27
Klebsiella/*Enterobacter*/ *Citrobacter*-ESBL	$1.86 \times 10^5 \pm 8.26 \times 10^3$	0	0
Proteus-ESBL	$5.59 \times 10^5 \pm 2.48 \times 10^4$	$3.76 \times 10^2 \pm 3.56 \times 10$	0.07
Other coliform-(ESBLs + non-ESBLs)	$3.72 \times 10^5 \pm 1.65 \times 10^4$	$2.63 \times 10^3 \pm 2.49 \times 10^2$	0.71
Non-coliform bacteria	$6.05 \times 10^6 \pm 2.68 \times 10^5$	$6.35 \times 10^4 \pm 6.01 \times 10^3$	1.05
Pseudomonas-ESBL	0	$7.30 \times 10^2 \pm 6.91 \times 10$	increased
Acinetobacter-ESBL	$1.58 \times 10^6 \pm 7.00 \times 10^4$	$1.46 \times 10^3 \pm 1.38 \times 10^2$	0.09
Other-(ESBLs + non-ESBLs)	$4.47 \times 10^6 \pm 1.98 \times 10^5$	$6.13 \times 10^4 \pm 5.81 \times 10^3$	1.37

the reduction of bacteria in biogas plant. Generally, organic wastes were processed at mesophilic (30 - 38 °C) or thermophilic (50 - 55 °C) temperatures in biogas plant, and thermophilic digestion has shorter pasteurization time of substrates than mesophilic digestion. The reduction time of bacteria can be expressed by the decimation reduction time (T_{90}), which indicates 90% reduction or a decrease by one logarithmic unit. For many bacteria, T_{90} can be counted in hours and in days in thermophilic and mesophilic digestion, respectively (Sahlstrom, 2003). However, reduction time is different between batchwise and continuous system in biogas plants. Most of biogas plants were performed with continuous system due to the economic and practical reasons. As fresh substrate is added continuously to the processing substrates, continuous system would need longer periods for pasteurization compared to the batchwise system. The digestion period of 15 days in thermophilic anaerobic digestion was thought to be sufficient to inactivate most bacteria. In this study, however, while the reduction rates of CEZ-R bacteria and ESBL-producers were 99.1% (decreased from 7.19×10^6 cfu/g-DM to 6.65×10^4 cfu/g-DM) and 99.2% (decreased from 3.38×10^6 cfu/g-DM to 3.08×10^4 cfu/g-DM), respectively, the reduction rate of CEZ-S bacteria was 89.5% (decreased from 1.56×10^8 cfu/g-DM to 1.63×10^7 cfu/g-DM), which indicated the possibility of insufficient inactivate bacteria other than CEZ-R bacteria and ESBL-producers. In general, the digestion period in biogas plant was mostly calculated based on the required time to complete the digestion of organic matters. This is usually marked by the completion of biogas production, while the digestion period would not be sufficient to reduce some bacteria completely. Actually, some researchers have reported the survival of Listeria, Campylobacter, and Yersinia in digestates (Sahlstrom, 2003), though it depends on the fed materials. Therefore, there are several biogas plants that have a separate pre- or post-pasteurization process in Austria, Denmark, Germany, Sweden, and Japan.

Pseudomonas-ESBL was the only group which increased from a non-detectable amount to $7.30 \times 102 \pm 6.91 \times 10$ cfu/g-DM after thermophilic anaerobic digestion. Spore-forming bacteria, such as *Bacillus* or *Clostridium*, are known to resist to heat-stress, and some *Pseudomonas* species were reported to be relatively resistant to heat stress (Palleroni, 2005). However, considering that *Pseudomonas* species are not spore-forming bacteria, the increase of the amount was supposed to be the result of analysis errors. On the other hand, *Bacillus* species are known to be present in dairy manure at relatively high amount (Qi, et al., 2018) and possible to survive over a wide temperature range even under anaerobic conditions, the other-(ESBLs + non-ESBLs) group might comprise that sort of bacteria.

Other coliform-(ESBLs + non-ESBLs) and non-coliform-(ESBLs + non-ESBLs) made up 5.18% and 62.2% of the CEZ-R bacteria in dairy manure, respectively. All these bacteria also significantly decreased after thermophilic anaerobic digestion. CEZ-susceptible bacteria or non-ESBL-producers can become host bacteria of ESBL-encoding plasmid. However, these bacteria decreased during thermophilic anaerobic digestion as shown in Fig. 1 and Table 1. These results

indicate the advantage of thermophilic anaerobic digestion in preventing the spread of plasmid-born resistance into environmental bacteria via dairy wastes.

The effect of thermophilic anaerobic digestion on AMP-R bacteria

In contrast to CEZ-R bacteria, the number of AMP-R bacteria was also quantified as a non-propagative resistance because of the absence of the plasmid-borne resistance gene. The numbers of AMP-R bacteria in dairy manure and digestate are shown in Fig. 3. The percentage of constituent AMP-R bacteria was low in the dairy manure (approximately 2%). The average number of AMP-R bacteria decreased from 3.30×10^6 cfu/g-DM to 4.63×10^4 cfu/g-DM after thermophilic anaerobic digestion, which was a 1.40% survival rate and the majority (99.7%) of surviving bacteria were AMP-S (Fig. 3a). These results show the preferential effectivity of thermophilic anaerobic digestion on AMP-R bacteria.

AMP-R bacteria in dairy manure consisted of 3.00% (9.91×10^4 cfu/g-DM) *E. coli*, 6.55% (2.17×10^5 cfu/g-DM) other coliforms and 90.4% (2.99×10^6 cfu/g-DM) non-coliform bacteria. On the other hand, AMP-R bacteria in digestate consisted of 0% *E. coli*, 6.50% (3.01×10^3 cfu/g-DM) other coliforms and 93.5% (4.33×10^4 cfu/g-DM) non-coliform bacteria (Fig. 3b). The survival rates of AMP-R of *E. coli*, other coliforms and non-coliform bacteria were 0%, 1.39% and 1.45%, respectively. These results showed that thermophilic anaerobic digestion treatment was especially effective on AMP-R *E. coli* and had a similar effect on other coliforms and non-coliform bacteria to that of AMP-R, as seen by the comparison of survival rates.

AMP-S bacteria in dairy manure ($1.60 \times 10^8 \pm 4.54 \times 10^7$ cfu/g-DM) consisted of 23.6% *E. coli*, 2.72% other coliforms and 73.7% non-coliform bacteria, which amounted to a total of approximately 1/4 coliform bacteria (Fig. 3c). This proportion of constituents was identical to that in CEZ-S bacteria. No AMP-S *E. coli* was detected in digestate, and AMP-S other coliforms were also not detected in digestate. The survival rate of AMP-S bacteria was 10.3%, and all residual bacteria were found to be non-coliform bacteria. AMP-S bacteria amounted to 99.7% among residual bacteria in digestate (Fig. 3a), and all were found to be non-coliform bacteria (Fig. 3b, 3c).

AMP-S non-coliform bacteria were found to have a marked tendency to survive compared to AMP-R bacteria after thermophilic anaerobic digestion (Fig. 3a), and it was also clear that the high survival rate in AMP-S bacteria was reflected by the high survival of non-coliform bacteria. The high reduction of AMP-R bacteria by thermophilic anaerobic digestion was found in every group; however, only AMP-S survived after thermophilic anaerobic digestion, similar to the result of CEZ. Larger amounts of susceptible bacteria than antimicrobial-resistant bacteria in dairy manure may account for greater survival in digestate. However, other reasons could be considered to account for the difference in survival rates, for example, the difference in the composition of bacterial genera or species between the resistance group and the susceptible group or relative differences in the mechanisms between

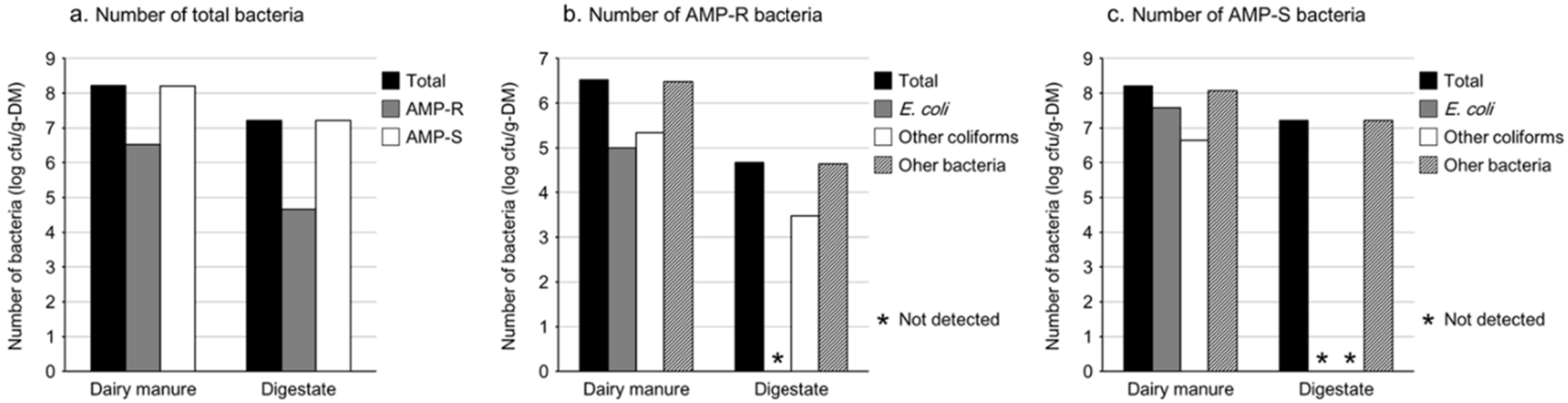

Fig. 3 Numbers of bacteria classified as susceptible to AMP in dairy manure and digestate

thermophilic anaerobic digestion resistance and antimicrobial resistance. To more clearly understand this point, more detailed analysis will be needed in another bacteria group.

3.4 CONCLUSIONS

TDA was shown to effectively eliminate not only plasmid-born but also non-plasmid-born resistant bacteria. In this study, the survival rates of bacteria were 0.93% in CEZ-R and 1.40% in AMP-R, while the survival rates were 10.5% in CEZ-S and 10.3% in AMP-S after thermophilic anaerobic digestion. Moreover, the survival rates of ESBL-producers, ~0.09%, were lower than those of antimicrobial-resistant bacteria. Thus, thermophilic anaerobic digestion eliminated ESBL-producers more effectively than other antimicrobial-resistant bacteria. In conclusion, the elimination effect of thermophilic anaerobic digestion was defined to be more prominent on antimicrobial-resistant bacteria and ESBL-producers than antimicrobial-susceptible bacteria. Anaerobic digestion treatment can prevent the spread of antimicrobial-resistant bacteria into the environment and will be used to fill gaps in the realization of the philosophy of One Health.

Chapter 4

Quantity Changes in *Pseudomonas* Species in Dairy Manure During Anaerobic Digestion at Mesophilic and Thermophilic Temperatures

ABSTRACT

Anaerobic digestion is known to eliminate many kinds of microorganisms in livestock wastes. In this study, the quantitative changes of *Pseudomonas* species during mesophilic and thermophilic anaerobic digestions were investigated with focus on *P. aeruginosa* and *P. fluorescens*, which are known as a pathogen and a plant growth-promoting rhizobacterium, respectively. Furthermore, quantitative changes in antimicrobial-resistant *Pseudomonas* species against cefazolin were also investigated as a representative of antimicrobial resistance in dairy farms. The quantitative measurement of *Pseudomonas* species was performed by a plating method with modified *Pseudomonas*-selective agar medium. The colonies on the agar plates were classified into three groups by their colour development and constitutional fluorescence, and the group to which *P. aeruginosa* and *P. fluorescens* belong was confirmed by colony PCR using species-specific primers. The results revealed that while the number of total *Pseudomonas* decreased, the number of fluorescent *Pseudomonas* including *P. fluorescens* increased after anaerobic digestion. The abundance of cefazolin-resistant *Pseudomonas* also decreased, and no *P. aeruginosa* could be detected in any samples. These results suggested that the digestate has benefits such as the absence of pathogenic risks led by the *Pseudomonas* species and is expected to have a plant growth-promoting effect facilitated by fluorescent *Pseudomonas*.

The research in this chapter was reported in Journal of Material Cycles and Waste Management in 2019 by Iwasaki et al. (Iwasaki, et al., 2019b).

4.1 INTRODUCTION

As the number of livestock waste-treating biogas plants being constructed increases, digestate have taken an important position as an organic fertilizer. Livestock wastes include many kinds of microorganisms, such as intestinal and environmental bacteria, fungi, protozoa, nematodes, and often include antimicrobial-resistant microorganisms. Therefore, it is important to be aware of the biological hygiene of digestates. In the studies about the effects of anaerobic digestion on the fate of bacteria in dairy manure in Chapter 3, it has been revealed that most of the bacteria such as *Escherichia coli*, *Enterococcus*, other coliform bacteria, CEZ-R and AMP-R bacteria, and ESBL-producing bacteria were reduced during anaerobic digestion. *Pseudomonas* species, however, were found to

survive during mesophilic anaerobic digestion, and the abundance of ESBL-producing *Pseudomonas* increased slightly in mesophilic digestate (Iwasaki, et al., 2019).

In the dairy industry, some *Pseudomonas* species (e.g., *Pseudomonas aeruginosa*) can act as a pathogen causing cow mastitis, which is one of the major diseases of dairy cows (Sumathi, et al., 2008; Jackson, et al., 2011; Ohnishi, et al., 2011). The main therapeutic agent for cow mastitis is CEZ following ampicillin, orbifloxacin, kanamycin, and erythromycin (Wagner and Erskine, 2013). Because cow mastitis caused by CEZ-R *Pseudomonas* such as ESBL-producing *Pseudomonas* is unsuccessful in therapeutic efficiency, the spread of digestate to the farm environment entails the risk of diseases when digestate includes such bacteria. In addition, some *Pseudomonas* species were found to be plant pathogens (Misaghi and Grogan, 1969). *P. corrugate* and *P. viridiflava* are phytopathogens that cause pith necrosis of tomato plants (Alippi, et al., 2003), and *P. syringae* pv. *tomato* is a phytopathogen for bacterial leaf spots of tomato (Zaccardelli, et al., 2005). Therefore, digestate must suffer from the contamination of pathogenic *Pseudomonas* when spreading to fields.

However, many researchers have also reported that some fluorescent *Pseudomonas* strains have beneficial characteristics for plants and are well known as plant growth-promoting rhizobacteria (PGPR) (Cawoy, et al., 2011; De Brito, et al., 1995; Hameeda, et al., 2006). Similar to some *Bacillus* strains such as *B. thuringiensis* KR1 and *B. subtilis* GBO3 (Mishra, et al., 2009; Ongena and Jacques, 2008), many plant growth-promoting fluorescent *Pseudomonas* strains have been isolated from the rhizosphere of the plants (Cawoy, et al., 2011), and the mechanism for promoting plant growth and suppressing plant diseases has been revealed. *P. fluorescens* is one of the most commonly studied strains among the fluorescent *Pseudomonas*. Plant growth promotion of PGPR was derived directly by the production of plant hormones (Brandl, et al., 2001) and indirectly by the production of phytopathogen-suppressive factors such as hydrogen cyanide (HCN), antibiotics (pyrrolnitrin, DAPG, etc.), phenazines, cyclic lipopeptides, siderophore compounds and fluorescent pigments (pyoverdin) (Haas and Défago, 2005), and the inhibition of the colonization of other fungi and phytopathogens by competition for limiting nutrients and space (Laville, et al., 1992; Vidhyasekaran and Muthamilan, 1995; Botelho and Mendonça-Hagler, 2006; Iwamoto and Aino, 2008). Plant growth-promoting fluorescent pseudomonads were also found in composts (De Brito, et al., 1995; Hameeda, et al., 2006), so it is possible to predict the existence of plant growth-promoting fluorescent *Pseudomonas* in digestate of dairy manure.

In this research, I focused on *Pseudomonas* in dairy manure and analysed the quantity changes in *Pseudomonas* with dividing *P. aeruginosa*, *P. fluorescens*, other fluorescent *Pseudomonas* (fluorescent pseudomonads), and other non-fluorescent *Pseudomonas* after mesophilic and thermophilic anaerobic digestion. Additionally, the quantity of CEZ-R *Pseudomonas* in each group was analysed. As a measurement of quantities of *Pseudomonas*, the plating method (plate spread method) using modified cetrimide agar (mCA) culture plates was adopted for these simple and easy

methods. The quantity of beneficial bacteria such as *Pseudomonas* will possibly be an additional standard of the fertilizer value of digestate.

4.2 MATERIALS AND METHODS

Anaerobic digestion of cow manure

The continuously fed anaerobic digestion was carried out using two 10-L laboratory-scale digesters (Yamashiro, et al., 2013) that were kept at 37 °C (mesophilic) and 55 °C (thermophilic) in water baths. Five litres of digestate from mesophilic biogas plants located at Shihoro-cho, Hokkaido (43°10'58"N, 143°00'55"E), and thermophilic biogas plants located at Asyoro-cho, Hokkaido (43°18'10"N, 143°41'07"E), were inoculated into these digesters as starters of anaerobic digestion. Fresh dairy manure was collected weekly from Obihiro University Farm, where CEZ was used to treat cow mastitis, and 300 g of dairy manure was added daily to the mesophilic digester, whereas 600 g was added to the thermophilic digester after adjusting the TS content of the manure to 10% with water dilution. The TS concentration of the manure was determined gravimetrically after heat-drying at 105 °C for 24 h. Digestate flowed out through the outlet by continuously feeding manure into the digester. HRT of manure were 30 and 15 days in mesophilic and thermophilic digesters, respectively. These parameters of the TS content of feeder manure and HRT were similar to the parameters of general biogas plants for digesting organic matter completely (Iwasaki, et al., 2011). After stabilizing anaerobic digestion (approximately 1-2 months after starting continuous feed), digestates from both digesters were collected and immediately processed for measurement of amount of *Pseudomonas*. The stability of continuously fed anaerobic digestion was assessed based on produced biogas yield (approximately 0.3 L/g-TS in both digesters) and the methane concentration of the produced biogas (approximately 57% in both digesters).

Quantification of *Pseudomonas* species

Stanbridge and Board (1994) improved selection efficiency of the CFC medium, which has been widely used as a *Pseudomonas*-selective medium. They noticed the differences in the utilization of L-arginine among *Pseudomonas* and other bacteria (Stalon and Mercenier, 1984) and identified not all but some *Pseudomonas* species by red colour development of colonies on L-arginine and phenol red-containing CFC agar medium. Based on their report, the amounts of *Pseudomonas* in samples were quantified by the plate spreading method with a modified cetrimide agar (mCA) culture plate, which consists of cetrimide agar base with 10 ml/L glycerin, 10 g/L L-arginine hydrochloride, 20 mg/L phenol red and 100 mg/L cycloheximide. In addition, *Pseudomonas aeruginosa*-selective agar (PASA) medium was used for stand-alone detection of *P. aeruginosa*. For the detection of CEZ-R *Pseudomonas*, cefazolin sodium salt (CEZ, Sigma-Aldrich Co. LLC, St Louis, MO) was added to

mCA at the concentration of 50 mg/L (mCA + CEZ). Cetrimide agar base and PASA medium were purchased from BBL (Becton, Dickinson and Company, New Jersey, USA), and other chemical components were purchased from WAKO Pure Chemical Industries Ltd. (Osaka, Japan). Dairy manure, mesophilic and thermophilic digestates were diluted 1:10 with phosphate buffered saline (PBS) and smeared on mCA, mCA + CEZ culture plates and PASA medium at 100 µl/plate. All cultures were performed in triplicate. After incubating the plates at 35 °C for 24 h, colonies were observed under room light and UV (302 nm) light and counted. By these methods, each colony grown on mCA culture plates could be classified into 4 groups; 1) red colour developed colony with fluorescence (red/fluor), 2) red colour developed colony with non-fluorescence (red/non-fluor), 3) white colour (non-colour development) with fluorescence (white/fluor), and 4) white colour with non-fluorescence (white/non-fluor). For the correct comparison of bacterial numbers in each sample, the unit of bacterial number was shown as cfu/g-dry matter (DM) because different volumes of diluent water were added to dairy manure every time when the manure was sampled from the farm during experimental periods. DM weights of samples were calculated from the weight of wet raw samples and post-dried samples heated at 105 °C for 24 h. Statistical analyses of quantified bacteria were performed with Microsoft Excel 2010 (Microsoft Corporation, Seattle, WA, USA).

Identification of *Pseudomonas* species by colony PCR

Bacterial colony PCR was conducted to identify *P. aeruginosa* and *P. fluorescens* on mCA culture plates. Referenced bacterial strains and their GeneBank accession numbers, target genes, PCR primer annealing sites, and primer names were shown in Fig. 1. Specific primers for two outer membrane lipoprotein genes, *oprI* and *oprL* genes (Fig. 1, A and B), and specific PCR primers for the GDP-mannose dehydrogenase (*algD*) gene (Fig. 1, C) were used to distinguish *P. aeruginosa* from other fluorescent *Pseudomonas* species and to detect *P. aeruginosa* directly (da Silva Filho, et al., 1999). De Vos et al. (1997) observed in their report that it was possible to distinguish *P. aeruginosa* from other fluorescent *Pseudomonas* species from the results of PCR using these two primer sets. While the *oprI* gene commonly exists in the fluorescent *Pseudomonas* genome, the *oprL* gene is only in the *P. aeruginosa* genome, so it is possible to distinguish between *P. aeruginosa* and other fluorescent *Pseudomonas* from the gel electrophoresis pattern of PCR products. In addition, two specific primer sets were designed for the direct detection of *P. fluorescens* (Fig. 1 D and E). The complete (CP000094.2) and partial (X74218.2, AJ583090, DQ146946.1, X80001) genome sequences of *P. fluorescens* obtained from GenBank (http://www.ncbi.nlm.nih.gov/genbank/) were referenced for the design of specific primers. Duan et al. (2000) reported that *tolB* gene, a member of the gene cluster consistent with *orf1*, *tolQ*, *tolR*, *tolA*, *tolB*, *oprL*, and *orf2* genes, has a specific sequence for each *Pseudomonas* species. Referring to their report, one primer set (PFL-OPRL and PFL-TOLB, Fig. 1, D) for the direct detection of *P. fluorescens* was designed within *tolB* and *oprL* gene by primer search

software GENETYX-Win ver.10 (Software Development Co., Tokyo, Japan) and the GeneBank data base. Additionally, another primer set for the direct detection of *P. fluorescens* (Fig. 1, E) was designed by primer search between *16S rRNA* and *23S rRNA* based on the report of Locatelli et al. (2002). The specificities of these designed primers were confirmed by using the Basic Local Alignment Search Tool [BLAST, The National Center for Biotechnology Information (NCBI), http://blast.ncbi.nlm.nih.gov/Blast.cgi?CMD=Web&PAGE_TYPE=BlastHome]. Synthetic oligonucleotide primers were purchased from Life Technologies, Inc. (Gaithersburg, MD). The primer sequences and expected PCR amplicon sizes are summarized in Table 1.

Colonies were selected from the mCA culture plates with dairy manure using sterilized toothpicks and suspended in 100 μL of distilled deionized water (DDW). After boiling for 5 min, the bacterial lysates were cooled, and PCR was performed using AmpliTaq Gold® DNA Polymerase and 10×PCR Gold Buffer/$MgCl_2$ with dNTPs (Applied Biosystems, Foster City, CA). The total volume of the PCR reaction mixture was 50 μL containing 5 μL of colony lysate (template), 5 μL of 10×PCR buffer, 0.5 μL of forward and reverse primers (100 mM each), 5 μL of dNTP mixture (10 mM each), 4 μl of $MgCl_2$ (25 mM), 0.5 μL of AmpliTaq Gold® DNA Polymerase (5 u/μL) and DDW. The thermal cycling conditions were 4 min at 94 °C, followed by 30 cycles of 94 °C for 1 min, 55 °C for 1 min, 72 °C for 2 min and extension for 7 min at 72 °C. After PCR reaction, the amplicons were analysed by agarose gel electrophoresis with 1.5% (w/v) agarose (Nippon Gene Co., Ltd., Tokyo, Japan) gel. Marker 5 (Nippon Gene Co., Ltd., Tokyo, Japan) was used as a DNA size marker.

Calculations of Td and T_{90} of *Pseudomonas* groups during anaerobic digestion

Population doubling times (Td) and decimation reduction time (90% reduction time: T_{90}) of bacteria are important indicators to find the optimum operating conditions for anaerobic digestion systems. The averages of Td and T_{90} in each classified group were calculated from the number of *Pseudomonas* by the following formula:

$$Td = (t2 - t1) \times \log(2) / \log(q2/q1)$$

$$T_{90} = (t2 - t1)/(\log q1 - \log q2), \text{ where}$$

q1 = the number of *Pseudomonas* at time t1

q2 = the number of *Pseudomonas* at time t2

In this study, Td and T_{90} values of *Pseudomonas* could be calculated by exchanging factors as follows:

t2 – t1 = HRT of digester [mesophilic: 30 (days), thermophilic: 15 (days)]

q1 = number of *Pseudomonas* in dairy cow manure

q2 = number of *Pseudomonas* in digestate

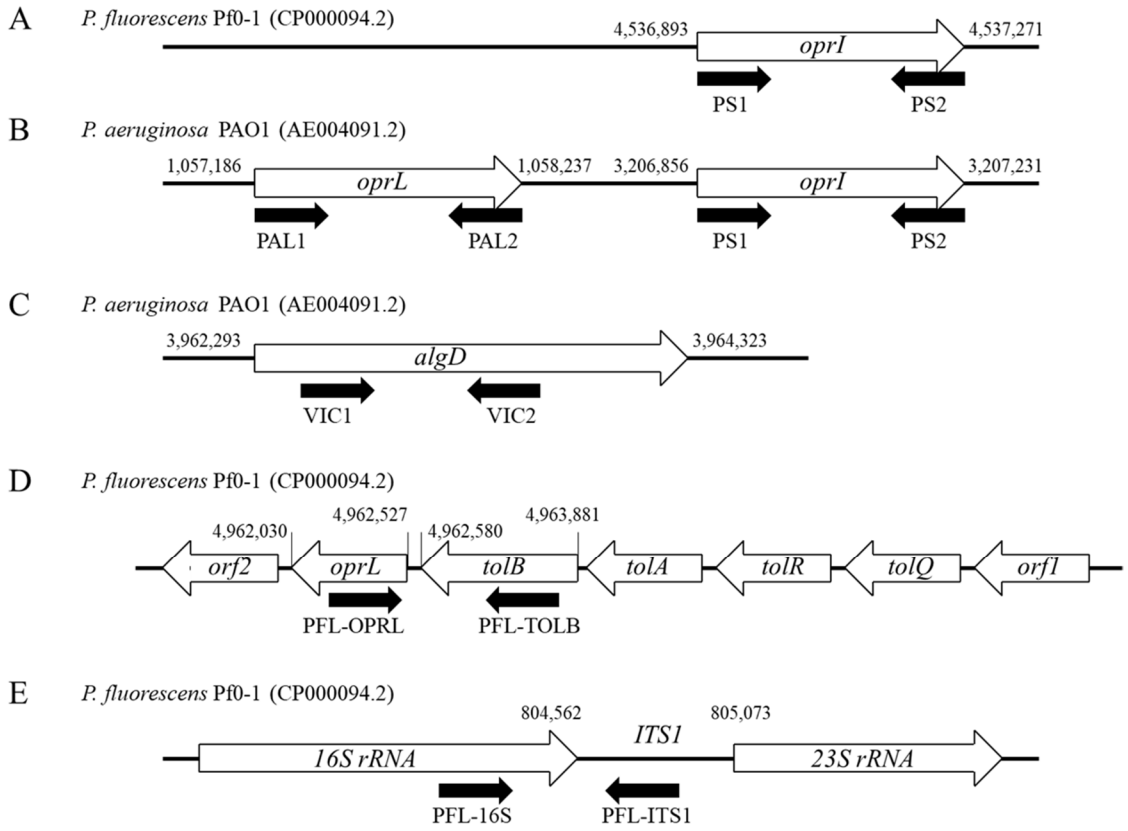

Fig. 1 PCR primer annealing sites on *Pseudomonas* genomes

Table 1 Summary of PCR primers

	Primer name	Direction	Sequence	Specificity	Amplicon size (bp)
A	PS1	Forward	5'-ATGAACAACGTTCTGAAATTCTCTGCT-3'	Fluorescent *Pseudomonas* species common *oprI* gene	249
	PS2	Reverse	5'-CTTGCGGCTGGCTTTTTCCAG-3'		
B	PAL1	Forward	5'-ATGGAAATGCTGAAATTCGGC-3'	*P. aeruginosa oprL* gene	504
	PAL2	Reverse	5'-CTTCTTCAGCTCGACGCGACG-3'		
C	VIC1	Forward	5'-TTCCCTCGCAGAGAAAACATC-3'	*P. aeruginosa* GDP-mannose dehydrogenase (*algD*) gene	520
	VIC2	Reverse	5'-CCTGGTTGATCAGGTCGATCT-3'		
D	PFL-OPRL	Forward	5'-CCGTTTGCTTTCAGGTCTTTG-3'	*P. fluorescens oprL* gene	518
	PFL-TOLB	Reverse	5'-AAATCCTCACTGATAGCACTCT-3'	*P. fluorescens tolB* gene	
E	PFL-16S	Forward	5'-TGGTGCCTTCGGGAACATTGAGAC-3'	*P. fluorescens 16S rRNA* gene	725
	PFL-ITS1	Reverse	5'-TGAGCTATGGCCCCGTATTTCTACAGC-3'	*P. fluorescens* ITS1 region	

4.3 RESULTS AND DISCUSSION

Identification of *P. aeruginosa* and *P. fluorescens* on mCA culture plates by colony PCR

The characteristics of *Pseudomonas* colonies grown on mCA culture plates were classified into 3 resulting types: 1) red/fluor colony, 2) red/non-fluor colony, and 3) white/non-fluor colony. No white/fluor colonies were observed in dairy manure, mesophilic and thermophilic digestates; all fluorescent colonies indicated red colour development on mCA culture plates. However, there were no colonies grown on PASA medium with any samples. *P. aeruginosa* was estimated to indicate red/fluor characteristics on mCA culture plates. However, I considered that no or an undetectable amount of *P. aeruginosa* was in any samples because no bacterial colonies grew on the PASA medium.

Eight typical colonies among the red/fluor colonies (colonies 1-1 to 1-8), 2 typical colonies among red/non-fluor colonies (colonies 2-1, 2-2), and 6 typical colonies among white/non-fluor colonies (colonies 3-1 to 3-6) were selected from the mCA culture plates with dairy manure, and colony PCR was performed. At the same time, colour development and fluorescence of the selected colonies were re-confirmed by culturing with fresh mCA culture plates.

The results of agarose gel electrophoresis of colony PCR amplicons are summarized in Fig. 2. The 249 bp product amplified using the PS1/PS2 primer set was detected only with fluorescent colonies, and no amplicon was detected with non-fluorescent colonies (lane 1). However, no product amplified using the PAL1/PAL2 primer set (expected amplicon size is 504 bp) was detected among all red/fluor colonies (lane 2). In addition, the direct detection of *P. aeruginosa* using VIC1/VIC2 primer set also resulted in no amplicons (lane 3). These results were consistent with the results of culturing with PASA medium that suggested no or an undetectable amount of *P. aeruginosa* in dairy manure.

In the results of agarose gel electrophoresis of PCR products amplified with PFL-OPRL/PFL-TOLB primer set (lane 4) and PFL-16S/PFL-ITS1 primer set (lane 5) for the direct detection of *Pseudomonas fluorescens* colonies and amplified genes, the expected PCR amplicon size was 518 bp with the PFL-OPRL/PFL-TOLB primer set and 725 bp with the PFL-16S/PFL-ITS1 primer set; these were detected in one of 8 red/fluor colonies, colony 1-1. This colony was thought to be *P. fluorescens*. However, the other 7 red/fluor colonies were thought not to be *P. fluorescens* because no amplicon with these primer sets could be detected (colony 1-2). Therefore, the red/fluor colony group was thought to include *P. fluorescens*, but no phenotypic differences between *P. fluorescens* and other fluorescent pseudomonads were observed on mCA culture plates.

Quantitative measurement of *P. aeruginosa*, *P. fluorescens*, and other *Pseudomonas* spp. in dairy cow manure and digestates

From the coincidence of the results of culturing of *Pseudomonas* with colony PCR, it was revealed that *P. aeruginosa* was not detected in any samples and that *P. fluorescens* was a constituent

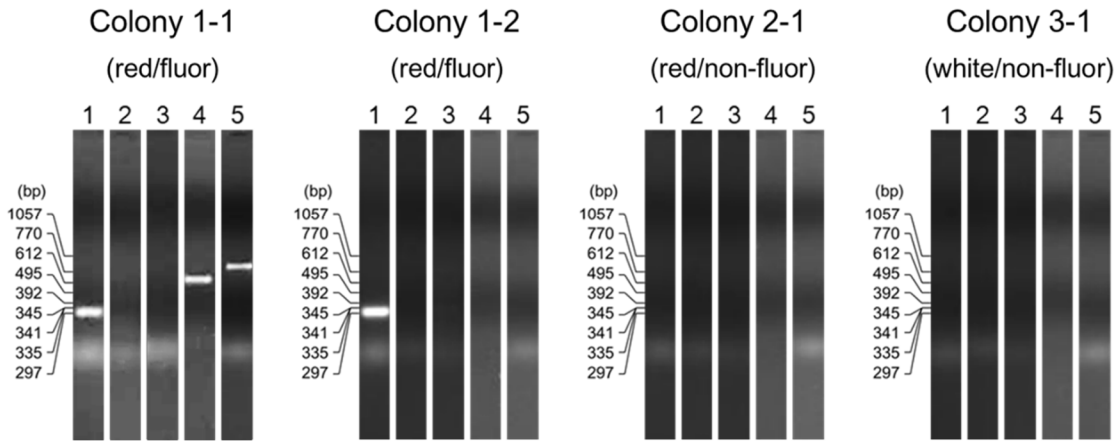

Fig. 2 Agarose gel electrophoretic patterns of colony PCR products

This Figure shows representative results among 16 executed *Pseudomonas* colonies. Extracted bacterial genomic DNA was amplified with primer sets (forward/reverse primer) as below; lane 1: PS1/PS2, lane 2: PAL1/PAL2, lane 3: VIC1/VIC2, lane 4: PFL-OPRL/PFLTOLB, lane 5: PFL-16S/PFL-ITS1

of the red/fluor group. Apart from these colonies, there were red/non-fluor and white/non-fluor groups that were thought to be non-fluorescent *Pseudomonas*. Because of the difference in the ability to metabolize L-arginine, different colour development on mCA culture plates was observed between these two groups. Therefore, each group can be expressed by the biological function of L-arginine metabolisms; the red colour developed colony group is the L-arginine-metabolizing group, and the white colony group is the L-arginine-non-metabolizing group. In summarizing the above results, colonies on PASA and mCA culture medium were classified into four groups as follows: *P. aeruginosa* (which was not actually detected in this study), other fluorescent *Pseudomonas* (which included not only *P. fluorescens* but also other fluorescent pseudomonads except for *P. aeruginosa*), L-arginine-metabolizing non-fluorescent *Pseudomonas*, and L-arginine-non-metabolizing non-fluorescent *Pseudomonas*.

Figure 3 shows the numbers of classified *Pseudomonas* with additional dividing to CEZ-R and CEZ-S. The total number of *Pseudomonas* in dairy manure was significantly decreased during anaerobic digestion. The reduction rate was 40.4% in mesophilic digestion and 51.7% in thermophilic digestion. However, the population of CEZ-R *Pseudomonas* increased approximately 1.3 times by mesophilic digestion at an increasing rate of 30.3%. Contrastingly, the number of CEZ-R *Pseudomonas* decreased during thermophilic digestions, and the reduction rate was 69.4%. The number of CEZ-S *Pseudomonas* decreased in mesophilic and thermophilic digestates, and the reduction rates were 54.8% and 48.1% by mesophilic and thermophilic digestion, respectively, which indicated the slightly higher survival in thermophilic digestate than that in mesophilic digestate (Fig. 3, A). *P. aeruginosa* was not detected through this experiment (Fig. 3, B). While the number of other fluorescent *Pseudomonas* significantly increased during anaerobic digestion, the number of CEZ-R other fluorescent *Pseudomonas*, which was the majority in other fluorescent *Pseudomonas* in dairy manure, decreased both in the mesophilic and thermophilic digestates. The reduction rate of CEZ-R other fluorescent *Pseudomonas* in mesophilic and thermophilic digestates was 43.2% and 71.3%, respectively. The total number of other fluorescent *Pseudomonas* was approximately 1.9 times and 2.5 times larger than that in the dairy manure in the mesophilic and thermophilic digestates, respectively, and these increasing numbers were caused by increasing numbers of CEZ-S other fluorescent *Pseudomonas*. The numbers of CEZ-S other fluorescent *Pseudomonas* were approximately 5.3 times and 8.4 times larger in the mesophilic and thermophilic digestates, respectively, than those in dairy manure. Consequently, CEZ-S became the majority of other fluorescent *Pseudomonas* in both digestates (Fig. 3, C). While other fluorescent *Pseudomonas* increased in digestates, the prevalence rates of fluorescent *Pseudomonas* in total *Pseudomonas* were as low as 2.49%, 7.76%, and 12.9% in dairy manure, mesophilic, and thermophilic digestate, respectively. The majority of the total *Pseudomonas* was non-fluorescent *Pseudomonas* in each sample, and the reduction in the total number of *Pseudomonas* was caused mainly by significant reduction in the number of non-fluorescent

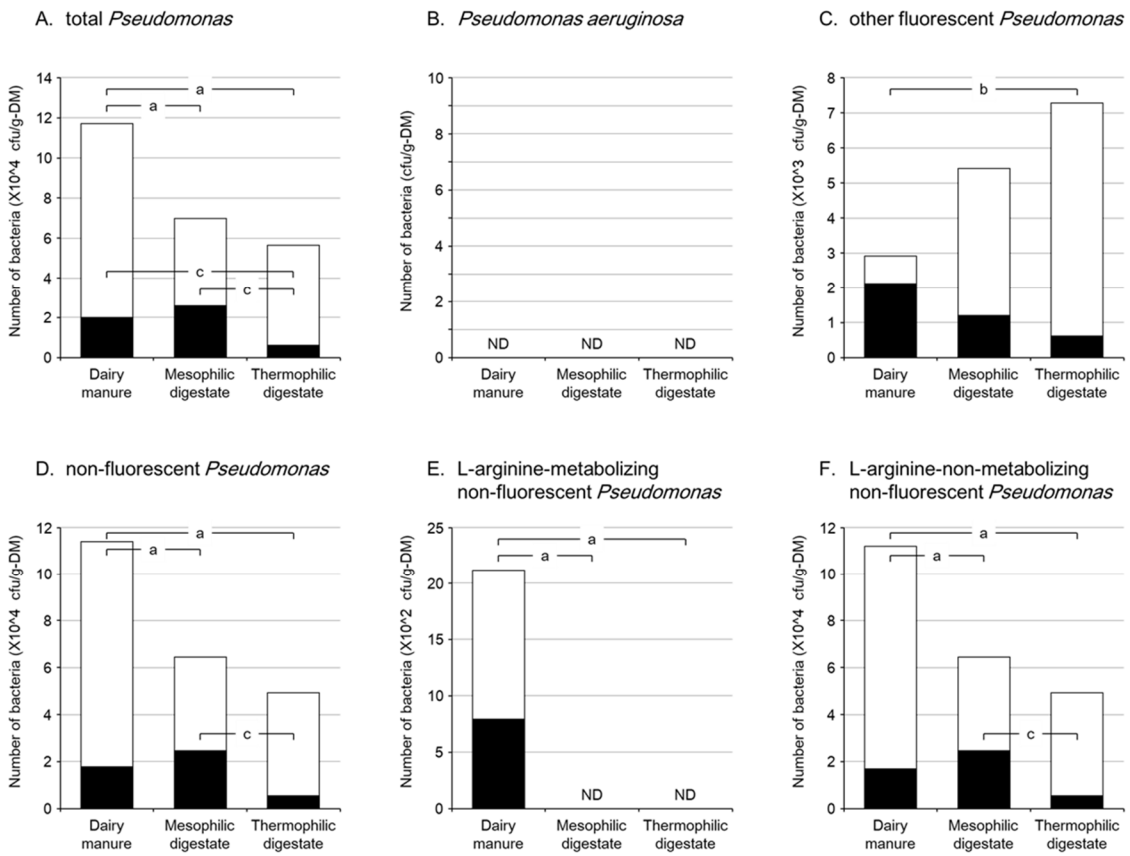

Fig. 3 Quantitative measurement of *Pseudomonas* species in dairy manure and digestates

These Figures showed the averaged values of CEZ-R (closed square) and CEZ-S (open square) among total *Pseudomonas* species (A), *P. aeruginosa* (B), other fluorescent *Pseudomonas* (C), non-fluorescent *Pseudomonas* (D), L-arginine-metabolizing non-fluorescent *Pseudomonas* (E), and L-arginine-non-metabolizing non-fluorescent *Pseudomonas* (F). The statistical significance was a: $p < 0.01$, b: $p < 0.05$ among total (CEZ-R + CEZ-S) bacterial numbers, and c: $p < 0.05$ among CEZ-R bacterial numbers. ND, not detected

Pseudomonas during anaerobic digestion. The reduction rates of non-fluorescent *Pseudomonas* were 43.6% in mesophilic digestate and 56.9% in thermophilic digestate. The reduction rate of non-fluorescent *Pseudomonas* in the thermophilic digestate was higher than the reduction rate in the mesophilic digestate. However, the reduction rates of CEZ-S non-fluorescent *Pseudomonas* were 58.8% and 54.6% in the mesophilic and thermophilic digestates, respectively, which showed relatively a higher survival of CEZ-S non-fluorescent *Pseudomonas* in the mesophilic digestate than that in the thermophilic digestate. The population of CEZ-R non-fluorescent *Pseudomonas* increased slightly in the mesophilic digestate, and the increasing rate was 39.1% (approximately 1.4 times that of dairy manure). In thermophilic digestate, the number of CEZ-R non-fluorescent *Pseudomonas* decreased at a reduction rate of 69.2% (Fig. 3, D). In the classification of non-fluorescent *Pseudomonas*, the populations of both L-arginine-metabolizing and non-metabolizing non-fluorescent *Pseudomonas* decreased. No L-arginine-metabolizing non-fluorescent *Pseudomonas* was detected in both the mesophilic and thermophilic digestate (Fig. 3, E). The numbers of L-arginine-non-metabolizing non-fluorescent *Pseudomonas* decreased in the digestates, and the reduction rate in the mesophilic and thermophilic anaerobic digestion was 42.5% and 56.1%, respectively. The abundance of CEZ-R L-arginine-non-metabolizing non-fluorescent *Pseudomonas* increased in the mesophilic digestion among the L-arginine-non-metabolizing non-fluorescent *Pseudomonas*, which showed a rate of increase of 45.6% (approximately 1.5 times that in dairy manure). However, the number of L-arginine-non-metabolizing non-fluorescent *Pseudomonas* decreased in the thermophilic digestion, and the reduction rate was 67.8%. The population of CEZ-S L-arginine-non-metabolizing non-fluorescent *Pseudomonas* decreased in both digestates, and the reduction rates were 58.2% and 54.0% in the mesophilic and thermophilic digestate, respectively (Fig. 3, F). In the overview of these results, the number of total *Pseudomonas* and CEZ-R *Pseudomonas* was found to be reduced. However, the number of CEZ-S fluorescent *Pseudomonas* increased in correlation with the temperature during anaerobic digestion.

Increased numbers of fluorescent *Pseudomonas* were able to survive and grow under low-nutrient anaerobic conditions. As these *Pseudomonas* are thought to serve as processors of organic matter in dairy manure, cellulolytic *Pseudomonas* species such as *P. putida* and *P. alcaligenes*, which degrade monosaccharides into acetate (Noike, et al., 1985; Baserba, et al., 2012; Gopinath, et al., 2014; Resende, et al., 2014), and other organic fermentative fluorescent pseudomonads such as *P. stutzeri* and *P. luteola* (Resende, et al., 2014; Carlson and Ingraham, 1983), can be suggested to be the increased fluorescent *Pseudomonas* populations. However, Redfearn et al. (1966) and Ali Shah et al. (2014) reported *P. pseudomallei* and *P. mendocina*, respectively, as anaerobic digestion-related non-fluorescent *Pseudomonas* species. In addition, because of the properties of cetrimide agar medium admitting the growth of some bacteria other than *Pseudomonas* species, the white/non-fluor group might include not only *Pseudomonas* but also other bacteria that are not able to decompose L-arginine.

These white/non-fluor group bacteria decreased in number during the mesophilic and thermophilic anaerobic digestions in this study. However, white/non-fluor group bacteria must be identified by other methods.

Td and T_{90} of *Pseudomonas* species during anaerobic digestion

As shown in Fig. 3, the reduction in the *Pseudomonas* populations was the sum of the competition of increasing numbers of fluorescent *Pseudomonas* and decreasing numbers of non-fluorescent *Pseudomonas*. The calculated Td values of increased and T_{90} values of reduced *Pseudomonas* group abundances are summarized in Table 2. L-Arginine-metabolizing non-fluorescent *Pseudomonas* was not detected after anaerobic digestion because of the sufficient number of experimental digestion periods to reduce the population of this group, so it was considered that the T_{90} of this group was shorter than the HRT (mesophilic: 30 days, thermophilic: 15 days). The number of CEZ-R among L-arginine-non-metabolizing non-fluorescent *Pseudomonas* was observed to respond in a thermo-sensitive manner, increased with mesophilic digestion and decreasing with thermophilic digestion. The Td value at mesophilic digestion in this group (55.4 days) was much longer than that in fluorescent *Pseudomonas* (33.5 days) and the period of general mesophilic biogas plant (30 days), and the increasing rate at day 30 was not remarkable, at 45.6%. However, this fact should be noted because supplying these digestates to the field will lead to supplying antibiotic-resistant bacteria to the field.

Because the genus *Pseudomonas* consists of pathogenic (e.g., *P. aeruginosa*), phytopathogenic (e.g., *P. corrugate* and *P. viridiflava*), and PGPR (e.g., fluorescent *Pseudomonas* species) species, the ideal digestate for environments and plants is thought not to contain pathogenic, phytopathogenic species or antimicrobial-resistant bacteria but to contain higher amounts of PGPR species. L-Arginine-non-metabolizing non-fluorescent *Pseudomonas* was the only group of increased abundance among CEZ-R *Pseudomonas* during mesophilic digestion. Because this *Pseudomonas* group has a relatively long doubling time (Td = 55.4 days), it may possible to finish digestion of organic matter before fully increasing the CEZ-R *Pseudomonas* population in an actual mesophilic biogas plant (HRT = 30 days). However, the T_{90} value of CEZ-R other fluorescent *Pseudomonas* was 122.2 days; thus, most of the CEZ-R other fluorescent *Pseudomonas* would be considered to survive in the digestate of an actual mesophilic biogas plant. Overall, the T_{90} values of thermophilic-treated *Pseudomonas* were shorter than the T_{90} values of mesophilic-treated *Pseudomonas*. However, these values were longer than the general HRT of thermophilic digestion (15 days). Therefore, a longer treatment period will be required to reduce CEZ-R *Pseudomonas* and whole *Pseudomonas* species populations other than fluorescent *Pseudomonas*. Interestingly, a higher increasing rate and shorter Td value of fluorescent *Pseudomonas* was observed in thermophilic digestion than that in mesophilic digestion. Because the extension of HRT (i.e., digestion time) to eliminate CEZ-R, other fluorescent *Pseudomonas* may lead to increased numbers of CEZ-R L-arginine-non-metabolizing non-fluorescent

Table 2 Td and T_{90} values of classified group of *Pseudomonas* during anaerobic digestion

	Td (days)		T_{90} (days)	
	Mesophilic	Thermophilic	Mesophilic	Thermophilic
Total *Pseudomonas*	−	−	133.7	47.4
fluorescent *Pseudomonas*	33.5	11.3	−	−
Pseudomonas aeruginosa	−	−	−	−
Other fluorescent *Pseudomonas*	33.5	11.3	−	−
CEZ-R	−	−	122.2	27.6
CEZ-S	12.5	4.9	−	−
non-fluorescent *Pseudomonas*	−	−	120.7	41.0
L-arginine-metabolizing	−	−	< 30	< 15
CEZ-R	−	−	< 30	< 15
CEZ-S	−	−	< 30	< 15
L-arginine-non-metabolizing	−	−	124.8	42.0
CEZ-R	55.4	−	−	30.5
CEZ-S	−	−	79.1	44.5

−: increased after anaerobic digestion

Pseudomonas. Digestion at thermophilic temperature or post-sanitary treatment of digestate is better for the safe use of digestate. Generally, digestate is further treated in sterilization tanks by hyper-heat treatment in a biogas plant if necessary. In the case of thermophilic digestion, while there was no increase in the CEZ-R *Pseudomonas* group population in the digestate, the T_{90} values indicated that it is better to extend digestion time to obtain biological safety and PGPR-containing digestate.

From another point of view, while anaerobic digestion under optimum conditions to make the balance between suppressing pathogenic *Pseudomonas* and gaining plant growth-promoting *Pseudomonas* is important, it might also be important to compromise the survival of *Pseudomonas* species after applied to the field. If it is impossible to remove pathogenic and antimicrobial-resistant *Pseudomonas* completely, they might be eliminated by the homeostasis of microflora in the rhizosphere (Ramadan, et al., 2016). Similar principles might be applied to surviving CEZ-R *Pseudomonas* species. In the case of inherent resistance, antimicrobial-resistant bacteria would suffer the same fate of pathogens after application to an agricultural field. However, a remarkably different aspect regarding CEZ-R bacteria is the inclusion of resistant genes. Many β-lactamase genes, which lead to a resistant phenotype to cefems such as CEZ, have been found and are plasmid-mediated (Jacoby, 2006). These plasmid-mediated genes can be transferred into other bacteria after the extinction of host bacteria. Therefore, the attention to surviving CEZ-R *Pseudomonas* species and other bacteria, especially non-identified CEZ-R *Pseudomonas* species populations increased in the mesophilic digestate, will be necessary.

The quantity of *Pseudomonas* species will be an evaluation indicator of digestate. The pathogenic *P. aeruginosa* risks may be avoided, as they were not detected in dairy cow manure or the mesophilic and thermophilic digestate, and increased fluorescent *Pseudomonas* numbers can be expected to be affected as PGPR. However, the details of species and actual plant growth-promoting effect of digestates by fluorescent *Pseudomonas* were not examined in this study. In addition, it will also be needed to confirm whether the amount detected here is an effective value to promote the growth and suppress diseases of plants. Therefore, the effect of increased fluorescent *Pseudomonas* species numbers against phytopathogens was worthy of analysis.

4.4 CONCLUSIONS

Here, a bacterial indicator for healthy use of digestate for agricultural crops was presented. It is important to determine the operational conditions in a biogas plant by referring to the bacterial quantity in the digestate. The plating method using selection medium is a relatively simple and reliable method to quantify bacteria in the digestate. In the case of quantifying *Pseudomonas*, the classification into *P. aeruginosa*, fluorescent *Pseudomonas* and other *Pseudomonas* species was thought to be useful because this genus contains both beneficial and harmful species. In addition, it is also important to

quantify antimicrobial-resistant bacteria in the digestate. In this study, while the numbers of total and CEZ-R *Pseudomonas* were more reduced by anaerobic digestion performed at thermophilic temperature than they were at mesophilic temperature, the number of fluorescent *Pseudomonas* increased more in thermophilic anaerobic digestion. This increased fluorescent *Pseudomonas* population was thought to have a beneficial effect on plant growth. These results indicate that thermophilic digestate has an advantage both in the absence of harmful bacteria and in the presence of biological control bacteria against crops. However, energy input is required to heat and stir substrates in biogas plants. Furthermore, a large-scale digester tank and a digestate storage tank are necessary for the extension of HRT. Therefore, the balance between the benefits and construction costs of correlating the equipment or energy input must be considered to develop the plants.

General Discussion and Conclusions

At the 26th UN Climate Change Conference of the Parties (COP26) held from October 31 to November 12, 2021, more than 100 countries signed a commitment to reduce methane emissions by 30% from 2020 levels by 2030. Moreover, Japan has set the goal to achieve Carbon Neutrality by 2050 and supports local efforts by establishing a roadmap to reduce GHG emissions to net-zero by 2050. Although the sources of methane emissions are mainly wetlands, rice paddies, livestock, natural gas, and biomass combustion, methane emissions from the livestock environment are mainly those from fermentation in the digestive tract (burps) of ruminants and those associated with rice cultivation (rice paddies) and livestock waste management. When all these factors are considered together, they account for nearly 1.8% of Japan's total GHG emissions (GHG emissions from the overall agricultural field in 2019 account for 2.6% of Japan's total GHG emissions) ("National Greenhouse Gas Inventory Report of Japan 2021," Chapter 5, Agriculture Sector). Owing to the fact that methane from livestock manure is emitted during open-air deposition, composting, and drying processes, anaerobic digestion facilities for collecting methane through anaerobic processing in an enclosed space may effectively reduce methane emissions to the atmosphere. The CO_2 emitted from such biomass is carbon neutral, although CO_2 is emitted by burning the collected methane.

In this way, although anaerobic digestion is a superior technology for processing livestock manure, there are some problems using digestate as liquid fertilizer, and one example is contamination with weed seeds. Not only digestate but organic fertilizers derived from dairy cow manure often lead to the spread of weed seeds on agricultural land. This is because the seeds are excreted without being digested in the digestive tracts of dairy cows. Although there are several reports on weed seed mortality when processing livestock manure contaminated with weed seeds by composting, there are many uncertain points related to the anaerobic digestion process. The mortality effects of the anaerobic digestion process on weed seeds mixed into dairy cow manure are shown in Chapter 1. The viability of weed seeds depends on digestion temperature. Under mesophilic digestion, the survival rate of weed seeds was higher than that in thermophilic condition, with 0% in thermophilic digestion but a high percentage (81.6%) of seeds surviving in mesophilic digestion. Most seeds that survived in the mesophilic digestion process were dormant. Therefore, thermophilic anaerobic digestion is considered desirable to treat dairy cow manure to completely reduce weed growth after digestate fertilization.

Another issue is the spreading pathogens. In the livestock environment, humans, livestock, crops, and the environment are mutually interrelated. If there is a loss of health in any of them, it is possible to threaten the health of others. Therefore, the principle of One Health has been proposed– that there is a need to view the health of the ecosystem as an integrated whole. From the standpoint of "spread of pathogens from livestock manure," this study examined the effects of treatment temperatures during mesophilic and thermophilic anaerobic digestion processes on the survival of

pathogens, as shown in Chapter 2. In Japan, there are many cases where raw or untreated manure is often spread directly on agricultural fields. However, appropriate methods of handling manure are needed to minimize the risk of pathogen exposure to crops. Digestates from mesophilic and thermophilic biogas plants operating in Hokkaido, Japan, were collected to examine for residual counts of the *Coli-aerogenes* and *Enterococcus*. *Coli-aerogenes* and *Enterococcus* were not detected in digestate from the two thermophilic biogas plants, but *Coli-aerogenes* and *Enterococcus* were detected at low levels in digestate from the two mesophilic biogas plants. Therefore, the processing temperature is important to keep the safety of the digestate. This study showed that the thermophilic process was more effective in killing *Coli-aerogenes* and *Enterococcus*, suggesting that the thermophilic anaerobic digestion process is also effective for most of all pathogenic bacteria in dairy cow manure.

In addition to pathogens, antimicrobial-resistant bacteria are a problem. Residual antimicrobial-resistant bacteria can also lead to the spread of antimicrobial resistance. Through the lateral spread of transmissible antimicrobial resistance genes carried by plasmid resistance genes and transposons, antimicrobial-susceptible bacteria can become antimicrobial-resistant. Although dairy farming mostly uses cefazolin (CEZ) as a treatment for bovine mastitis, the substrate-specific extended-spectrum β-lactamase (ESBL) gene among CEZ-resistant (CEZ-R) genes responsible for CEZ resistance is a plasmid resistance gene, suggesting that there is a large risk of spreading CEZ-R bacteria in the livestock environment. The experiments in Chapter 3 showed the reduction effects of thermophilic anaerobic digestion on plasmid-born (plasmid-mediated) and non-plasmid-born resistant bacteria. The survival rate of antimicrobial-susceptible bacteria in digestate of thermophilic anaerobic digestion was 10.5% for CEZ-susceptible bacteria and 10.3% for ampicillin-susceptible bacteria, whereas that of resistant bacteria was 0.93% for cephazolin-resistant bacteria and 1.40% for ampicillin-resistant bacteria, indicating that resistant bacteria were eliminated more in thermophilic anaerobic digestion. Furthermore, the survival rate of bacteria with ESBLs (ESBL-producers) among CEZ-R bacteria was ~0.09%, a much lower survival rate among antimicrobial-resistant bacteria. Thus, the elimination effect among antimicrobial-resistant bacteria of thermophilic anaerobic digestion on bacteria is greater for ESBL-type-resistant bacteria.

Therefore, many antimicrobial-resistant bacteria could be reduced by the anaerobic digestion process. Factors that may eliminate general bacteria and pathogens include environmental stresses, such as temperature with protein denaturation, acid/alkali, and desiccation, as well as hydrogen sulfide as an intermediate product, higher fatty acids, ammonia, volatile fatty acids (VFAs), or competition for nutrients with other bacteria. Hydrogen sulfide penetrates the bacterial cell membrane, denatured proteins, and destroys bacterial metabolism. Higher fatty acids tend to accumulate with high-fat content in raw materials and can inhibit enzyme activity by adhering to the bacterial surface or penetrating the cell wall or membrane and, in some cases, also cause bacterial lysis. The longer the carbon chain is, the more toxic it is. Ammonia and VFAs are highly toxic in the state

of free ammonia and free VFAs, penetrating the bacterial cell membrane and being dissociated intracellularly, causing changes in intracellular pH, intracellular and extracellular proton imbalance, increased maintenance energy requirements, and inhibition of enzyme activity, resulting in cell death. Free ammonia tends to increase in thermophilic anaerobic digestion and dry anaerobic digestion, and VFA becomes rapidly more toxic at pH <7.

The factors associated with bacterial decrease are type of bacteria, digestion temperature, digestion method (batch or continuous, fully mixed or plug flow), hydraulic retention time (HRT), type of raw material (solid or liquid, mono-digestion or mixed digestion), and higher mortality is observed in higher temperature rather than anaerobic digestion, dry rather than wet, and batches rather than consecutive. The change in operating parameters given by the input of subcomponents in mixed digestion affects the bacterial flora. Moreover, the number of residual pathogens may be affected when chemicals, antibiotics, and heavy metals are mixed in raw and secondary materials. Although a similar mechanism could be established for antimicrobial-resistant bacteria, it is unclear why they exhibit a greater elimination effect on antimicrobial-resistant bacteria in anaerobic digestion. This is probably due to different bacterial repertoires between antimicrobial-resistant and -susceptible bacteria. Thus, it is expected that the anaerobic digestion process is a technology that can stop the spread of antimicrobial-resistant bacteria into the environment and make the One Health philosophy more practical.

Although the number of residual microorganisms is one indicator of safety in using digestate for growing crops, the number of bacteria in digestate can also be an indicator of appropriate operating conditions in biogas plants. However, there are not only harmful microorganisms but also bacteria that are beneficial for plant growth in digestate. These bacteria could promote plant growth by producing plant growth hormones or inhibiting infections of plant pathogenic microorganisms. The best known among these are *Bacillus* spp. and fluorescent pseudomonads. In *Pseudomonas* spp., there is a mixture of pathogenic species, such as *Pseudomonas aeruginosa*, and fluorescent species, such as *Pseudomonas fluorescens* and *Pseudomonas putida*, which are advantageous for crop growth. Some *Bacillus* spp. and fluorescent pseudomonads are commercially available and used as biological pesticides. Experimental results in Chapter 3 showed that *Pseudomonas*-ESBL slightly increased in digestate of the thermophilic biogas plant. *Pseudomonas* spp. relieve environmental stress by forming viscous biofilms, suggesting a resistant effect on high-temperature anaerobic conditions. Moreover, biofilms cause low antimicrobial penetration, indicating antimicrobial-resistant factors. However, for the same reason, the acquisition of resistance may be unlikely to occur through the incorporation of resistance genes. This study focused on survived *Pseudomonas* spp. in digestate and developed a simple and reliable quantitative method to classify and analyze *Pseudomonas* spp. into *P. aeruginosa*, fluorescent pseudomonads, and other *Pseudomonas* spp. using a culture method with selective media and polymerase chain reaction (PCR) method, as shown in Chapter 4. As a result of analyzing digestate

by conducting anaerobic digestion at thermophilic and mesophilic on a laboratory scale, there was an increase in fluorescent *Pseudomonas* in mesophilic and thermophilic digestate, with a significant increase in thermophilic digestate. This suggests that digestate could possibly have applications as a biological pesticide. *P. aeruginosa* was also not detected in either the raw material (dairy cow manure) or digestate, indicating a low risk of infection caused by *Pseudomonas* spp. As a result of examining the number of remaining CEZ-R bacteria in digestate, similar to the results in Chapter 3, there was a slight increase in mesophilic digestate but without significant difference. Therefore, there are few harmful bacteria and an increase in beneficial bacteria in thermophilic digestate with respect to *Pseudomonas* spp.

To use livestock manure safely and effectively, microbial risks should be avoided, and it is important to understand the characteristics of treatment methods. Although many pathogens are reduced by mesophilic and thermophilic treatment, considering the effectiveness of pathogen reduction, the anaerobic digestion process of livestock manure should be conducted at high temperatures. However, there is a need for more energy to maintain the biogas plant at higher temperatures and larger volume digester tanks to increase the throughput and extend HRT. Therefore, when constructing a biogas plant, it is important to understand the properties of livestock manure and organic waste (secondary raw material) to be mixed and treated and select an appropriate treatment method considering the balance between the profit to be gained from digestate, construction costs, and energy input. Because it is difficult to easily change an existing biogas plant once a system has been established, it is possible to obtain safer digestate by adding attached equipment, such as sterilization tanks. Some biogas plants install a sterilization tank to obtain safe digestate and treat digestate at a high temperature of 70 °C for ~1 h. In some foreign countries, including the European Union (EU), the United Kingdom, the United States, and China, hygiene standards for microorganisms in digestate have been established, and sterilization of digestate is mandatory in the EU and other countries.

Anaerobic digestion is a technology that produces renewable energy through effective processing of livestock manure. Furthermore, digestate is widely recognized as a good fertilizer and is epidemiologically safe. This study presented the possibility of anaerobic digestion to reduce the risks as spreading weed seeds, pathogens, antimicrobial-resistant bacteria, antimicrobial-resistant genes to dairy and agricultural field, and to enhance the number of bacteria which promote crop growth. I hope that this study may contribute to society by resolving issues with effective use of resources, reducing methane and CO_2 emissions, and livestock manure disposal issues.

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Japanese Abstract

第 1 章では，嫌気発酵処理が乳牛ふん尿に混入する雑草種子の生存に与える影響を明らかにするため，乳牛ふん尿に雑草（エゾノギシギシ）種子を投入し，中温（35 °C）および高温（55 °C）嫌気発酵処理を行った後の種子の発芽率，休眠種子の割合を調査した。エゾノギシギシ種子を 35 °C と 55 °C で熱処理した場合の生存率はそれぞれ 75.5%と 0%であったのに対し，同じ温度の嫌気発酵処理では 81.6%と 0%であった。35 °C の処理における生存率は嫌気発酵処理と熱処理では同程度であったが，1 次休眠および 2 次休眠の状態にある種子の割合は熱処理と比較して嫌気発酵処理で高い値であった。この結果は，嫌気発酵消化液は液肥としての利用価値が高い一方，中温発酵消化液では休眠状態にある雑草種子が残存する可能性があるため注意が必要であることを示している。

第 2 章では，家畜ふん尿を原材料として実規模のバイオガスプラントで嫌気発酵を行った場合における家畜ふん尿中の病原菌の死滅に関して検討を行った。北海道で現在稼動中である中温発酵槽（38 °C）と高温発酵槽（55 °C）を備えた計 4 カ所のバイオガスプラントの受け入れ槽，発酵槽，発酵後消化液貯留槽から糞尿を採取し，その中に含まれる大腸菌群と腸球菌を培養して発酵処理後の生存率を解析した。その結果，高温発酵プラントでは発酵槽内の糞尿，発酵後の消化液のどちらからも大腸菌群，腸球菌は検出されなかったが，中温発酵プラントでは発酵の進行に従い大腸菌群，腸球菌の生存数は減少するものの，発酵槽内の糞尿と発酵後の消化液に大腸菌群，腸球菌の生存が認められた。中温発酵における大腸菌群と腸球菌の 90％死滅時間（T_{90}）の平均値は，それぞれ 13.3 日と 16.7 日であった。

畜産業では家畜の疾病の治療や予防のために大量の抗菌性物質が使用されており，それによる畜産系廃棄物中の薬剤耐性菌の出現が世界的な問題となっている。セファゾリンは乳牛乳房炎の治療等に最も多く使用されている抗生物質であり，投与された乳牛の糞尿からはセファゾリン耐性菌が検出される。第 3 章では，高温嫌気発酵が乳牛ふん尿中のセファゾリン耐性および感受性菌，さらに基質特異性拡張型 β ラクタマーゼ（ESBL）産生菌（大腸菌，クレブシエラ/エンテロバクター/シトロバクター，プロテウス，シュードモナス，アシネトバクター）の残存に与える影響を検討した。その結果，セファゾリン耐性菌数は高温嫌気発酵によって 0.93%に減少し，その内訳は，大腸菌数，その他の大腸菌群数，その他の細菌数はそれぞれ，検出限界以下，0.27%，1.05%に減少したが，ESBL 産生シュードモナスは僅かに増加が認められた。これらの結果は畜産系廃棄物の高温嫌気発酵処理はセファゾリン耐性菌の拡散リスクを低減するが，一部の薬剤耐性菌は残存する可能性があることを示唆するものである。

高温嫌気発酵で ESBL 産生シュードモナスの僅かな増加が認められたことから，第 4 章ではセファゾリン耐性シュードモナス属菌を重点的に解析した。シュードモナス属菌には病原性のもの，植物の生育を助長するものなどの種が混在しているため，市販の PASA 培地と新しく作製した CAP 培地（市販の Cetrimide 培地に L-アルギニンとフェノールレッドを

添加し，発色の違いによって菌種を分類する培地）を用いて，*P. aeruginosa*，*P. fluorescens*，その他のシュードモナス属菌に分類し，定量的な解析を行った。これらの分類の裏付けはPCR による確認で行った。中温，高温いずれの嫌気発酵後にはシュードモナス属菌は減少し，嫌気発酵処理の効果は中温嫌気発酵に比べて高温嫌気発酵が大きいことが分かった。*P. aeruginosa* は採取した糞尿，中温・高温嫌気発酵処理後の発酵消化液のいずれにも検出されなかった。一方，中温嫌気発酵消化液中のセファゾリン耐性シュードモナスは発酵処理前の糞尿よりも増加していた。*P. fluorescens* は糞尿中に存在し，すべてセファゾリン耐性であったが，中温，高温嫌気発酵により検出されなくなった。*P. aeruginosa* と *P. fluorescens* 以外の蛍光性シュードモナスは嫌気発酵処理後に増加が認められ，その多くはセファゾリン感受性であった。また，蛍光性シュードモナス以外のセファゾリン耐性シュードモナス属菌も中温嫌気発酵処理により増加が認められた。細菌数の割合から，中温嫌気発酵処理により増加が認められたセファゾリン耐性シュードモナス属菌の多くは蛍光性シュードモナス以外の菌であると考えられた。このように，中温嫌気発酵では蛍光性シュードモナス以外のセファゾリン耐性シュードモナス属菌が増加したことから，嫌気発酵消化液を液肥として利用する際には環境への耐性菌拡散のリスクを考慮しなければならないと考えられた。

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