Energy Recovery and Pathogen Inactivation with Dry Co-digestion of Food Waste and Pig Manure

by **Yan Jiang**

Civil Engineering, College of Engineering & Informatics, National University of Ireland, Galway, Ireland

Research Supervisor:

Prof. Xinmin Zhan, Civil Engineering, NUI Galway

Dr. Peadar G. Lawlor, Teagasc, Animal & Grassland Research & Innovation Centre, Cork

Dr. Gillian E. Gardiner, Department of Science, Waterford Institute of Technology

Professor of Civil Engineering: Padraic E. O'Donoghue

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Abstract

Anaerobic digestion is one of the best available technologies for food waste (FW) and pig manure (PM) management by producing methane-rich biogas. However, mono digestion of FW or PM is easily inhibited by high volatile fatty acid (VFA) or ammonia. Co-digestion of FW and PM can provide an effective solution to address these issues due to the buffering interactions between the VFA and ammonia. Compared with wet digestion, dry digestion can reduce the digester volume significantly, thereby decreasing initial capital expenditure and the energy consumption required for heating.

In this research, batch dry co-digestion of FW and PM was conducted in laboratory-scale digesters at the total solid (TS) content of 20%. The research objectives were to assess (1) the feasibility and optimal operation conditions of dry co-digestion systems; (2) methane production kinetics and the inhibition mechanisms; (3) the biosafety of digestate, i.e. the inactivation of enteric indicator bacteria, including total coliforms, *E. coli*, enterococci and *Salmonella*; and (4) microbial community structure evolution in dry co-digestion systems.

The results showed that preferable operation conditions were obtained at a digestate inoculum rate of 50% and a FW/PM ratio of 50:50, with an average specific methane yield (SMY) of 252 mL/g VS_{added} (volatile solids). Using digestate as inoculum didn't increase the total amount of SMY but significantly decreased the lag phase from 28 days to 13 days compared with using dewatered anaerobic sludge as inoculum. Total VFA was the main inhibition factor on methane production ($P<0.001$), and the total VFA concentration was suggested to be < 20.0 g/L to avoid complete inhibition.

Dry co-digestion of FW and PM can effectively inactivate the enteric indicator bacteria, as *E. coli* and total coliform counts decreased below the limit of detection (LOD, 10^2 CFU/g) within 4-7 days, with free VFA being identified as a significant inactivation factor. *Enterococci* decreased to below the LOD within 12-31 days, with digestion time the most significant inactivation factor. *Salmonella* was completely eliminated within 6-7 days. Statistical analysis results showed that pH, VFA type, VFA/ammonia concentration and *Salmonella* serotype all significantly impacted *Salmonella* inactivation ($P < 0.01$). In VFA minimum inhibitory concentration (MIC) tests, the inhibitory effect sequence was in the order of pH > VFA concentration > VFA type > *Salmonella* serotype; and in ammonia MIC tests, the inhibitory effect sequence was in the order of ammonia concentration > pH > *Salmonella* serotype. At the same concentration, the inhibition effect of VFA was much greater than that of ammonia.

The inoculum was more significant in determining the microbial community structure than the FW/PM ratio. Hydrogenotrophic methanogenesis was an important methane production pathway, with *Methanoculleus* the dominant methanogen. Significant correlation was observed between the relative abundance of specific microbial taxa and digesters' physicochemical parameters. Based on correlation analysis, the dry co-digestion associated functions of some previously poorly reported bacteria were predicted here for the first time.

The results in this study indicate that dry co-digestion of FW and PM is an effective way for treatment of both substrates, with recovery of methane-rich biogas and safe digestate. The data obtained can provide guidance for on-farm engineering practice. The preliminary prediction on the functionality of previously poorly described bacteria will provide reference for further study.

Keywords: Co-digestion, dry digestion, *E. coli*, enterococci, food waste, hydrogenotrophic methanogenesis, inoculum, minimum inhibitory concentration (MIC), pig manure, *Salmonella*, syntrophic oxidation, total coliforms, volatile fatty acid (VFA) inhibition

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Declarations

This thesis or any part thereof, has not been, or is not currently being submitted for any degree at any other university.

Yan Jiang

The work reported herein is as a result of my own investigations, except where acknowledged and referenced.

Yan Jiang

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List of Abbreviations

AC: antimicrobial agent control

ANOVA: analysis of variance

ATP: adenosine triphosphate

ATR: acid tolerance response

BGA: brilliant green agar

BHI: brain heart infusion

BMW: biodegradable municipal solid waste

BPW: buffered peptone water

CFU: colony forming unit

CM: cattle manure

CTBX: ChromoCult tryptone bile X-glucuronide

DM: dry matter

DRT: decimal reduction time

FAN: free ammonia nitrogen

FEW: family-wise error rate

FW: food waste

GC: gas chromatography

GC: growth control

GRG: generalized reduced gradient

HAC: acetic acid

HB: butyric acid

HIB: isobutyric acid

HIV: isovaleric acid

HPLC: high performance liquid chromatography

HPR: propionic acid

HRT: hydraulic retention time

HV: valeric acid

LOD: limit of detection

MANOVA: Multivariate analysis of variance

MC: moisture content

MIC: minimum inhibitory concentration

MPN: most probable number

MRD: maximum recovery diluent

MSW: municipal solid waste

MVFA: equimolar mixture of the individual volatile fatty acid

NA: nutrient agar

NIC: non-inhibition concentration

NOB: nitrite-oxidizing bacteria

OD: optical density

OFMSW: organic fraction of municipal solid waste

OLR: organic loading rate

PCoA: principal coordinate analysis

PFU: plaque forming unit

PM: pig manure

PMF: proton motive force

RSD: relative standard deviation

SC: sterility control

SCOD: soluble chemical oxygen demand

SD: standard deviation

SDMY: specific daily methane yield

SHF: continuous separated hydrolysis and fermentation

SHY: specific hydrogen yield

SMY: specific methane yield

SRT: sludge retention time

SSE: sum of square error

SSF: simultaneous saccharification and fermentation

STP: standard temperature and pressure

TAN: total ammonia nitrogen

TS: total solids

TSA: tryptone soy agar

VFA: volatile fatty acid

VS: volatile solids

XLD: xylose lysine deoxycholate

Chapter 1
Introduction

1.1 Background

Human activity produces biowastes that can be recovered as sources of bioenergy. Examples of these include animal manure generated in the farming sector, municipal waste, catering waste in restaurants, yard waste produced in residential accommodation, and so on. Food Harvest 2020 predicts a 50% increase in milk production, and 20%, 20%, 50% and 10% increases in the output values of the Irish beef, sheep, piggery and poultry sectors, respectively (DAFM, 2018). It will lead to expansion of the Irish livestock industry and consequently an increase in the amount of animal manure generated. According to the CSO livestock survey, there were 7.36, 5.25 and 1.56 million cattle, sheep and pigs, respectively, in Ireland in 2017. The average slurry/manure production for cattle, sheep and pigs is reported to be 5.08, 0.05 and 1.45 tonnes/annum/head, respectively (Singh et al., 2010). As a result, the total amounts of manure generated by cattle, sheep and pig farming in 2017 were estimated to be 37.4, 0.26 and 2.26 million tonnes, respectively. The commonly used method for disposal of animal manure in Ireland is land spreading. However, untreated manure is a reservoir of rich nutrients (nitrogen, phosphorous, potassium, organic matter, etc), various pathogenic microorganisms (bacteria, viruses and protozoa), and genes encoding resistance to antibiotics, heavy metals and biocides (Manyi-Loh et al., 2016). The improper use of untreated manure may therefore cause pollution to water, soil and air, resulting in serious environmental and public health problems (Manyi-Loh et al., 2016). A number of studies have shown that land application of manure transmits pathogens, increases the abundance of antibiotic-resistant bacteria in soil and disseminates antibiotic and metal resistance genes to the farm environment (Chee-Sanford et al., 2009; Udikovic-Kolic et al., 2014; Venglovsky et al., 2017). As a result of the environmental and health concerns outlined above, legislations including the Nitrates Directive (91/676/EEC), the Water Framework Directive (2000/60/EC) and policies on greenhouse gas (GHG) emission mitigation have made it necessary to develop alternative strategies for the management of livestock manure.

The other main types of organic waste generated in Ireland is biodegradable municipal waste (BMW), which is typically composed of food and garden waste, wood, paper, cardboard and textiles (EPA, 2018a). Landfilling used to be a main treatment method for BMW. The EU Landfill Directive (1999/31/EC) requires a decrease in the maximum BMW allowed to be landfilled from 916 kilotons in 2010 to 610 kilotons in 2013 and 427 kilotons in 2016. Accordingly, the landfill levy was increased from €50/tonne of waste disposed in 2011 to €65/tonne in 2012 and €75/tonne in 2013. The increasing cost together with increasingly stringent regulations make the diversion of BMW from landfill necessary. Besides, the BMW may host pathogenic microorganisms and resistance genes, thus biosafety should also be considered when selecting alternatives to landfilling.

The high volatile solids (VS) to total solids (TS) ratios (VS/TS) of food waste (FW, 80% - 98%) and pig manure (PM, 71% - 82%) indicate the high potential of energy recovery from these substrates via anaerobic digestion (Dennehy et al., 2017; Forster-Carneiro et al., 2008b; Jiang et al., 2018; Lee et al., 2009). Anaerobic digestion is one of the best available treatment methods for them by achieving energy recovery as a methane-rich biogas, waste stabilization, GHG emissions mitigation and nutrient recovery by using the digested waste as a fertilizer. O'Shea et al. (2016) studied the biomethane potential of waste substrates in Ireland, including animal manure, slaughterhouse waste, milk processing waste and household organic waste, and estimated that the total biomethane resource could replace 7.6% of natural gas usage, 7% of energy consumption in transport, 26.5% of industrial gas use, or 52% of residential gas use. However, mono-digestion of FW or PM is easily inhibited by high volatile fatty acid (VFA) or ammonia concentration (Hansen et al., 1998; Zhang et al., 2011a). Co-digestion of PM and FW can solve these problems because of the buffering effect between ammonia and VFA, and the trace elements in PM and high organic matters in FW can result in synergistic effects (Dennehy et al., 2016; Mata-Alvareza et al., 2014). Wet co-digestion is currently most widely used. However, compared with wet digestion, dry anaerobic digestion is of interest because it (1) can reduce the digester volume significantly, (2) requires less energy for heating, and (3) avoids the need for post-digestion separation of liquid digestate. It has been reported that it is very expensive and complicated to treat liquid digestate if local land spreading is not available (Carney et al., 2013; Zhang et al., 2012; Zhang et al., 2011b). Therefore, dry co-digestion of FW and PM is expected to provide a promising solution to their management.

Animal manure is frequently referred to as a reservoir of pathogenic microorganisms. The most prevalent zoonotic pathogens reported in PM include *Escherichia coli* O157:H7, *Salmonella*, *Yersinia*, *Campylobacter*, *Giardia* and *Cryptosporidium* (Guan & Holley, 2003). These pathogens can be transmitted from animals to humans and cause various diseases, all of which can be life-threatening (Scallan et al., 2015). McCarthy et al. (2013) reported *Salmonella* was detected in the manure from half of the 33 Irish pig farms sampled and that most of the *Salmonella* recovered was multi-drug resistant *S. Typhimurium*. A follow-on study then showed that *Salmonella* inoculated into stored manure was capable of surviving for as long as 84 days (McCarthy et al., 2015). Ziemer et al. (2010) also reported that pathogens such as *Salmonella*, *E. coli*, *Campylobacter*, *Yersinia enterocolitica* and *Listeria* survived during manure storage and subsequently survived in soil and water after the land application for considerable time. Food waste also harbours various disease-causing organisms. Seven pathogens have been identified as the leading causes of foodborne diseases across the United States, Canada and Europe, including non-typhoidal *Salmonella*, *Toxoplasma*, *Campylobacter*, norovirus, *Listeria monocytogenes*, *Clostridium perfringens* and *E. coli* O157 (Scallan et al., 2015). Therefore, biosafety risks during dry co-digestion of FW and PM require further investigation.

Anaerobic digestion is performed by different microbes, including hydrolysis and acidification bacteria, acetogens accompanied by acetoclastic methanogens and/or syntrophic oxidizing bacteria accompanied by hydrogenotrophic methanogens (Nelson et al., 2011). Compared with acetoclastic methanogens, hydrogenotrophic methanogens are more resistant to stress conditions such as high VFA and ammonia concentrations

(Demirel & Scherer, 2008; Hori et al., 2006). Hydrogenotrophic methanogens need to cooperate with syntrophic oxidizing bacteria to relieve the inhibition from hydrogen partial pressure and produce methane (Hattori, 2008; Müller et al., 2010). Syntrophic oxidation together with hydrogenotrophic methanogenesis might be an important methane production pathway during dry co-digestion of FW and PM due to the high VFA and ammonia accumulation. However, the microbial community structure during dry co-digestion of FW and PM was rarely studied.

1.2 Objectives

In this study, dry co-digestion of FW and PM was conducted to investigate:

(1) the effects of inoculum rates (dewatered sludge at the rate of 25% and 50% based on VS) and FW/PM ratios (0:100, 25:75, 50:50, 75:25 and 100:0 based on VS) on methane production kinetics, system stability and microbial community structure.

(2) the effects of inoculum types (digestate and dewatered sludge at a rate of 50% based on VS) and FW/PM ratios (50:50 and 75:25 based on VS) on system performance and the inhibition of high VFA concentrations on methane production.

(3) the effects of inoculum types and substrate ratios on the structure of the microbial community and to explore the potential roles of microbes, whose functionality in dry co-digestion systems is previously poorly described.

(4) the effects of different inocula and FW/PM ratios on the inactivation of enteric indicator bacteria, including *E. coli*, total coliforms and enterococci, and the possible mechanisms of their inactivation.

(5) the inactivation of *Salmonella* in dry co-digestion systems and the effects of pH, VFA and ammonia on its inactivation.

1.3 Procedures

The structure of the research project is shown in Figure 1-1.

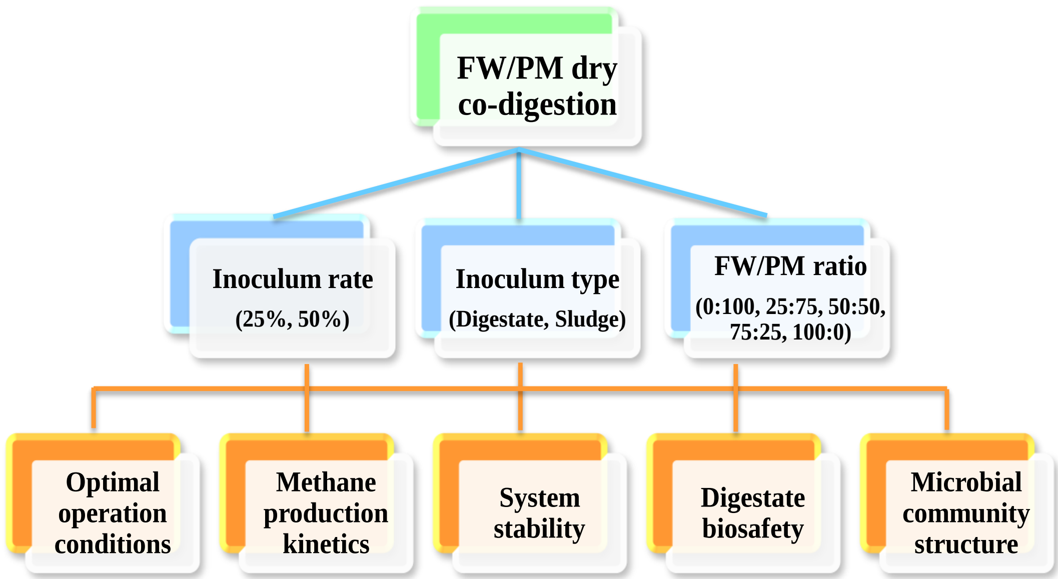


Figure 1-1: Flow chart of the research

1.3.1 Batch dry co-digestion experiment

Batch dry co-digestion of FW and PM was conducted in 1 L glass digesters. In the first batch experiment, the inoculum was dewatered anaerobic sludge collected from a local wastewater treatment plant in Galway City, Ireland. The substrates and inoculum were fed into the reactors according to the required inoculum rates (25% and 50% based on VS) and FW/PM ratios (0:100, 25:75, 50:50, 75:25 and 100:0 based on VS). Tap water was added to adjust the TS content in each digester to 20%. After feeding all the digesters were placed in an incubator at 37 ^{o}C and shaken by hand every day.

In the following experiments, the digestate obtained from previously operated dry co-digestion systems was also used as the inoculum to assess the effect of inoculum types. The digesters were operated at the selected operation conditions based on previous experiment results.

1.3.2 Statistical analysis

Statistical analysis was performed using R (version 3.3.2) and SPSS 22.0 (IBM, USA). Differences and correlations were considered significant at $P < 0.05$.

Shapiro-Wilk test was used to test the normality of the data, with $P > 0.05$ indicating normal distribution. Comparisons between two factors were performed using the Mann-Whitney U test (non-normality) or Student T test (normality) according to the normality test results. Comparisons among different levels in one factor (≥ 3) were conducted using one-way analysis of variance (ANOVA) followed by Bonferroni post hoc test for pairwise comparison to control the family-wise error rate (FWE). The effects of multi-factors and their interactions on dependent variables were conducted by multivariate analysis of variance (MANOVA).

Correlation analysis was performed using a two-tailed Spearman's rank order correlation. Multiple linear regression analysis was performed to determine the effects of multiple independent variables on dependent variables. The stepwise method was used to avoid co-linearity among the variables.

Range analysis was performed to assess the effect sequence of different factors, and Bonferroni post hoc multiple comparisons were used to explore the effect sequence of different levels in each factor.

1.4 Structure of thesis

This dissertation comprises 8 chapters:

Chapter 1 is the introduction. The background to the research, main objectives and research procedures are presented.

Chapter 2 reviews the arising and treatment of FW and PM, dry anaerobic digestion and the pathogen inactivation during anaerobic digestion systems.

Chapter 3 studies the effects of inoculum rate (dewatered sludge at the rate of 25% and 50% based on VS) and FW/PM ratios (0:100, 25:75, 50:50, 75:25 and 100:0 based on VS) on the system performance, stability and microbial community structure during dry co-digestion.

Chapter 4 studies the effects of inoculum types (digestate and dewatered sludge at a rate of 50% based on VS) and FW/PM ratios (50:50 and 75:25 based on VS) on methane production kinetics and VFA inhibition.

Chapter 5 details the effects of inoculum types and FW/PM ratios on the microbial community structure, and explores the possible functionality of previously poorly described microbes associated with dry co-digestion of FW and PM.

Chapter 6 studies the inactivation of enteric inhibitor bacteria (*E. coli*, total coliforms and enterococci) and their inactivation mechanisms in dry co-digestion systems.

Chapter 7 studied the inactivation of *Salmonella* during dry co-digestion of FW and PM, and the effects of pH, VFA and ammonia on *Salmonella* inactivation are analyzed.

Finally, Chapter 8 presents the conclusions drawn from all the studies described in Chapters 3-7. Recommendations for further research are also put forward.

Chapter 2
Literature review

Both FW and PM are biodegradable wastes produced in Ireland. In this chapter, the literature of management of both biowastes, dry anaerobic digestion and the pathogen inactivation during anaerobic digestion systems are reviewed.

2.1 Introduction

The Global Food Loss and Food Waste report (Gustavsson et al., 2011) states that ~1.3 billion tonnes of food ends up as waste globally every year. This is equivalent to almost one third of global food production. The exact amount of FW is not available in Ireland, but the amount of BMW can be used to approximately estimate the amount of biowaste which mainly consists of FW. In 2017, about 538 kilotons BMW was produced in Ireland, among which 307 kilotons was disposed to landfills (EPA, 2018a); this was under the Landfill Directive (1999/31/EC) limit of 427 kilotons. The amounts of BMW sent for composting and anaerobic digestion were 182 and 49 kilotons, respectively (EPA, 2018b). Therefore, the proportion of BMW treated by anaerobic digestion was < 10%.

Pig manure is an organic by-product of agricultural systems. As mentioned in Chapter 1, Section 1.1, the total PM production was estimated to be 2.26 million tonnes in 2017, with land spreading the main application method. However, the Nitrates Directive (91/676/EEC) requires the amount of livestock manure applied on land be limited at 170 kg organic nitrogen per hectare per year, which limits the amount of manure used on land. It therefore asks for a substitute treatment method.

In 2015, the contribution of total renewable energy to the gross final consumption (GFC) was 9.1% in Ireland, just over halfway of the 2020 target of 16% (Howley & Holland, 2016). The proportions of electricity, transport and heating generated from renewable energy were 25.3%, 5.7% and 6.5%, respectively, still a long way from the 2020 targets of 40%, 10% and 12% (Howley & Holland, 2016). The increasing amounts of FW and PM production together with the demand for renewable energy indicate recovery of energy from biowastes is crucial, thereby necessitating the development of alternative strategies for the management of FW and PM.

2.2 Treatment methods of food waste and pig manure

2.2.1 Food waste treatment

2.2.1.1 Anaerobic digestion

Anaerobic digestion of FW for methane production is commonly studied, but the specific methane yield (SMY) varied greatly under different operation conditions. The effects of temperature (30 - 55 ^{o}C at a step of 5 ^{o}C) and hydraulic retention time (HRT, 8, 10 and 12 days) on anaerobic digestion of liquor FW (TS content of 12.38%) were studied by Kim et al. (2006), and the highest SMY (223 mL/g $SCOD_{degraded}$) was obtained at 50 ^{o}C and the HRT of 12 days. Forster-Carneiro et al. (2008b) assessed the effects of TS content (20%, 25% and 30%) and inoculum percentage (20% - 30%) on anaerobic digestion of FW, and the best performance was obtained at the TS content of 20% and inoculum percentage of 30%, with a SMY of 490 mL/g VS. Izumi et al. (2010) studied the impact of the particle size of FW on anaerobic digestion and found after beak milling at 1000 rpm, the methane yield was improved by 28% than control. Grimberg et al. (2015) found two phase mesophilic digestion of FW increased the SMY to 446 mL/g VS compared with that of 380 mL/g VS in the single phase system. A SMY of 402 mL/g VS was reported when treating source-segregated domestic FW at 42 ^{o}C in a full scale biogas plant (Banks et al., 2011). Zhang et al. (2007) conducted batch anaerobic digestion of FW at 50 ^{o}C, and obtained the SMY of 435 mL/g VS after 28 days. El-Mashad and Zhang (2010) reported the SMY of 353 mL/g VS from batch anaerobic digestion of FW at 35 ^{o}C after 30 days.

However, the mono-digestion of FW is not stable and easily inhibited by high VFA concentrations. Zhang et al. (2011a) found the methane production from mono-digestion of FW started to decrease from Day 18 and ceased on Day 35 due to the accumulation of VFA (18,000 mg/L). Lin et al. (2011) also reported almost no methane was produced from mono-digestion of FW at the VFA concentration of 8,887 mg/L. Co-digesting FW with other substrates may relieve the VFA inhibition, increase the methane yield, shorten the lag phase and improve the system stability compared with mono-digestion systems, due to the buffering effect, optimization of the carbon to nitrogen ratio (C/N) and the addition of trace elements (Dennehy et al., 2016; Dennehy et al., 2017). Zhang et al. (2011a) found the methane production ceased on Day 35 during mono-digestion of FW with the SMY not given in the text, while addition of 17% piggery wastewater resulted in a SMY of 388 mL/g VS_{added} due to the introduction of trace elements. The effects of trace elements were also proved by Banks et al. (2012), because addition of 0.16 and 0.22 mg/kg Se and Co to fresh substrate effectively relieved VFA accumulation during FW anaerobic digestion and obtained the specific biogas production of 750 mL/g VS_{added}. Zhang et al. (2013) indicated at the FW/cattle manure (CM) ratio of 2, the SMY was improved by 41.1% and 55.2% in batch and semi-continuous tests, respectively, because of the adjusted C/N ratio and enhanced buffer capacity between ammonia and VFAs. Co-digestion of fruit and vegetable waste with FW at the optimum ratio of 1:1 had the SMY of 490 mL/g VS (Lin et al., 2011). Co-digestion of FW and straw at 5:1 increased the SMY by 39.5% to 392 mL/g VS compared with mono-digestion of FW (Yong et al., 2015).

2.2.1.2 Hydrogen production

Fermentative hydrogen production from FW is also widely studied. Temperature is an important factor affecting hydrogen production. Shin et al. (2004) reported hydrogen production from FW at thermophilic condition was much higher than that at mesophilic condition. At the HRT of 5 days, the specific hydrogen yields (SHY) were 28.4 - 46.3

mL/g VS at 55 ^oC and 1.3 - 5.0 mL/g VS at 35 ^oC, respectively. At the VS concentration of 6 g/L, the highest SHY of 91.5 mL/g VS was obtained with the HRT and temperature of 5 days at 55 ^oC, respectively (Shin et al., 2004). Algapani et al. (2016) reported thermophilic temperature (55 ^oC) was advantageous than hyperthermophilic temperature (70 ^oC) to pretreat FW, with suspended solids solubilization of 47.7% and 29.5% and total VFA/ SCOD ratio of 15.2% and 4.9%, respectively; the stable SHY of 70.7 mL/g VS was obtained at 55 ^oC with the HRT of 5 days and pH of 5.5. Kim et al. (2004) indicated proper addition of sewage sludge to FW can enrich the protein content so as to enhance the hydrogen production potential, and the maximum SHY (122.9 mL/g carbohydrate-COD) was observed at the FW/sludge ratio of 87:13 and the VS concentration of 3.0%. Han and Shin (2004a) reported a high FW fermentation efficiency was obtained at the initial dilution rate of 4.5 d^{-1}, and when it was adjusted to 2.3 d^{-1}, the fermentation efficiency was improved to 70.8%, with 19.3%, 36.5% and 15.0% of the removed COD being converted to hydrogen, VFA and ethanol, respectively. Shin and Youn (2005) got the optimal operation conditions at the pH of 5.5, HRT of 5 days and the organic loading rate (OLR) of 8 g VS/L/d for hydrogen production from FW, with the SHY of 2.2 mol /mol-hexose consumed. A combined solid-state fermentation + dark fermentative hydrogen production from FW resulted in the best hydrogen production of 39.14 mL/g wet weight (219.91 mL/g VS_{added}) (Han et al., 2015).

Simultaneous hydrogen and methane production was studied in thermophilic - mesophilic two-stage systems. Chu et al. (2008) studied combined thermophilic hydrogen production [HRT = 1.3 days, OLR = 38.4 kg VS/(m^3 d)] and mesophilic methane production [HRT = 5.0 days, OLR = 6.6 g kg VS/(m^3 d)] in a two stage process, and obtained the hydrogen and methane yields of 205 mL/g VS_{added} and 464 mL/g VS_{added}, respectively. Algapani et al. (2018) reported the optimal HRT of 5 days and 15 days for hydrogen (104.5 L/kg VS_{added}) and methane (526 L/kg VS_{added}) productions, which accounted for 4% and 55% of the COD, respectively. In another two-stage hydrogen and methane production systems, the optimum OLR and sludge retention time (SRT) were 22.65 kg VS/ (m^3 d) and 6.7 d for hydrogen production and 4.61 kg VS/(m^3 d) and 26.7 d for methane production, and the SHY and SMY were 65 mL/g VS and 546 mL/g VS, respectively, accounting for 5.8% and 82.2% of the influent COD (Wang & Zhao, 2009). Han and Shin (2004b) developed an innovative two-stage process to produce hydrogen and methane from FW; at the OLR of 11.9 kg VS/(m^3 d), the SHY and SMY were 0.31 m^3/kg VS_{added} and 0.21 m^3/kg VS_{added}, with the COD conversion of 28.2% and 69.9%, respectively. Hydrothermal pretreatment of 140 ^oC was reported to greatly promote the solubilization of FW, which increased the energy conversion efficiency from 46.9% to 78.6% and resulted in the SHY and SMY of 43.0 mL/g VS and 511.6 mL/g VS, respectively (Ding et al., 2017). Silva et al. (2018) reported co-digestion of FW, sewage sludge and glycerol maximized energy production, and the optimal conditions were the FW/sludge ratio of 2:1 and glycerol addition of 1%, with the SHY and SMY of 140 mL /g VS and 342 mL /g VS, respectively.

2.2.1.3 Bioethanol production

The feasibility of ethanol production from FW was studied by Kim et al. (2011), and the ethanol yields were reported to be 0.3 g/g TS and 0.2 g/g TS in continuous separated hydrolysis and fermentation (SHF) process and simultaneous saccharification and fermentation (SSF) process, respectively. Kim et al. (2008) reported the optimal pH, temperature and enzyme concentration were 5.2, 46.3 ^oC and 0.16% (v/v) for enzymatic saccharification, and the optimal pH, temperature and fermentation time were 6.85, 35.3 ^oC and 14 h for ethanol fermentation, with the reducing sugar and ethanol production of 120.1 g/L and 57.6 g/L, respectively. Le Man et al. (2010) studied ethanol production from FW leachate, and the maximum ethanol production of 24.17 g/L was obtained at the pH, temperature and reducing sugar concentration of 5.45, 38 ^oC and 75 g/L, respectively. The SHF ethanol production from FW at high TS contents led to the final ethanol yield of 107.58 g/kg TS (Matsakas et al., 2014). Yan et al. (2012) studied the production of ethanol from concentrated FW hydrolysates with yeast cells immobilized on corn stalk, and the maximum ethanol yield of 0.43 g/g of reducing sugar was achieved at the HRT of 3.1 h. Huang et al. (2015) studied ethanol production from high TS content FW (35%) with vacuum recovery; the results showed the ethanol inhibition was effectively relieved and the ethanol yield increased from 327 g/kg TS to 358 g/kg TS. The effect of FW pretreatment on the production of ethanol was also studied. Ma et al. (2016) reported after acid pretreatment, the reducing sugar of FW increased from 46% (w/w) to 62%, and the maximum ethanol yield of 0.047 g/g soluble solid in liquid hydrolysate was obtained at the fermentation time of 40 h and the immobilized bead ratio of 54:100 (w/v).

2.2.1.4 Composting

Composting of FW can provide nutrient enriched soil conditioner. Chang et al. (2006) studied the effects of air suction rate, seeding and agitation on the composting of synthetic FW, and the optimal operating parameters were 1.6 L air/kg dry solid, 32% of seeding and 50% of agitation time, respectively. While FW was co-composted with green waste, the moisture content (MC) of 60% and C/N ratio of 19.6 were reported as optimal conditions, which reduced VS by 33% in 12 days. The selection of bulking agents is also important for composting of FW. Adhikari et al. (2009) indicated FW/chopped wheat straw ratio of 8.9:1 and FW/chopped hay ratio of 8.6:1 were effective for composting. During composting the water loss through evaporation and leaching was 0.67 - 0.71 kg/kg, much higher than the N loss of 2.7 - 3.2 g/kg, thereby resulting in higher total nitrogen contents in the composts than those in the original substrates. Rice husk was also reported as a good bulking agent for FW composting; sawdust could be mixed with rice husk but the proportion should be controlled < 50%, while rice bran was not a suitable agent (Chang & Chen, 2010). Adhikari et al. (2008) indicated chopped wheat straw and chopped hay showed best properties as bulking agents, with a water absorption capacity of > 500% and high C/N ratio of ~ 50 at a neutral pH.

Odor is a significant problem to be concerned during composting of FW. Ammonia, amines, dimethyl sulfide, acetic acid, ethyl benzene and p-Cymene were reported as critical components of odors during FW composting (Mao et al., 2006; Tsai et al., 2008). Komilis and Ham (2006) reported composting of FW produced 370 g CO_2-C and 34 g NH_3-N per kg dry substrate. He et al. (2000) found the emission of N_2O was relatively high at the beginning of FW composting, and decreased to 0.53 ppmv after 2 days. The addition of composted cattle manure increased N_2O emissions.

2.2.2 Pig manure treatment

2.2.2.1 Anaerobic digestion

Anaerobic digestion of PM is commonly studied. However, mono-digestion of PM is prone to inhibition caused by high ammonia concentration and a subsequent low C/N ratio of 5 - 8 (Murto et al., 2004). Co-digestion with energy-rich substrates is a promising method to solve this problem, which can optimize the C/N ratio, enhance system stability, increase SMY yield and decrease lag phase.

The efficiency of co-digestion is affected by various operation conditions, e.g. pH, C/N ratio and OLR. Zhang et al. (2015) studied the thermophilic co-digestion of PM and maize stalk, and found pH 6.81and the C/N ratio of 26:1 were optimal operation conditions, with the maximum biogas production of 146 mL/g VS. Wu et al. (2010) studied co-digestion of PM with corn stalks, oat straw or wheat straw, and found the optimal C/N ratio was obtained at 20:1, with the daily maximum biogas volumes being increased by 11.4, 8.45 and 6.12-fold, respectively. The OLR also affect the stability of PM digestion. Glanpracha and Annachhatre (2016) reported during co-digestion of cyanide-containing cassava pulp and PM, the OLR of 2 - 6 kg VS/(m^3 d) led to an average SMY of 380 mL/g VS_{added}, but incompletely hydrolysis occurred at the OLR of 7 kg VS/(m^3 d) and system failed at the OLR of 9 kg VS/(m^3 d) due to VFA accumulation.

Similarly, during co-digestion of PM and rice straw, the average specific biogas yield was 413 mL/g VS at the OLR of 3 - 8 kg VS/(m^3 d), foam was formed at >8 kg VS/(m^3 d) and severe VFA inhibition appeared at 12 kg VS/(m^3 d).

The selection of co-substrates and mixing ratios are also crucial for co-digestion systems. When cassava pulp was co-digested with PM at a ratio of 60:40, the SMY was increased by 41% to 306 mL/g VS_{added} (Panichnumsin et al., 2010). Zhang et al. (2011a) reported a low SMY of 187 mL/g VS_{added} in mono-digestion of piggery wastewater due to ammonia inhibition, but when PM was co-digested with FW at the ratio of 17/83, the SMY increased to 388 mL/g VS_{added}. Co-digestion of PM and grass silage at the ratio of 1:1 resulted in the SMY of 304 mL/g VS compared with 279 mL/g VS in mono-digestion of PM (Xie et al., 2011). The PM and potato waste ratio of 80:20 produced the SMY of 280 - 330 mL/g VS_{added} compared with 120 - 150 mL/g VS_{added} in mono-digestion of PM (Kaparaju & Rintala, 2005). Astals et al. (2012) reported compared with mono-digestion of PM, addition of 4% crude glycerol increased the biogas production from 450 mL/g VS to 740 mL/g VS.

2.2.2.2 Composting

The C/N ratio is one of the most important factors for composting of PM, and the composting efficiency is low at low C/N ratios. Szanto et al. (2007) reported the straw-rich PM with low C/N ratio (7-13) could be composted directly but it required as long as four-month when being turned monthly. Nolan et al. (2011) indicated the addition of bulking agent (sawdust, chopped straw and shredded green waste) and < 60% initial moisture content were essential for composting of PM at C/N ratio of 12.0 - 23.3. High C/N ratio can greatly promote the composting of PM. Wu et al. (2017) studied the effects of C/N ratio (15, 20 and 25) on speciation of Zn and Cu and enzymatic activity during PM composting, and the results showed that high C/N ratio of 25 immobilized 90% of Zn and Cu in the residues and decreased the urease activity, leading to a safe and high quality compost for land application. Huang et al. (2004) reported during co-composting of PM and sawdust, the C/N ratio of 30 achieved mature compost after 49 days, while at C/N ratio of 15, the compost was still immature after 63 days.

Odor emission is a serious problem during PM composting, which limits the scale of composting facilities and is the leading cause for closure of manure composting plants (Tsai et al., 2008). Zang et al. (2016) found sulfur compounds were responsible for odors during PM composting, the PM/corn stalk ratio and aeration rate significantly affected the emissions of Me_2SS and Me_2S, respectively. Zang et al. (2017) reported addition of nitrogen chemicals (2 mM HHT + 40 mM NO_2^-) can reduce Me_2S and Me_2SS by 92.3% and 82.3%, respectively, but the emissions of nitrogen oxides were not assessed. N_2O is one of the most important greenhouse gases, whose global warming potential is 298 times higher than CO_2 (Solomon et al., 2007). Thus reduction of N_2O emission during composting of PM is highly concerned. Fukumoto et al. (2006) found addition of nitrite-oxidizing bacteria (NOB) in PM composting greatly decreased N_2O emission from 88.5 g N-N_2O/kg $TN_{initial}$ to 17.5 - 20.2 N-N_2O/kg $TN_{initial}$. Jiang et al. (2015) also reported that adding 1% nitrogen turnover bacteria (NTB) agent at the beginning of PM composting can significantly reduce nitrogen loss, facilitate organic carbon degradation and improve the compost quality. Besides, the addition of 10% medical stone (MS) based on dry PM reduced 48.8% NH_3 loss and 85.3% N_2O emission (Wang et al., 2016b), and 10% Ca-bentonite (CB) amendment before PM composting can promote organic matter degradation, improve nitrogen and phosphorus transformation , and enhance immonilization of Zn and Cu (Wang et al., 2016a).

2.3 Dry anaerobic digestion

Depending on the TS content of the substrate, anaerobic digestion can be classified as wet digestion (TS < 15%) or dry digestion (TS ≥ 15%) (Kothari et al., 2014; Li et al., 2011). Dry anaerobic digestion is of interest because it can reduce the digester volume significantly and require less energy for heating, but it also has limitations such as poor mixing in the reactor, high VFA and ammonia inhibitions and much longer HRT (Jiang et al., 2018; Li et al., 2011). Studies on dry anaerobic digestion mainly focused on FW, municipal solid waste (MSW) or organic fraction of municipal solid waste (OFMSW) (Table 2-1). The accumulation of VFA is a critical inhibition factor during dry digestion, and the VFA concentrations observed in dry anaerobic digestion systems can reach as high as 60 g/L (Li et al., 2014). Therefore NaOH is usually added to adjust pH to prevent system instability or failure (Table 2-1).

The TS content is one of the most important factors to be concerned during dry anaerobic digestion. High TS content decreases the system efficiency due to the mass transfer limitation. Fernandez et al. (2010) found during the anaerobic digestion of OFMSW, when the TS increased from 20% to 30%, the lag phase incresed from 14 days to 28 days, the SMY decreased from 110 mL/g VS to 70 mL/g VS, and VS removal rates decresed from 49.94% to 40.91%. Benbelkacem et al. (2015) reported the SMY of 202, 181 and 120 mL/g VS at the inlet TS content of 22%, 26% and 30%, respectively, and the optimal TS content was estimated to be about 20%. Abbassi-Guendouz et al. (2012) studied the effect of TS content (10%-35%) during mesophilic anaerobic digestion of cardboard, and found the SMY decreased slightly from the TS content of 10% to 25% with an average value of 176 mL/g VS, but severe VFA inhibition was observed at the TS content of 30%. In the anaerobic digestion of source-sorted biowaste, Li et al. (2014) reported the SMYs were about 350 mL/g VS at the TS content of 20% under both mesophilic and thermophilic conditions, and the SMY started to be inhibited at the TS content of 25% and 30% at 37 ^{o}C and 55 ^{o}C, respectively (Table 2-1). Riya et al. (2018) studied the effect of TS content (18% - 31%) on dry-thermophilic anaerobic co-digestion of PM and rice straw, and no significant difference was observed on SMY (282-295 mL/g VS) at the TS content of 18% - 27%, but VFA inhibition was observed at TS > 28%. As a result, the TS content of 25% could be considered as the threshold concentration for dry anaerobic digestion systems, while the TS content of > 25% is prone to VFA inhibition. According to Table 2-1, the average SMY at the TS content < 25% and > 25% digesters were about 284 mL/g VS and 152 mL/g VS, respectively.

Table 2-1 Summary of research of dry anaerobic digestion in the literature

No.	Substrate	T (℃)	TS (%)	VS (%)	VS/TS (%)	pH	HRT (d)	VFA_{max} (g/L)	Ammonia (mg/L)	SMY (mL/gVS_{added})	VS removal rate (%)	pH control	Reference
1	SS-OFMSW [a]	55	21.1	17	80.57	6.2-8.0		~4	1,788-2,400	25 L	45	NaOH	(Forster-Carneiro et al., 2008a)
	MS-OFMSW [b]	55	19.2	9.8	51.04	7.1-8.5		~5	1,800-2,400	29.9 L	56		
2	SS-OFMSW	36.7	23		50	7.9		2	-	400	40-45		(Bolzonella et al., 2006)
	Grey waste + MS-OFMSW + Sludge	38.6	28		55	8		2.5	-	140	35-40		
3	MSW [c]	35	32.1-39.7		33.9-46.5	8		8	1,200-2,000	200			(Guendouz et al., 2010)
4	OFMSW [d]	55	28	25.1	89.64	8.5		6.478		290	43	NaOH	(Carneiro et al., 2007)
5	OFMSW	55	31	25	80.65	8.7			2,800	100	83.98	NaOH	(Fdez-Guelfo et al., 2010)
6	OFMSW	35	20		42.3	6.68		2.308		110	49.95	NaOH	(Fernandez et al., 2010)
		35	30		36.31	6.46		2.743		70	40.91		
7	WS-OFMSW [e]	30	16		51.5	5.4-7.1		22.399	1,200	273	26.1	NaOH	(Dong et al., 2010)
8	FW [f] (Two phase)	37	20	19	95	3.5-8		~15		360-373	89.8-90.0	Recirculation of effluent	(Cho et al., 1995)
9	FW	35	19.2-21.1		94.8-97.8	5.0-6.0		30					(Lim et al., 2008)
10	MSW		15			8.1	21	1		202	46.0		(Benbelkacem et al., 2015)
			22			8.3		8		181	48.9		
			26			7.3		25		120	44.4		
11	FW	35	21.1	17.4	82.5	~7.5	60			1.39 $m^3/(m^3\ d)$	65.3		(Cho et al., 2013)
						~7.5	40			2.51 $m^3/(m^3\ d)$	65.8		
						~6.7	20	4					

No.	Substrate	T (℃)	TS (%)	VS (%)	VS/TS (%)	pH	HRT (d)	VFAmax (g/L)	Ammonia (mg/L)	SMY (mL/gVSadded)	VS removal rate (%)	pH control	Reference
12	Cardboard	35	10 - 25			7.43				176			(Abbassi-Guendouz et al., 2012)
			30 [g]			7.43				142			
			30 [h]			5.95		29		37			
			35			6.10		36		24			
13	OFMSW(C/N=27)	55	16-21		79-90	6.95-7.53	54-153	35.92	2,671-3,040	176-327		NaOH	(Zeshan et al., 2012)
	OFMSW(C/N=32)		15-25		80-90	7.29-7.75	13-45	11.725	1,758-2,360	121-222			
14	SS-OFMSW	37	20		65-67	6-8		~ 30		350		NaOH	(Li et al., 2014)
		55	20					~ 30		350			
		37	25					~ 35		210			
		55	25					~ 38		350			
		37	30					~ 60		30			
		55	30					~ 38		75			
15	PM and rice straw	55	18			8.5-9.0		0	1011	295			(Riya et al., 2018)
			23					0	1067	278			
			27					0	790	282			
			31					~ 1.2	911	241			

[a] SS-OFMSW: Source sorted organic fractions of municipal solid waste
[b] MS-OFMSW: Mechanically selected organic fractions of municipal solid waste
[c] MSW: Municipal solid waste
[d] OFMSW: Organic fractions of municipal solid waste
[e] WS-OFMSW: Water sorted organic fractions of municipal solid waste
[f] FW: Food waste
[g,h] Two replicates at 30% were stable (g) and the other two other replicates were inhibited (h)

2.4 Pathogen inactivation during anaerobic digestion

2.4.1 Background on pathogen inactivation

2.4.1.1 Commonly reported pathogens and their risks

The pathogens in digestate derive from the substrates. Livestock manure, FW and sewage sludge are all referred to as reservoirs of pathogenic microorganisms including bacteria, viruses and protozoa. Sahlström et al. (2004) surveyed the presence of bacteria pathogens in 8 Swedish sewage treatment plants, and found 67% of the 64 raw samples and 55% of the 69 treated samples were positive for *Salmonella*, followed by *Campylobacter*, which was 29% and 6% in the raw and treated samples, respectively. Côté et al. (2006) analyzed samples in 32 hog operations in Quebec and found *E.coli* concentration ranged 0 - 5.52 $\log_{10}$ colony forming unit (CFU)/g, and *Salmonella* spp., *Y. enterocolitica* and *Cryptosporidium* spp. were detected in 37%, 9% and 3% of the samples, respectively. McCarthy et al. (2013) surveyed 30 pig farms in Ireland and found 50% of them were *Salmonella* positive. In general, the most prevalent zoonotic pathogens in biowastes include bacteria such as *Salmonella, Escherichia coli* O157, *Listeria, Yersinia, Campylobacter*, *Enterococcus* and *Clostridium* (Bonetta et al., 2011; Horan et al., 2004; Massé et al., 2011); nematode such as *Ascaris* (Aitken et al., 2005), protozoa such as *Giardia* and *Cryptosporidium* (Chauret et al., 1999; Kato et al., 2003); and viruses such as poliovirus, norovirus, and rotavirus (Aitken et al., 2005; Pandey & Soupir, 2011; Scheuerman et al., 1991).

Zoonotic pathogens can be transmitted from animals to humans, and cause various life-threatening diseases. Seven leading foodborne pathogens cause about 112,000 disability adjusted life years (DALYs) annually in the United States; they are non-typhoidal *Salmonella* (29.4%), *Toxoplasma* (29.2%), *Campylobacter* (20.1%), norovirus (8.8%), *Listeria monocytogenes* (7.9%), *Clostridium perfringens* (3.6%) and *Escherichia coli O157* (1.1%) (Scallan et al., 2015). Greig and Ravel (2009) reported of 4093 foodborne outbreaks occurred globally between 1998 and 2007, with 46.9% attributed to *Salmonella*, followed by norovirus (13.5%), *E. coli* (9.5%), *Clostridium* (8.7%), *Campylobacter* (4.6%) and *Staphylococcus aureus* (4.4%). Adak et al. (2002) reported in England and Wales in 2000, 1,338,772 cases, 20,759 hospital admissions, and 480 deaths were due to indigenous foodborne diseases, and the leading pathogens were *Campylobacter, Salmonella*, *Clostridium perfringens,* verocytotoxin producing *E. coli* (VTEC) O157, and *Listeria monocytogenes*. The diseases caused by the above common pathogens are summarized in Table 2-2.

Table 2-2 Diseases caused by manure and foodborne pathogens

Pathogens	Diseases, symptoms, sequelae	Reference
Bacteria		
Non-typhoidal *Salmonella*	Salmonellosis, acute gastroenteritis, diarrhea, fever, abdominal cramps, reactive arthritis, post-infectious irritable bowel syndrome	(Finstad et al., 2012; Scallan et al., 2015)
Campylobacter	Campylobacteriosis, acute gastroenteritis, fever, vomiting, headaches, watery or bloody diarrhoea, Guillain-Barre syndrome, reactive arthritis and post-infectious irritable bowel syndrome	(Dasti et al., 2010; Manyi-Loh et al., 2016; Scallan et al., 2015)
Listeria monocytogenes	Listeriosis, flu-like symptoms, vomiting, diarrhea, meningitis, septicemia, encephalitis, pregnancy-associated neurological complications in infant, abortion or stillbirth	(Low & Donachie, 1997; Manyi-Loh et al., 2016; Scallan et al., 2015)
Clostridium perfringens	Acute gastroenteritis, tetanus, botulism, black leg	(Sahlström, 2003)
Escherichia coli O157	Acute gastroenteritis, mild to bloody diarrhea, vomiting, haemolytic uremic syndrome, hemorrhagic colitis, end-stage renal disease	(Karmali, 2009; Scallan et al., 2015)
Yersinia enterocolitica	Diarrhea, lymphadenitis, pneumonia, abortions, acute enteritis	(Manyi-Loh et al., 2016; Sahlström, 2003)
Enterobacter sakazakii	Neonatal meningitis and sepsis	(Back et al., 2009)
Protozoa		
Toxoplamsa gondii	Chorioretinitis, intracranial calcifications, hydrocephalus, and central nervous system abnormalities	(Scallan et al., 2015)
Cryptosporidium parvum	Gastroenteritis and cryptosporidiosis, diarrhoea	(Manyi-Loh et al., 2016; Savioli et al., 2006)
Giardia lamblia	Giardiasis, diarrhoea and abdominal cramps	(Manyi-Loh et al., 2016; Thompson, 2000)

Pathogens	**Diseases, symptoms, sequelae**	**Reference**
Nematode		
Ascaris lumbricoides	Ascariasis, cognitive and societal impairment, higher susceptibility to infection, decreased responsiveness to vaccination, and malnutrition	(Pecson & Nelson, 2005)
Viruses		
Norovirus	Gastroenteritis	(Lopman et al., 2004)
Poliovirus	Paralytic poliomyelitis	(Kim-Farley et al., 1984)
Rotavirus	Gastroenteritis, diarrhea	(Gleizes et al., 2006; Ramig, 2004)
Enterovirus	Gastroenteritis	(Venglovsky et al., 2006)
Adenovirus	Respiratory infections	(Venglovsky et al., 2006)
Sapovirus	Gastroenteritis, diarrhea	(Ziemer et al., 2010)

2.4.1.2 Resistance mechanisms of various pathogens

The commonly concerned pathogens in anaerobic digestion systems are classified as Gram-negative bacteria, Gram-positive bacteria, protozoa and viruses. Fecal coliforms, notably *E.coli*, *Salmonella*, *Campylobacter*, *Enterobacter*, *Shigella*, and *Yersinia* are all Gram-negative bacteria, while *Enterococcus* is the most common Gram-positive bacteria. The inactivation efficiencies of different pathogens vary greatly according to their respective physiological structures and features.

Mechanical integrity and permeability barrier are two main protection mechanisms of bacteria owing to the functions of peptidoglycan and cell membrane, respectively (Dijkstra & Keck, 1996). In Gram-negative bacteria, the cell wall is very thin, and constitutes only a monolayer of peptidoglycan and a lipid-rich outer membrane, which offers little protection to the bacteria against the toxic factors in the environment (Beveridge, 1999). Thus Gram-negative bacteria are sensitive to various stress factors, e.g. thermophilic temperature, VFA and ammonia (Himathongkham et al., 2000; Jiang et al., 2018; Watcharasukarn et al., 2009).

Compared with Gram-negative bacteria, the cell wall of Gram-positive bacteria is much thicker, consisting of highly cross-linked, multi-layered peptidoglycan and teichoic acids. Apart from the cell membrane, they can also act as a permeability barrier to prevent the toxic factors getting into the cell (Dijkstra & Keck, 1996). Gram-positive bacteria are more resistant to various stress conditions than Gram-negative bacteria, which can grow at a range of temperature (5-50 ℃), pH (4.6-9.9), high free VFA concentration (375 mg/L) and high osmotic pressure (40% bile salts and 6.5% NaCl) (Fisher & Phillips, 2009; Jiang et al., 2018).

Clostridium perfringens is also Gram-positive bacteria but it can form spores. The vegetative cells of *Clostridium perfringens* are sensitive to thermophilic temperature but the spore is highly heat-resistant. Byrne et al. (2006) reported the decimal reduction time (DRT) of vegetative cells were 16.3 min and 0.9 min at 55 and 65 ^{o}C, respectively, while the DRT of spores were 34.2 min and 2.2 min at 90 and 100 ^{o}C, respectively. In normal environment, the spores can survive for many years (Sahlström, 2003).

As a typical nematode, the resistance of *Ascaris suum* egg derives from its eggshell, which is a four-layer structure. The inner is a lipoprotein layer responsible for the impermeability of the shell, and the next is a chitin/protein layer giving mechanical strength, followed by a lipoprotein vitelline layer and the outer acidic mucopolysaccharide/protein layer (Wharton, 1980). The structure of the shell makes *Ascaris suum* egg resistant to various stress conditions such as acid, alkaline, osmotic pressure and desiccation (Butkus et al., 2011; Clarke & Perry, 1980; Wharton, 1979).

Poliovirus is a non-enveloped virus which provides an idea model for understanding how viruses enter cells and initiate infections (Hogle, 2002). It contains a single-strand plus-sense RNA genome enclosed in an icosahedral protein capsid (Bubeck et al., 2005). The inactivation of poliovirus at high temperature can be caused by RNA cleavage, capsid protein denaturation and changes of isoelectric points (Ward, 1978; Woese, 1960). Ward and Ashley (1976) studied the inactivation mechanism of poliovirus and found the RNA of inactivated particles was nicked, and some breakdown was also observed in the two largest viral proteins. Popat et al. (2010) studied the inactivation of poliovirus in sludge anaerobic digestion at the temperature ranging 51.1 - 55.5 ^{o}C, he found the activation energy of poliovirus was 39 kJ/mol, which fell in the range for RNA inactivation. However, Aitken et al. (2005) reported the inactivation energy of 510 kJ/mol during sludge anaerobic digestion at 49 - 53 ^{o}C, which was the characteristic of capsid protein denaturation. Ward (1978) reported heat inactivation of poliovirus at 45 ^{o}C caused 3 log_{10} reductions in viral infectivity and changed the isoelectric points from 7.5 and 4.5 to 4.4.

2.4.1.3 Survival of pathogens in biowastes and in soil after land spreading

Direct spreading of fresh biowastes to land poses risks to environment and human health due to pathogen transfer. Some biowaste, e.g., livestock manure, is required to be stored before land spreading, and the storage method and period are specified by local regulations (Côté et al., 2006; McCarthy et al., 2015). The survival of pathogens in storage of biowastes and in soil after land application needs to be examined to assess the potential risks.

The reduction of pathogens during storage is affected by many factors, i.e. storage method, initial pathogen concentrations in biowastes, microbial interactions, temperature, pH, aeration and dry matter (DM) content (Nicholson et al., 2005; Ziemer et al., 2010). McCarthy et al. (2015) studied the survival of *Salmonella* in the storage of PM at 10.5 ^{o}C, and found *Salmonella* was capable of surviving for as long as 84 days. Nicholson et al. (2005) reported *E. coli* O157, *Salmonella* and *Campylobacte*r in stored slurries can survive for up to 3 months, while *Listeria* can survive for up to 6 months. All these pathogens survived for less than one month in solid manure when being heaped at the temperature of > 55 ^{o}C. Gaasenbeek and Borgsteede (1998) found *Ascaris suum* eggs in pig slurry could be reduced by 80% after 4 weeks and were not detectable after 16 weeks in storage. Côté et al. (2006) surveyed 32 hog operations in Quebec and found it took 54-114 days for *E. coli* to reach undetectable levels in storage, the maximal observed survival time for *Salmonell*a was 88 days and *Y. enterocolitica* was detectable in a 73-day storage.

Survival of pathogens in soil after land spreading can be affected by both physic-chemical and biological factors, such as pH, MC and initial pathogen concentrations in the biowastes and soil, the application rate and spreading method, the type of the soil, temperature, exposure to the air and sunlight and the interactions between microorganisms (Nicholson et al., 2005; Pourcher et al., 2007). Pourcher et al. (2007) reported after land spreading of sewage sludge, enteroviruses were not detected after 2 weeks, both *E. coli* [initially 2.3×10^3 most probable number (MPN)/g DM] and enterococci (8.9×10^2 MPN/g DM) decreased to about 40 MPN/g DM after 2 months, while the concentration of *Clostridium perfringens* was stable during the 2 months application. Nicholson et al. (2005) reported after land spreading of manure, *E. coli* O157, *Salmonella* and *Campylobacter* survived for one month, while *Listeria* survived for more than one month. Gaasenbeek and Borgsteede (1998) reported *Ascaris suum* eggs survived for 2-4 weeks in dry and sunny field conditions, but 90% of the eggs were viable after 8 weeks under wet and shady conditions.

From the above, the survival time of pathogens in storage and land spreading vary depending on types of pathogens, biowastes, storage methods, and land application conditions. Neither biowaste storage nor land spreading is effective for inactivation of pathogenic microorganisms. Therefore, before land spreading, biowastes must be treated and the pathogens must be reduced to levels not posing a threat to public health and environment.

2.4.1.4 Regulations and indicator organisms

Because of the diversity of pathogens and the impracticality of testing all of them, the analyses of indicator organisms are essential to indicate the biosafety of the digestate. However, the indicator organisms and their threshold levels vary in regulations from country to country, with a brief summary shown in Table 2-3. The most common indicator bacteria are *Salmonella*, *E. coli*, fecal coliform and *Enterococcus*. *Salmonella* is required to be absent in 25 g or 50 g fresh matter in a variety of regulations, while *E. coli*, fecal coliform and *Enterococcus* are required to be less than 10^3 CFU/g dry or wet weight in different countries (BSI, 2014; EU, 2011; IRBEA, 2012; Milieu et al., 2008; USEPA, 1994). *Clostridium perfringens* is concerned as an indicator of spore-producing bacterium which is more resistant to environment; the standard for *Clostridium perfringens* is not common but an EU consulting company suggests it to be less than 3×10^3 spores/g dry weight (Milieu et al., 2008). The eggs of parasitic worms *Helminth ova*, notably *Ascaris ova*, are highly resistant to stress conditions such as desiccation, acid and base because of the protection of the eggshell (Pecson & Nelson, 2005). Because of the resistance, *Ascaris* eggs are also used to indicate pathogen inactivation and are required to be absent in the digestate (Lepeuple et al., 2004; Milieu et al., 2008; USEPA, 1994). Viruses are not clearly stated in these regulations, with only parvovirus being required for a 3-$\log_{10}$ reduction during treatment by EU (EU, 2011).

Table 2-3 Threshold levels of pathogens in digestate

Country	Pathogen	Threshold	Reference
EU	*E. coli*	4 log_{10} reduction	(Milieu et al., 2008)
		< 10^3 CFU/g DW	
	Ascaris ova	Absent	
	Clostridium perfringens	< 3 ×10^3 spores/g DW	
	Salmonella spp.	Absent in 50 g wet weight	
	E. coli	< 10^3 CFU/g fresh matter	(EU, 2011)
	Enterococcaceae	< 10^3 CFU/g fresh matter	
	Salmonella	Absent in 25 g fresh matter	
	Enterococcus faecalis	5 log_{10} reduction	
	Salmonella Senftenberg	5 log_{10} reduction	
	Parvovirus	3 log_{10} reduction	
	Ascaris eggs	3 log_{10} reduction	
USA Class A	Fecal coliform	< 10^3 MPN/g TS	(USEPA, 1994)
	Salmonella	< 3MPN/ 4g TS	
	Helminth	< 1 viable egg/4g TS	
	Enteric viruses	< 1 PFU/ 4g TS	
USA Class B	Fecal coliform	< 2 ×10^6 MPN/g TS	
UK	*E. coli*	< 10^3 CFU/g fresh matter	(BSI, 2014)
	Salmonella spp.	Absent in 50 g fresh matter	
Ireland	*E. coli*	< 10^3 CFU/g fresh matter	(IRBEA, 2012)
	Salmonella spp.	Absent in 25 g fresh matter	
Switzerland	*Enterobacteria*	< 10^2 MPN/g DW	(Lepeuple et al., 2004)
	Helminth ova	Absent	

MPN: Most probable mumbers

DM: Dry matter

CFU: Colony-forming unit

PFU: Plaque-forming unit

2.4.2 Effects of temperature - time (Batch - Continuous)

In anaerobic digestion systems, pathogens are exposed to complex environment, with the inactivation efficiency being affected by many factors, e.g. bacteria species, substrate, digestion time, operation method (batch or continuous), temperature, pH, VFA and ammonia (Pecson et al., 2007; Sahlström, 2003; Salsali et al., 2006). Interactions exist among different factors, for example, the toxicity of VFA and ammonia is usually reported to be pH dependent, as free VFA and free ammonia (NH_3) are considered more toxic to pathogens (Pecson & Nelson, 2005; Salsali et al., 2006). The changes of temperature affect the dissociation constants of VFA and ammonia, causing changes on the concentrations of free VFA and NH_3 (Bates & Pinching, 1949; Harned & Ehlers, 1933a; Harned & Ehlers, 1933b). Besides, the inactivation effects of temperature, VFA and ammonia are all related to digestion time.

2.4.2.1 Inactivation mechanism of temperature

Studies on anaerobic digestion are usually conducted under ambient (20-25 ^oC), mesophilic (30-40 ^oC) and thermophilic (50-55 ^oC) temperatures (Scaglia et al., 2014; Varel et al., 2012), while mesophilic and thermophilic anaerobic digestion are widely used in engineering practice. Besides, in many countries such as the UK, Austria, Denmark, Germany, and Sweden, a separate pasteurisation step is carried out before anaerobic digestion, which heats the substrates at 70 ^oC for 1 h to realize pathogens inactivation (EU, 2011). Thus the temperature related to AD ranges 20-70 ^oC.

Temperature acts on pathogen inactivation directly and indirectly. Firstly, temperature has an impact on the composition of the cell membrane. The increase of temperature (from 37 ^oC to 70 ^oC) can increase the fluidity and permeability of the cell membrane by changing the membrane lipid phase, denaturing the membrane proteins and collapsing the cytoskeletal proteins (Bischof et al., 1995). The increase of cell membrane permeability enables more rapid diffusion of toxic chemicals, e.g. VFA and ammonia, into the cytoplasm so as to increase the inhibitory effects (Salsali et al., 2006). Secondly, temperature can affect the dissociation constants and diffusion rates of VFA and ammonia, thereby affecting the concentrations of free VFA and NH_3 which cause the pathogen inactivation (Bates & Pinching, 1949; Harned & Ehlers, 1933a; Harned & Ehlers, 1933b; Ouattara et al., 2000). Two or more mechanisms may act simultaneously on the inactivation, however, at the thermophilic conditions, thermal inactivation is usually the primary factor. Pecson et al. (2007) reported at 50 ^oC, the effect of temperature on *Ascaris* eggs was dominant and the effects of free NH_3 (0-5000 mg N/L) and pH (7-12) were neglectable. The inactivation effect of temperature is time-dependent. Four time-temperature regimes (Table 2-4) are regulated by the USEPA to indicate the time required to meet the Class A standard (USEPA, 1994). However, these guidelines require excessively long holding time and are considered extremely conservative (Aitken et al., 2005; Popat et al., 2010).

Table 2-4 Four time - temperature regimes for Class A pathogen reduction

Regime	Applies to:	Requirement	Time-temperature relationship
A	Biosolids with TS [a] $\geq$ 7% (except those covered by Regime B)	T [b] $\geq$ 50 ^oC for at least 20 minutes	$D^c = \frac{131{,}700{,}000}{10^{0.14T}}$
B	Biosolids with TS $\geq$ 7% in form of small particles and heated by contact with either immiscible liquid or warmed gases	T $\geq$ 50 ^oC for at least 15 seconds	$D = \frac{131{,}700{,}000}{10^{0.14T}}$
C	Biosolids with TS $\leq$ 7%	Heated for 15 seconds - 30 minutes	$D = \frac{131{,}700{,}000}{10^{0.14T}}$
D	Biosolids with TS $\leq$ 7%	T $\geq$ 50 ^oC for at least 30 minutes	$D = \frac{50{,}070{,}000}{10^{0.14T}}$

[a] TS: total solids

[b] T: temperature in degrees Celsius (oC)

[c] D = time in days

It’s well known that increasing the temperature can accelerate the inactivation of pathogens significantly. As shown in Table 2-5, even for the same pathogen, the inactivation rate varied greatly in different studies. Generally, at ambient and mesophilic conditions, the inactivation rates are low, with slight increase while increasing the temperature. Pandey and Soupir (2011) found the inactivation rate of *E. coli* increased by 1.1 times when increasing the temperature from 25 ^{o}C to 37 ^{o}C. Pecson et al. (2007) found the inactivation of *Ascaris* increased by 2.5 times with the increasing of temperature from 20 ^{o}C to 30 ^{o}C, while it increased by 13.2 times from 30 ^{o}C to 40 ^{o}C; however, when the temperature increased from mesophilic to thermophilc conditions, the inactivation rates increased as much as 1-2 orders of magnitude. Watcharasukarn et al. (2009) found when increasing the temperature from 37 ^{o}C to 55 ^{o}C, the inactivation rates for sensitive fractions of *E. coli*, *Enterococcus faecaliss* and *Clostridium perfringens* increased by 400, 50 and 100 times, respectively. But Pandey and Soupir (2011) only observed an increase of 15 times for *E. coli* from 37 ^{o}C to 52.5 ^{o}C. The inactivation rate of *Ascaris* was increased by 394 times with the increase of temperature from 37 ^{o}C to 55 ^{o}C (Pecson et al., 2007). Great increase of the inactivation rate was also observed when changing the temperature from thermophilic to pasteurization temperature. About 100 times increases were observed for the inactivation rates of *E. coli* from 55 ^{o}C to 60/70 ^{o}C (Watcharasukarn et al., 2009; Ziemba & Peccia, 2011). But only 4-8 time increases were observed for *Enterococcus faecaliss* and *Clostridium perfringens* from 55 ^{o}C to 70 ^{o}C (Watcharasukarn et al., 2009; Ziemba & Peccia, 2011). It's difficult to compare the inactivation efficiency of different pathogens based on results obtained in different studies, but according to the data obtained in the same study, the thermal resistance of *Clostridium perfringens* > *Enterococcus faecalis* > *E. coli* (Watcharasukarn et al., 2009; Ziemba & Peccia, 2011), *Ascaris* eggs > poliovirus (Aitken et al., 2005; Popat et al., 2010), and porcine parvovirus > fecal enterococci > bovine enterovirus (Lund et al., 1996).

Table 2-5 Summary of inactivation rate constants (k, d^{-1}) of pathogens

Pathogen	Ambient (20-25 ^oC)	Mesophilic (30-40 ^oC)	Thermophilic (50-55 ^oC)	Pasteurization (60-70 ^oC)	Reference
E. coli	0.12	0.13	2.01		(Pandey & Soupir, 2011)
	~ 0	~ 0	0.06-0.28	27.08	(Ziemba & Peccia, 2011)
		2.5	10^3	10^5	(Watcharasukarn et al., 2009)
Enterococcus	~ 0	~ 0	1.0-6.7	177	(Ziemba & Peccia, 2011)
		2	100	800	(Watcharasukarn et al., 2009)
Ascaris eggs	0.01	0.025-0.33	130		(Pecson et al., 2007)
			1.25-17.3		(Aitken et al., 2005)
			2.10-3.74		(Popat et al., 2010)
Clostridium perfringens		1	100	400	(Watcharasukarn et al., 2009)
Poliovirus				1.79-38.9	(Aitken et al., 2005)
				4.09-4.54	(Popat et al., 2010)

2.4.2.2 Inactivation effect of temperature in anaerobic digestion

The effects of temperature on various pathogens during anaerobic digestion are widely reported, and a short summary is shown in Table 2-6. However, the reductions were highly variable among different conditions, such as pathogen species, initial concentrations, substrates and operation methods.

Table 2-6 Summary of the effects of temperature on pathogen inactivation during anaerobic digestion

Substrate	T (^oC)	TS (%)	Initial count (CFU/g or mL)	Finial count (CFU/g or mL)	Log_{10} reduction	Tc/HRT (d)	Meet standard (Yes/no)	Reference
E. coli - Batch								
Dairy manure	25	1.39	6.5×10^7	$< 10^2$	>6	60	Y	(Pandey & Soupir, 2011)
Nutrient broth /centrifuged liquid raw sludge	35		10^8-10^9	10^6-10^8	1-2	20	N	(Smith et al., 2005)
Dairy manure	35		3.6×10^5	Below LOD	3.6	62	Y	(Manyi-Loh et al., 2014)
Dairy manure	37	2.05	1.85×10^7	$< 10^3$	6	41	Y	(Pandey & Soupir, 2011)
Cow manure	37	6.6	6×10^4	Below LOD	4.94	6	Y	(Watcharasukarn et al., 2009)
Dairy manure	52.5	1.75	2.5×10^7	Below LOD	>7	3.5	Y	(Pandey & Soupir, 2011)
Cow manure	55	6.6	6×10^4	Below LOD	4.37	40 min	Y	(Watcharasukarn et al., 2009)
Nutrient broth /centrifuged liquid raw sludge	55		10^8	Below LOD	8	20-60 min	Y	(Smith et al., 2005)
Cow manure	70	6.6	6×10^4	Below LOD	2.6	2 min	Y	(Watcharasukarn et al., 2009)
E. coli - Continuous								
Swine manure	24	6.2-7.2	4.0×10^2-5.8×10^5		0.9-2.9	7, 14	N	(Massé et al., 2011)
Cattle manure	33	7	10^3	3×10^2	~0.5	55	Y	(Popova et al., 2013)
Primary sludge	35	3-5	5.02×10^5-1.3×10^6		1.48-1.68	12	N	(Horan et al., 2004)
Sludge	37.8	3.2	10^5-10^7	10^2-10^4	~2	50	N	(Lloret et al., 2013)
Sewage sludge	38	3-4	3.6×10^5	8.4×10^3	1.6	14-20	N	(Sahlström et al., 2004)
Manure and agriculture waste	39	6.3-9.3	1.5×10^4-8.5×10^4	0-1.9×10^3	0.9-4.2	20-28	N	(Orzi et al., 2015)
Manure and agriculture waste	39-42	4.5-5.6	1.5×10^1-2×10^5	0-7×10^2	0-4.6	35-45	Y	(Orzi et al., 2015)
Manure and agriculture waste	39	6.1-7.3	1.55×10^4-4.65×10^5	Below LOD	4.2-5.7	70	Y	(Orzi et al., 2015)
Cattle manure	45	7		Below LOD	3	55	Y	(Popova et al., 2013)
Sludge	52.3-53.9	1.9-2.7	10^5-10^7	Below LOD	5-7	16-28	Y	(Lloret et al., 2013)
Dairy cattle manure	55		0.5410^6-1.44×10^6	$< 10^2$	>4	7.8	Y	(Aitken et al., 2007)
Sewage sludge	55	3-4%	1.0×10^6	2	~6	9	Y	(Sahlström et al., 2004)

Pathogen	Substrate	T (^{o}C)	TS (%)	Initial count (CFU/g or mL)	Finial count (CFU/g or mL)	Log_{10} reduction	Tc/HRT (d)	Meet standard (Yes/no)	Reference
Total coliforms and fecal coliform - *Batch*									
Fecal coliforms	Sludge	20	12	3.55×10^{7} MPN/g DM	4.86×10^{3} MPN/g DM	3-4	60	N	(Scaglia et al., 2014)
Fecal coliforms	Sludge	21	3.6-7.8	10^{7}-10^{8} MPN/g TS	Below LOD	7-8	5	Y	(Puchajda et al., 2006)
Coliforms	swine slurry	22	12.5-13.5	10^{5}-10^{6}	>10^{3}	2-3	25	N	(Varel et al., 2012)
Coliforms	cattle slurry	22	4	10^{4}-10^{5}	10^{2}	2-3	28	Y	(Varel et al., 2012)
Fecal coliforms	Sludge	35	12	3.55×10^{7} MPN/g DM	< 10^{3} MPN/g DM	4-5	20	Y	(Scaglia et al., 2014)
Coliforms	swine slurry	38	12.5-13.5	10^{5}-10^{6}	Below LOD	3-4	3	Y	(Varel et al., 2012)
Coliforms	cattle slurry	38	4	10^{4}-10^{5}	Below LOD	2-3	14	Y	(Varel et al., 2012)
Fecal coliforms	Sludge	38	3.8-8.3	10^{6}-10^{8} MPN/g TS	>10^{3} MPN/g TS	3-5	5	N	(Puchajda et al., 2006)
Coliforms	swine slurry	55	12.5-13.5	10^{5}-10^{6}	Below LOD	3-4	1	Y	(Varel et al., 2012)
Coliforms	cattle slurry	55	4	10^{4}-10^{5}	Below LOD	2-3	1	Y	(Varel et al., 2012)
Fecal coliforms	Sludge	55	12	3.55×10^{7} MPN/g DM	< 10^{3} MPN/g DM	4-5	20	Y	(Scaglia et al., 2014)
Total coliforms and fecal coliform - *Continuous*									
Total coliforms	Swine manure	24	6.2-7.2	6.0×10^{2}-1.5×10^{6}		1-2.9	7, 14	N	(Massé et al., 2011)
Fecal coliforms	Swine manure	24	6.2-7.2	5.0×10^{2}-1.6×10^{6}		0.9-3	7, 14	N	(Massé et al., 2011)
Coliforms	Cattle manure	33	7	10^{3}	10^{2}	1	55	Y	(Popova et al., 2013)
Fecal coliforms	Sludge	35		10^{8}-10^{9} MPN/g TS	>10^{6} MPN/g TS	2-3	15	N	(Riau et al., 2010)
Fecal coliforms	sludge	35	3-5	10^{8} MPN/g TS	10^{6}-10^{7} MPN/g TS	1-2	15	N	(Cheunbarn & Pagilla, 2000)
Total coliforms	Sludge	35	4.3-5.9	10^{8}-10^{9}	10^{7}-10^{8}	~1	20, 15, 10, 5	N	(Coelho et al., 2011)
Total coliforms	Microwaved sludge	35	4.3-5.9	10^{5}-10^{7}	10^{4}-10^{5}	1-2	20, 15, 10, 5	N	(Coelho et al., 2011)
Total coliforms	sludge	37.8	3.2	10^{6}-10^{8}	10^{2}-10^{5}	~2	50	N	(Lloret et al., 2013)
Coliforms 37 ℃	Sewage sludge	38	3-4	1.6×10^{6}	5.0×10^{4}	1.5	14-20	N	(Sahlström et al., 2004)
Coliforms 44 ℃	Sewage sludge	38	3-4	4.4×10^{5}	1.4×10^{4}	1.5	14-20	N	(Sahlström et al., 2004)
Fecal coliforms	Manure + AW	39	6.3-9.3	1.8×10^{4}-6.1×10^{4}	0-2.15×10^{3}	0.9-4.3	20-28	N	(Orzi et al., 2015)

Pathogen	Substrate	T (oC)	TS (%)	Initial count (CFU/g or mL)	Finial count (CFU/g or mL)	Log_{10} reduction	Tc/HRT (d)	Meet standard (Yes/no)	Reference
Total coliforms and fecal coliform - *Continuous*									
Fecal coliforms	Manure + AW	39-42	4.5-5.6	2.0×10^2-1.55×10^5	0-1.15×10^3	1.1-4.1	35-45	N	(Orzi et al., 2015)
Fecal coliforms	Manure + AW	39	6.1-7.3	1.0×10^4-3.9×10^5	0	4.0-5.6	70	Y	(Orzi et al., 2015)
Coliforms	Cattle manure	45	7	10^3	10^2	1	55	Y	(Popova et al., 2013)
Total coliforms	sludge	52.3-53.9	1.9-2.7	10^6-10^8	Below LOD	6-8	16-28	Y	(Lloret et al., 2013)
Coliforms 37 ℃	Sewage sludge	55	3-4	4.5×10^6	6	>6	9	Y	(Sahlström et al., 2004)
Coliforms 44 ℃	Sewage sludge	55	3-4	1.4×10^6	2	>6	9	Y	(Sahlström et al., 2004)
Total coliforms	Sludge	55	4.3-5.9	10^8-10^9	Below LOD	6-7	20, 15	Y	(Coelho et al., 2011)
Total coliforms	Microwaved sludge	55	4.3-5.9	10^5-10^7	Below LOD	3-5	20, 15	Y	(Coelho et al., 2011)
Total coliforms	Sludge	55	4.3-5.9	10^8-10^9	10^4-10^6	2-3	10, 5	N	(Coelho et al., 2011)
Total coliforms	Microwaved sludge	55	4.3-5.9	10^5-10^7	10^3-10^4	2-3	10, 5	N	(Coelho et al., 2011)
Total coliforms	Sludge	55(2d)+35(3-8d)	4.3-5.9	10^8-10^9	10^5-10^6	~3	10, 5	N	(Coelho et al., 2011)
Total coliforms	Sludge	55(2d)+55 (3-8d)	4.3-5.9	10^8-10^9	10^3-10^4	~5	10, 5	N	(Coelho et al., 2011)
Total coliforms	Sludge	55(2d)+35(13-18d)	4.3-5.9	10^8-10^9	Below LOD	6-7	20, 15	Y	(Coelho et al., 2011)
Total coliforms	Sludge	55(2d)+55(13-18d)	4.3-5.9	10^8-10^9	Below LOD	6-7	20, 15	Y	(Coelho et al., 2011)
Total coliforms	Microwaved sludge	55(2d)+35(3-18d)	4.3-5.9	10^5-10^7	Below LOD	3-5	20, 15, 10, 5	Y	(Coelho et al., 2011)
Total coliforms	Microwaved sludge	55(2d)+55 (3-18d)	4.3-5.9	10^5-10^7	Below LOD	3-5	20, 15, 10, 5	Y	(Coelho et al., 2011)
Fecal coliforms	Sludge	55(15d)+35(15d)		10^8-10^9 MPN/g TS	>10^4 MPN/g TS	4-5	30	N	(Riau et al., 2010)
Fecal coliforms	Sludge	55(5d)+35(15d)		10^8-10^9 MPN/g TS	>10^3 MPN/g TS	5-6	20	N	(Riau et al., 2010)
Fecal coliforms	Sludge	55(3d)+35(15d)		10^8-10^9 MPN/g TS	10^3 MPN/g TS	5-6	18	N	(Riau et al., 2010)
Fecal coliforms	Sludge	55(3d)+35(12d)		10^8-10^9 MPN/g TS	10^3 MPN/g TS	5-6	15	N	(Riau et al., 2010)
Fecal coliforms	Dairy manure	55(4d)+35(10d)	3.5-10.4	10^5-10^7 MPN/g TS	Below LOD	5-7	14	Y	(Harikishan & Sung, 2003)
Fecal coliforms	sludge	62(1d)+35(14d)	1.6-1.8	10^8 MPN/g TS	<10^2 MPN/g TS	>5	15	Y	(Cheunbarn & Pagilla, 2000)
Fecal coliforms	sludge	35(14d)+62(1d)	1.6-1.8	10^8 MPN/g TS	<10^2 MPN/g TS	>5	15	Y	(Cheunbarn & Pagilla, 2000)

Substrate	T (^oC)	TS (%)	Initial count (CFU/g or mL)	Finial count (CFU/g or mL)	Log_{10} reduction	Tc/HRT (d)	Meet standard (Yes/no)	Reference
Salmonella - Batch								
Sludge	20	12	81 MPN/g DM	27 MPN/g DM		60	N	(Scaglia et al., 2014)
Sludge	35	12	81 MPN/g DM	0 MPN/g DM		13	Y	(Scaglia et al., 2014)
Centrifuged liquid raw sludge	35		10^8-10^9	10^5-10^8	1-3	20	N	(Smith et al., 2005)
Dairy manure	35		7.4×10^3	Below LOD	1.9	133		(Manyi-Loh et al., 2014)
Sludge	55	12	81 MPN/g DM	0 MPN/g DM		3	Y	(Scaglia et al., 2014)
Centrifuged liquid raw sludge	55		10^8	0	8	20-60 min	Y	(Smith et al., 2005)
Salmonella - Continuous								
Swine manure	24	6.2-7.2	$<1\times10^2$-5.0×10^4		0.9-1.4	7, 14	N	(Massé et al., 2011)
Primary sludge	35	3-5	4.6×10^6-1.3×10^7 CFU/g DM		1.91-2.23	12	N	(Horan et al., 2004)
Sludge	35		6-10 MPN/4g DM	4 MPN/4g DM		15	N	(Riau et al., 2010)
sludge	35	3-5	2-12 MPN/4g DM	<1 MPN/4g DM		15	Y	(Cheunbarn & Pagilla, 2000)
1:1 primary + WAS	35	3.24	16,000 CFU/100 mL	11,000 CFU/100 mL		15	N	(Salsali et al., 2006)
sludge	37.8	3.2	Detected in 100% samples	Detected in 45% samples		50	N	(Lloret et al., 2013)
1:1 primary + WAS	42	3.17	16,000 CFU/100 mL	8,000 CFU/100 mL		15	N	(Salsali et al., 2006)
1:1 primary + WAS	49	3.15	16,000 CFU/100 mL	5,000 CFU/100 mL		15	N	(Salsali et al., 2006)
sludge	52.3-53.9	1.9-2.7	Detected in 100% samples	Not detected		16-28	Y	(Lloret et al., 2013)
Sludge	55(15d)+35(15d)		6-10 MPN/4g DM	2 MPN/4g DM		30	Y	(Riau et al., 2010)
Sludge	55(5d)+35(15d)		6-10 MPN/4g DM	1 MPN/4g DM		20	Y	(Riau et al., 2010)
Sludge	55(3d)+35(15d)		6-10 MPN/4g DM	1 MPN/4g DM		18	Y	(Riau et al., 2010)
Sludge	55(3d)+35(12d)		6-10 MPN/4g DM	2 MPN/4g DM		15	Y	(Riau et al., 2010)
Dairy manure	55(4d)+35(10d)	3.5-10.4	1000-1500 MPN/g DM	<1 MPN/g DM	>3	14	Y	(Harikishan & Sung, 2003)
sludge	62(1d)+35(14d)		2-12 MPN/4g DM	<1 MPN/4g DM		15	Y	(Cheunbarn & Pagilla, 2000)
sludge	35(14d)+62(1d)		2-12 MPN/4g DM	<1 MPN/4g DM		15	Y	(Cheunbarn & Pagilla, 2000)

Substrate	T (^{o}C)	TS (%)	Initial count (CFU/g or mL)	Finial count (CFU/g or mL)	Log_{10} reduction	Tc/HRT (d)	Meet standard (Yes/no)	Reference
***Enterococcus/Enterococci* - Batch**								
Cow manure	37	6.6	6.6×10^{5}		3.13	15	N	(Watcharasukarn et al., 2009)
Cow manure	55	6.6	6.6×10^{5}		1.7	2	N	(Watcharasukarn et al., 2009)
Cow manure	70	6.6	6.6×10^{5}		1.77	1	N	(Watcharasukarn et al., 2009)
Pig and cow manure	35	4 (VS)			4	12.5	N	(Lund et al., 1996)
Pig and cow manure	55	4 (VS)			4	1-2 h	N	(Lund et al., 1996)
***Enterococcus/Enterococci* - Continuous**								
Swine manure	24	6.2-7.2	1.8×10^{4}-1.7×10^{6}		0.6-1	7, 14	N	(Massé et al., 2011)
Cattle manure	33	7	10^{6}-10^{7}	10^{4}	2-3	55	N	(Popova et al., 2013)
Bovine manure and AW	35		4.6 log_{10} CFU/g	4.6 log_{10} CFU/g	0		N	(Bonetta et al., 2011)
Sewage sludge	38	3-4	3.6×10^{4}	2.0×10^{3}	1.3	14-20	N	(Sahlström et al., 2004)
Sewage sludge	55	3-4	3.2×10^{4}	0	>4	9	Y	(Sahlström et al., 2004)

Substrate	T (^{o}C)	TS (%)	Initial count (CFU/g or mL)	Finial count (CFU/g or mL)	Log_{10} reduction	Tc/HRT (d)	Meet standard (Yes/no)	Reference
Clostridium perfringens - Batch								
Cow manure	37	6.6	10^3-10^4		1.35	15		(Watcharasukarn et al., 2009)
Cow manure	55	6.6	10^3-10^4		<1	2		(Watcharasukarn et al., 2009)
Cow manure	70	6.6	10^3-10^4		<1	1		(Watcharasukarn et al., 2009)
Clostridium perfringens - Continuous								
Swine manure	24	6.2-7.2	$<1\times10^3$-3.7×10^6		0-0.2	7, 14	N	(Massé et al., 2011)
Bovine manure and AW	35		Observed in 50% samples	Observed in 75% samples				(Bonetta et al., 2011)
1:1 primary + WAS	35	3.24	7.24×10^6 CFU/100mL	1.55×10^7 CFU/100mL	-0.33	15	N	(Salsali et al., 2008)
sludge	37.8	3.2	5.28 log_{10} spores/mL	5.40 log_{10} spores/mL	-0.12	50	N	(Lloret et al., 2013)
Sewage sludge	38	3-4	1.7×10^5	6.1×10^4	0.4	14-20	N	(Sahlström et al., 2004)
Manure and AW	39	6.3-9.3	2.1×10^4-9.5×10^4	7×10^2-9.75×10^4	-1.31-0.89	20-28	N	(Orzi et al., 2015)
Manure and AW	39	6.1-7.3	2.1×10^5-6×10^5	7.5×10^2-1.3×10^4	-0.09-3.86	70	N	(Orzi et al., 2015)
Manure and AW	39-42	4.5-5.6	8×10^3-4×10^6	5.5×10^2-3.75×10^5	-0.34-2.45	35-45	N	(Orzi et al., 2015)
1:1 primary + WAS	42	3.17	7.24×10^6 CFU/100mL	2.00×10^7 CFU/100mL	-0.44	15	N	(Salsali et al., 2008)
1:1 primary + WAS	49	3.15	7.24×10^6 CFU/100mL	2.03×10^7 CFU/100mL	-0.45	15	N	(Salsali et al., 2008)
sludge	52.3-53.9	1.9-2.7	5.28 log_{10} spores/mL	4.63 log_{10} spores/mL	0.06	16-28	N	(Lloret et al., 2013)
1:1 primary + WAS	55	3.6	7.24×10^6 CFU/100mL	1.72×10^7 CFU/100mL	-0.38	15	N	(Salsali et al., 2008)
Sewage sludge	55	3-4	1.7×10^5	1.1×10^5	0.2	9	N	(Sahlström et al., 2004)

Substrate	T (^oC)	TS (%)	Initial count (CFU/g or mL)	Finial count (CFU/g or mL)	Log_{10} reduction	Tc/HRT (d)	Meet standard (Yes/no)	Reference
Ascaris suum eggs - Batch								
Sludge	20	6.0-6.5	173.5 eggs/2g TS		~0.2	80	N	(Pecson et al., 2007)
Sludge	30	6.0-6.5	173.5 eggs/2g TS		~0.2	24	N	(Pecson et al., 2007)
Sludge	37	4		75% reduction	<1	10	N	(Kato et al., 2003)
Sludge	40	6.0-6.5	77.7 eggs/2g TS	Below LOD	1.9	12	Y	(Pecson et al., 2007)
Sludge	47	4		95% reduction	<1	2	N	(Kato et al., 2003)
Sludge	49	2	4×10^5 viable eggs/L	1.5 log_{10} viable eggs/L	~3	6 h		(Aitken et al., 2005)
Sludge	50	6.0-6.5	77.7 eggs/2g TS	Below LOD	1.9	110 min	Y	(Pecson et al., 2007)
Sludge	51	2	4×10^5 viable eggs/L	Below LOD	~3	1.5 h	Y	(Aitken et al., 2005)
Sludge	51.1	2	10^4 ova/4g DW	Below LOD	4	4 h	Y	(Popat et al., 2010)
Sludge	53	2	4×10^5 viable eggs/L	Below LOD	~3	0.5 h	Y	(Aitken et al., 2005)
Sludge	53.3	2	10^4 ova/4g DW	Below LOD	4	4 h	Y	(Popat et al., 2010)
Sludge	55	2	4×10^5 viable eggs/L	Below LOD	~3	0.5 h	Y	(Aitken et al., 2005)
Sludge	55	4		> 99% reduction	2	1 h		(Kato et al., 2003)
Sludge	55.5	2	10^4 ova/4g DW	Below LOD	4	4 h	Y	(Popat et al., 2010)

Substrate	T (^{o}C)	TS (%)	Initial count (CFU/g or mL)	Finial count (CFU/g or mL)	Log_{10} reduction	Tc/HRT (d)	Meet standard (Yes/no)	Reference
Poliovirus - Batch								
Sludge	49	2	10^4-10^6 PFU/L		2.5	4 h		(Aitken et al., 2005)
Sludge	51	2	10^4-10^6 PFU/L	Below LOD	~3	0.5 h	Y	(Aitken et al., 2005)
Sludge	51.1	2	10^6 PFU/4g DW	Below LOD	6	4 h	Y	(Popat et al., 2010)
Sludge	53	2	10^4-10^6 PFU/L	Below LOD	~3	0.5 h	Y	(Aitken et al., 2005)
Sludge	53.3	2	10^6 PFU/4g DW	Below LOD	6	4 h	Y	(Popat et al., 2010)
Sludge	55	2	10^4-10^6 PFU/L	Below LOD	~3	0.5 h	Y	(Aitken et al., 2005)
Sludge	55.5	2	10^6 PFU/4g DW	Below LOD	6	4 h	Y	(Popat et al., 2010)
Poliovirus - Continuous								
Sludge	34	0.91	10^6 PFU/mL		1-2	5		(Sanders et al., 1979)
Sludge	34	1.36	10^6 PFU/mL		2-3	10		(Sanders et al., 1979)
Sludge	35	3.99			~ 2	14		(Bertucci et al., 1977)
Sludge	37	0.97	10^6 PFU/mL		1-2	5		(Sanders et al., 1979)
Sludge	37	2.09	10^6 PFU/mL		3-4	10		(Sanders et al., 1979)
Sludge	37	1.79	10^6 PFU/mL		5-6	15		(Sanders et al., 1979)
Sludge	50	1-2	10^6 PFU/mL		1-2	5		(Sanders et al., 1979)
Sludge	50	1-2	10^6 PFU/mL		3-4	10		(Sanders et al., 1979)

(a) Gram-negative bacteria

E.coli, fecal coliforms and *Salmonella* are commonly used Gram-negative indicator bacteria. In continuous operation, the reactors are daily fed in continuous or semi-continuous modes, indicating consistent introduction of pathogens into the reactors. It should take more time for pathogens to be inactivated in continuous or semi-continuous digesters.

E. coli

During anaerobic digestion, *E. coli* is required to be reduced by 4 $\log_{10}$ reductions with the final concentration $<10^3$ CFU/g DM (Milieu et al., 2008), or $< 10^3$ CFU/g fresh matter in the digestate according to different regulations (BSI, 2014; EU, 2011; IRBEA, 2012). In batch experiment, *E. coli* can be reduced by ~ 5 - 6 $\log_{10}$ reductions during 40-60 days in ambient and mesophilic conditions (Manyi-Loh et al., 2014; Pandey & Soupir, 2011), with the final pathogen concentration $< 10^3$ CFU/g. At the shorter digestion time of 20 days, only 1 - 2 $\log_{10}$ reductions were observed at 35 ^oC (Smith et al., 2005). The rapid elimination of *E. coli* (4.94 $\log_{10}$ reduction) within 6 days in mesophilic anaerobic digestion of cow manure was attributed to the accumulation of free VFA (Watcharasukarn et al., 2009). While at thermophilic conditions, *E. coli* was reduced to below LOD from 20 - 60 minutes (Smith et al., 2005; Watcharasukarn et al., 2009) to 3.5 days (Pandey & Soupir, 2011), and it took only 2 minutes at 70 ^oC (Watcharasukarn et al., 2009). Therefore, in batch ambient and mesophilic experiments, usually a 60 - day digestion time is required to meet the required standards ($< 10^3$ CFU/g).

As to continuous or semi-continuous systems, in ambient and mesophilic conditions, 0.9 - 2.9 $\log_{10}$ reductions were obtained at the HRT of 7 - 55 days, and in most cases the system didn't meet the standard of < 10^3 CFU/g (Horan et al., 2004; Lloret et al., 2013; Massé et al., 2011; Popova et al., 2013; Sahlström et al., 2004). At the relative higher mesophilic temperature of 39 - 42 ^oC in 10 full-scale biogas plants in Italy, Orzi et al. (2015) reported the final *E. coli* concentrations of 0-1.9×10^3 CFU/g at the HRT of 20-28 days, <10^3 CFU/g at the HRT of 30-40 days, and below LOD at the HRT of 70 days. It indicated increasing the HRT can promote the inactivation of *E. coli*. At thermophilic conditions, 4 - 6 $\log_{10}$ reductions were observed at the HRT of 7.8 - 9 days, which resulted in the final concentrations of < 10^3 CFU/g (Aitken et al., 2007; Sahlström et al., 2004). At the HRT of 16 - 28 days, complete eliminations were obtained with 5 - 7 $\log_{10}$ reductions (Lloret et al., 2013). The complete elimination time was much longer than the values of 20 minutes - 3.5 days obtained in batch experiment; this is consistent with the assumption that a longer inactivation time should be required to meet the standards in continuous or semi-continuous systems.

Total coliforms and fecal coliforms

Coliform bacteria are rod-shaped Gram-negative non-spore forming bacteria. Total coliforms include coliform bacteria found in the soil, waste and human/animal excreta, while fecal coliforms are a sub-group of total coliforms and exist in the intestines and feces of human and animals, with *E. coli* a sub-group of fecal coliforms (Scheuttpelz, 1969). Fecal coliforms are required to be < 10^3 MPN/g TS in Class A standard and < 2×10^6 MPN/g TS in Class B standard in the USA (USEPA, 1994). Class A standard means pathogens are below the detectable levels; while Class B standard means pathogens in biowastes are detectable but have been reduced to levels that don't pose a threat to the environment and public health, provided actions are taken to prevent exposure to the biowastes after their use or disposal.

In batch studies, the time required to meet the coliform standards varied greatly. At ambient conditions, Scaglia et al. (2014) reported fecal coliforms didn’t meet the Class A standard after digestion of 60 days. While, a complete elimination of 7 - 8 log_{10} reductions within 5 days was observed by Puchajda et al. (2006), while it was attributed to the effect of free VFA rather than temperature. Varel et al. (2012) studied the AD of swine and cattle slurries at 22 ^oC, and the final coliform concentration were above and below 10^3 MPN/g TS after 25 days and 28 days, respectively. The difference may derive from the different substrates, TS contents and initial coliform concentrations. At mesophilic conditions, coliforms were reduced to below the LOD within 3 days and 14 days in anaerobic digestion of swine and cattle slurries at 38 ^oC, respectively (Varel et al., 2012). In sludge digesters, final fecal coliforms did not meet the standard until 20 days in mesophilic conditions (Puchajda et al., 2006; Scaglia et al., 2014). At thermophilic conditions, reduction of coliforms met the Class A standard within 1-20 days (Scaglia et al., 2014; Varel et al., 2012).

In continuous or semi-continuous digesters, total coliforms or fecal coliforms didn’t meet the Class A standard in ambient and mesophilic conditions with the HRT ranging from 5 to 50 days (Cheunbarn & Pagilla, 2000; Lloret et al., 2013; Massé et al., 2011; Orzi et al., 2015; Popova et al., 2013; Riau et al., 2010; Sahlström et al., 2004). But Orzi et al. (2015) reported at the HRT of 70 days during anaerobic digestion of pig slurry and agriculture waste, fecal coliforms were completely eliminated at 39 ^oC. In thermophilic conditions, coliforms were reduced to below the LOD at the HRT of 9 - 28 days (Coelho et al., 2011; Lloret et al., 2013; Sahlström et al., 2004), but coliforms at the HRT of 5 - 10 days didn’t meet the Class A standard (Coelho et al., 2011). The inactivation on indicator pathogens showed that the inactivation is caused by both temperature and digestion time. In the temperature phased AD systems, the total HRT of less than 10 days is not enough for coliform inactivation. Coelho et al. (2011) observed the coliform concentration of > 10^3

MPN/g TS at a total HRT of 5 and 10 days at 55 ^{o}C/35 ^{o}C of 2d/3d and 2d/8d. The fecal coliforms were reported < 10^2 MPN/g TS at the total HRT of 14 - 17 days at 55 ^{o}C/35 ^{o}C of 2d/13d, 2d/15d and 4d/10d; 62 ^{o}C/35 ^{o}C of 1d/14d and 35 ^{o}C/62 ^{o}C of 14d/1d (Cheunbarn & Pagilla, 2000; Coelho et al., 2011; Harikishan & Sung, 2003). Riau et al. (2010) studied anaerobic digestion of sludge at 55 ^{o}C/35 ^{o}C with the HRT of 15d/15d, 5d/15d, 3d/15d and 3d/12d, fecal coliforms were reduced to < 10^3 MPN/g TS in the thermophilic stage, but the recurrence of fecal coliforms was observed in the HRT 15d/15d and 5d/15d digesters, which exceed the Class A limit of 10^3 MPN/g TS.

Salmonella

Salmonella is required to be absent in 50 g fresh matter (BSI, 2014; Milieu et al., 2008), or in 25 g fresh matter (EU, 2011; IRBEA, 2012), or < 3 MPN/4g TS in the digestate (USEPA, 1994) according to different regulations. In EU, the species *Salmonella Senftenberg* is required to be reduced by 5 $\log_{10}$ reductions (EU, 2011).

In batch experiment, the inactivation of *Salmonella* can't meet the standard at 20 ^{o}C after 60 days (Scaglia et al., 2014). At 35 ^{o}C, the final *Salmonella* concentration met the standard after 13 days at the low initial concentration of 81 MPN/g DM (Scaglia et al., 2014), but didn't at the high initial concentration of 10^8-10^9 CFU/mL after 20 days (Smith et al., 2005). Manyi-Loh et al. (2014) reported *Salmonella* decreased to below the LOD (10^2 CFU/g) from 7.4×10^3 CFU/g after 133 days, but didn't clearly indicated whether it was absent in the digestate. At thermophilic conditions, *Salmonella* was reported to be completely eliminated in 20-60 min or 3 days (Scaglia et al., 2014; Smith et al., 2005).

In continuous or semi-continuous digesters, the inactivation of *Salmonella* didn't meet the standards at the temperature ranging 24 - 49 ^{o}C at the HRT of 7 - 15 days

(Cheunbarn & Pagilla, 2000; Horan et al., 2004; Massé et al., 2011; Riau et al., 2010; Salsali et al., 2006). Lloret et al. (2013) reported *Salmonella* was detectable in the digestate at 37.8 ^{o}C and HRT of 50 days. Thus ambient and mesophilic temperatures are not effective in *Salmonella* inactivation in absence of other factors. While at thermophilic conditions, *Salmonella* was not detected at the HRT of 16-28 days (Lloret et al., 2013). At temperature-phased anaerobic digestion systems, the digestate met the standard of < 3 MPN/4g DM with the total HRT ranging 14 - 30 days (3 - 15 days at 55^{o}C and 10 - 15 days at 35 ^{o}C) (Harikishan & Sung, 2003; Riau et al., 2010). The digestate also met the standard at the combination of 1 day at 62^{o}C and 14 days at 35 ^{o}C, or 14 days at 35 ^{o}C and 1 day at 62^{o}C (Cheunbarn & Pagilla, 2000). To sum up, the *Salmonella* concentration can meet the standard at thermophilic or combined thermophilic and mesophilic digestion systems with total HRT > 14 days.

(b) Gram-positive bacteria

Enterococcus

Enterococcus is Gram-positive non-spore forming bacteria, and is used as an indicator for pathogen inactivation during anaerobic digestion. In EU, the family *Enterococcaceae* is required to be < 10^3 CFU/g fresh matter, and 5 $\log_{10}$ reductions are required for the species *Enterococcus faecalis* (EU, 2011).

In batch studies, Watcharasukarn et al. (2009) reported *Enterococcus faecalis* was reduced by 3.13, 1.7 and 1.77 $\log_{10}$ reductions at 35 ^{o}C (15 days), 55 ^{o}C (2 days) and 70 ^{o}C (1days), respectively, from the initial concentration of 6.6×10^5 CFU/mL. Lund et al. (1996) found 4 $\log_{10}$ reductions were obtained after 12.5 days and 1-2 hours at 35 ^{o}C and 55 ^{o}C, respectively. The final *Enterococcus* concentrations were unknown in both studies, but 5 $\log_{10}$ reductions were not achieved.

In continuous or semi-continuous systems, 0 - 3 $\log_{10}$ reductions were observed in ambient and mesophilic digesters with the HRT ranging 7 - 55 days (Bonetta et al., 2011; Massé et al., 2011; Popova et al., 2013; Sahlström et al., 2004). It indicates ambient and mesophilic conditions are not effective in *Enterococcus* inactivation. However, *Enterococcus* was completely eliminated in thermophilic digesters at the HRT of 9 days (Sahlström et al., 2004). Similarly, Watanabe et al. (1997) surveyed 17 wastewater treatment plants in Japan, and found *Enterococcus* ranged 10^2 - 10^5 MPN/g in mesophilic digestion systems but was < 10 MPN/g in thermophilic digesters.

Clostridium perfringens

Clostridium perfringens is also Gram-positive bacterium but is spore-forming. In EU, *Clostridium perfringens* is required to be less than 3×10^3 spores/g DM (Milieu et al., 2008). *Clostridium perfringens* is one of the most resistant bacteria and can't be effectively inactivated by anaerobic digestion even at thermophilic conditions (Table 2-6). In batch experiment, *Clostridium perfringens* was reduced by 1.35 $\log_{10}$ reductions at 35 ^oC for 15 days, and < 1 $\log_{10}$ reductions were observed at 55^oC (2 days) and 70 ^oC (1 day). In continuous or semi-continuous digesters, the $\log_{10}$ reduction of *Clostridium perfringens* ranged (-1.31) - 3.86 at ambient to thermophilic temperatures (24 - 55 ^oC) and various HRT (7 - 70 days), and almost none of them met the standard of < 3×10^3 spores/g DM (Lloret et al., 2013; Massé et al., 2011; Orzi et al., 2015; Sahlström et al., 2004; Salsali et al., 2008). It indicates *Clostridium perfringens* is highly thermal resistant, and increasing temperature is not an effective way to inactivate it.

(c) Nematode and viruses

Ascaris suum is a kind of helminth which usually infects pigs. During anaerobic digestion, helminth is required to be < 1 viable egg/4g TS (USEPA, 1994) or to be absent in the digestate (Lepeuple et al., 2004), and *Ascaris* eggs are required to be reduced by 3 log_{10} reductions (EU, 2011) or be absent in the digestate (Milieu et al., 2008). The inactivation of *Ascaris* eggs during anaerobic digestion is mainly studied in batch experiment. Pecson and Nelson (2005) reported *Ascaris* eggs were not inactivated by the temperature of < 40 ^oC in the absence of other factors, and Wharton (1979) indicated the impermeability of *Ascaris* eggs was destroyed at 63 - 65 ^oC. It was demonstrated that at 20 - 30 ^oC, minor reductions (~ 0.2 log_{10}) were observed during the digestion time of 24 - 80 days (Pecson et al., 2007). At 37 - 47 ^oC, less than 2 log_{10} reductions were obtained within 2 - 12 days (Kato et al., 2003; Pecson et al., 2007). At thermophilic conditions of 50 - 55 ^oC, *Ascaris* eggs were decreased to below LOD with 2 - 4 log_{10} reductions within 0.5 - 4 hours (Aitken et al., 2005; Kato et al., 2003; Pecson et al., 2007; Popat et al., 2010). As a summary, the ambient and mesophilic temperatures can't inactivate *Ascaris* eggs effectively; while at thermophilic condition, *Ascaris* eggs can be inactivated within 4 hours.

During anaerobic digestion, enteric viruses are required to be < 1 plaque-forming unit (PFU)/ 4g TS in the USA (USEPA, 1994), and parvovirus is required to be reduced by 3 log_{10} reductions in EU (EU, 2011). In batch experiment, Aitken et al. (2005) and Popat et al. (2010) studied the inactivation of parvovirus in sludge anaerobic digestion at the temperature of 49 - 55.5 ^oC, and found poliovirus was reduced to below LOD within 0.5 - 4h. But in continuous/semi-continuous digesters, it usually takes much longer time to meet the standards. Bertucci et al. (1977) spiked poliovirus into sludge and digested at 35 ^oC with a digestion time of 14 days, and found Poliovirus I (Sabin) was inactivated by 98.8% after 48 hours. Sanders et al. (1979) studied the inactivation of poliovirus during anaerobic digestion of sludge at 34 ^oC, 37 ^oC and 50 ^oC at the HRT of 5 - 15 days. It showed that the inactivation of poliovirus was a function of time and temperature, but time's importance decreased as the temperature increased. At 50 ^{o}C, temperature was the dominant inactivation factor, accounting for nearly 100% of the poliovirus inactivation, while at 34 ^{o}C and 37 ^{o}C, the contributions of temperature accounted for only 46% and 56%, respectively.

As a summary, generally, ambient and mesophilic temperatures are not effective in pathogen inactivation in the absence of other factors. At thermophilic or temperature phased (55^{o}C + 35^{o}C) systems, the total HRT of > 15 days are usually necessary for the completely elimination of Gram-negative bacteria (*E. coli*, fecal coliforms and *Salmonella*) and non-spore forming Gram-positive bacteria (*Enterococcus*). However, the recurrence of pathogens must be noted in the mesophilic phase of temperature phased systems. Protozoa and viruses can also be inactivated at thermophilic conditions, but the data on the inactivation in continuous anaerobic digestion systems are little and further study is needed. The spore-forming Gram-positive bacterium, *Clostridium perfringens*, is highly thermal resistant and can't be effectively inactivated in anaerobic digestion even at thermophilic conditions.

2.4.3 Effects of VFA on pathogen inactivation

2.4.3.1 Inactivation mechanism of VFA

Volatile fatty acids are important intermediate products during anaerobic digestion. In digesters, VFA exists in forms of free VFA and ionized VFA, whose equilibrium is pH dependent (Kunte et al., 2000a). At present, the inactivation mechanisms of VFA on pathogens are not fully understood, and the inhibition effects of VFA are summarized as: (1) to affect the electrophysiology and metabolism of the cell, (2) to acidify the cytoplasm and (3) to cause osmotic problems.

Free VFA is usually considered more toxic to pathogen than ionized VFA, because it is lipophilic and can permeate across the cell membrane freely, while ionized VFA is lipophobic and cannot pass across the cell membrane (Puchajda et al., 2006). Most bacteria carry a negative charge and a neutral or slightly alkaline interior pH, which can form a specific proton motive force (PMF, Δp) across the cell membrane (Kashket, 1985). When the environmental pH is low, free VFA passes across the cell membranes and dissociates in the more alkaline interior, producing H^+ and ionized VFA, which decrease the PMF and acidify the cytoplasm. The PMF, consisting of pH gradient (ΔpH) and electrical potential ($\Delta\psi$) across the cell membrane, plays important roles in metabolism of bacteria (Kashket, 1985). For example, PMF was reported to affect the sensory transduction of bacteria (Taylor, 1983), drive the synthesis of adenosine triphosphate (ATP) (Armitage et al., 1985), energize the active transport of ions and metabolites (Armitage et al., 1985), provide a mechanical force for flagella motors (Armitage et al., 1985), control the *Staphylococcus aureus lrgAB* operon expression (Patton et al., 2006) and drive the DNA uptake in genetic transformation (van Nieuwenhoven et al., 1982). The decreased PMF may pose great harm to the metabolism of pathogens, but the detailed mechanisms are not clear up to date. Russell (1991) observed the decreased protein production of *Streptococcus bovis* JB1 at the acetate concentration of 100 - 360 mM and pH 5.2, and this reduction may be due to a shift in fermentation products and a decline in ATP production. Sheu and Freese (1972) assumed VFA can alter the cell membrane structure or uncouple the electron transport system, so as to obstruct the generation of ATP and the transport of needed compounds. He also reported that VFA strongly inhibited the uptake of L-serine or other L-amino acids by *Bacillus subtilis* (Sheu et al., 1972).

The dissociated H^+ in the cells can acidify the cytoplasm. To overcome this problem, the cells must pump out H^+ via a mechanism called H^+-ATPase pump, which consumes energy and may cause energy depletion of the cell (Puchajda et al., 2006).

If the H^+ efflux is not rapid enough, pH may drop to below the threshold pH of the cells and the cellular functions are ceased (Kashket, 1987; Russell & Diez-Gonzalez, 1997). For example, the threshold pH for *E. coli O157:H7* was reported to be 6.1 before the growth ceased (Russell & Diez-Gonzalez, 1997).

On the other hand, the dissociation of VFA in the cells leads to a logarithmic accumulation of ionized VFA in the cytoplasm, increasing the osmotic pressure (Roe et al., 1998). Roe et al. (1998) reported at pH 6.0 and the environmental acetate concentration of 8 mM, the intracellular acetate concentration of acetate was greater than 240 mM. The severe accumulation of acetate must be compensated by a great reduction of other anions. Potassium glutamate is the dominant compound to maintain the osmotic pressure in most Gram-negative pathogens (McLaggan et al., 1994). The accumulation of VFA anions in cytoplasm replaced glutamate to maintain the constant osmotic pressure and resulted in the reduction of glutamate in the cell. However, the reduction of glutamate can only partially compensate for the accumulated VFA anions (Roe et al., 1998).

The inhibitory effects of VFA on pathogen inactivation are attributed to all the three factors, but their contributions are not clear and vary according to various conditions, such as pathogen species, VFA type, VFA concentration and pH level. Salmond et al. (1984) studied the effect of low benzoate concentration (2.5 mM) on *E. coli* at pH 5.0 - 7.6, and indicated although the inhibition of cytoplasm acidification was significant, the effect of benzoic acid on cell metabolism was more potent. Eklund (1983) studied the inhibition of sorbic acid on various bacteria, and found both free and ionized acids contributed to the inhibition. Although the inhibitory effect of free acid was 10 - 600 times greater than ionized acid, the ionized acid was observed to cause >50% growth inhibition at pH > 6 to most bacteria tested.

The above theory assumes that the bacteria, e.g. *E. coli*, have a neutral or slightly alkaline interior to form a large PMF across the cell membrane (Salmond et al., 1984). However, some fermentative bacteria, e.g. *Clostridium perfringens*, are acid-resistant and able to decrease their intracellular pH to form lower Δp, and they have the ability to generate ATP at lower intracellular pH (Russell & Diez-Gonzalez, 1997; Salsali et al., 2008). These bacteria are usually Gram-positive and have a high interior K^+ concentration, which can counteract the accumulation of ionized VFA and allow the pathogen to tolerate even higher VFA anions (Russell & Diez-Gonzalez, 1997). But the modest decreased Δp can only partially counteract the inhibitory effect of VFA (Russell & Diez-Gonzalez, 1997).

Several questions on VFA inhibition need to be answered: (1) the inhibitory effect may be mitigated by some glycolytic compounds; (2) the pathogens may recover by transferring to no VFA medium; and (3) the acid tolerance response (ATR) mechanism of pathogens may be induced at low pH. Sheu and Freese (1972) found the inhibition of 0.2 M acetate on *Bacillus subtilis* was reduced but not eliminated by the addition of 0.2% glucose or fructose. He also observed that when the cells were transferred to no VFA medium after exposure to 200 mM acetate for 2 hours, the inhibited cells grew at the same rate as non-treated ones (Sheu & Freese, 1972). But Roe et al. (1998) reported VFA could acidify the cytoplasm of *E. coli* and caused the reduction of glutamate in the cell, when transferred the cells from VFA to no-VFA media, the recovery of the cytoplasmic pH was depended on the synthesis of glutamate. Baik et al. (1996) reported the ATR of *Salmonella typhimurium* was induced at an extreme acid shock of pH 4.4 but not in sub-inhibitory concentrations of VFA.

2.4.3.2 Effects of various factors on VFA caused pathogen inactivation

The inhibitory effect of VFA on pathogens is not independent, but impacted by many other factors such as the resistance of pathogens, pH, temperature, digestion time, VFA type, concentration and chain-length (Salsali et al., 2006; Watcharasukarn et al., 2009).

(a) Effects of pH on VFA caused pathogen inactivation

The effect of pH on pathogen inactivation is significant at mesophilic conditions by affecting the concentration of free VFA. Usually little inhibition is observed at pH 7.0, while high VFA concentrations and acidic pH pose greater inhibition on pathogens (Kunte et al., 2000a; Salsali et al., 2006; Wolin, 1969). Wolin (1969) found at pH 7.0 and 40 ^{o}C, no inhibition was observed on the growth of *E. coli* by either acetate (45 mM) or VFA mixtures (60 mM acetate + 20 mM propionate + 15 mM butyrate) after 24 hours. But the acetate caused 41% and 80% inhibitions, and the VFA mixtures caused 69% and 96% inhibitions on *E. coli* at pH 6.5 and 6.0, respectively. Similarly, Kunte et al. (2000a) found at 37 ^{o}C and neutral pH, even the VFA (C2-C6) concentrations of 3,000 mg/L didn't inhibit the growth of *Shigella dysenteriae*. The toxicity of VFA increased dramatically with the decrease of pH, for example, the minimum inhibitory concentration (MIC) of acetate on *Shigella dysenteriae* were 3000, 1000,500 and 10 mg/L at the pH of 6.5, 6.0, 5.5 and 5.0, respectively (Kunte et al., 2000a). Salsali et al. (2006) also observed at 35 ^{o}C and neutral pH of 6.5 and 7.5, the growth of *Salmonella* was not inhibited in 750 mg/L VFA mixture after 24 hours, but great reductions were observed at the pH of 5.5 and 4.5.

(b) Effects of temperature on VFA caused pathogen inactivation

The increase of temperature usually enhances the inhibitory effect of VFA. Firstly, the increasing temperature affects the diffusion rate of VFA. Ouattara et al. (2000) found when the temperature rose from 4 ^{o}C to 24 ^{o}C, the diffusion coefficients of acetic and propionic acids increased by 2.18 and 2.05 times, respectively. Secondly, temperature affects the dissociation constant of VFA, thereby affecting the concentration of free VFA. Harned and Ehlers (1933a) reported with the increase of temperature (0-60 ^{o}C), the dissociation constant of acetic acid increased first and then decreased with the peak value obtained at 25 ^{o}C. The dissociation constants of acetic acid at 25, 35 and 55 ^{o}C were 1.754, 1.729 and 1.590 $\times 10^{-5}$, respectively. A similar trend was observed for propionic acid, with the dissociation constants of 1.336, 1.310 and 1.195 $\times 10^{-5}$ at 25, 35 and 55 ^{o}C, respectively (Harned & Ehlers, 1933b). According to the ionization equilibrium of VFA [$c(HA)/c(A^{-})=c(H^{+})/K_a$], the decrease of K_a from 25 ^{o}C to 55 ^{o}C increases the proportion of free VFA, so as to post greater harm. At the similar pH and total VFA concentrations, Puchajda and Oleszkiewicz (2006) observed about 1 more $\log_{10}$ reduction of fecal coliforms at 38 ^{o}C than that at 24 ^{o}C in semi-continuous sludge digesters. Similarly, Salsali et al. (2006) reported at the same total VFA concentration and pH level, the inactivation coefficients of *Salmonella* increased with the increase of temperature (35 - 49 ^{o}C).

(c) Effects of VFA chain-length

The inhibitory effect of VFA usually increases with the increase of chain length. Sheu and Freese (1972) studied the inhibition of C1-C18 acids on the growth of *Bacillus subtilis*, and found the longer the chain-length, the lower molar concentration was required to achieve a specific inhibition level. The inhibitory sequence of short chain VFA (C1-C6) on *Bacillus subtilis* growth was formic acid < acetic acid < propionic acid < n-butyric acid < n-pentanoic acid < n-hexanoic acid (Sheu & Freese, 1972). Besides, VFA inhibited the uptake of amino acids, and similar with the inhibition on *Bacillus subtilis* growth, the inhibitory effect of VFA increased with the increase of chain length (Sheu et al., 1972). Wolin (1969) found at similar concentrations, propionic (10 mM), butyric (11 mM) and valeric (10 mM) acids inhibited *E. coli* growth by 57%, 74% and 79%, respectively. Back et al. (2009) indicated the inhibitory effect of organic acids against *Enterobacter sakazakii* was propionic acid > acetic acid > formic acid. But different results were reported by Salsali et al. (2006), who studied the impact of VFA on *Salmonella* inactivation and indicated the inhibitory effect of mixed VFA > acetic acid > propionic acid > butyric acid. However, the inhibitory effects of different VFAs in this study were compared based on the same mass concentrations rather than molar concentrations. For example, at the same mass concentration of 6,000 mg/L tested, the molar concentration of acetic acid was 100 mmol/L, which was higher than those of propionic acid (81 mmol/L) and butyric acid (68 mmol/L). Thus the high inhibitory effect of acetic acid on *Salmonella* may be caused by its higher molecular concentrations rather than the chain length.

2.4.3.3 Inactivation of pathogens during anaerobic digestion

The effects of VFA on the inactivation of pathogens are shown in Table 2-7. These research results indicate that the inhibitory effect of VFA is affected by various factors, and the results varied a lot. Therefore it’s difficult to have a universal threshold inhibition concentration for VFA.

In batch experiment, the lowest inhibitory concentration of free VFA ranged 42 - 78 mg/L, which decreased *E. coli* and total coliforms by 4.9 - 7.2 $\log_{10}$ reductions within 6 - 7 days (Jiang et al., 2018; Watcharasukarn et al., 2009). Jiang et al. (2018) conducted dry co-digestion of food waste and pig manure at 37 ^{o}C, and indicated free VFA was the significant inactivation factor for *E. coli* and total coliforms.

Watcharasukarn et al. (2009) studied the inactivation of pathogens at various temperatures, and reported the higher inactivation of *Clostridium perfringens* at 37 ^{o}C than at 55 ^{o}C or 70 ^{o}C was attributed to free VFA. Retention time is another important factor for pathogen inactivation. Salsali et al. (2006) reported free VFA of ~133 mg/L only caused 18% reduction on *Salmonella* in 24 hours, while the concentration of ~ 1066 mg/L resulted in a 73% reduction. Puchajda et al. (2006) found during anaerobic digestion of sludge, the free VFA concentration of 200 - 600 mg/L resulted in ~ 4 log_{10} reductions on fecal coliforms in 5 days, while the concentration of 600 - 1400 mg/L caused complete elimination. Puchajda and Oleszkiewicz (2006) also reported the free VFA of 2300 - 2500 mg/L resulted in 5-6 log_{10} reductions on fecal coliforms in 9 days regardless temperature (24 ^{o}C and 38 ^{o}C) in sludge anaerobic digestion. Similar with temperature, the VFA resistance of Gram-positive bacteria was higher than Gram-negative bacteria, with the sequence of *Clostridium perfringens* > *Enterococcus faecalis* > *E. coli* and total coliforms (Jiang et al., 2018; Watcharasukarn et al., 2009).

In continuous or semi-continuous anaerobic digestion systems with the HRT of 20 - 30 days, the VFA concentrations were usually low (90 - 700 mg/L at pH 6.7 - 7.6) with almost no free VFAs (1 - 2 mg/L), so the pathogens were not effectively inactivated (Kunte et al., 1998; Kunte et al., 2000a; Kunte et al., 2000b). However, when decreasing the HRT to 10 - 15 days, the VFA was accumulated (4500 - 9000 mg/L) due to acidification and pH decreased to 5.8 - 6.5, resulting in the free VFA concentration of 160 - 270 mg/L, which effectively inactivated Gram-negative bacteria such as *Shigella dysenteriae*, *Vibrio cholera* and *Salmonella typhi* within 12 - 20 days (Kunte et al., 1998; Kunte et al., 2000a; Kunte et al., 2000b). The initial pathogen concentration in the substrates is an important factor affecting the biosafety of digestate. Kunte et al. (1998) indicated if the digester was daily dosed with 10^6 CFU/mL *Salmonella*, even though free VFA concentration of ~ 270 mg/L was obtained from high VFA concentration (4800 - 5700 mg/L) and low pH (6.0), the final concentration stayed in the digestate at 10^2 CFU/mL with ~ 4 $\log_{10}$ reductions. Puchajda and Oleszkiewicz (2006) reported at the HRT of 9 - 11 days, high VFA (6531 - 9058 mg/L) and low pH (5.2 - 5.4) resulted in high free VFA concentrations of 1342 - 1980 mg/L, but only 1 - 2 and 3 - 4 $\log_{10}$ reductions of total coliforms were observed at 24 ^{o}C and 38 ^{o}C, respectively. It may be due to the introduction of high total coliforms concentrations (10^6 - 10^8 MPN/g TS) every day.

Table 2-7 Effects of volatile fatty acids on the inactivation of pathogens

Pathogens/virus	Substrate	T (℃)	TS (%)	pH	VFA (mg/L)	Free VFA (mg/L)	Initial (CFU/mL)	Final (CFU/mL)	Log_{10} reduction	Tc (d)	HRT (d)	DRT (d)	Meet standard (Yes/no)	Reference
Batch														
Salmonella	Sludge	35		5.5	6000	1066 [a]	11000	3000		24 h			N	(Salsali et al., 2006)
					750	133 [a]	CFU/100mL	9000		24 h			N	
Fecal coliforms	Sludge	38	3.8-8.3	5.5-6.5	3297-7182	200-600	10^6–10^8	10^2-10^4	~4	5			N	(Puchajda et al.,
		21	3.6-7.8	5.4-6.0	3565-7020	600-1400	MPN/g TS	Below LOD	6-8	5			Y	2006)
Fecal coliforms	Sludge	38, 24	4.2	5.2	9200-10000	2300-2500	10^6–10^7 MPN/g TS	10^1-10^2	5-6	9			Y	(Puchajda & Oleszkiewicz, 2006)
E. coli	Cow Manure	37	6.6	6.9-7.5	5022-8712	11-78	6×10^4	Below LOD	4.94	6		0.92	Y	(Watcharasukarn et al., 2009)
E.faecalis							6.6×10^5		3.13	15		1.15	N	
C. perfringens							10^3-10^4		1.35	15		4.05		
E. coli	Food waste & pig manure	37	20	7.7-8.4	7695-31033	1-42	6.20×10^8	Below LOD	6.8	7		1.1	Y	(Jiang et al., 2018)
Total coliforms				7.7-8.4	7695-31033	1-42	1.56×10^9	Below LOD	7.2	7		1	Y	
Enterococci				7.0-8.4	7695-31033	1-134	6.10×10^8	Below LOD	6.8	31		2.4	Y	
Continuous														
Shigella dysenteriae	Human night soil	37	8	6.5	9000	160 [a]	10^6	Below LOD	6	18	10	2.2		(Kunte et al., 2000a)
				7.4	700	2 [a]		10^2	4		20	3.7		
Vibrio cholerae	Human night soil	23-27	8	6.4	8000	178 [a]	10^6	Below LOD	6	20	10	1.53		(Kunte et al., 2000b)
				7.6	500	1 [a]		10^2	4		20	2.63		
Salmonella typhi	Cattle dung	37	6	5.9-6.1	4500-5000	270 [a]	10^6–10^7	Below LOD	6-7	12	15	2.44	Y	(Kunte et al., 1998)
				6.7-6.9	90-140	1 [a]	Single dose	Below LOD	6-7	26	30	4.80	Y	
				5.8-6.2	4800-5700	270 [a]	10^6–10^7	10^2	4		15	4.22	N	
				6.7-7.1	100-125	1 [a]	Daily dose	10^4	3		30	18.63	N	
Fecal coliforms	Sludge	24		5.2-5.4	6565-8294	1342-1980		10^5-10^6	1-2		4.5+4.5		N	
		24		5.3	6841-8294	1443-1948	10^6–10^8	10^4-10^6	~2		4.5+4.5		N	(Puchajda &
		38		5.3	7781-8127	1777-1827	MPN/g TS	10^3-10^5	~3		4.5+6.5		N	Oleszkiewicz, 2006)
		38		5.3	6531-9058	1543-2127		10^2-10^4	~4		4.5+6.5		N	

a. Estimated according to equivalent acetic acid.

2.4.4 Effects of ammonia on pathogen inactivation

2.4.4.1 Inactivation mechanism of ammonia

Ammonia, released due to anaerobic degradation of protein, is another important inhibitory factor reported during anaerobic digestion. Similar with VFA, the inhibition mechanism of ammonia on pathogens is not clearly explicated. Free ammonia (NH_3) is considered more toxic to pathogens than ammonium ion (NH_4^+) because it's lipophilic, which can pass the cell membrane freely and dissociate inside the cell to change ΔP across the cell membrane and produce a harmful pH (Pecson & Nelson, 2005). Ammonia was also reported virucidal, which caused cleavage to RNA within poliovirus, but didn't affect the capsid proteins or isoelectric point of viral particles (Ward, 1978).

The effect of ammonia is pH dependent, and high pH promotes the conversation of NH_4^+ to NH_3 (Pecson & Nelson, 2005; Scaglia et al., 2014). Temperature affects the inhibitory effect of ammonia by influencing the dissociation constants. Both K_a for NH_4^+ and K_b for NH_3 increase with the rising of temperature from 0 ^oC to 50 ^oC, as pK_w changes with the temperature as well (Bates & Pinching, 1949). The changes of pK_w and pK_a with time were shown in Eq. 2-1and 2-2 (Bates a& Pinching, 1949; Emerson et al., 1975). The pK_a at 25 ^oC and 35 ^oC were 9.25 and 8.95, respectively. Martinelle and Häggström (1997) indicated the calculated NH_3 concentration was almost doubled using the pK_a at 35 ^oC than using the value at 25 ^oC, so it's important to use the correct pK_a value while calculating NH_3.

$$pK_w = \frac{4470.99}{T} - 6.0875 + 0.01706\,T \qquad \text{2-1}$$

$$pK_w = pK_a = \frac{2729.92}{T} + 0.090181T \qquad \text{2-2}$$

2.4.4.2 Effects of ammonia - pH

It is commonly considered pH doesn't inactivate pathogens directly but by affecting the conversion of NH_4^+ to NH_3. Cramer et al. (1983) studied the effect of ammonia on Poliovirus type 1 (strain CHAT) and bacteriophage f 2, and found OH^- had no significant effect on the inactivation of viruses and the effect of NH_4^+ was similar with Na^+, while NH_3 was the real inactivation factor. Fidjeland et al. (2015) reported in absence of ammonia, pH had no direct effect on the inactivation of *Ascaris* eggs, but had an indirect effect by affecting NH_3 activity. Park and Diez-Gonzalez (2003) also indicated pH 8.5, 9.0 and 9.5 didn't inactivate *E. coli* O157:H7 and *S. Typhimurium* DT104 after 24 hours. Pecson and Nelson (2005) observed no significant difference on the inactivation of *Ascaris* eggs at pH 7, 9 and 11 after 24 hours. However, Pecson et al. (2007) found the effect of pH on the inactivation of *Ascaris* eggs varied with temperature: at 20 ^oC, no significant pH effect was observed over 80 days, while at 30 ^oC and 40 ^oC, increasing pH from 7 to 12 significantly decreased the 99% inactivation time (t_{99}) of *Ascaris* eggs by 7.8 and 4.1 times, respectively, but at 50 ^oC, the effect of temperature dominated, with the effects of pH and NH_3 not being observed in the extremely short time of < 2 hours. The different results may be caused by the prolonged operation time (up to 80 days) and larger pH range (from 7 to 12). But Scaglia et al. (2014) found at 20 ^oC, the initial pH of 8.5, 9.0 and 9.5 adjusted by adding NH_3 didn't inactivate *Salmonell*a and *Helminth* ova effectively after 60 days. It may be because pH dropped quickly and maintained similar low NH_3 (60.2 - 73.2 mg N/L) during the digestion period.

2.4.4.3 Effects of ammonia - temperature

The effect of temperature must be considered while assessing the inhibitory effect of ammonia. Pecson et al. (2007) indicated each 10 ^oC increase in temperature significantly enhanced the inactivation of *Ascaris* eggs; at the same NH_3 concentration of 5000 mg N/L, the t_{99} values were 25 d, 4.8 d, 0.39 d and 97 min at 20, 30, 40 and 50 ^oC, respectively. Fidjeland et al. (2015) also indicated each 16 ^oC increase in temperature caused 10 - fold decrease in the inactivation time of *Ascaris* eggs. Scaglia et al. (2014) observed after 60 - day anaerobic digestion of sludge at 20, 35 and 55 ^oC, the NH_3 concentrations were 73.2, 662 and 1003 mg N/L, respectively. As a result, fecal coliforms, *Salmonell*a and *Helminth* ova were not effectively inactivated at 20 ^oC, but met the biosafety standard after 20 days at 35 and 55 ^oC. Himathongkham et al. (2000) also reported *Salmonella. typhimurium* and *E. coli* O157:H17 in poultry manure had the DRT of 0.59 - 0.61, 1.45 - 1.59 and 8.56 - 16.39 days at 4, 20 and 37 ^oC, respectively, with the inactivation mainly caused by the accumulation of ammonia.

2.4.4.4 Threshold inhibitory concentration of ammonia

Similar with VFA, it's difficult to get a universal threshold inhibitory concentration for ammonia due to the complex experiment conditions and different pathogens. For example, Ottoson et al. (2008) indicated the NH_3 concentration of 46 mmol/L caused 5 log_{10} reductions on *Salmonella Typhimurim* and *Enterococcus faecalis* after 1.6 and 4.2 days, respectively, compared with the 16 and 19 days in control system (NH_3 of 1.6 mmol/L). But 46 mmol/L NH_3 didn't significantly increase the inactivation rate of MS2 coliphage (ssRNA phage), and the inactivation rates of DNA phages ΦX174 and *Salmonella* phage 28B were significantly higher in the control systems (NH_3 of 1.6 mmol/L).

Park and Diez-Gonzalez (2003) observed a threshold NH_3 concentration of 5 mM for *E. coli* O157:H7 and *S. Typhimurium* DT104 in Luria-Bertani (LB) broth at 37 ^oC, as they grew under this concentration. A more than 40 mM NH_3 can cause a complete *Salmonella* reduction after 6 hours, but 180 mM NH_3 was needed to get > 5 log_{10} reductions for *E. coli* O157:H7 after 6 hours. Himathongkham et al. (2000) indicated at 20 ^oC, the threshold NH_3 concentration for *E. coli* O157:H7 was 0.28% (28% NH_3 v/v), with the added ammonia concentration of 0.6%.

Nordin et al. (2009) reported at 24 ^oC, 20 mM NH_3 might be the threshold concentration for the inactivation of *Ascaris* eggs. At 24 ^oC, 66 mM NH_3 caused > 3 log_{10} reductions to *Ascaris* eggs after 6 months, while at 34 ^oC, 40 mM NH_3 caused 3 log_{10} reductions in 1.5 months. Ghiglietti et al. (1997) reported at 22 ^oC, 0.5% (30% NH_3 v/v) NH_4OH didn't inactivate *Ascaris suum* eggs in sludge up to 90 days, and *Ascaris suum* eggs started to be inactivated from NH_4OH concentration of 1% (pH = 9.8), which can be considered as the threshold inhibitory concentration. At NH_4OH concentrations of 1.5%, 2%, 3.5% and 4% (pH 10 - 10.5), *Ascaris suum* eggs were completely eliminated after 60, 60, 40 and 21 days, respectively.

Jenkins et al. (1998) studied the inactivation of NH_3 (7 - 148 mM) on *Cryptosporidium parvum* Oocysts, and found even 7 mM NH_3 could cause significant inactivation after 24 hours. The inactivation rates increased from 0.014 to 0.066 h^{-1} with the increase of NH_3 concentration.

2.5 Summary

The literature review indicates that dry co-digestion of FW and PM is an effective management method with recovery of methane-rich biogas and nutrient-rich digestate. High VFA and ammonia concentrations are significant factors attributing to the inactivation of pathogens during anaerobic digestion, especially VFA at low pH and ammonia at high pH. Therefore, the high VFA and ammonia concentrations during dry co-digestion are expected to enhance pathogen inactivation and produce pathogen-safe digestate, and it might be a relative low-cost and high-effective way for inactivation of pathogens.

Chapter 3
Effects of extremely high volatile fatty acid on methane production kinetics during dry co-digestion of food waste and pig manure

3.1 Introduction

In this chapter, dry co-digestion of FW and PM was conducted to investigate (1) the effects of inoculum rates (25% and 50% based on VS) and FW/PM ratios (0:100, 25:75, 50:50, 75:25 and 100:0 based on VS) on system stability; (2) the inhibition of VFA or ammonia on methane production kinetics; and (3) the interactions among syntrophic oxidizing bacteria and hydrogenotrophic methanogens.

3.2 Materials and methods

3.2.1 Experimental design

Food waste was collected from 10 residences in Galway City, Ireland and stored at -10 ^{o}C. Before use, it was defrosted, mixed and ground in a food processor (Kenwood FPP210 Multipro Food Processor, Hampshire, UK) to less than 2 mm. Pig manure was obtained from the finishing unit of a local pig farm and stored at -10 ^{o}C. Before use it was defrosted and centrifuged at 1,500 ×g for 5 minutes (MSE super minor centrifuge, London, UK) and the solid fraction was used in the experiment. The inoculum was dewatered anaerobic sludge collected from a local wastewater treatment plant in Galway City, Ireland and stored at 11 ^{o}C in the laboratory prior to commencing the experiment. The pictures of the reactors are shown in Figure 3-1, and the characteristics of FW, PM and inoculum are listed in Table 3-1.

Figure 3-1 Pictures of the dry co-digestion experiment

Table 3-1: Characteristics of the food waste, pig manure and inoculum added to the digesters

Characteristics	FW [a]	PM [b]	Inoculum
pH	4.5 ± 0.2	8.4 ± 0.1	8.4 ± 0.1
Moisture content (MC, %)	70.6 ± 0.5	74.4 ± 0.2	81.2 ± 0.1
Total solids (TS, g/kg)	293.8 ± 0.5	256.2 ± 0.2	188.0 ±0.1
Volatile solids (VS, g/kg)	283.7 ± 0.8	211.4 ± 0.2	128.3 ± 0.6
VS/TS (%)	96.6 ± 2.4	82.5 ± 1.2	68.2 ± 2.8
Soluble chemical oxygen demand (SCOD, g/kg)	92.6 ± 3.9	35.0 ± 3.1	16.4 ± 2.1
Total volatile fatty acids (VFA, mg/kg)	3,007 ± 351	9,821 ± 899	0 ± 0
Total ammonia nitrogen (TAN, mg/kg)	386 ± 35	3,666 ± 393	990 ± 109
Carbohydrates (g/g TS)	0.45 ± 0.06	0.18 ± 0.02	0.16 ± 0.03
Proteins (g/g TS)	0.18 ± 0.02	0.17 ± 0.02	0.34 ± 0.03
Fats (g/g TS)	0.10 ± 0.02	0.05 ± 0.01	0.06 ± 0.01

The samples were analyzed in duplicate.

[a] FW: food waste

[b] PM: pig manure

Sixteen 1 L glass digesters were operated in this study in batch mode, with the detailed addition of FW, PM and inoculum shown in Table 3-2. The inoculum rates were set at 25% and 50% based on VS, and the FW/PM ratios in the mixtures were 0:100, 25:75, 50:50, 75:25 and 100:0 on a VS basis. The co-digestion digesters were operated in duplicate and the mono-digestion of FW or PM was operated separately as control. Tap water was added to ensure the TS content in each digester was 20%. After adding the inoculum-substrate mixtures, all of the reactors were flushed with nitrogen gas for 5 minutes to remove oxygen, and then placed in an incubator at 37 ^{o}C. The digesters were shaken by hand every day.

Table 3-2 Qualities of food waste, pig manure and inoculum added to each digester

Sludge rate (VS basis) [a]	FW/PM (VS basis) [b]	FW (g)	PM (g)	Inoculum (g)	Total (g)
25%	0:100	-	555	305	860
	25:75	105	430	315	850
	50:50	220	295	325	840
	75:25	335	150	330	815
	100:0	462	-	340	802
50%	0:100	-	352	583	935
	25:75	65	270	590	925
	50:50	135	185	600	920
	75:25	210	95	615	920
	100:0	282	-	623	905

[a] VS: volatile solids

[b] FW: food waste; PM: pig manure

3.2.2 Physico-chemical analysis

Biogas from each digester was collected with a Tedlar bag (Restek Corporation, Bellefonte, PA, USA) 2 - 3times a week. The biogas volume was then measured with a flow meter (FMA-1620A-TOT, Omega, Deckenpfronn, Germany) and converted to standard temperature and pressure (STP). The methane content in the biogas was measured by gas chromatography (GC 7890 A, Agilent Technology, Santa Clara, CA, USA) with a 45-60 mesh, matrix molecular sieve 5A column (Sigma-Aldrich, St. Louis, MO, USA) and a thermal conductivity detector. Helium gas was used as the carrier gas and the flow rate was 30 mL/min. The operation temperatures of the injection inlet, oven and detector were 100 ^{o}C, 60 ^{o}C and 105 ^{o}C, respectively (Xie et al., 2012).

About 3 g digestate sample (in slurry form) was taken from the sampling port located at the bottom of each digester once a week using a lab spoon. Then pH, TS, VS, VFA and total ammonia nitrogen (TAN) were analysed. The weight of the sample was recorded, and while calculating the SMY, the decreased VS caused by sampling was subtracted from the total VS to exclude the effect of sampling. TS and VS were measured directly by standard methods (APHA, 1995). When measuring liquid parameters, 19 parts of deionized water (w/w) were added to 1 part of digestate sample (~1 g) and mixed well to achieve a 20-fold dilution. The pH was measured using a pH meter (pH 3210, WTW, Weilheim, Germany). The mixed liquor was centrifuged at 18,000 ×g for 10 min (Laborzentrifugen 2-15, Sigma, Osterode am Harz, Germany), and the supernatant was filtered with 0.45 μm cellulose nitrate membrane filter paper (Whatman, Maidstone, UK) for measurement of soluble chemical oxygen demand (SCOD), VFA and TAN. The SCOD was analyzed using the standard method (APHA, 1995). Total VFAs were measured by high performance liquid chromatography (HPLC, Agilent 1200, Agilent Technology, Richardson, TX, USA) with an Aminex HPX-87H column (Bio-Rad, Hercules, CA, USA) and a UV index detector. The mobile phase was 0.1% H_2SO_4 with a flow rate of 0.6 mL/min. The temperatures of the column and detector were 65 ^{o}C and 40 ^{o}C, respectively (Wu et al., 2009). The standard VFA sample was a mixture of acetic, propionic, isobutyric, butyric, isovaleric and valeric acids with a concentration of 10 mmol/L each (Sigma-Aldrich, St. Louis, MO, USA). Concentrations of the other acids were converted to acetic acid equivalents when calculating the total VFA concentrations. The TAN was analyzed using a nutrient analyzer (Thermo Clinical Labsystems, Vantaa, Finland).

The concentrations of SCOD, total VFA and TAN in the digestate samples in terms of the liquid phase were converted from the values measured in the mixed liquor (described above) according to Eq. 3-1. Parameters were presented as g/L or mg/L in order to compare them with the data from the literature. Because the digestate sample was in a slurry form, it was difficult to measure SCOD, total VFA and TAN concentrations directly, so dilution of the digestate sample was necessary, as is commonly performed in dry digestion research.

$$C_s = \frac{C(W_H + W_s \cdot MC)}{W_s \cdot MC} = C(19/MC + 1) \qquad \textbf{3-1}$$

where: C_s represents the concentration (mg/L) of SCOD, total VFA or TAN in the liquid phase of the digestate sample; C represents the measured concentrations (mg/L) of SCOD, total VFA or TAN in the supernatant of the mixed liquor; W_s represents the weight (g) of the digestate sample; W_H represents the weight (g) of the deionized water added to the digestate sample ($W_H = 19W_s$); MC represents the moisture content (%) of the digestate sample. Water in the slurry digestate sample consisted of free water, interstitial water, vicinal water and hydration water. Because centrifugation only removes free and interstitial water but not vicinal and hydration water (Vesilind, 1994), the liquid phase of the digestate sample obtained by centrifugation did not include vicinal and hydration water, with the proportion in the digestate sample being less than the MC. As a result, the concentrations of these parameters obtained using Eq. 3-1 are conservative, and the actual concentrations of these parameters in the liquid portion of the digestate should be higher than C_s calculated. The concentrations calculated using Eq. 3-1 were used for data analysis.

Digestate samples (in slurry form) were dried at 105 ^{o}C overnight before being ground for analysis of carbohydrate, protein and lipid contents. The carbohydrate content was measured by the 3, 5 - dinitrosalicylic acid (DNS) method (Millier, 1959). The protein content was determined by the Kjeldahl method after the sample was digested in a digestion unit (Digestion Unit K-424, Büchi, Switzerland). The lipid content was determined by the acid hydrolysis method (AOAC, 2016).

3.2.3 Models and calculations

Free ammonia nitrogen (FAN) concentration was calculated according to Eq. 3-2 (Hansen et al., 1998).

$$\frac{C_{FAN}}{C_{TAN}} = (1 + 10^{-pH}/10^{-(0.09018+2729.92/T)})^{-1} \quad \textbf{3-2}$$

where, C_{FAN} is the FAN concentration (mg/L), C_{TAN} is the TAN concentration (mg/L), and T is the temperature (Kelvin).

Free VFA concentrations were calculated according to Eq. 3-3 (Watcharasukarn et al., 2009):

$$\mathrm{c}_{\mathrm{VFA}_f}/\mathrm{c}_{\mathrm{VFA}_t} = 1/(1 + 10^{(-\mathrm{pK_a}+\mathrm{pH})}) \quad \textbf{3-3}$$

where, $\mathrm{c}_{\mathrm{VFA}_f}$ is the free VFA concentration (mg/L); $\mathrm{c}_{\mathrm{VFA}_t}$ is the total VFA concentration (mg/L); $\mathrm{pK_a}$ are the dissociation constants of individual VFAs, with values of 4.757, 4.874, 4.812, 4.840, 4.835 and 4.767 for acetic, propionic, butyric, isobutyric, valeric and isovaleric acids at 25 ^{o}C, respectively. All of the other acids were converted into acetic acid equivalents and the concentration of free VFAs was calculated as the sum of them.

The lag phase, λ, and the maximum specific daily methane yield, R_{max}, were calculated using the Gompertz model (Lay et al., 1998) (Eq. 3-4).

$$M = P \cdot exp\left\{-exp\left[\frac{R_{max} \cdot e}{P}(\lambda - t) + 1\right]\right\} \qquad \textbf{3-4}$$

where, M is the cumulative methane production (mL/gVS$_{added}$) at time t (day); P is the methane production potential (mL/gVS$_{added}$); R_{max} is the maximum methane production rate [mL/(gVS$_{added}$ d)]; λ is the lag phase (day); and e is exp(1) = 2.7183.

To quantify the effect of total VFA on methane production, a substrate inhibition model from Tseng and Wayman (1975) was modified to simulate the relationship between total VFA and the specific daily methane yield (SDMY) (Eq. 3-5).

$$D = \frac{D_{max} \mathrm{c}_{\mathrm{VFA}_t}}{K + \mathrm{c}_{\mathrm{VFA}_t}}, \text{when } \mathrm{c}_{\mathrm{VFA}_t} < c_{VFA_\theta}$$

$$D = \frac{D_{max} \mathrm{c}_{\mathrm{VFA}_t}}{K + \mathrm{c}_{\mathrm{VFA}_t}} - i\left(\mathrm{c}_{\mathrm{VFA}_t} - c_{VFA_\theta}\right)^n, \text{when } \mathrm{c}_{\mathrm{VFA}_t} \geq c_{VFA_\theta} \qquad \textbf{3-5}$$

where: D, SDMY, mL/(gVS$_{added}$ d); D_{max}, the maximum SDMY, mL/(gVS$_{added}$ d); K, the Monod saturation constant for total VFA, mg/L; c_{VFA_θ}, the threshold inhibition concentration of total VFA, above which methane production starts to be inhibited, mg/L; i and n, the constants associated with total VFA inhibition. Another important parameter, the maximal inhibition concentration of total VFA (c_{VFA_m},) is also calculated according to Eq. 3-5, which is the concentration above which methane production is terminated.

One sample was taken under each operation condition for DNA extraction on Day 100. For co-digestion systems, the sample was taken from one of the duplicate digesters, and totally 10 samples were snap-frozen in liquid nitrogen and stored at -80 ^{o}C. The DNA extraction was performed later. Detailed methodology on DNA extraction and 16S rRNA gene sequencing are described in Appendix A.

Statistical analysis was conducted using SPSS 22.0 (IBM, Rochester, MN, USA). Multiple linear regression analysis (stepwise method) was performed to assess the effects of total VFA, free VFA, TAN and FAN on SMY. The stepwise method was used to avoid collinearity of the variables. The significance level was 0.05.

3.3 Results and discussion

3.3.1 Overall performance of the dry co-digestion systems

Methane production and overall performance of the dry co-digestion systems are shown in Figure 3-2 and Table 3-3. Almost no methane was produced at high FW/PM ratio systems (FW/PM = 75:25 & 100:0, inoculum = 25%; and FW/PM = 100:0, inoculum = 50%), indicating the complete inhibition of the systems. The final pH ranged 5.3 - 6.9 at the end of the experiment, indicating the acidification of the reactors. In uninhibited systems, the increase of FW/PM ratio improved the specific methane yields (SMY) and VS removal rate, but prolonged the lag phase. The SMY and VS removal rates previously reported in MSW or OFMSW dry digestion systems ranged from 70 to 400 mL/gVS$_{added}$ and from 26.1% to 90.0%, respectively (Table 2-1). Both the SMYs and the VS removal rates obtained in uninhibited systems fell within these ranges (Table 3-3).

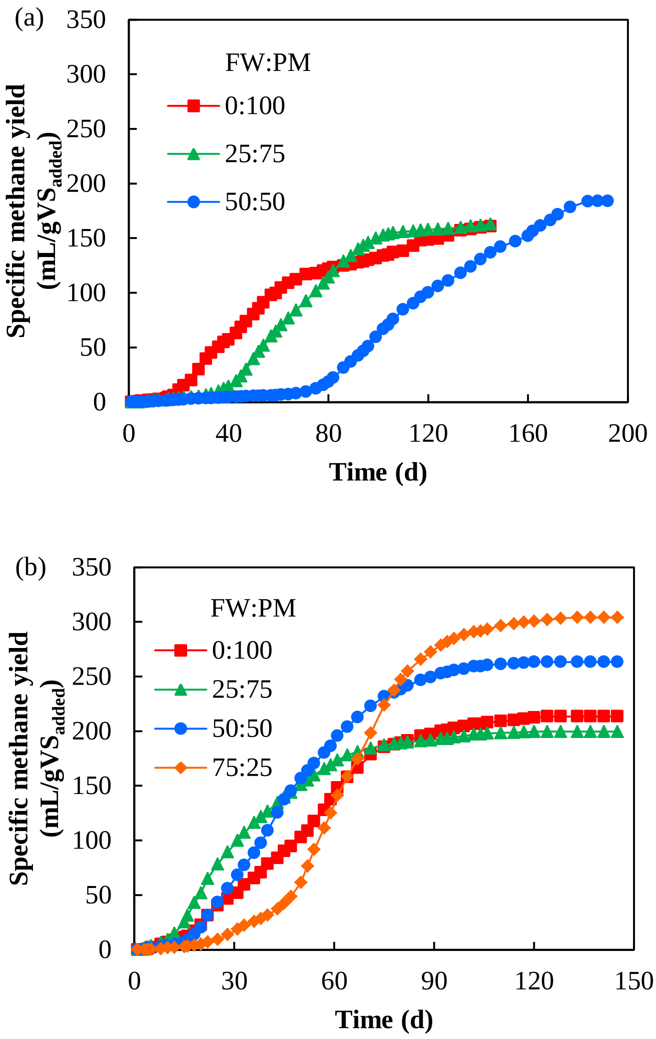


Figure 3-2: Variation in the specific methane yield over time during dry co-digestion of food waste and pig manure using inocula of 25% (a) and 50% (b)

Table 3-3 Overall performance of the food waste and pig manure dry co-digestion systems

Inoculum rate	25%					50%				
FW/PM ratio [a]	0:100	25:75	50:50	75:25	100:0	0:100	25:75	50:50	75:25	100:0
Lowest pH observed	8.1	7.3	6.3	6.0	4.5	8.3	8.0	7.2	6.2	5.4
Final pH	8.7	8.7	8.5	6.7	5.3	8.8	8.8	8.8	8.8	6.9
VS removal rate (%) [b]	30.2	24.0	30.6	39.6	51.9	49.0	40.2	60.9	66.9	70.5
SMY (mL/g VS_{added}) [c]	161	163	184	NA [d]	NA	213	200	263	304	NA
λ (d) [e]	18	36	72	NA	NA	17	9	18	39	NA
R_{max} (mL/(g VS_{added} d)) [f]	2.3	2.9	2.0	NA	NA	3.5	4.2	5.0	6.7	NA
Total VFA_{max} (mg/L) [g]	24,582	36,901	62,176	54,906	38,759	11,847	20,251	32,185	32,647	48,048
Free VFA_{max} (mg/L)	11	132	2,324	3,364	9,613	4	13	80	1,102	6,794
TAN_{max} (mg/L) [h]	5,109	5,997	5,311	4,376	2,299	4,277	4,647	5,039	4,882	3,802
FAN_{max} (mg/L) [i]	1,381	1,244	677	25	1	1,234	1,177	1,169	998	18

[a] FW: food waste; PM: pig manure

[b] VS: volatile solids

[c] SMY: Specific methane yield

[d] NA: Not available

[e] λ: Lag phase

[f] R_{max}: Maximum methane production rate

[g] VFA_{max}: Maximum volatile fatty acids through the experiment

[h] TAN_{max}: Maximum total ammonia nitrogen through the experiment

[i] FAN_{max}: Maximum free ammonia nitrogen through the experiment

At an inoculum rate of 25%, the VS removal rate, SMY and R_{max} ranged 24.0% - 39.6%, 161 - 184 mL/g VS_{added} and 2.0 - 2.9 mL/(g VS_{added} d), respectively, in uninhibited systems. But as the FW/PM ratio increased, the lag phase extended from 18 to 72 days. When the inoculum rate was 50%, the VS removal rates (40.2% - 70.5%), SMYs (200 - 304 mL/g VS_{added}) and $R_{\max}$ [3.5 - 6.7 mL/(g VS_{added} d)] in uninhibited systems were much higher than those obtained at the inoculum rate of 25%, and the lag phases of 9 - 39 days were much lower. A higher inoculum rate meant more microorganisms were added to the system, which may promote the extent of hydrolysis and methanogenesis. Hence, during the continuous operation of dry digestion in engineering, it is important to retain sufficient inoculum in the reactor when fresh substrate is added. This helps to shorten the lag phase and increase the methane yield.

At the inoculum rate of 50%, there were no increases in the SMY and VS removal rate when the FW/PM ratio was changed from 0:100 to 25:75, but the lag phase decreased from 17 to 9 days. The reason for this may be that the addition of FW enabled the rapid hydrolysis of easily degradable substrate, and the VFAs produced mitigated the ammonia inhibition in the mono-PM digestion system, thereby shortening the lag phase. Compared with the mono-PM digestion system, the SMYs at the FW/PM ratios of 50:50 and 75:25 were increased by 23.5% and 42.7%, the VS removal rates were increased by 24.3% and 36.5%, and R_{max} were increased by 42.8% and 91.4%, respectively. This illustrates the synergistic effect of co-digesting FW with PM. Even though the highest SMY and R_{max} were obtained at the FW/PM ratio of 75:25, the lag phase was prolonged to 39 days (18 days at the FW/PM ratio of 50:50), thereby, limiting the practicality of using high FW proportion in the substrate mixture. As a result, the FW/PM ratio must be appropriately controlled to prevent inhibition.

3.3.2 System stability and inhibition factors

The methane producing lag phase indicated the inhibition during dry co-digestion of FW and PM. It's essential to investigate the inhibition mechanism so as to provide reference for engineering application. High VFA and ammonia concentrations are widely reported inhibition factors during anaerobic digestion (Benabdallah El Hadj et al., 2009; Karthikeyan & Visvanathan, 2012). In this study, multiple linear regression analysis (stepwise method) was performed to assess the effects of total VFA, free VFA, TAN and FAN on SMY. The results showed that total VFA had significant negative correlation with SMY under all conditions ($P \leq 0.001$), indicating its inhibition on methane production. Besides, significant linear correlation was observed between the lag phase duration, λ, and the maximum total VFA ($P < 0.05$, Figure 3-3), indicating the inhibition of total VFA again.

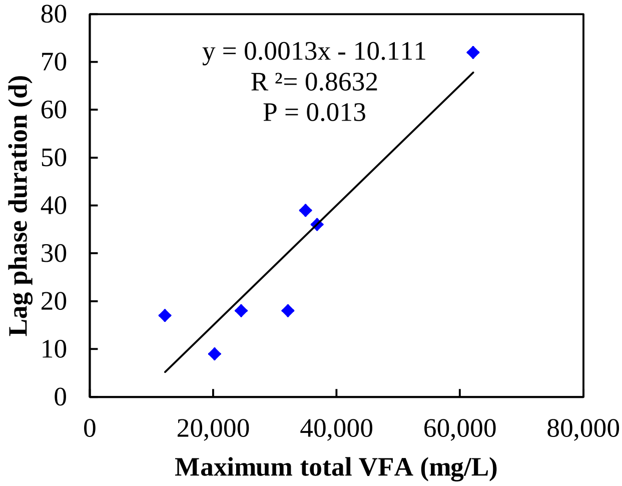


Figure 3-3: Relationship of lag phase duration with the maximum total volatile fatty acid during dry co-digestion of food waste and pig manure

A substrate inhibition model from Tseng and Wayman (1975) was modified to quantify the effect of total VFA on methane production (Eq. 3-5), with the results shown in Figure 3-4 and Table 3-4. The Generalized Reduced Gradient (GRG) non-linear method in the Solver function of Microsoft Excel was used to estimate the kinetic parameters of D_{max}, K, c_{VFA_θ}, i and n (Table 3-4). The scatter plots in Figure 3-4 represent the specific daily methane yields obtained from the experiment. The solid lines are responses of the model with the parameters (D_{max}, K, c_{VFA_θ}, i and n) obtained. When the total VFA concentration was higher than c_{VFA_m}, the specific daily methane yield was almost zero, so the data are not shown in Figure 3-4. The small sum of square errors (SSE, ≤ 6.45) indicated the good fitness between simulated and experimental data.

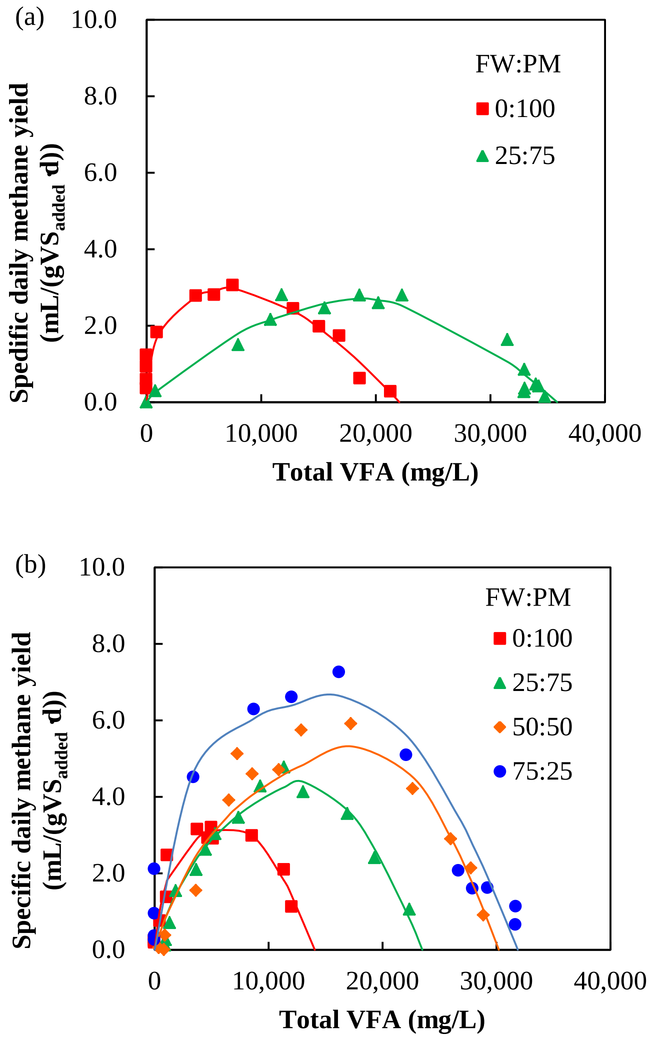


Figure 3-4: Simulation of specific daily methane yields at different total volatile fatty acid concentrations during dry co-digestion of food waste and pig manure using inocula of 25% (a) and 50% (b)

Table 3-4 Summary of the kinetic parameters according to Eq. 3-5.

Inoculum rate	25%		50%			
Food waste/pig manure ratio	0:100	25:75	0:100	25:75	50:50	75:25
D_{max} (mL/(g VS_{added} d)) [a]	3.33	4.79	4.69	6.82	7.72	7.51
K (mg/L) [b]	894	13,375	1,271	6,885	7,784	2,112
c_{VFA_θ} (mg/L) [c]	6,755	16,799	6,295	12,068	17,736	16,580
c_{VFA_m} (mg/L) [d]	21,826	35,820	14,053	23,497	30,210	31,874
i [e]	5.75×10^{-7}	5.08×10^{-7}	5.95×10^{-7}	5.08×10^{-7}	5.08×10^{-7}	5.65×10^{-7}
n [f]	1.61	1.60	1.74	1.73	1.73	1.70
SSE [g]	3.12	1.19	1.08	1.68	5.56	6.45
r [h]	0.936	0.968	0.970	0.981	0.971	0.945

[a] D_{max}: Maximum specific daily methane yield, mL/(gVS_{added} d)

[b] K: Monod saturation constant for VFAs, mg/L

[c] c_{VFA_θ}: Threshold inhibition concentration of VFAs, mg/L

[d] c_{VFA_m}: Maximal inhibition concentration of VFAs, mg/L

[e] i: Constant associated with VFA inhibition

[f] n: Constant associated with VFA inhibition

[g] SSE: Sum of square errors

[h] r: Pearson correlation coefficient

As shown in Table 3-4, the values of c_{VFA_θ} and c_{VFA_m} increased with the increase of FW/PM ratio and the decrease of the inoculum rate. It might be because increasing the FW/PM ratio accelerated the hydrolysis and acidification of the substrate, and resulted in rapid accumulation of total VFAs. This rapidly exposed the bacteria to extremely high total VFA concentrations, which enhanced the selection for acidophilic bacteria. Under the same FW/PM ratio, c_{VFA_θ} and c_{VFA_m} at the lower inoculum rate were much higher, because the substrate/inoculum ratio at the inoculum rate of 25% was three times that at the inoculum rate of 50%. The c_{VFA_θ} and c_{VFA_m} at the preferable conditions (inoculum rate of 50% and FW/PM ratio of 50:50) were 17,736 mg/L and 30,210 mg/L, respectively. The c_{VFA_θ} and c_{VFA_m} at the inoculum rate of 50% and FW/PM ratios of 50:50 and 75:25 were similar to those obtained at the inoculum rate of 25% and FW/PM ratio of 25:75. The similar values obtained using different inoculum rates and FW/PM ratios indicated that the critical c_{VFA_θ} and c_{VFA_m} values for dry co-digestion of FW and PM systems were about 16.5-18.0 g/L and 30.0-36.0 g/L, respectively.

The total VFA inhibition concentrations reported in the literature varied according to the operation conditions and substrates. Ghanimeh et al. (2012) found 10.3 g/L total VFA decreased the SMY from 280 mL/g VS_{added} to 100 mL/g VS_{added} during dry digestion of source-sorted organic fractions of municipal solid waste (SS-OFMSW) at 55 ^oC. Karthikeyan and Visvanathan (2012) found total VFA >8,000 mg/L negatively affected the overall methanogenesis process during dry anaerobic digestion of biowaste. Total VFA concentrations in the present study were much higher than those reported in the literature. The highest total VFA at the inoculum rates of 25% and 50% reached 62,176 mg/L (FW/PM=50:50) and 32,647 mg/L (FW/PM=75:25), respectively (Table 3-3), but the methane production recovered after long lag phases of 72 and 39 days (Figure 3-2). This illustrates the high tolerance of methanogens to high total VFA concentrations, which is a striking feature of this study.

Overall, taking the SMY, VS removal rate, R_{max} and lag phase into consideration, an inoculum rate of 50% and a FW/PM ratio of 50:50 appeared preferable in this study, with the SMY and VS removal rate of 263 mL/g VS_{added} and 60.9% achieved, respectively.

3.3.3 Microbial community structure

3.3.3.1 Overall biodiversity and richness of the microbes

Overall, 82,814-199,541 reads were obtained from each biomass sample. Totally 26 phyla (3 archaea and 23 bacteria), 53 classes (5 archaea and 48 bacteria), 93 orders (6 archaea and 87 bacteria), 165 families (11 archaea and 154 bacteria) and 248 genera (15 archaea and 233 bacteria) were observed. The beta diversity of samples was visualized by principal coordinate analysis (PCoA) using weighted UniFrac matrices (Figure 3-5). Samples were separated clearly into two clusters (Cluster I and Cluster II) along the axis of PC1. Distinct microbial community structures were observed at high FW/PM ratio samples (Cluster I, FW/PM = 75:25 & 100:0, inoculum = 25%; and FW/PM = 100:0, inoculum = 50%) compared with the others (Cluster II).This was corresponded to the observation of methane production: almost no methane was produced in the reactors in Cluster I, and methane started to increase after the lag phases in Cluster II. It means FW/PM ratio played an important role in the formation of microbial communities. At the same FW/PM ratio of 75:25, samples at the inoculum rate of 25% and 50% were located in different clusters, indicating the effect of inoculum. The PD whole tree and observed species were used to assess the diversity and richness of the dry co-digestion systems, respectively. The PD whole tree and observed species in Cluster II ranged 50.9 - 53.5 and 660 - 699, respectively, which were much higher than those of 44.4 - 48.8 and 537 - 612 in Cluster I ($P<0.05$). It indicated that the extremely high total VFAs decreased both the diversity and richness of microbes.

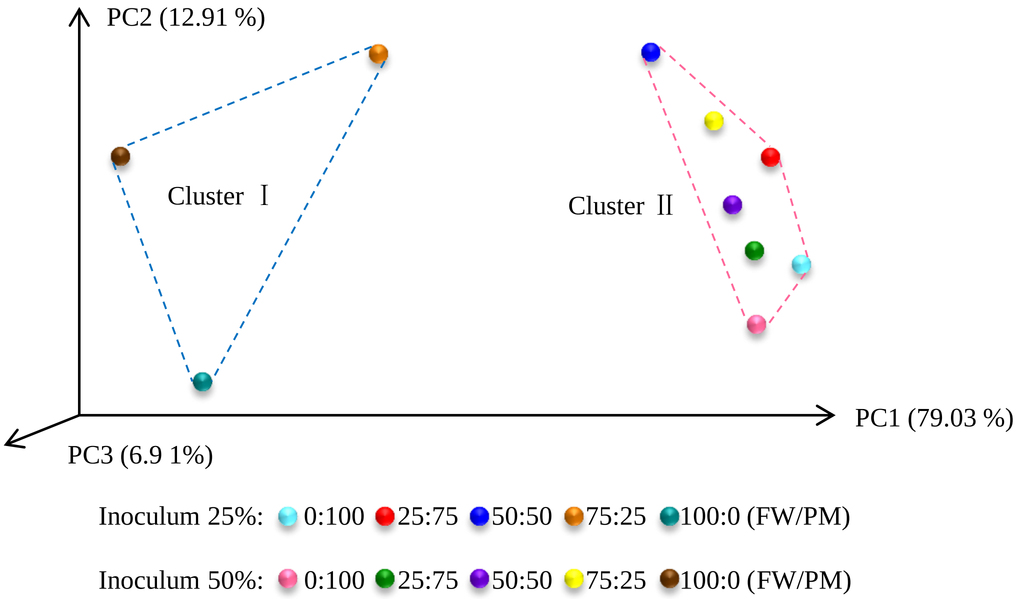


Figure 3-5: Principal coordinate analysis (PCoA) of microbial community structure during dry co-digestion of food waste and pig manure

3.3.3.2 Bacterial community structure

In all the reactors, phyla *Firmicutes* (18.6% - 61.7%) and *Proteobacteria* (6.6% - 23.6%) were dominant in bacteria, followed by *Bacteroidetes*, *Chloroflexi*, *Thermotogae*, *Synergistetes*, *Actinobacteria* and *WWE1* (Figure 3-6 a). The bacterial community structure varied greatly at the class and order levels. At the class level, classes *Clostridia* (13.5% - 60.1%) and *Bacilli* (0.8% - 37.1%) in phylum *Firmicutes* were most abundant (Figure 3-6 b). However, *Clostridia* was dominant in Cluster II, while *Bacilli* was mainly observed in Cluster I. At the order level, orders *Lactobacillales* (0.3% - 36.3%) and *Clostridiales* (13.5% - 34.3%) in phylum *Firmicutes* were abundant (Figure 3-6 c). *Lactobacillales* was dominant in Cluster I, whereas the relative abundance of *Clostridiales* was much higher in Cluster II. The distinct distributions of bacteria in different clusters indicated their different functions in dry co-digestion systems.

The composition of bacteria at the genus level (relative abundance >1% in at least one sample) is shown in Figure 3-7. The unclassified genera in order *Lactobacillales* (0.0%-29.4%) and candidate order *MBA08* (0.1%-15.7%), the genus *Leuconostoc* (0.0%-11.9%), an unclassified genus in family *Clostridiaceae* (2.9%-10.5%) and the genus *Clostridium* (0.3%-

9.1%) were dominant. All these belong to the phylum *Firmicutes*, indicating its important role during dry co-digestion of FW and PM.

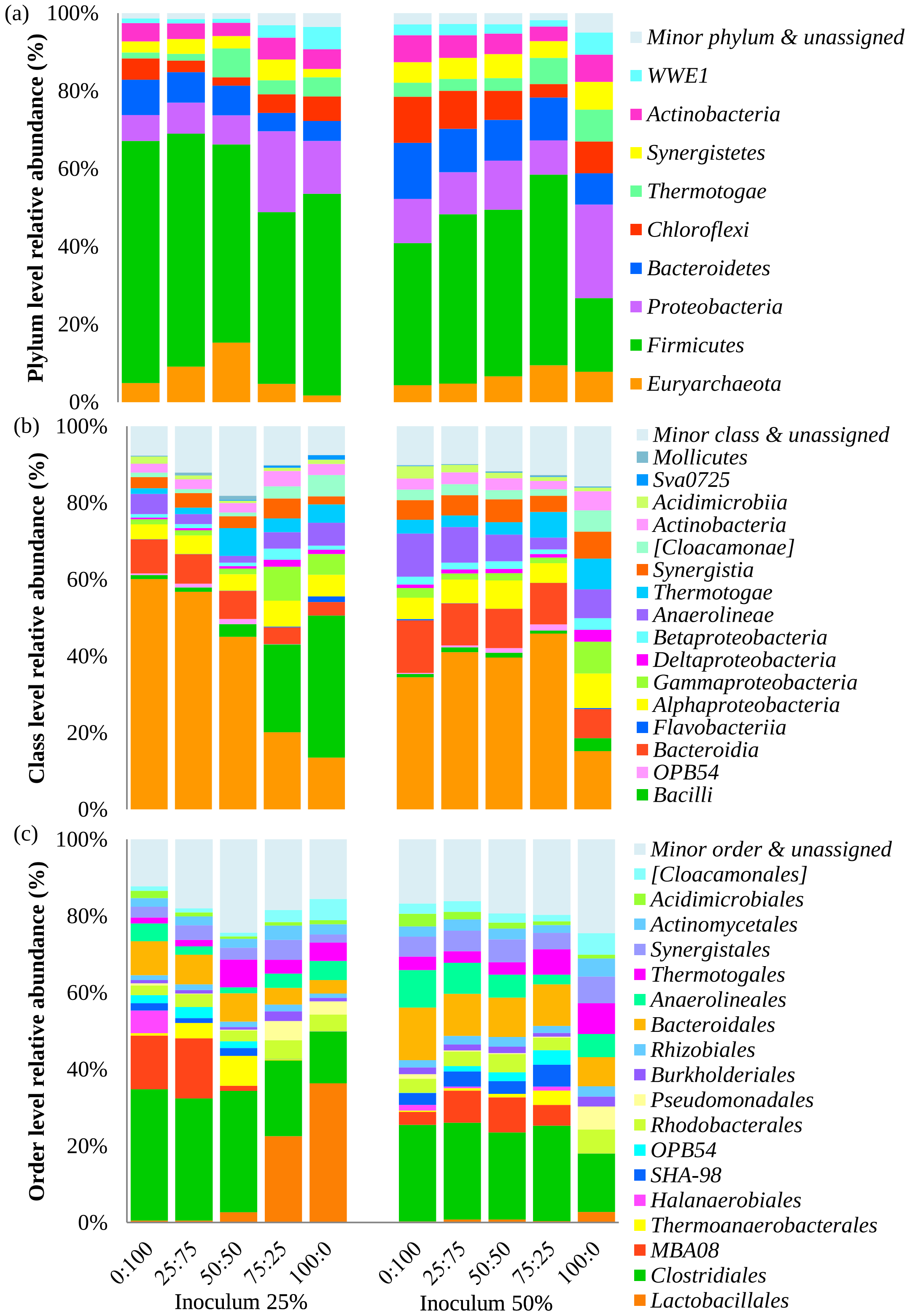


Figure 3-6: Microbial relative abundance in dry co-digestion of food waste and pig manure at (a) phylum level, (b) class level, and (c) order level (>5% in at least one sample)

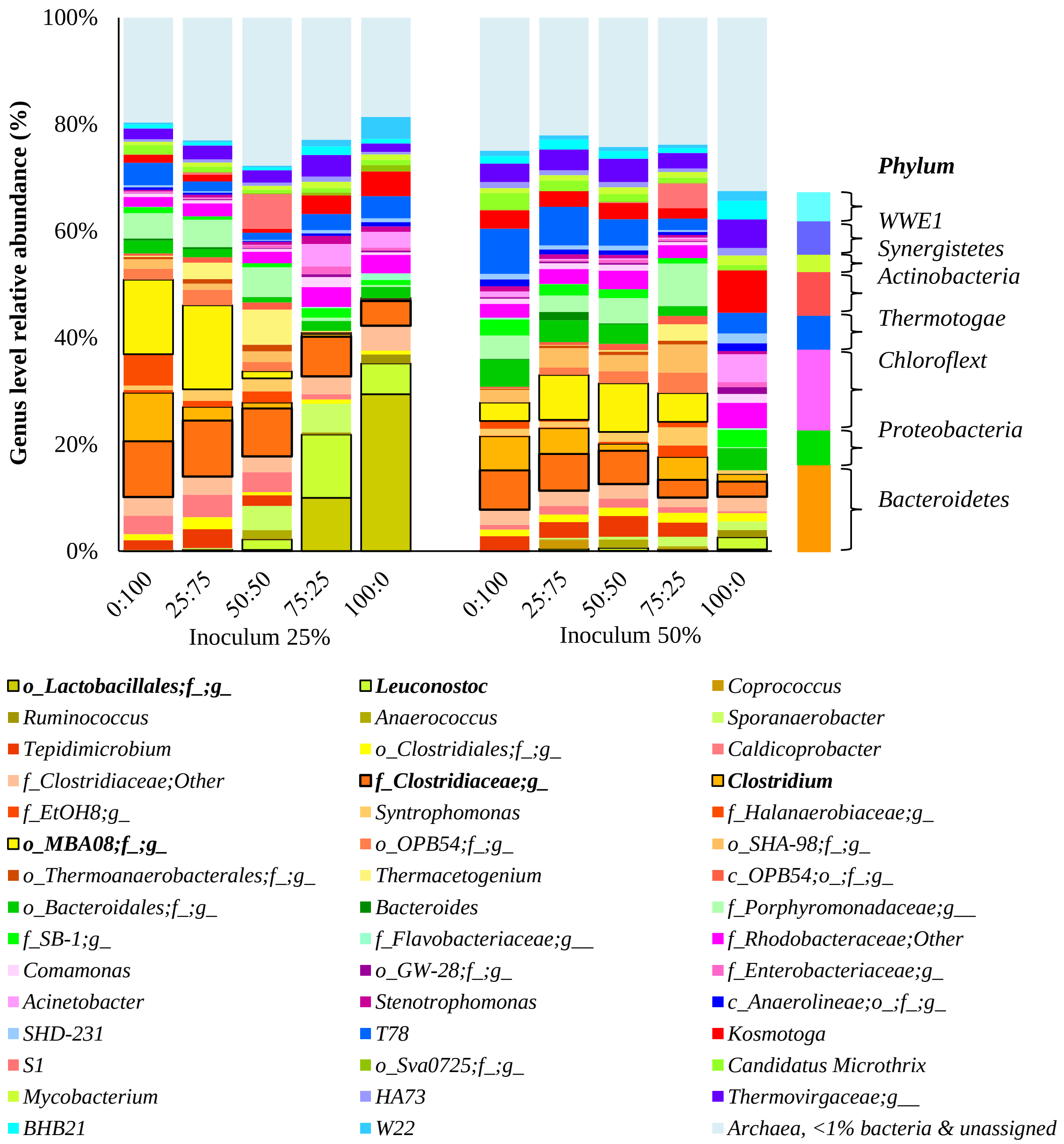


Figure 3-7: Genus level relative abundance (>1% in at least one sample) in dry co-digestion of food waste and pig manure

To explore the relationships between the bacteria (relative abundance >1% in at least one sample) and various VFA types, Spearmen's correlation analysis was conducted. The results with significant correlations (P<0.05) are shown in Table 3-5.

Table 3-5 Spearman's correlations between bacteria and physicochemical parameters during dry co-digestion of food waste and pig manure

Phylum	*Class*	*Genus*	Acetate	Propionate	Butyrate	Valerate	VFA [a]	SMY [b]
Firmicutes	Bacilli	*o_Lactobacillales;f_;g_*	*	*	**	*	**	*
		Leuconostoc	*		**	*	**	*
	Clostridiales	*o_Clostridiales;f_;g_*	*			*		*
		Clostridium		*	*	**	**	
		Syntrophomonas	**		*			*
		*f_Halanaerobiaceae;g_*	*	*	*		**	*
		*o_MBA08;f_;g_*	**		**			
		*o_OPB54;f_;g_*	***		*			*
		*o_SHA-98;f_;g_*	*		*		*	***
		*o_Thermoanaerobacterales;f_;g_*	*					
		Thermacetogenium	*					
Actinobacteria	*Acidimicrobiia*	*Candidatus Microthrix*		**	**	*	**	
Bacteroidetes	*Flavobacteriia*	*f_Flavobacteriaceae;g__*	*					*
Proteobacteria	*Gammaproteobacteria*	*Stenotrophomonas*	*					
Acidobacteria	*Sva0725*	*o_Sva0725;f_;g_*			*	**		

*** P<0.001, ** P<0.01, * P<0.05

[a] VFA: Volatile fatty acid on Day 100

[b] SMY: Specific methane yield

Correlations were determined using a two-tailed pairwise Spearman's correlation at the significant level of P<0.05.

Red box indicates a negative correlation, and green box indicates a positive correlation.

According to the positive and negative correlations with various VFAs, the bacteria can be divided into two groups. The bacteria in the first group showed positive correlations with various VFAs, for example, the genus *Leuconostoc* and an unclassified genus in order *Lactobacillales* (phylum *Firmicutes*); an unclassified genus in family *Flavobacteriaceae* (phylum *Bacteroidetes*); the genus *Stenotrophomonas* and an unclassified genus in family *Enterobacteriaceae* (phylum *Proteobacteria*); and an unclassified genus in order *Sva0725* (phylum *Acidobacteria*). These bacteria may work on hydrolysis and fermentation in dry co-digestion systems, which decompose organic matters and produce VFAs. *Leuconostoc* and other members in the order *Lactobacillales* are well reported as lactic acid bacteria (Carr et al., 2002). The genus *Flavobacterium* in family *Flavobacteriaceae* could be responsible for cellulose degradation (Dechrugsa et al., 2013). The genus *Stenotrophomonas* was reported to be responsible for hydrolysis and fermentation of carbohydrate and amino acids (Ma et al., 2015). All these were in line with the results observed in this study. The order *Sva0725* is rarely reported in literature, and the positive correlation with butyrate and valerate in this study indicates that some members in this order might be responsible for hydrolysis and fermentation of organic matter and production of VFAs.

In the second group, the bacteria were negatively correlated with various VFAs and positively correlated with SMY, indicating these bacteria may consume VFAs and benefit the production of methane. Syntrophic oxidation is the main VFA consuming method under stress environment such as high ammonia or VFA concentrations (Karakashev et al., 2006; Müller et al., 2010). During this process, VFAs such as acetate, propionate, butyrate and valerate are converted into H_2, CO_2 and formate (Table 3-6), and the products are then utilized by hydrogen- or formate-using methanogens (Hatamoto et al., 2007; Hattori, 2008; Müller et al., 2010).

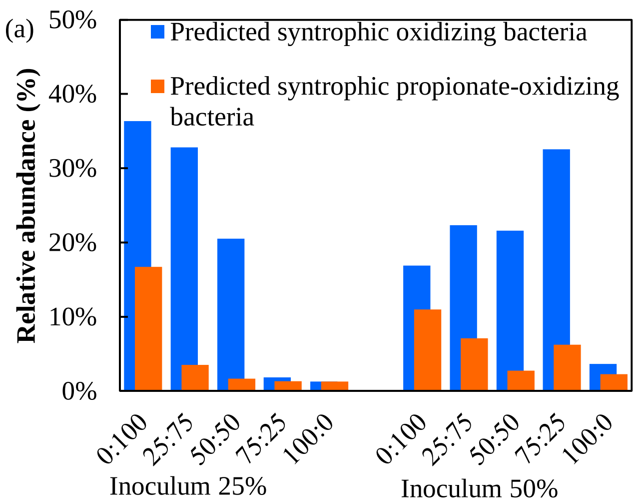
Table 3-6 Reactions involved in syntrophic oxidation of acetate, propionate, butyrate and valerate (Hatamoto et al., 2007; Hattori, 2008; Müller et al., 2010)

Syntrophic reactions	$\Delta G^{0'}$ (kJ/mol)
$CH_3COO^- + 4H_2O \rightarrow 2HCO_3^- + 4H_2 + H^+$	+104.6
$CH_3CH_2COO^- + 2H_2O \rightarrow CH_3COO^- + CO_2 + 3H_2$	+76.0
$CH_3CH_2COO^- + 2HCO_3^- \rightarrow CH_3COO^- + 3HCOO^- + H^+$	+72.0
$CH_3CH_2CH_2COO^- + 2H_2O \rightarrow 2CH_3COO^- + 2H_2 + H^+$	+48.1
$CH_3CH_2CH_2COO^- + 2HCO_3^- \rightarrow 2CH_3COO^- + 2HCOO^- + H^+$	+45.5
$CH_3CH_2CH_2CH_2COO^- + 2H_2O \rightarrow CH_3COO^- + CH_3CH_2COO^- + 2H_2 + H^+$	+48.4

As shown in Table 3-5, the genus *Syntrophomonas* was negatively correlated with acetate and butyrate, which conformed to the report that *Syntrophomonas wolfei* is syntrophic butyrate-oxidizing bacteria (Wallrabenstein & Schink, 1994). The genus *Thermacetogenium* in the order *Thermoanaerobacterales* was reported to be syntrophic acetate-oxidizing bacteria (Hattori et al., 2000), corresponding to the negative correlations with acetate in Table 3-5. Fermentative hydrogen production is also a VFA consuming method during the hydrogenotrophic methanogenesis system (Stieb & Schink, 1985). Species in genus *Clostridium* are widely reported as hydrogen producing bacteria by fermentation of carbohydrates (Chen et al., 2005; Jo et al., 2008). The species *Clostridium bryantii* is reported to be able to degrade even-numbered (up to 10-C) fatty acids to acetate and H_2, and odd-numbered (up to 11-C) fatty acids to acetate, propionate and H_2 (Stieb & Schink, 1985), *Clostridium ultunense* is reported as syntrophic acetate-oxidizing bacterium (Hattori, 2008;

Schnurer et al., 1996). These accorded to the negative correlations with propionate, butyrate, valerate and TVFA observed in this study. The unclassified genus in family *Halanaerobiaceae* was negatively correlated with acetate, propionate, butyrate and VFA. Members in the family *Halanaerobiaceae* are usually reported as alkaliphilic bacteria, which can ferment simple sugars and glycerol to produce acetate, formate and H_2 (Cono et al., 2015; Sousa et al., 2015). This is in line with the results obtained in this study and can provide substrates for hydrogen- or formate-using methanogens.

The relative abundances of the predicted syntrophic oxidizing bacteria and the composition of total VFAs on day 100 are shown in Figure 3-8. In Cluster I, the relative abundances of predicted syntrophic oxidizing bacteria ranged 1.3%-3.7%, which led to the accumulation of total VFAs and the subsequent inhibition on methanogen. The relative abundances of syntrophic oxidizing bacteria in Cluster II (16.9%-36.3%) were much higher than those in Cluster I. The little accumulation of acetate and butyrate in Cluster II corresponded to the abundance of predicted syntrophic acetate- and butyrate- oxidizing bacteria. However, the lack of syntrophic propionate- oxidizing bacteria at the inoculum rate of 25% and FW/PM ratio of 25:75 and 50:50 resulted in the accumulation of propionate. It indicates that syntrophic oxidizing bacteria can link fermentative bacteria and hydrogenotrophic methanogens and play important roles in the stability of the whole dry co-digestion system.



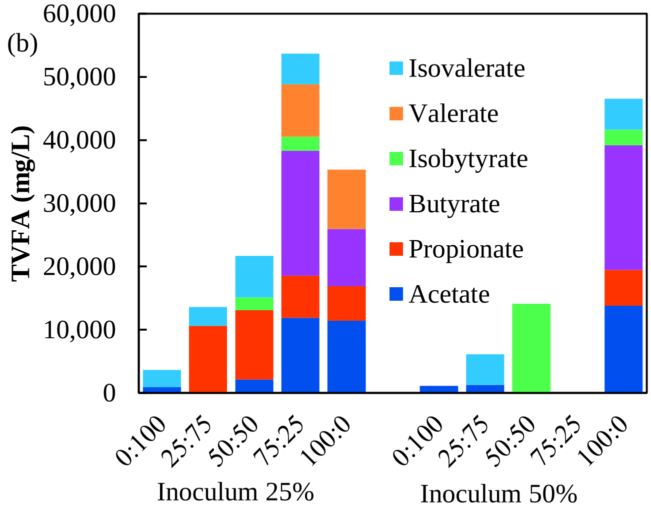


Figure 3-8: Relative abundances of the predicted syntrophic oxidizing bacteria (a) and the composition of TVFAs on Day 100 (b)

3.3.3.3 Archaeal community structure

The revival of methanogenesis at extremely high total VFA concentrations indicated the high tolerance of methanogens in dry co-digestion systems. The composition of the methanogen communities on Day 100 is shown in Figure 3-9. The relative abundances of methanogens first increased with the increase of FW/PM ratios, and then decreased at high FW/PM ratios, indicating the inhibition of high total VFA concentrations.

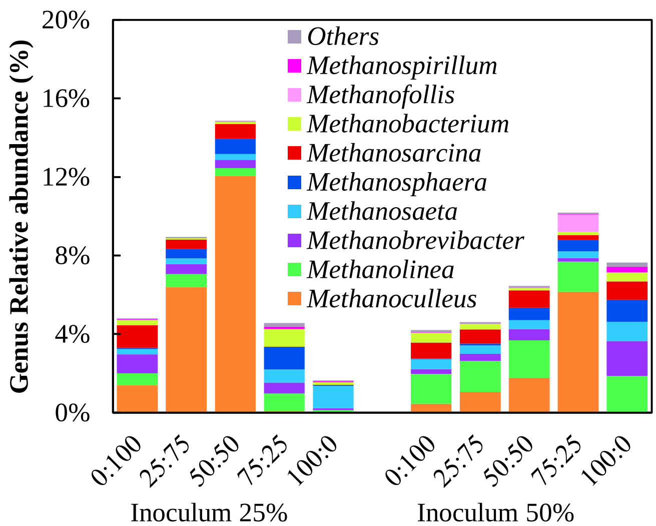


Figure 3-9: Composition of methanogens during dry co-digestion of food waste and pig manure

The methanogens in Cluster I were diverse, including *Methanolinea* (0.9%-1.9%), *Methanobrevibacter* (0.1%-1.8%), *Methanosphaera* (0.1%-1.2%), *Methanosaeta* (0.7%-1.2%), *Methanosarcina* (0.0%-1.0%) and other minor methanogens (<1.0%). Both *Methanolinea* and *Methanobrevibacter* are hydrogenotrophic methanogens, which can utilize H_2/CO_2 or formate for growth and methane production (Imachi et al., 2008; Savant, 2002). *Methanobrevibacter* was reported to be acid-tolerant, which could grow at the pH of 5.0-7.5 (Savant, 2002). The pH range of 4.5 - 6.9 in high FW/PM ratio reactors was consistent with this report (Table 3-3). *Methanosaeta* is exclusively an acetate-utilizing (acetoclastic) methanogen, while *Methanosarcina* is versatile and can use H_2/CO_2, acetate, methanol as well as methylamine (Guo et al., 2015). *Methanosphaera* produces methane by reducing methanol with hydrogen as the electron donor (Fricke et al., 2006). However, the activities of all these methanogens were inhibited as very low amount of methane was produced from the reactors in Cluster I.

In Cluster II, *Methanoculleus* (1.4%-12.1%) was the dominant methanogen at the inoculum rate of 25%, while *Methanoculleus* (0.5%-6.2%) and *Methanolinea* (1.5%-1.9%) were abundant at the inoculum rate of 50%. *Methanoculleus* is also a hydrogenotrophic methanogen, and is reported as a biomarker of methane production in swine manure (Barret et al., 2012). In Cluster II, accumulation of propionate was observed at the inoculum rate of 25% but not at 50% (Figure 3-8 b); this may be related to the dominance of *Methanolinea* at the high inoculum rate systems. *Methanolinea tarda* and *Methanolinea mesophila* were reported to be isolated from propionate-degradation enrichment culture, in which syntrophic propionate-oxidizing bacteria and propionate were added to produce H_2 for *Methanolinea* (Imachi et al., 2008; Sakai et al., 2012). This agreed with the abundance of predicted syntrophic propionate-oxidizing bacteria at the high inoculum rate (Figure 3-8 a).

The relative abundances of hydrogenotrophic methanogens accounted for 72.2% - 91.8% of the total methanogens in Cluster II, indicating the dominance of hydrogenotrophic methanogenesis in dry co-digestion of FW and PM.

3.4 Summary

Dry co-digestion of FW and PM was investigated with sludge as the inoculum. The optimal operation conditions were obtained at an inoculum rate of 50% and a FW/PM ratio of 50:50. Total VFAs were main inhibition factors on methane production. Syntrophic oxidation of VFAs with hydrogenotrophic methanogenesis was an important methane production pathway.

Chapter 4
Effects of inoculum on system performance and stability during dry co-digestion of food waste and pig manure

4.1 Introduction

In the present study, dry co-digestion of FW and PM was conducted to assess: (1) the effects of different inocula (digestate and sludge) on system performance and (2) the inhibition of high VFA concentrations on methane production. The results should provide reference for large-scale engineering of dry co-digestion systems.

4.2 Materials and methods

4.2.1 Experimental design

Fresh FW and PM were collected and pretreated as described in Chapter 3. Two inocula were used in order to assess the effect of inoculum on dry co-digestion performance. One was solid digestate taken from the previously operated laboratory-scale batch FW/PM co-digestion systems. The digesters have been operated for ~5 months, with no methane production any longer. The other was dewatered anaerobic sludge obtained from a local wastewater treatment plant. The sludge was placed at 37 ^{o}C for two weeks to release biogas prior to use. The physicochemical properties of the FW, PM, digestate and sludge are shown in Table 4-1.

Table 4-1 Physicochemical properties of food waste and pig manure substrates and the digestate and sludge used as inocula

Feature	FW [a]	Solid fraction of PM [b]	Digestate	Sludge
pH	5.0	8.6	8.9	8.2
Moisture content (MC, %)	73.8	76.2	83.4	81.0
Total solids (TS, %)	26.3	23.8	16.6	19.0
Volatile solids (VS, %)	25.0	19.4	11.7	12.6
VS/TS (%)	95.4	81.5	70.2	66.3
Total volatile fatty acids (VFAs, mg/L)	4,657	5,314	NA [c]	1,758
Free VFA calculated (mg/L)	1,907	1	NA	1
Total ammonia nitrogen (TAN, mg/L)	621	5,052	7,149	2,773
Free ammonia nitrogen calculated (FAN, mg/L)	0	904	1,570	218

[a] FW: Food waste

[b] PM: Pig manure

[c] NA: Not available

The previous study in Chapter 3 showed that an inoculum ratio of 50% and a FW/PM ratio of 50:50 were preferable for dry co-digestion of FW and PM. Even though a higher FW/PM ratio (i.e. 75:25) resulted in a higher SMY, the lag phase was as long as 39 days because of VFA inhibition. However, high VFA concentrations were expected to be effective in inactivating pathogens in dry co-digestion systems (Kunte et al., 1998). Using digestate taken from previous FW/PM co-digestion systems as an inoculum was expected to be more resistant to high VFA concentrations compared with dewatered anaerobic sludge. Therefore, both inocula (digestate and dewatered anaerobic sludge at the inoculum ratio of 50% based on VS) and FW/PM ratios (50:50 and 75:25 based on VS) were investigated. It resulted in four conditions, which were denoted as R1 (digestate as inoculum, FW/PM=50:50 based on VS), R2 (digestate as inoculum, FW/PM=75:25), R3 (sludge as inoculum, FW/PM=50:50) and R4 (sludge as inoculum, FW/PM=75:25). Each condition was conducted in quadruplicate, so sixteen 1 L glass digesters were used. Totally 800 g FW, PM and inoculum mixtures were added to each digester, with the detailed quantities shown in Table 4-2. Tap water was added to control the TS content at 20%.

After feeding, all of the reactors were placed in a 37 ℃ incubator and were shaken once by hand every day. The reactors were operated for 120 days, biogas was collected twice weekly during the first 72 days and then weekly from all of the reactors, and the volume and methane content were analysed according to Chapter 3. As shown in Table 4-2, about 2 g samples were taken weekly from two of the digesters under each condition from the sampling port located at the bottom of the digester using a lab spoon. Then pH, TS, VS, VFA and TAN of the samples were analysed. The analysis methods of these parameters and the calculations of free VFA and FAN were detailed in Chapter 3. The two un-sampled digesters under each condition were used to assess the effect of decreasing substrate concentrations (due to digestate sampling) on biogas production.

Table 4-2 Experimental design and sampling in dry co-digestion of food waste and pig manure

Condition	Reactor	Inoculum	FW/PM (VS basis)	Digestate (g)	Sludge (g)	FW (g)	PM (g)	Sampling	Inhibition
R1	1	Digestate	50:50	522.2	-	122.0	157.3	No	No
	2							No	No
	3							Yes	No
	4							Yes	No
R2	5	Digestate	75:25	534.6	-	186.8	80.8	No	Yes
	6							No	No
	7							Yes	No
	8							Yes	No
R3	9	Sludge	50:50	-	508.2	128.4	165.6	No	No
	10							No	No
	11							Yes	No
	12							Yes	No
R4	13	Sludge	75:25	-	520.2	197.5	84.2	No	Yes
	14							No	Yes
	15							Yes	No
	16							Yes	Yes

[a] FW: Food waste

[b] PM: Pig manure

4.2.2 Models and calculations

The stability of the dry co-digestion systems was assessed by examining the pH, SMY, R_{max}, λ and the relative standard deviation (RSD) of the SMY. The R_{max} and λ are calculated from the Gompertz model (Lay et al., 1998) as described in Chapter 3.

The RSD_{SMY}, defined according to Eq. 4.1, is used to describe the variation of SMY:

$$\mathrm{RSD_{SMY}} = \frac{\sqrt{\frac{1}{n-1}\sum_{i=1}^{n}(x_i-\bar{x})^2}}{\bar{x}} \times 100\% \qquad 4\text{-}1$$

where, RSD $_{SMY}$ is the RSD of the SMY in %; n is the number of replicates per condition, i.e. 4; x_i is the individual SMY obtained in each digester under each condition in mL/g VS_{added}; and $\bar{x}$ is the average value of the SMY under each condition.

The c_{VFA_θ} on SDMY were obtained from a substrate inhibition model (Tseng & Wayman, 1975) as described in Chapter 3.

4.2.3 Statistical analysis

Statistical analysis was conducted using SPSS 22.0 (IBM, Rochester, MN, USA). Multiple linear regression analysis was performed to determine the effect of independent variables (digestion time, pH, total VFAs, free VFAs, TAN and FAN) on SMY. To avoid co-linearity among the variables, the stepwise method was used. At each iteration, the most significant independent variable ($P<0.05$) was included in the model and the selections stopped when no more variables were significant.

4.3 Results and discussion

4.3.1 Performance of the digesters and system stability

The overall performance of the digesters is shown inTable 4-3, and the profiles of methane production, COD_{VFA} concentration and $COD_{VFA+CH4}$ concentration are shown in Figure 4-1. The COD_{VFA} is the COD equivalent of VFA, which was 1.07 g COD/g acetic acid. The $COD_{VFA+CH4}$ is the sum of the COD equivalents of VFA and methane. The COD equivalent of methane is 4 g COD/g CH_4.

Table 4-3 Performance during dry co-digestion of food waste and pig manure [mean ± standard deviation (SD)]

Inoculum	Digestate		Sludge	
Food waste/pig manure	50:50	75:25	50:50	75:25
Specific methane yield [a] (SMY; mL/gVS_{added})	252±7	284 [b] ±27	246±2	- [c]
Relative standard deviation [a] (RSD_{SMY}; %)	2.9	59.9	0.9	145.4
Maximum specific methane production rate [a] [R_{max}; mL/(gVS_{added} d)]	4.0±0.3	5.5 [b] ±0.8	4.3±0.4	- [c]
Lag phase [a] (λ; d)	13±1	23 [b] ±2	28±1	- [c]
Lowest pH [d]	7.6±0.0	6.8±0.0	7.2±0.0	6.0±0.0
Stable pH [d]	8.7±0.0	8.6±0.0	8.6±0.0	8.6±0.3
Maximum total volatile fatty acids [d] (VFA; mg/L)	29,834±600	42,150±653	48,809±890	47,373±967
Maximum free VFAs calculated [d] (mg/L)	35±0	375±5	147±26	895±45
Maximum total ammonia nitrogen [d] (TAN; mg/L)	7,261±259	7,203±215	6,973±70	6,419±110
Maximum free ammonia nitrogen calculated [d] (FAN; mg/L)	3,317±181	3,275±362	2,898±119	2,863±96

[a] Values are the mean of data from four replicate reactors ± standard deviation (SD), except where indicated. ([b]) The mean of data from three uninhibited reactors, with the other reactor being inhibited. ([c]) Three reactors were inhibited, with only one producing gas.

[d] Values are the mean of data from two replicate reactors ± SD.

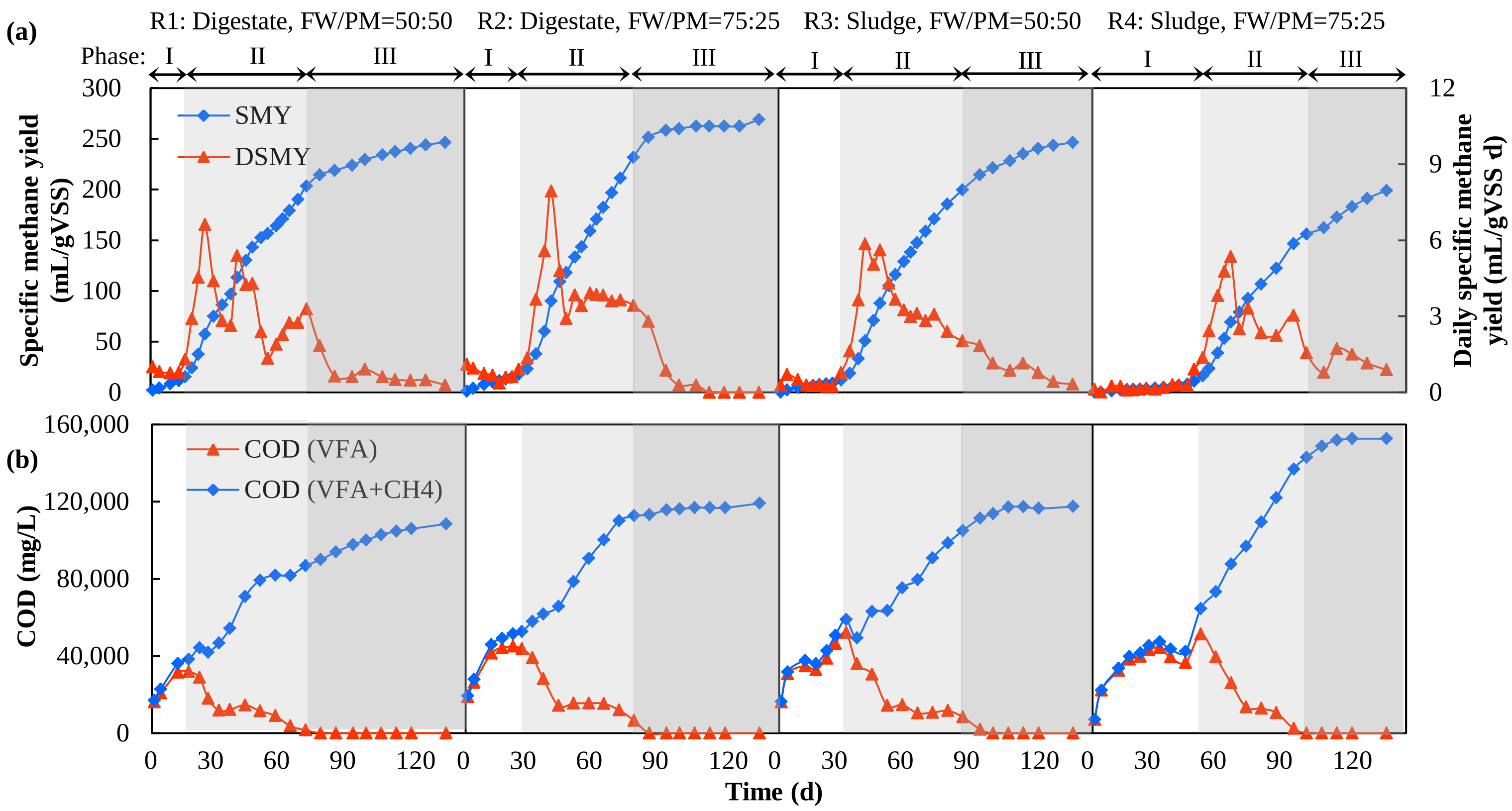


Figure 4-1: Performance of food waste/pig manure dry co-digestion systems (a) Specific methane yield (SMY) and daily specific methane yield (DSMY) and (b) COD_{VFA} and $COD_{VFA+CH4}$. SMY and DSMY values are the mean of data from four replicate digesters except at the FW/PM ratio of 75:25, as one of the four replicate digestate inoculum systems and three of the four replicate sludge inoculum systems were inhibited, with almost no methane production. Only the results from the uninhibited digesters are shown here. COD_{VFA} and $COD_{VFA+CH4}$ values are the mean of data from duplicate digesters where samples were taken.

As shown in Table 4-3, the lowest pH values at the FW/PM ratio of 50:50 (7.6 and 7.2 for the digestate and sludge inoculum systems, respectively) were higher than those at the FW/PM ratio of 75:25 (6.8 and 6.0 in the digestate and sludge inoculum systems, respectively). This indicated that a high FW enabled the rapid hydrolysis of substrates, which resulted in the accumulation of VFAs and decreased the pH. The pH values were stable at 8.6-8.7 at the end of the experiment in all operation conditions, but it took 26 and 36 days to reach the stable pH in digestate inoculum systems at FW/PM ratios of 50:50 and 75:25, respectively, and took 57 and 93 days in the sludge inoculum systems at the FW/PM ratios of 50:50 and 75:25, respectively. The shorter pH stable time indicated that the systems inoculated with digestate and with a FW/PM ratio of 50:50 were more stable than the other digestion systems.

According to the DSMY and COD_{VFA} concentration, the dry co-digestion process could be divided into three phases (Figure 4-1). Phase I was the lag phase, during which hydrolysis and acidification bacteria were active, with COD_{VFA} increasing rapidly and almost no methane production. In Phase II, COD_{VFA} were utilized and almost 80% of the methane yield was produced during this period. Hydrolysis and acidification continued as $COD_{VFA+CH4}$ continued to increase. In phase III, all of the COD_{VFA} was consumed and a reduced volume of methane was produced.

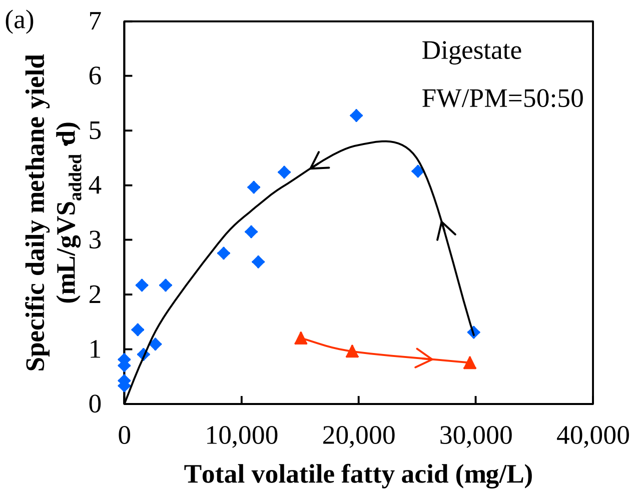
At a FW/PM ratio of 50:50, no significant difference was observed between the SMYs in the sampled compared to the un-sampled digesters (P>0.05); therefore, the average SMY values of all the four digesters under each condition were used here. As shown in Table 4-3, the average SMY and R_{max} in the digestate and sludge inoculum systems were 252 and 246 mL/g VS_{added} and 4.0 and 4.3 mL/(g VS_{added} d), respectively. There were no significant differences in SMY between the two inoculum systems (P>0.05), but the lag phase of the sludge inoculum system (28 days) was much longer than that of the digestate inoculum system (13 days). This is most likely because the digestate inoculum had already been adapted to the environment, as it was taken from previous dry co-digestion digesters, while the sludge inoculum had to undergo a selection and adaptation period before being adapted to the dry co-digestion environment. Hence, using digestate as the inoculum can reduce the start-up time and lag phase of dry co-digestion systems considerably, but does not affect the total methane yield.

At the FW/PM ratio of 75:25, one of the four reactors was inhibited in the digestate inoculum systems, and three of the four sludge inoculum reactors were inhibited. Almost no methane was produced from the inhibited digesters. The RSD_{SMY} was used to assess the stability of the systems, which was calculated from the SMY of the four replicate reactors. A low RSD_{SMY} should be obtained in stable systems because of good repeatability of replicates. As shown in Table 4-3, at a FW/PM ratio of 75:25, the RSD_{SMY} values were 59.9% and 145.4% in the digestate and sludge inoculum systems, respectively, which were much higher than those of 2.9% and 0.9% obtained at a FW/PM ratio of 50:50. This indicated the instability of reactors at high FW ratios, and the digestate inoculum reactors were more stable than the sludge inoculum reactors. Even though the average SMY at the FW/PM ratio of 75:25 increased by 12.7% than that of 50:50 in digestate inoculum systems, the increased lag phase from 13 days to 23 days reduced its practical applicability. These data demonstrate the importance of selecting a suitable inoculum as well as an optimal substrate ratio. Digestate inoculum and a FW/PM ratio of 50:50 were found to be the preferable conditions for dry co-digestion of FW and PM.

Dry anaerobic co-digestion of FW and PM is rarely reported in the literature, the limited studies of dry digestion focused on MSW or OFMSW. For example, Fernandez et al. (2008) reported the lag phases of 14 days and 28 days during dry anaerobic digestion of OFMSW at the TS content of 20% and 30%, respectively. These were similar with the results observed in this study (13 - 28 days). The SMY obtained in different experiments varied. Guendouz et al. (2010) studied dry anaerobic digestion of MSW and got the SMY of 200 mL/gVS at the TS content of 32.1%-39.7%. Dong et al. (2010) reported the SMY of 273 mL/gVS in semi-dry anaerobic digestion of water sorted OFMSW at the TS content of 16%. These results generally accorded with the values obtained in this study, the difference may be caused by different substrates and TS contents. The inhibition caused by rapid hydrolysis of FW during dry anaerobic digestion was also reported (Lim et al., 2008), which was in line with this study. The results observed in this study fill in the gap on dry co-digestion of FW and PM, and can provide reference for engineering application.

4.3.2 Inhibition factors for methane production

Multiple linear regression analysis (stepwise method) between SMY and physicochemical parameters (including pH, total VFAs, free VFAs, TAN and FAN) showed that total VFAs was the main factor inhibiting SMY during dry co-digestion of FW and PM (P<0.001). The relationships between total VFAs and the SDMYs are shown in Figure 4-2. As the experiment proceeded, the concentrations of total VFAs first increased and then decreased, as illustrated by the arrows in Figure 4-2. The total VFA concentrations increased rapidly at the beginning of the experiment because of hydrolysis and acidification of the substrates, during which the SDMY was low (red scatters in Figure 4-2). This may be because hydrolysis and acidification bacteria dominated in the reactors, with methanogens being not adapted to the high VFA environment. Once the methanogenic population was adapted to the environment, the total VFAs were utilized and started to decrease, and SDMY began to increase (blue scatters in Figure 4-2). However, the methanogens were inhibited when the total VFA concentrations were higher than the threshold concentration (c_{VFA_θ}). When the total VFAs decreased below this threshold concentration, the inhibition ceased and the subsequent decrease of SDMY was caused by the depletion of total VFAs.



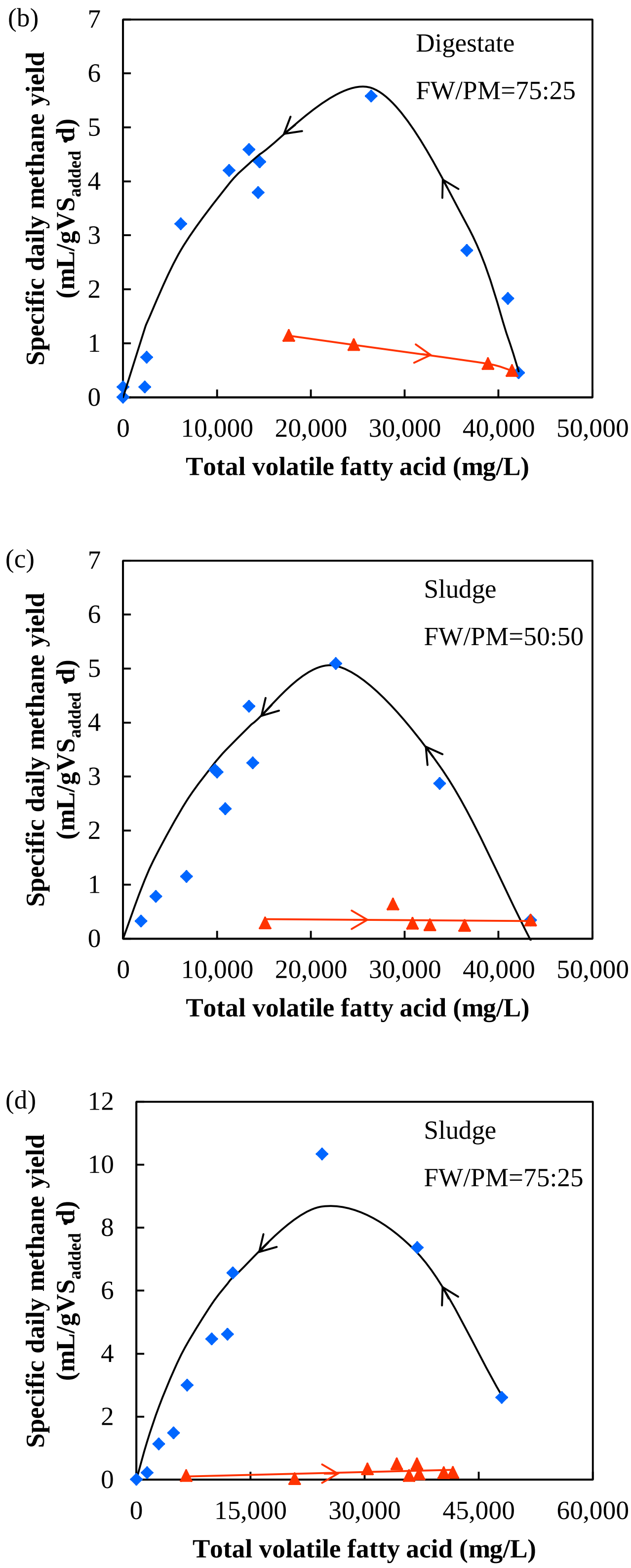


Figure 4-2: Effect of total volatile fatty acid concentrations on specific daily methane yields from dry co-digestion of food waste and pig manure in digestate (a, b) and sludge (c, d) inoculum systems. (The arrows indicate the changes with time, the scatters are experimental

data and the solid lines are responses of the models). Values are the mean of data from uninhibited reactors.

▲ Total VFAs increasing phase ◆ Total VFAs decreasing phase

The c_{VFA_θ} was obtained from a substrate inhibition model (Tseng & Wayman, 1975). The responses of the models are shown in Figure 4-2 (black solid lines). At a FW/PM ratio of 50:50, the c_{VFA_θ} values were 20.0 and 21.0 g/L in the digestate and sludge inoculum systems, respectively. The c_{VFA_θ} at a FW/PM ratio of 75:25 were 26.1 and 25.6 g/L in the digestate and sludge inoculum systems, respectively. A higher FW proportion in the feedstock led to greater VFA accumulation, which may select acidophilic bacteria with a higher tolerance to VFAs. However, a longer lag phase and unstable methane production followed when increasing the proportion of FW in the feedstock. Therefore, it is recommended to control the total VFA concentrations below 20.0 g/L during dry co-digestion of FW and PM to maintain system stability and prevent inhibition.

The reported VFA inhibition concentrations varied in different studies. Wang et al. (2009) found the methanogen's activity was severely inhibited at the total VFA concentration of 10.0 g/L in anaerobic digestion of swine manure, and the inhibition can be relieved when VFAs fell to 6.2 - 8.5 g/L. Ghanimeh et al. (2012) found the VFA concentration of 10.3 g/L decreased the methane yield by 64% during thermophilic anaerobic digestion of source-sorted OFMSW. The threshold VFA inhibition concentration of about 20.0 g/L during dry co-digestion of FW and PM was much higher than those reported in the literature. It can provide a tangible guidance for engineering practice of FW and PM dry co-digestion systems.

4.4 Summary

The effects of inoculum types and FW/PM ratios on the stability of dry co-digestion systems were evaluated. The use of digestate as an inoculum and a FW/PM ratio of 50:50 resulted in stable systems, with a SMY of 252 mL/gVS$_{added}$ being achieved. Total VFAs was the main factor inhibiting methane production; it was found that VFA concentrations needed to remain below ~ 20.0 g/L in order to prevent inhibition and to shorten the lag phase.

Chapter 5
Effect of biological and environmental factors on the microbial communities of dry anaerobic digesters co-digesting food waste and pig manure explored using high-throughput 16S rRNA gene amplicon sequencing

5.1 Introduction

The research objectives of this chapter were: (1) to investigate the effects of inoculum type (digestate and sludge) and FW/PM ratio (50:50 and 75:25) on the structure of the microbial community within dry FW/PM co-digestion systems, and (2) to explore the potential roles of microbes, whose functions in dry co-digestion systems have been poorly described.

5.2 Materials and Methods

5.2.1 Experimental design

Batch dry co-digestion of FW and PM was conducted in sixteen 1 L glass digesters as described in Chapter 4. The analysis of biogas, pH, TS, VS, SCOD, total VFA, TAN, calculation of free VFA and FAN were outlined in Chapter 3. The sampling method was detailed in Chapter 4. Totally 56 samples were taken for microbiota analysis (~ 2 g) from each of 2 replicate digesters per condition on Day 2, 17, 31, 50, 71, 93 and 120, snap-frozen in liquid nitrogen and stored at -80 ^{o}C for subsequent analysis. Detailed methodology on DNA extraction and 16S rRNA gene sequencing are described in Appendix A.

5.2.2 Statistical analysis

Statistical analysis was performed using R (version 3.3.2) and SPSS 22.0 (IBM, USA). Microbial richness, diversity and phylum level microbial relative abundance data were tested for normality using the Shapiro-Wilk test, with $P > 0.05$ indicating normal distribution. Microbial richness, diversity and phylum level relative abundance across three different phases [Phases I to III, determined based on the DSMY and COD_{VFA} concentration as described in Chapter 4] and the four operating conditions (R1 to R4 as described in Chapter 4) were compared using the Kruskal-Wallis test followed by the Dunn-Bonferroni post hoc test for pairwise comparison. Bonferroni correction was used to control the FWE and the adjusted P values were used in the results. Comparisons between the two inocula and the two FW/PM ratios were performed using the Mann-Whitney U Test. Correlations between microbial relative abundance at genus level and physicochemical parameters (pH, SCOD, total VFAs, free VFAs, acetate, propionate, butyrate, TAN, FAN and SMY) were performed using a two-tailed Spearman's rank order correlation. Differences and correlations were considered significant at $P < 0.05$. QIIME was used to generate the PCoA figures for both the weighted and unweighted unifrac distances.

5.3 Results and Discussion

5.3.1 Microbial richness and diversity

The number of reads per sample ranged from 57,866 to 222,595 in the 56 samples taken across all 8 reactors sampled over the 120-day operating period. A total of 1,987 OTUs were found, of which 1,934 were *Bacteria* and 53 were *Archaea*. In total, 33 phyla (30 *Bacteria* and 3 *Archaea*), 50 classes (45 *Bacteria* and 5 *Archaea*), 132 orders (125 *Bacteria* and 7 *Archaea*), 234 families (222 *Bacteria* and 12 *Archaea*) and 409 genera (391 *Bacteria* and 18 *Archaea*) were found.

Alpha diversity metrics were used to assess microbial richness and diversity, with the results shown in Table 5-1. The Chao1 Index and Observed Species indicate microbial richness, and PD Whole Tree and the Shannon Index indicate diversity. The Chao1 Index, Observed Species and PD Whole Tree in Phase I were lower than those in Phase II and III ($P<0.01$). This indicates that both microbial richness and diversity increased with time during the dry co-digestion process. However, microbial richness and diversity did not differ between Phase II and Phase III ($P>0.05$). Significant differences in microbial richness and diversity were observed between the different operating conditions ($P<0.01$). Both inoculum and FW/PM ratio contributed to the differences, but the diversity differences (PD Whole Tree and Shannon Index) were mainly influenced by the inoculum ($P<0.01$), while the richness differences (Chao1 Index and Observed Species) were mainly influenced by the FW/PM ratio ($P<0.01$). Microbial diversity in the digestate inoculum systems was lower than that in the sludge systems ($P<0.01$). This is most likely because the microbes in the digestate were already adapted to the digester environment, with the unadapted ones washed out. No significant differences were observed within the same inoculum type (i.e. R1 vs R2 and R3 vs

R4; P>0.05). Microbial richness at a FW/PM ratio of 50:50 was higher than that at 75:25 (P<0.01).

Table 5-1 Microbial richness and diversity at the genus level during dry co-digestion of food waste and pig manure (mean ± SD)

	Richness		Diversity	
	Chao1	Observed Species	PD Whole Tree	Shannon
Phase I	1415±125	1208±126	92.8±8.4	6.58±0.93
Phase II	1595±72	1366±64	102.2±3.9	6.85±0.69
Phase III	1637±54	1404±83	104.8±4.6	6.97±0.58
R1	1597±123	1312±112	98.7±7.2	6.09±0.41
R2	1570±118	1294±123	97.5±7.6	6.30±0.49
R3	1582±122	1411±124	104.9±7.6	7.47±0.41
R4	1473±111	1310±93	99.8±5.5	7.37±0.31
	Statistical analysis (P values)			
	Chao1	Observed species	PD whole tree	Shannon
Phase	0.000 ***	0.000 ***	0.000 ***	0.667
Phase I vs Phase II	0.001 ***	0.002 **	0.008 **	1.000
Phase I vs Phase III	0.000 ***	0.000 ***	0.000 ***	1.000
Phase II vs Phase III	0.500	0.927	0.424	1.000
Condition	0.008 **	0.004 **	0.002 **	0.000 ***
Inoculum	0.057	0.022 *	0.008 **	0.000 ***
FW/PM	0.007 **	0.009 **	0.013 *	0.941
R1 vs R2	1.000	1.000	1.000	1.000
R1 vs R3	1.000	0.078	0.048 *	0.000 ***
R1 vs R4	0.007 **	1.000	1.000	0.000 ***
R2 vs R3	1.000	0.003 **	0.002 **	0.000 ***
R2 vs R4	0.309	1.000	1.000	0.007 **
R3 vs R4	0.069	0.039 *	0.070	1.000

*** P<0.001, ** P<0.01, * P<0.05.

The PCoA was performed to analyse beta diversity within the reactor samples, and weighted UniFrac matrices were used. This showed that the microbial communities within the sludge and digestate inoculum systems were clearly distinct (Figure 5-1), indicating that the inoculum played an important role in shaping microbial community structure. However, the microbiota did not appear to cluster on the basis of FW/PM ratio, except to some extent for each inoculum type.

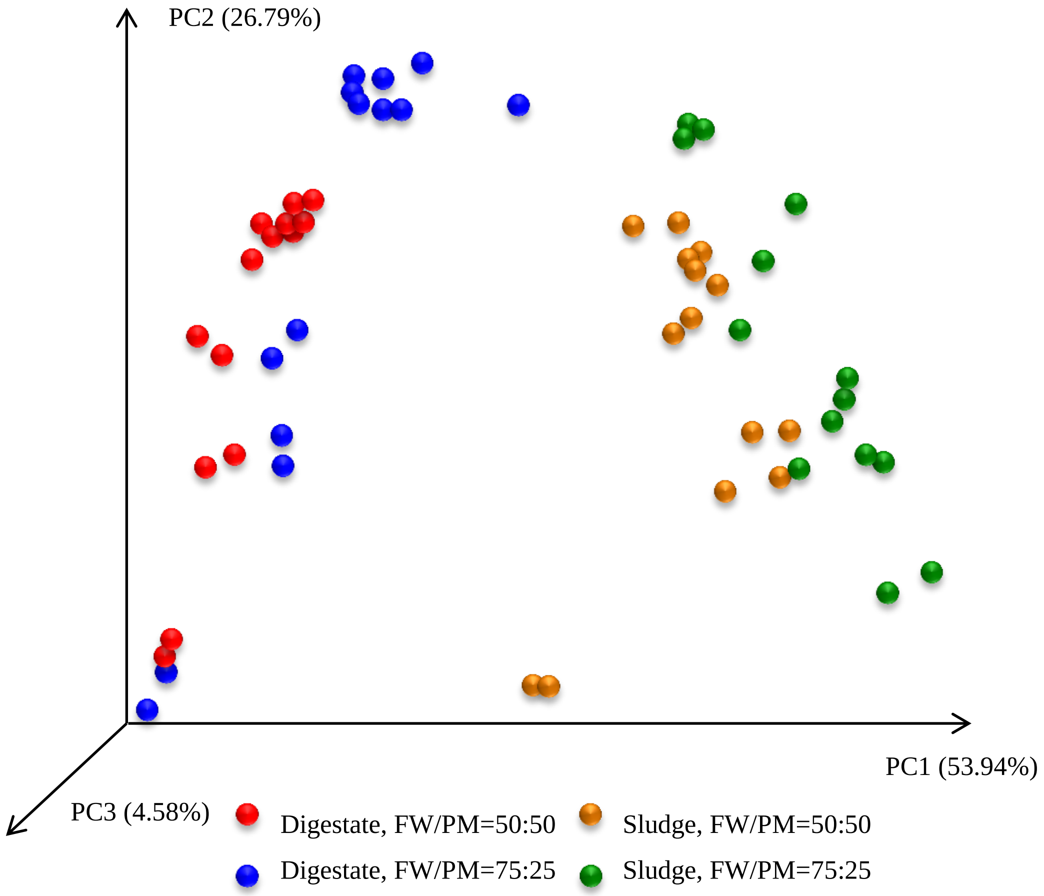


Figure 5-1 Principal coordinate analysis (PCoA; based on weighted UniFrac distances) of microbial community structure during dry co-digestion of food waste and pig manure

5.3.2 Microbial community composition at the phylum level

The phylum-level relative abundances of the microbial communities for the four different operating conditions are shown in Figure 5-2. Nine abundant phyla (relative abundance >1% in at least one sample) were found in all reactors.

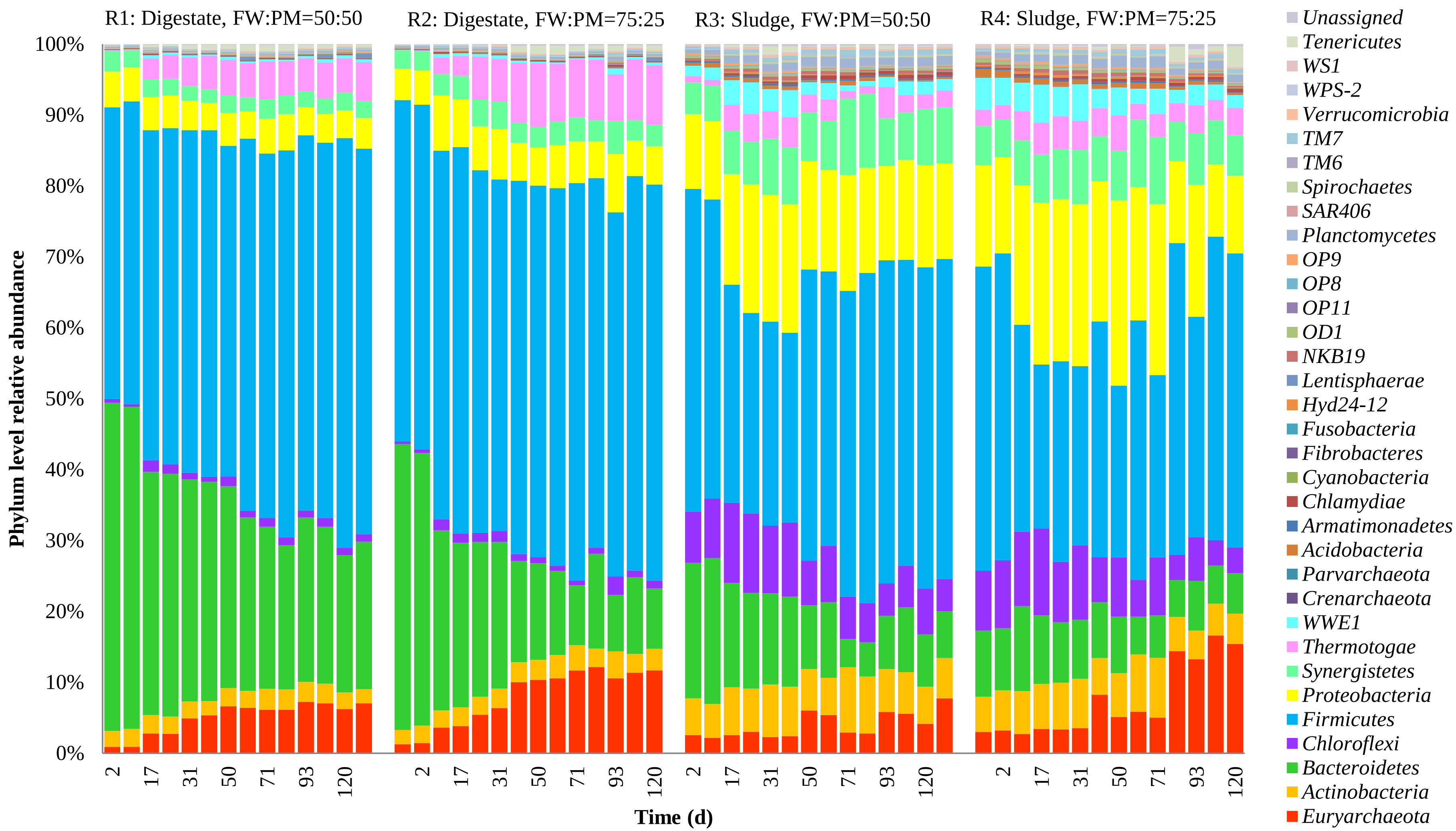


Figure 5-2 Phylum-level relative abundance during dry co-digestion of food waste and pig manure using four different operating conditions. The nine abundant phyla are in bold. Data are from two replicates of each condition at each time point.

In the digestate inoculum systems, *Firmicutes* (41.9%-56.1% relative abundance), *Bacteroidetes* (10.9%-46.7%) and *Euryarchaeota* (0.9%-11.5%) were the most abundant phyla. The orders *Clostridiales* (23.0%-35.5%), *Bacteroidales* (9.7%-45.9%) and *Methanomicrobiales* (0.4%-11.0%) dominated, respectively within these phyla (Table 5-2). Other abundant phyla were *Thermotogae* (0.1%-8.5%), *Proteobacteria* (4.0%-7.3%), *Synergistetes* (2.0%-3.9%) and *Actinobacteria* (2.2%-3.4%).

In the sludge inoculum systems, the most abundant phyla were *Firmicutes* (26.2%-45.2%), *Proteobacteria* (10.6%-22.9%), *Bacteroidetes* (4.4%-19.8%) and *Euryarchaeota* (2.3%-16.0%). The orders *Clostridiales* (10.8%-28.0%), *Pseudomonadales* (0.1%-11.5%), *Bacteroidales* (4.3%-19.6%) and *Methanomicrobiales* (0.1%-14.3%) dominated in the above phyla, respectively (Table 5-2). Other abundant phyla were *Chloroflexi* (3.6%-11.4%), *Synergistetes* (4.7%-10.6%), *Actinobacteria* (4.4%-8.6%), *Thermotogae* (0.9%-4.5%) and candidate phylum *WWE1* (0.8%-4.7%).

Table 5-2 Heat map for orders found at a relative abundance >1% in at least one sample during dry co-digestion of food waste and pig manure

Phylum	Class	Order	Digestate, FW:PM=50:50							Digestate, FW:PM=75:25							Sludge, FW:PM=50:50							Sludge, FW:PM=75:25						
			2	17	31	50	71	93	120	2	17	31	50	71	93	120	2	17	31	50	71	93	120	2	17	31	50	71	93	120
Euryarchaeota	*Methanobacteria*	*Methanobacteriales*																												
	Methanomicrobia	*Methanomicrobiales*																												
		Methanosarcinales																												
Acidobacteria	*Sva0725*	*Sva0725*																												
Actinobacteria	*Acidimicrobiia*	*Acidimicrobiales*																												
	Actinobacteria	*Actinomycetales*																												
Bacteroidetes	*Bacteroidia*	*Bacteroidales*																												
	Flavobacteriia	*Flavobacteriales*																												
Chloroflexi	*Anaerolineae*	*Unclassified*																												
		Anaerolineales																												
Firmicutes	*Bacilli*	*Bacillales*																												
		Lactobacillales																												
	Clostridia	*BSA2B-08*																												
		Clostridiales																												
		Halanaerobiales																												
		MBA08																												
		OPB54																												
		SHA-98																												
		Thermoanaerobacterales																												
	OPB54	*Unclassified*																												
Planctomycetes	*Planctomycetia*	*Pirellulales*																												
Proteobacteria	*Alphaproteobacteria*	*Rhizobiales*																												
		Rhodobacterales																												
	Betaproteobacteria	*Burkholderiales*																												
	Gammaproteobacteria	*Pseudomonadales*																												
		Xanthomonadales																												
Synergistetes	*Synergistia*	*Synergistales*																												
Tenericutes	*Mollicutes*	*Acholeplasmatales*																												
	RF3	*ML615J-28*																												
Thermotogae	*Thermotogae*	*Thermotogales*																												
WWE1	*Cloacamonae*	*Cloacamonales*																												

0% 10% 20% 30% 40% 50%

Significant differences in relative abundance of the most abundant phyla for each operating condition are shown in Table 5-3. Differences were observed between different operating conditions for both bacteria ($P<0.01$) and *Archaea* ($P<0.05$). However, the differences in bacterial phyla were mainly caused by the different inocula ($P<0.01$), while the difference in *Archaea* resulted from the different FW/PM ratios used ($P<0.05$). No significant differences were observed within the same inoculum type (R1 vs R2 and R3 vs R4; $P>0.05$), indicating the lack of effect of the FW/PM ratio. The relative abundances of *Bacteroidetes*, *Firmicutes* and *Thermotogae* were significantly higher in the digestate inoculum systems than in the sludge inoculum systems, while the relative abundances of *Actinobacteria*, *Chloroflexi*, *Proteobacteria*, *Synergistetes* and *WWE1* were significantly higher in the sludge inoculum systems. Changes to abundance of the archaeal phylum, *Euryarchaeota*, were mainly in response to the reaction phase ($P<0.001$); abundance increased over time, being significantly higher in phase III than in Phase II ($P<0.05$) or Phase I ($P<0.001$).

Table 5-3 Differences in microbial composition at the phylum level during dry co-digestion of food waste and pig manure using four different operating conditions (mean ±SD). Only phyla with a relative abundance > 5% in at least one sample are shown.

Relative abundance (%)	R1 (Digestate, FW/PM=50:50)	R2 (Digestate, FW/PM=75:25)	R3 (Sludge, FW/PM=50:50)	R4 (Sludge, FW/PM=75:25)
Euryarchaeota	5.0 ±2.2	7.9 ±4.0	4.0 ±1.8	7.4 ±5.2
Actinobacteria	2.5 ±0.3	2.8 ±0.5	6.3 ±1.2	5.9 ±1.3
Bacteroidetes	28.9 ±8.8	18.5 ±10.5	10.9 ±5.0	7.6 ±2.0
Chloroflexi	1.1 ±0.3	1.0 ±0.4	7.5 ±2.3	7.5 ±2.8
Firmicutes	49.9 ±4.7	52.4 ±2.5	39.4 ±7.4	33.7 ±8.0
Proteobacteria	4.4 ±0.5	6.0 ±1.1	14.8 ±2.3	18.3 ±5.3
Synergistetes	2.4 ±0.3	3.1 ±0.4	7.0 ±1.5	6.7 ±1.1
Thermotogae	4.2 ±1.5	5.9 ±3.3	2.6 ±1.3	3.5 ±1.0
WWE1	0.3 ±0.1	0.3 ±0.2	2.2 ±1.1	3.4 ±1.2

Statistical analysis (P values)

Groups	Condition	Inoculum	FW/PM	R1 vs R2	R1 vs R3	R1 vs R4	R2 vs R3	R2 vs R4	R3 vs R4	Phase	Phase I vs II	Phase I vs III	Phase II vs III
Euryarchaeota	0.040 *	0.149	0.014 *	0.945	1.000	1.000	0.035 *	1.000	0.235	0.000 ***	0.003 **	0.000 ***	0.016 *
Actinobacteria	0.000 ***	0.000 ***	0.706	1.000	0.000 ***	0.000 ***	0.000 ***	0.001 ***	1.000	0.725	1.000	1.000	1.000
Bacteroidetes	0.000 ***	0.000 ***	0.019 *	0.235	0.000 ***	0.000 ***	0.228	0.005 **	1.000	0.023 *	0.669	0.019 **	0.229
Chloroflexi	0.000 ***	0.000 ***	0.935	1.000	0.000 ***	0.000 ***	0.000 ***	0.000 ***	1.000	0.157	1.000	1.000	1.000
Firmicutes	0.000 ***	0.000 ***	0.922	1.000	0.007 **	0.000 ***	0.000 ***	0.000 ***	1.000	0.030 *	0.264	0.025 *	0.715
Proteobacteria	0.000 ***	0.000 ***	0.039 *	0.278	0.000 ***	0.000 ***	0.012 *	0.000 ***	1.000	0.276	0.320	0.105	0.490
Synergistetes	0.000 ***	0.000 ***	0.195	0.171	0.000 ***	0.000 ***	0.002 **	0.007 **	1.000	0.983	1.000	1.000	1.000
Thermotogae	0.003 **	0.001 **	0.105	1.000	0.099	1.000	0.003 **	0.119	1.000	0.002 **	0.004 **	0.006 **	1.000
WWE1	0.000 ***	0.000 ***	0.251	1.000	0.001 ***	0.000 ***	0.001 ***	0.000 ***	0.866	0.265	1.000	1.000	1.000

*** $P<0.001$, ** $P<0.01$, * $P<0.05$.

5.3.3 Genus-level microbial community composition and correlations between bacterial taxa and physicochemical parameters

Bacteria play important roles in hydrolysis, acidogenesis and acetogenesis in anaerobic digestion systems. However, the specific function of many of the resident bacteria has not yet been elucidated. In this study, correlation analysis between relative abundance of the dominant bacterial taxa found in the digesters and physicochemical parameters over the 120-day operating period was performed in order to explore the possible functionality of these microbes (Table 5-4). The physicochemical parameters included in the analysis were: pH, SCOD, total VFA, free VFA, acetate, propionate, butyrate, TAN, FAN and SMY. The main findings of the correlation analysis are summarised in the sections below. When looking at the relative abundance of bacteria within the digesters at the genus level (Figure 5-3), in general, it can be seen that the sludge inoculum systems were more complex, but specific differences are outlined below when discussing correlations.

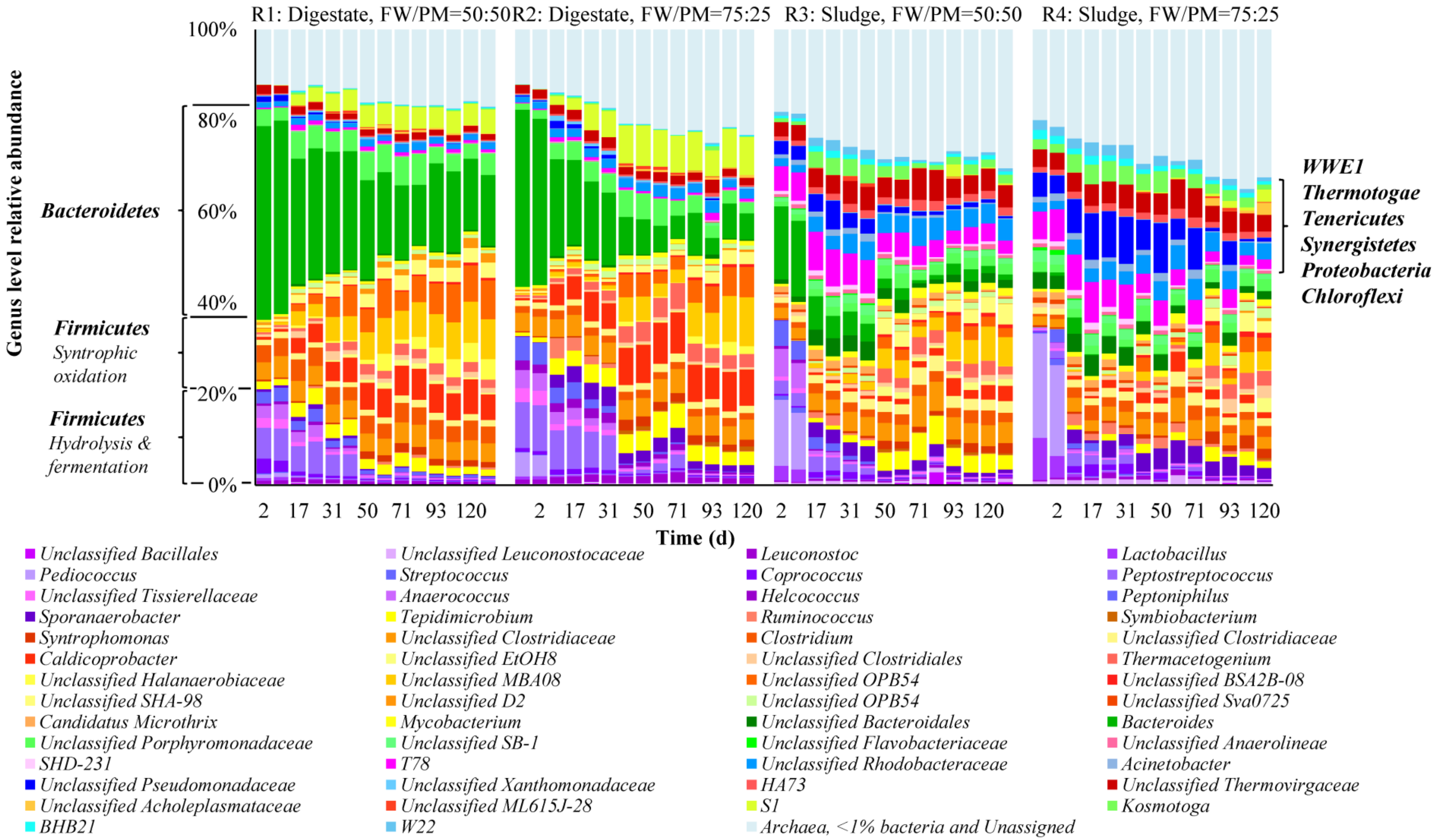


Figure 5-3 Genus-level relative abundance of bacteria during dry co-digestion of food waste and pig manure using four different operating conditions. Data are from two replicates of each condition at each time point. Only genera with a relative abundance > 1% in at least one sample are shown.

Table 5-4 Correlations between the relative abundance of microbial taxa and physicochemical parameters during dry co-digestion of food waste and pig manure under the four different operating conditions: (a) *Firmicutes* and (b) other phyla.

(a) Phylum	Class	Order	Family	Genus	R1: Digestate, FW:PM=50:50							R2: Digestate, FW:PM=75:25							R3: Sludge, FW:PM=50:50							R4: Sludge, FW:PM=75:25						
					SCOD [a]	Total VFA [b]	Free VFA	Acetate	Propionate	Butyrate	SMY [c]	SCOD	Total VFA	Free VFA	Acetate	Propionate	Butyrate	SMY	SCOD	Total VFA	Free VFA	Acetate	Propionate	Butyrate	SMY	SCOD	Total VFA	Free VFA	Acetate	Propionate	Butyrate	SMY
Firmicutes	*Bacilli*	*Lactobacillales*	*Leuconostocaceae*	*Leuconostoc*																			**				*			**		
			Lactobacillaceae	*Lactobacillus*	**	**	**	*		*	**	*	*	**	*		*	**										**				**
				Pediococcus	**	**	**	*		*	**	*	*	**	*		*	**			*				**			**				**
			Streptococcaceae	*Streptococcus*	**	*	**	*		*	*																	**				**
	Clostridia	*Clostridiales*	*Lachnospiraceae*	*Coprococcus*	**	**	**	*		*	**	**	**	**	**		**	*	*	*	*	*		**			*				*	
			Peptostreptococcaceae	*Peptostreptococcus*	**	**	**	*		*	**	*	*	**	*		*	**	*	*	*			*	**			*				*
			Tissierellaceae	*Unclassified*	**	**	**	*		*	**	*	*	**	*		*	**							*			*				*
				Anaerococcus	*	**	**	*		*	**	*	*	**	*		*	**			*			*	**			**				**
				Helcococcus	*	*	*	*		*	**	*	*	*	*		*	*	*	*		*		*	*			*				*
				Peptoniphilus	**	**	**	*		*	**	**	*	**	*		*	**			*			*	**			**				**
				Sporanaerobacter	**	**					*																					
				Tepidimicrobium																				*				**				**
			Clostridiaceae	*Unclassified*																					*							
				Clostridium															*	*	**	*		*	**							
				Other	*	*			*			*	*	*	*		*	*							*							
			Unclassified	*Unclassified*			*	*		*					*		*													*		
			Symbiobacteriaceae	*Symbiobacterium*										*	*		*	*						*	*			**				**
			EtOH8	*Unclassified*				*		*					*		*							*	*			**				
			Syntrophomonadaceae	*Syntrophomonas*	*	*		*		*					*		*							*	**							**
			Caldicoprobacteraceae	*Caldicoprobacter*	*	*	*	*		*	**				*		*		*	*	*	*		*	**							
		Thermoanaerobacterales	*Thermoanaerobacteraceae*	*Thermacetogenium*	*	*	*	*		*	*				*		*							*				**				**
		MBA08	*Unclassified*	*Unclassified*	**	**	**	*		*	**	*	*	**	*		*	**	*	*	*			*	**			**				**
		OPB54	*Unclassified*	*Unclassified*	*	*	*	*		*	**	*	*	**	*		*	**						*	*			**				**
		BSA2B-08	*Unclassified*	*Unclassified*	**	**	**	*		*	**	*	*	**	*		*	**	*	*	*			*	**			**				**
		SHA-98	*Unclassified*	*Unclassified*	**	**	**	*		*	**	*	*	**	*		*	**						*	**			**				**
			D2	*Unclassified*	**	**	*		*		**													*	*					*		

(b)

Phylum	Class	Order	Family	Genus	R1: Digestate, FW:PM=50:50							R2: Digestate, FW:PM=75:25							R3: Sludge, FW:PM=50:50							R4: Sludge, FW:PM=75:25						
					SCOD	Total VFA	Free VFA	Acetate	Propionate	Butyrate	SMY	SCOD	Total VFA	Free VFA	Acetate	Propionate	Butyrate	SMY	SCOD	Total VFA	Free VFA	Acetate	Propionate	Butyrate	SMY	SCOD	Total VFA	Free VFA	Acetate	Propionate	Butyrate	SMY
Euryarchaeota	*Methanomicrobia*	*Methanosarcinales*	*Methanosaetaceae*	*Methanosaeta*																	*				**			*				**
			Methanosarcinaceae	*Methanosarcina*															**	**	**	*		*	**							
		Methanomicrobiales	*Methanomicrobiaceae*	*Methanoculleus*	**	**	**	*		*	**	*	**	**	*		*	**						*	*			**				**
				Methanofollis																								**				**
Acidobacteria	*Sva0725*	*Sva0725*	*Unclassified*	*Unclassified*																								*				*
Actinobacteria	*Acidimicrobiia*	*Acidimicrobiales*	*Microthrixaceae*	*Candidatus Microthrix*															*	*	*			*		*						
	Actinobacteria	*Actinomycetales*	*Mycobacteriaceae*	*Mycobacterium*									*	*	*		*	*	*	*		*		*	*			*				**
Bacteroidetes	*Bacteroidia*	*Bacteroidales*	*Porphyromonadaceae*	*Unclassified*												*								*	*							
			Bacteroidaceae	*Bacteroides*	*	*	*	*		*	**		*	*	*		*	*	*	*		*		*	*			**				**
			Unclassified	*Unclassified*																		*				*		*	*			**
			SB-1	*Unclassified*																		*						**				**
	Flavobacteriia	*Flavobacteriales*	*Flavobacteriaceae*	*Unclassified*															**	**	**	**		**	**			*				**
Chloroflexi	*Anaerolineae*	*o__*	*Unclassified*	*Unclassified*																						**	*	*	**		*	
		Anaerolineales	*Anaerolinaceae*	*SHD-231*															*	*	*	**		*	*	*		*	*			**
				T78															*	*	**	*		*	*	*		*	*			**
Proteobacteria	*Alphaproteobacteria*	*Rhodobacterales*	*Rhodobacteraceae*	*Other*																		*			*							
	Gammaproteobacteria	*Pseudomonadales*	*Moraxellaceae*	*Acinetobacter*	*	*	*	*		*	*								**	**	**	**		**	*							
			Pseudomonadaceae	*Unclassified*	**	**	**	*		*	**	*	*	*	**		**	*	**	**	**	**		*	*	**	**		**		*	
		Xanthomonadales	*Xanthomonadaceae*	*Other*																										*		
Synergistetes	*Synergistia*	*Synergistales*	*Dethiosulfovibrionaceae*	*HA73*																										*		
			Thermovirgaceae	*Unclassified*																										*		
Tenericutes	*Mollicutes*	*Acholeplasmatales*	*Acholeplasmataceae*	*Unclassified*				*		*		**	*	**	*		*	**										*				*
	RF3	*ML615J-28*	*Unclassified*	*Unclassified*											*		*											*				*
Thermotogae	*Thermotogae*	*Thermotogales*	*Thermotogaceae*	*S1*	*	*	*	*		*	**			*	*		*	*			*			*	**			*				**
				Kosmotoga																		*		*		*	*	*	*		*	
WWE1	*[Cloacamonae]*	*[Cloacamonales]*	*Cloacamonaceae*	*BHB21*																								**				**
				W22															**	**	*	**		*	*	**		**	**			*

* P<0.05; ** P<0.01

[a] SCOD: Soluble chemical oxygen demand; [b] VFA: Volatile fatty acid; [c] SMY: Specific methane yield

The relative abundance and physicochemical parameters are the mean of data from the duplicate digesters sampled from R1-R3, and are the data from the uninhibited digester from R4.

Red boxes indicate negative correlations, green boxes indicate positive correlations, and blank boxes indicate no correlations.

Correlations were determined using a two-tailed pairwise Spearman's rank order correlation at a significance level of P<0.05.

5.3.3.1 Firmicutes

Firmicutes are prevalent in co-digestion systems treating substrates such as restaurant, household and slaughterhouse waste (Sundberg et al., 2013). Several members are well known fermentative and syntrophic oxidizing bacteria (Guo et al., 2015). In this study, correlations for *Firmicutes* members were more evident in the digestate inoculum than in the sludge inoculum systems (Table 5-4 a). In these systems, the genera *Lactobacillus*, *Pediococcus* and *Streptococcus* (in order *Lactobacillale*), and *Coprococcus, Peptostreptococcus*, an unclassified genus from the family *Tissierellaceae, Anaerococcus*, *Helcococcus* and *Peptoniphilus* (in order *Clostridiales*) had positive correlations with SCOD, total VFA, free VFA, acetate and butyrate, and had negative correlations with SMY. This indicates that these bacteria may play a role in hydrolysis and fermentation during dry co-digestion of FW and PM, likely degrading organic matter to SCOD and further converting this into various VFAs, most notably acetate and butyrate. This is corroborated, at least to some extent, when we look at the metabolic traits of these bacteria. *Lactobacillus*, *Pediococcus* and *Streptococcus* are well known as lactic acid producers (Carr et al., 2002), while *Coprococcus* can ferment carbohydrate with the resultant production of acetate, butyrate and other VFAs (Holdeman & Moore, 1974). *Peptoniphilus* and *Anaerococcus* are derived from the genus *Peptostreptococcus*; members of *Peptoniphilus* are reported to be non-saccharolytic, using peptone as a major energy source, while *Anaerococcus* members are reportedly saccharolytic. They all produce butyrate as a terminal VFA (Ezaki et al., 2001). Two species within the genus *Helcococcus* (*kunzii* and *sueciensis)* produce acids from lactose and trehalose (Ulger-Toprak et al., 2010), while *Helcococcus ovis* can produce volatile fatty acids from glucose (Collins et al., 1999). Unlike the genera outlined above, *Sporanaerobacter* only had a positive correlation with SCOD and total VFA, indicating its possible function in hydrolysis. *Sporanaerobacter acetigene* is reported to be sugar- and peptide-utilizing bacteria and can produce various VFAs (Hernandez-Eugenio et al., 2002).

On the contrary, the genera *Clostridium, Syntrophomonas, Caldicoprobacter*, an unclassified genus in the candidate family *EtOH8* and some unclassified genera in the order *Clostridiales*, the genus *Thermacetogenium* and some genera in the candidate orders *MBA08, OPB54, BSA2B-08* and *SHA-98* had negative correlations with SCOD, total VFA, free VFA, acetate and butyrate, and had positive correlations with SMY (Table 5-4 a). These bacteria may be responsible for syntrophic oxidation, which is the main process consuming VFAs, such as acetate, propionate and butyrate, under high ammonia or VFA conditions (Karakashev et al., 2006; Müller et al., 2010). In syntrophic oxidation reactions, propionate and butyrate are converted into acetate, formate, hydrogen and carbon dioxide (Müller et al., 2010). The formate and acetate can be further converted into hydrogen and carbon dioxide (Hattori, 2008). Syntrophic oxidations are endergonic reactions ($\Delta G^{0'}>0$) and thermodynamically unfavorable under standard conditions (P=1 atm, T=298 K). These reactions occur only when the products are consumed by hydrogenotrophic methanogens, which are exoergic reactions resulting in low partial pressure of hydrogen and low concentrations of acetate and formate (Demirel & Scherer, 2008). The dominance of hydrogenotrophic methanogens in the dry co-digestion FW/PM systems made this possible, as outlined in Section 5.3.4.

Clostridium is one of the genera listed above which may be responsible for syntrophic oxidation within the reactors. Varying functions have been reported for the 203 species and 5 subspecies of this genus (Horino et al., 2014). Many can ferment organic matter with the resultant production of butyrate (Mead, 1971). *Clostridium ultunense* is a syntrophic acetate-oxidizing bacterium, but it can also produce acetate from the syntrophic products H_2/CO_2 , which means it is also homoacetogenic (Hattori, 2008). The negative correlation of *Clostridium* with acetate and butyrate in this study suggests that acetate- and butyrate-oxidizing species might have dominated during the dry co-digestion of FW and PM. The genus *Syntrophomonas* is well known as butyrate-oxidizing bacteria (Ariesyady et al., 2007), and the species *Thermacetogenium phaeum* isolated from thermophilic digesters was previously reported as a syntrophic acetate-oxidizing bacterium (Hattori et al., 2000). These traits are in agreement with the results observed in this study. Looking at the other genera outlined above, *Caldicoprobacter* has been reported to be abundant at high TAN concentrations (5.0- 25.0 g/L) (Poirier et al., 2016) and in thermophilic conditions (Sun et al., 2015b). In this study, the TAN concentrations ranged from 3.9 to 7.2 g/L and incomplete mixing in the dry co-digestion reactors may have caused local thermophilic temperatures (hot spots). Some species of *Caldicoprobacter* isolated from hot springs or sheep's faeces, such as *algeriensis, oshimai* and *guelmensis* are reported to be able to ferment various sugars with the resultant production of acetate, lactate, ethanol, CO_2, and H_2 (Bouanane-Darenfed et al., 2013; Bouanane-Darenfed et al., 2011; Yokoyama et al., 2010). However, the negative correlations of *Caldicoprobacter* with acetate and butyrate in this study indicate that some species within this genus may function as syntrophic oxidisers of acetate and butyrate, which has not previously been reported. The function of the candidate family *EtOH8* is poorly described in the literature; however, its negative correlation with acetate and butyrate in this study indicate that some of its members are also likely acetate and butyrate syntrophic oxidisers.

The candidate order *MBA08* is mainly observed in thermophilic conditions (Li et al., 2015b) and, together with the order *SHA-98*, also in anaerobic digesters treating agricultural waste (Weithmann et al., 2016). The order *OPB54* was previously found in thermophilic digesters and at high TAN concentrations (7.0 g/L) (Hao et al., 2015; Sun et al., 2015a). But the candidate order *BSA2B-08* has not previously been reported in anaerobic digestion systems. Moreover, the functions of these candidate orders (*MBA08*, *OPB54*, *SHA-98* and *BSA2B-08*) in anaerobic digesters have not previously been reported. However, their negative correlations with various VFAs (especially acetate and butyrate) and their positive correlation with SMY mean that some of their members likely function as syntrophic acetate- and butyrate-oxidizing bacteria, which produce CO_2 and H_2 as substrates for hydrogenotrophic methanogens. The candidate family *D2* within the order *SHA-98* had a negative correlation with propionate, indicating that it may contain propionate-oxidizing bacteria.

Other genera within the phylum *Firmicutes* were detected in the dry digestion systems, but they did not have obvious correlations with the operating parameters include; *Leuconostoc*, a lactic acid bacterium (Bouanane-Darenfed et al., 2013), *Tepidimicrobium,* reported to contain homoacetogens, and *Ruminococcus,* members of which are fibrolytic (Li et al., 2015a).

5.3.3.2 Bacteroidetes

Like *Firmicutes*, the phylum *Bacteroidetes* includes species active in the hydrolytic and acidogenic steps of anaerobic digestion, where organic matter is hydrolysed and fermented to produce VFAs, CO_2 and H_2 (Regueiro et al., 2012; Traversi et al., 2012). In the digestate inoculum systems, the genus *Bacteroides* predominated (6.7%-42.5% relative abundance), followed by an unclassified genus from the family *Porphyromonadaceae* (1.6%-6.4%) (Figure 5-3). In the sludge inoculum systems, *Bacteroides* (0.1%-16.3%) and three unclassified genera from the family *Porphyromonadaceae* (1.2%-3.2%), family *SB-1* (0.5%-2.4%) and order *Bacteroidales* (1.0%-4.3%), respectively, dominated together (Figure 5-3).

The genus *Bacteroides* had positive correlations with SCOD, total VFA, free VFA, acetate and butyrate, and negative correlations with SMY (Table 5-4 b). This indicates that *Bacteroides* members may function as hydrolysers and fermenters of organic matter, producing various VFAs. In line with this, *Bacteroides cellulosolvens* has been reported to ferment cellulose and cellobiose to produce acetic acid, ethanol, CO_2/H_2 and small amounts of lactic acid (Murray et al., 1984) and other species of *Bacteroides* can degrade starch (Delbès et al., 2000). The only correlations observed for the unclassified genus from *Porphyromonadaceae* were positive correlations with pH and propionate in the digestate inoculum system at the FW/PM ratio of 75:25. It is difficult to draw conclusions from this as the *Porphyromonadaceae* family is a diverse group, comprising 11 different genera (Sakamoto, 2014). However, one member, *Paludibacter propionicigenes,* has been reported to utilize various sugars, producing propionate and acetate as major end products (Ueki et al., 2006).

5.3.3.3 Proteobacteria

The *Alphaproteobacteria* and *Gammaproteobacteria* classes were abundant within the phylum *Proteobacteria*. In the digestate inoculum systems, members of the *Rhodobacteraceae* (1.1%-2.4%) and *Pseudomonadaceae* (0.1%-1.3%) families dominated (Figure 5-3). In the sludge inoculum systems, the relative abundances of these two families were much higher (1.7%-5.5% and 0.6%-10.2%, respectively), followed by the genus *Acinetobacter* from the family *Moraxellaceae* (0.3%-1.7%, Figure 5-3). As shown in Table 5-4 b, the genus *Acinetobacter* and an unclassified genus from the family *Pseudomonadaceae* had positive correlations with SCOD, total VFA, free VFA, acetate and butyrate, indicating the likely hydrolysis and fermentation functions of these bacteria. The functions of these taxa within anaerobic digestion systems have not been clearly reported previously. However, *Pseudomonas putida,* belonging to the family *Pseudomonadaceae,* reduced the COD by 44.4% during the anaerobic treatment of distillery spent wash (Ghosh et al., 2002), and strains within the genus *Acinetobacter* reduced the COD by 44% when treating molasses spent wash anaerobically (Ghosh et al., 2004), however, the products of hydrolysis were not reported. Furthermore, Thangaraj et al. (2007) reported that of 31 *Acinetobacter* isolates assayed, 11 could utilize aromatic compounds and produce acidic intermediates which decreased the pH of the culture medium, but the detailed products were not clear. All of these reports support the possible hydrolysis and fermentation functions of members of the family *Pseudomonadaceae* and the genus *Acinetobacter* in this study, and the positive correlations with VFA, especially acetate and butyrate, indicate the end products.

5.3.3.4 Chloroflexi

The phylum *Chloroflexi*, was mainly detected within the sludge inoculum systems, and was reported to be able to utilize glucose (Ariesyady et al., 2007). In these systems, the candidate genus *T78* predominated (2.1%-8.4%), followed by *SHD-231* (0.3%-1.3%), both of which belong to the family *Anaerolinaceae* (Figure 5-3). These two candidate genera had positive correlations with SCOD, total VFA, free VFA, acetate and butyrate, which was indicative of their possible function in hydrolysis and fermentation in the dry co-digestion systems (Table 5-4 b). The family *Anaerolinaceae* was previously reported to ferment carbohydrate to produce acetate and hydrogen (Rui et al., 2015; Yi et al., 2014). The candidate genus *T78* has the potential to decompose carbohydrates (Li et al., 2015b) and it can degrade long chain petroleum hydrocarbons (Wang et al., 2016c). These traits are in line with the results observed in the current study. However, the detailed function of candidate genus *SHD-231* has not yet been reported. Based on the results of the current study, its members may function as hydrolysers and fermenters of organic matters, producing various VFAs, especially acetate and butyrate.

5.3.3.5 Synergistetes

The phylum *Synergistetes* also dominated in the sludge inoculum systems. An unclassified genus within the family *Thermovirgaceae* was most abundant (3.3%-6.8%), followed by the candidate genus *HA73* (1.0%-2.2%, Figure 5-3). Detailed functions of the genus *HA73* and the family *Thermovirgaceae* have not yet been reported in literature. Both belong to the order *Synergistales*, and most members in *Synergistales* are considered amino acid-degrading, with only members in the genera *Anaerobaculum* and *Thermanaerovibrio* having the ability to utilize sugars (Militon et al., 2015). In this study, positive correlations were found between these two genera and propionate (Table 5-4 b), indicating that they may function as fermenters of organic matters which produce propionate as an end product.

5.3.3.6 Thermotogae

Two genera from the phylum *Thermotogae* predominated within the digesters; the candidate genus *S1* was mainly found in the digestate inoculum systems (0.1%-8.6% relative abundance), and *Kosmotoga* mainly in the sludge inoculum systems (0.8%-4.3%, Figure 5-3). However, the phylum *Thermotogae* is reported to be dominant in thermophilic anaerobic digestion systems (Lin et al., 2016). The existence of *Thermotogae* in this study is again indicative of the occurrence of localised hot spots during the dry co-digestion of FW and PM. Positive correlations were observed between the genus *Kosmotoga* and SCOD, total VFA, acetate and butyrate (Table 5-4 b), indicating probable hydrolysis or fermentation functions. DiPippo et al. (2009) isolated a thermophilic *Kosmotoga* species from an oil field and found it was capable of fermenting carbohydrates, peptides and pyruvate. L'Haridon et al. (2014) reported that *Kosmotoga* can utilize various organic compounds such as casamino acids, glycerol, maltose, pyruvate, ribose, starch, sucrose, tryptone and xylose. This is in agreement with the results observed in this study. The function of the candidate genus *S1* has not previously been reported. In the present study, the candidate genus *S1* was negatively correlated with SCOD, total VFA, free VFA, acetate and butyrate, and positively correlated with SMY (Table 5-4 b), indicating its possible function as a syntrophic oxidiser of acetate and butyrate with the resultant production of CO_2 and H_2 for use as substrates by hydrogenotrophic methanogens.

5.3.3.7 WWE1

The candidate phylum *WWE1* was reported to play a role in either hydrolysis or fermentation in anaerobic digesters (Limam et al., 2014). In this study, *WWE1* was mainly found in the sludge inoculum systems, with its relative abundance in the digestate inoculum systems very low (≤0.3%). Two candidate genera *BHB21* and *W22* dominated, with abundances of 0.3%-2.0% and 0.4%-2.6%, respectively (Figure 5-3). Positive correlations were observed between the candidate genus *W22* and SCOD, total VFA, free VFA, acetate and butyrate (Table 5-4 b), indicating a possible role in hydrolysis and/or fermentation. The functionality of the candidate genus *W22* in anaerobic digesters has not yet been clearly reported in literature.

While correlation analysis between the relative abundances of specific bacterial taxa and physicochemical parameters alone is not sufficient to explore the function of these taxa within anaerobic digesters, the microbial functions predicted by this method in the present study were compared with results reported in the literature and were highly consistent with them. Moreover, the predicted functions of some bacterial taxa whose functionalities were previously poorly described in anaerobic digestion systems are outlined for the first time in the present study. These can provide reference for the investigation of bacteria in anaerobic digesters to some extent. They are summarized in Table 5-5.

Table 5-5 Predicted anaerobic digestion-associated functions of bacterial taxa whose roles within anaerobic digesters have not previously been reported in the literature

Phylum	Class	Order	Family	Genus	Predicted function
Firmicutes	*Clostridia*	*Clostridiales*	*Caldicoprobacteraceae*	*Caldicoprobacter*	Syntrophic oxidation of acetate and butyrate
			EtOH8	*Unclassified*	Syntrophic oxidation of acetate and butyrate
		MBA08	*Unclassified*	*Unclassified*	Syntrophic oxidation of acetate and butyrate
		OPB54	*Unclassified*	*Unclassified*	Syntrophic oxidation of acetate and butyrate
		BSA2B-08	*Unclassified*	*Unclassified*	Syntrophic oxidation of acetate and butyrate
		SHA-98	*Unclassified*	*Unclassified*	Syntrophic oxidation of acetate and butyrate
			D2	*Unclassified*	Syntrophic oxidation of propionate
Proteobacteria	*Gammaproteobacteria*	*Pseudomonadales*	*Moraxellaceae*	*Acinetobacter*	Hydrolysis and fermentation, produce VFA, especially acetate and butyrate
			Pseudomonadaceae	*Unclassified*	Hydrolysis and fermentation, produce VFA, especially acetate and butyrate
Chloroflexi	*Anaerolineae*	*Anaerolineales*	*Anaerolinaceae*	*SHD-231*	Hydrolysis and fermentation, produce VFA, especially acetate and butyrate
Synergistetes	*Synergistia*	*Synergistales*	*Dethiosulfovibrionaceae*	*HA73*	Fermentation, produce propionate
			Thermovirgaceae	*Unclassified*	Fermentation, produce propionate
Thermotogae	*Thermotogae*	*Thermotogales*	*Thermotogaceae*	*S1*	Syntrophic oxidation of acetate and butyrate
WWE1	*Cloacamonae*	*Cloacamonales*	*Cloacamonaceae*	*W22*	Hydrolysis and fermentation, produce VFA, especially acetate and butyrate

In a summary, almost all of the taxa with likely syntrophic oxidation functionality belonged to the phylum *Firmicutes*; however, the distribution of the hydrolysis- and fermentation-associated taxa varied with the different operating conditions (Figure 5-4). In the digestate inoculum systems, the phyla *Firmicutes* and *Bacteroidetes* were the main contributors to hydrolysis and fermentation, while in the sludge inoculum systems many more phyla contributed, including *Firmicutes*, *Bacteroidetes*, *Proteobacteria*, *Chloroflexi*, *Synergistetes*, *Thermotogae* and *WWE1*.

Overall the findings of this study provide possible references for exploring the possible function of bacterial taxa in anaerobic digesters. Further work will be conducted to prove the functionality of the bacteria (i.e. shortgun sequencing) and study the causation between bacterial taxa and physicochemical parameters (i.e. observe the changes of bacteria by adding a specific substrate such as acetate, propionate and butyrate).

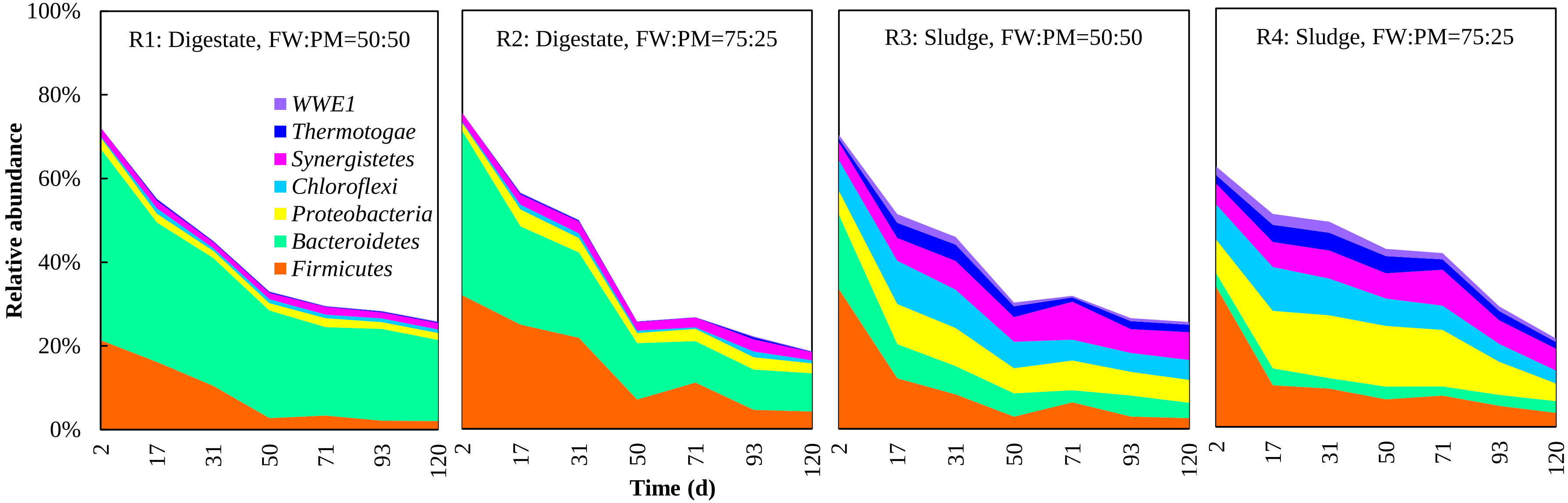


Figure 5-4 Phylum-level relative abundance of the hydrolysis- and fermentation-associated bacteria during dry co-digestion of food waste and pig manure using four different operating conditions

5.3.4 Methanogen composition and correlations with physicochemical parameters

The relative abundances of methanogens under the different operating conditions are shown in Figure 5-5. In the digestate inoculum systems, the genus *Methanoculleus* was dominant, with relative abundances of 5.6% and 10.5% at the end of the experiment at the FW/PM ratios of 50:50 and 75:25, respectively, accounting for 85.3% and 92.6% of the total methanogens. Members of the genus *Methanoculleu*s isolated thus far are hydrogenotrophic methanogens and can utilise H_2/CO_2, formate, 2-propanol/CO_2, 2-butanol/CO_2 and cyclopentanol/CO_2 but not acetate as substrates for methanogenesis (Pap et al., 2015; Wang et al., 2015). Significant positive correlations were established between the relative abundance of *Methanoculleu*s and SMY in the digestate inoculum systems (Figure 5-6, $P<0.01$). This indicates that *Methanoculleu*s was the main contributor to methane production in these systems, which is in line with previous reports that *Methanoculleu*s was the active methanogen in swine manure storage tanks (Barret et al., 2013).

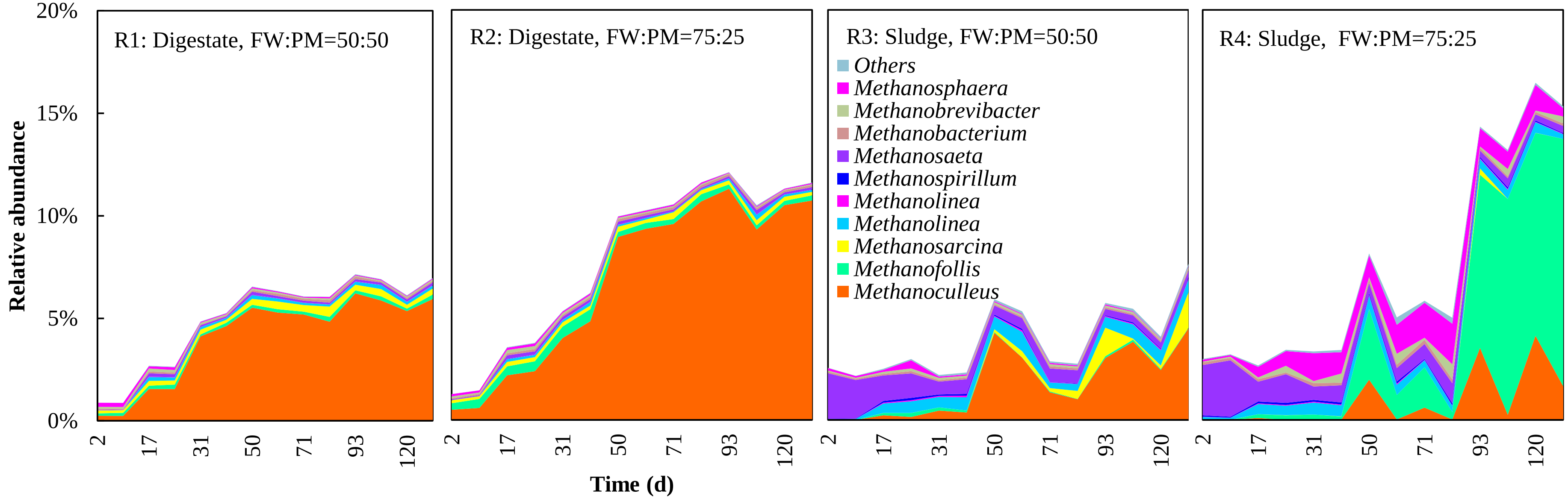


Figure 5-5 Relative abundances of methanogens at the genus level during dry co-digestion of food waste and pig manure using four different operating conditions

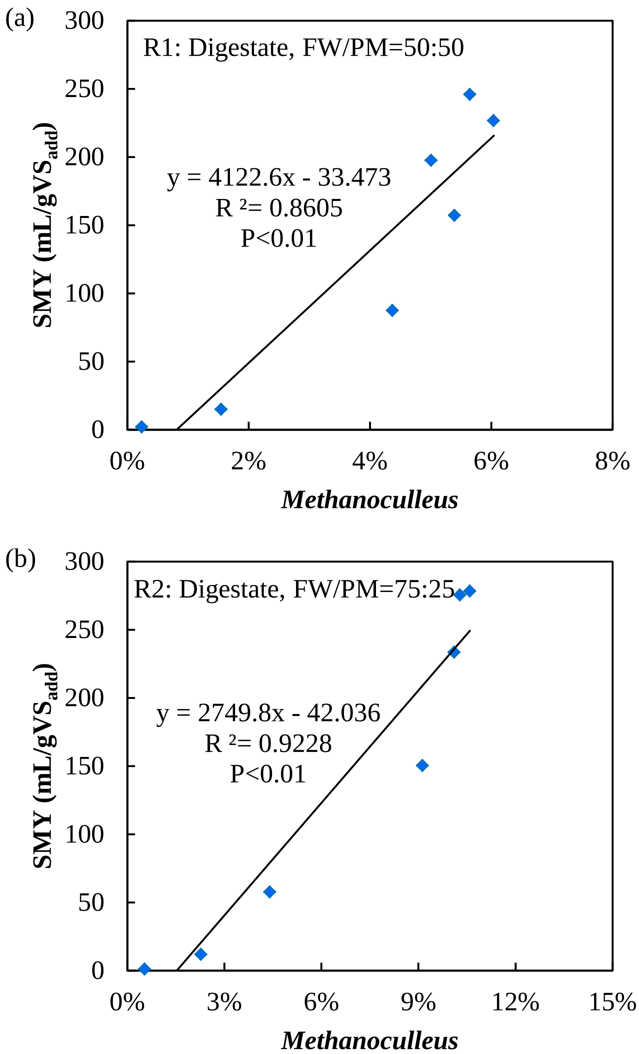


Figure 5-6 Correlation between the mean relative abundance of Methanoculleus and the mean specific methane yield in R1 (a) and R2 (b)

In the sludge inoculum systems, the methanogen composition was much more diverse and their relative abundance fluctuated more compared with those in the digestate systems, indicating the instability of the sludge inoculum systems. The genus *Methanosaeta* dominated at the beginning of the experiment, accounting for 82.3%-87.2% of the total methanogens, and the proportions decreased to < 5% at the end of the experiment. The relative abundances of *Methanoculleus* and *Methanofollis* increased as that of *Methanosaeta* decreased. At the FW/PM ratio of 50:50, *Methanoculleus* was abundant at the end of the experiment, accounting for 53.0%-70.3% of the total methanogens present in phase III. At a FW/PM ratio of 75:25, *Methanofollis* and *Methanoculleus* dominated in phase III, accounting for 59.0%-79.9% and 10.8%-25.0% of all methanogens, respectively.

Methanosaeta is an acetoclastic methanogen which uses only acetate as a substrate for methane production (Guo et al., 2015). It dominates only at a low acetate concentration and is highly sensitive to changes in environmental conditions (Zheng & Raskin, 2000). Similar to *Methanoculleus*, *Methanofollis* is also a hydrogenotrophic methanogen, which can utilize H_2/CO_2, formate, methanol, ethanol, 1-propanol, 1-butanol, and trimethylamine but not acetate for growth and methane production (Imachi et al., 2009; Wu et al., 2005). Hydrogenotrophic methanogens are reported more resistant to stress factors compared with acetoclastic methanogens. Calli et al. (2005) found that *Methanosaeta* was substituted by *Methanosarcina* as TAN increased from 1.0 g/L to 2.5 g/L. Ziganshin et al. (2011) reported that *Methanosaeta* prevailed at low OLRs and were outcompeted by *Methanosarcina* at high acetate concentrations and then dominated by *Methanoculleus* with even higher propionate and acetate accumulations. The high VFA (up to 51.3 g/L) and TAN (up to 7.2 g/L) concentrations found in dry co-digestion systems select for more robust hydrogenotrophic methanogens. The substitution of *Methanosaeta* by *Methanoculleus* and *Methanofollis* in the present study is in agreement with this. The negative correlation between *Methanosaeta* and SMY in the sludge inoculum systems can be explained by the inhibition of *Methanosaeta*, while the positive correlations between *Methanoculleus/Methanofollis* and SMY indicated their major contribution to methane production (Table 5-4 b).

In summary, hydrogenotrophic methanogenesis conducted by *Methanoculleus* and *Methanofollis* was the dominant pathway for methane production in the dry co-digestion systems, with the acetoclastic pathway being inhibited. This result is supported by the observation of syntrophic oxidation bacteria, as discussed in Section 5.3.3.1.

5.4 Summary

The research shows that the biological factor (inoculum) was more significant in determining the microbial community structure than the environmental factor (FW/PM ratio). In the digestate inoculum systems, the phyla *Firmicutes* and *Bacteroidetes* were the main contributors to hydrolysis and fermentation, while in the sludge inoculum systems, *Firmicutes*, *Bacteroidetes*, *Proteobacteria*, *Chloroflexi*, *Synergistetes*, *Thermotogae* and *WWE1* all contributed. *Methanoculleus* was the most abundant methanogen in both the digestate and sludge inoculum reactors, while *Methanofollis* predominated in the sludge inoculum and high FW/PM ratio reactors. Correlation analysis between the relative abundance of specific bacterial/archeal taxa and physicochemical parameters during anaerobic digestion proved a simple and effective tool for the exploration of possible microbial functions. In this way the anaerobic digestion-associated functionalities of some bacterial taxa were predicted for the first time.

Chapter 6
Inactivation of enteric indicator bacteria during dry co-digestion of food waste and pig manure

6.1 Introduction

In this chapter, dry co-digestion of FW and PM was conducted to assess: (1) the effects of different inocula (digestate and sludge) and FW/PM ratios (50:50 and 75:25 based on VS) on the inactivation of total coliforms, *E. coli* and enterococci; and (2) the efficiencies and possible mechanisms of their inactivation.

6.2 Materials and methods

6.2.1 Enumeration of enteric indicator bacteria

About 1 g samples were taken weekly from two of the four duplicate digesters under each condition as described in Chapter 4, which is denoted as Experiment 1. The enteric indicator bacteria, including total coliforms, *E. coli* and enterococci, were analysed. As the rapid inactivation of total coliforms and *E. coli* in the dry digestion systems was unexpected, an additional Experiment 2 was conducted to confirm the finding and to obtain detailed inactivation data. Experiment 2 was conducted in quadruplicate for 31 days. As no enough digestate left in the lab, dewatered anaerobic sludge was used as the inoculum at a rate of 50% and the FW/PM ratio was 50:50 based on VS; this also would help to elucidat the impacts of high VFAs on the inactivaiton efficiency. Samples were taken from four digesters three times in the first week and then twice a week for analysis of pH, VFAs, TAN and enteric indicator bacteria.

For enumeration of total coliforms, *E. coli* and enterococci, 9 parts (w/w) of buffered peptone water (BPW, Oxoid, Hampshire, UK) was added to 1 part of digestate sample (~1 g) in order to obtain a 1 in 10 dilution. From this, 10-fold serial dilutions (up to 10^{-9}) were performed in maximum recovery diluent (MRD; Oxoid). For the enumeration of total coliforms and *E. coli*, the 10^{-1} to 10^{-9} dilutions were pour-plated in duplicate onto McConkey agar (Sigma-Aldrich, St. Louis, MO, USA) and ChromoCult tryptone bile X-glucuronide (CTBX) agar (VWR, Dublin, Ireland), respectively, and incubated at 37 ℃ for 24 hr. Enterococci was enumerated by pouring plating in duplicate onto Kanamycin azide aesculin (KAA) agar (Oxoid) and incubated at 45 ℃ for 24 hr. The plates with between 30 and 300 colonies were counted after incubation, and the counts were averaged and presented as CFU/g of the original sample. Counts were considered below the LOD when the number of colonies on the 10^{-1} dilution plates were < 10, i.e. <100 CFU/g.

6.2.2 Models and calculations

The inactivation kinetics of the enteric indicator bacteria are explained using a modified Gompertz model in Eq. 6-1 (Linton et al., 1995):

$$\log\frac{N}{N_0} = Se^{-e^{BM}} - Se^{-e^{-B(t-M)}} \qquad 6\text{-}1$$

where, N is the number of surviving bacteria at time t in CFU/g; N_0 is the initial number of pathogens in CFU/g; S is the difference between the upper and lower asymptote values, i.e., between N_0 and N_{final} in $\log_{10}$ CFU/g; M is the time required to reach the maximal absolute inactivation rate, d; B is the relative inactivation rate at time M, (d^{-1}). The maximum inactivation rate μ_{max} ($\log_{10}$ CFU/d) is described as follows:

$$\mu_{max} = \frac{BS}{e} \qquad 6\text{-}2$$

The GRG non-linear method in the Solver function of Microsoft Excel was used to simulate the kinetic parameters. The sum of square error (SSE) and R^2 were used to evaluate the goodness of fit of the model.

6.2.3 Statistical analysis

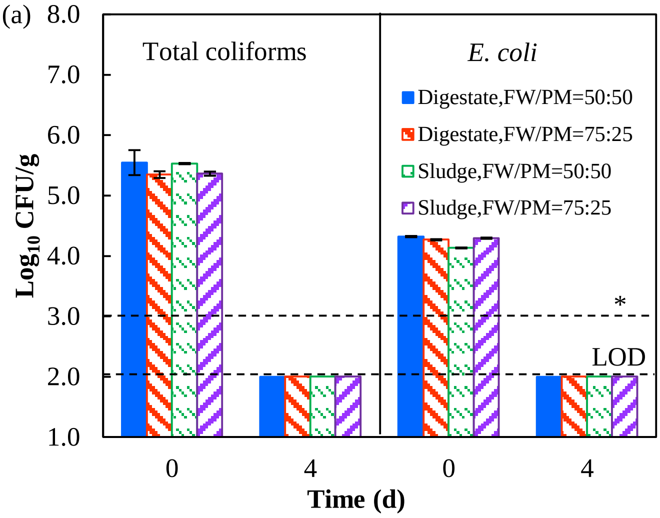
Statistical analysis was conducted using SPSS 22.0 (IBM, Rochester, MN, USA). Multiple linear regression analysis was performed to determine the effects of independent variables (digestion time, pH, total VFAs, free VFAs, TAN and FAN) on enteric indicator bacteria. To avoid co-linearity among the variables, the stepwise method was used. At each iteration, the most significant independent variable (P<0.05) was included in the model and the selections stopped when no more variables were significant.

6.3 Results and discussion

6.3.1 Inactivation of pathogens

Endogenous *E. coli*, total coliforms and enterococci were used as indicator bacteria in order to assess the inactivation of pathogens during dry co-digestion of FW and PM. As shown in Figure 6-1, *E. coli* and total coliform counts declined rapidly to below the limit specified in digestate standard (1000 CFU/g), and in fact to below LOD (100 CFU/g), within 4 days in all reactors. The $\log_{10}$ reductions were 2.1-2.3 and 3.4-3.6 for *E. coli* and total coliforms, respectively (Figure 6-1 a). Enterococci were more resistant compared with *E. coli* and total coliforms and took much longer to be inactivated in all of the reactors. In the digestate inoculum systems, counts decreased below both the limit of the digestate standard and the LOD within 12 days, irrespective of the substrate ratio used. In comparison, when sludge was used as the inoculum, it took 22 days for *Enterococcus* counts to decrease below the limit of digestate standard in both substrate ratios, and took 31 and 26 days to decrease below the LOD at the FW/PM ratios of 50:50 and 75:25, respectively (Figure 6-1 b). The reductions in *Enterococcus* counts ranged from $\log_{10}$ 2.8 to 3.2, with the digestate inoculum systems more effective in terms of inactivation compared with the sludge inoculum systems.

(a)



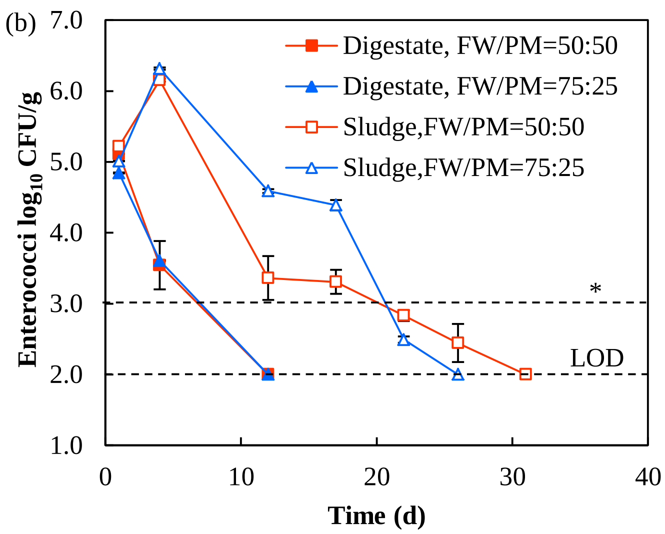


Figure 6-1: Inactivation of enteric indicator bacteria over time during dry co-digestion of food waste and pig manure. Values are the mean of data from two replicate reactors. The limit of detection (LOD) was 100 CFU/g (values below the limit of detection were recorded as $\log_{10}$ 2.0 CFU/g). * = limit for *E. coli* and *Enterococcus* in anaerobic digestate according to digestate standard.

As the rapid inactivation of total coliforms and *E. coli* in the dry co-digestion systems was unexpected, an additional dry co-digestion experiment was conducted to confirm this finding and to obtain detailed inactivation data. The dewatered anaerobic sludge was used as the inoculum (50% based on VS) and the FW/PM ratio was 50:50. The initial numbers of *E. coli*, total coliforms and enterococci were $\log_{10}$ 8.9, 9.0 and 9.2 CFU/g, respectively, much higher than the counts of $\log_{10}$ 4.8-5.5 CFU/g in the first experiment (Figure 6-1). This was due to the variability of new FW, PM and sludge used. As shown in Figure 6-2, *E. coli* and total coliform counts decreased below the limit of digestate standard and the LOD within 7 days, with $\log_{10}$ reductions of 7.2 and 7.0, respectively. The longer inactivation time compared to that observed in the previous experiment was most likely due to the higher initial counts. *Enterococcus* counts decreased below the limit set out in the digestate standard within 24 days and to below the LOD within 31 days, which was in line with the results obtained in the first experiment, although the $\log_{10}$ reduction was higher at 6.7 due to the higher initial count.

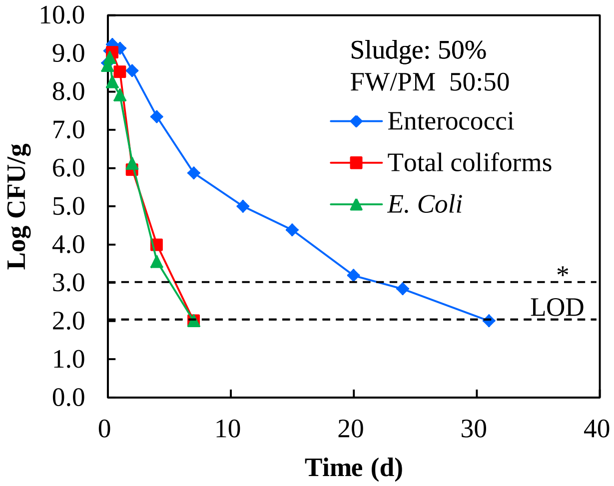


Figure 6-2: Inactivation of enteric indicator bacteria over time during dry co-digestion of food waste and pig manure. Values are the mean of data from four replicate reactors. The limit of detection (LOD) was 100 CFU/g (values below the limit of detection were recorded as $\log_{10}$ 2.0 CFU/g). * = limit for *E. coli* and *Enterococcus* in anaerobic digestate according to digestate standard.

6.3.2 Inactivation mechanisms

The inactivation of pathogens in anaerobic digestion systems can be affected by many factors, including bacterial characteristics, microbial competition, nutrient availability, temperature, VFAs, ammonia, pH and digestion time (Kunte et al., 1998; Pecson et al., 2007; Smith et al., 2005). A multiple linear regression analysis (stepwise method) was performed to determine the effects of operation factors (including digestion time, pH, total VFAs, free VFAs, TAN and FAN) on the inactivation of enteric indicator bacteria in the present study. The results are shown in Table 6-1.

Table 6-1 Summary of multiple linear regression analysis (stepwise method) on inactivation of enteric indicator bacteria during dry co-digestion of food waste and pig manure

Dependent variable	Constant	Coefficient [a]						R^2
		Digestion time (d)	pH	Total VFA (mg/L)	Free VFA (mg/L)	TAN (mg/L)	FAN (mg/L)	
Experiment 1								
Sludge, FW/PM=50:50								
Enterococci	5.685	-0.126						0.932
Sludge, FW/PM=75:25								
Enterococci	7.033	-0.086			-0.006			0.932
Experiment 2								
Sludge, FW/PM=50:50								
Enterococci	12.886	-0.165						0.929
Total coliforms	9.422				-0.184			0.975
E. coli	8.994				-0.174			0.961

[a] Only the coefficients for significant factors ($P < 0.05$) are shown in the table.

In Experiment 1, due to the unexpectedly rapid inactivation of *E. coli* and total coliforms in all of the reactors and enterococci in the reactors inoculated with digestate, only the inactivation of enterococci in the sludge inoculum systems was analysed. The negative correlations showed that at the FW/PM ratio of 50:50, digestion time was the only significant inactivation factor for enterococci, with each additional day associated with a 0.126 $\log_{10}$ CFU/g reduction in the count. It explained 93.2% of the variation in enterococci counts. On the other hand, at a FW/PM ratio of 75:25, digestion time and free VFAs were both significant in the model, with inactivation rates of 0.086 $\log_{10}$ CFU/g per day and 0.006 $\log_{10}$ CFU/g per mg/L free VFAs. The digestion time was the most significant factor, explaining 79.5% of the variation. When the factor free VFAs was added in the model, 93.2% of the variation in *Enterococcus* counts could be explained by the effect of digestion time and free VFAs. A high FW proportion accelerated hydrolysis and acidification of the substrates, resulting in a pH reduction and a rapid accumulation of free VFAs, thereby leading to a more rapid inactivation of enterococci compared with lower FW/PM ratio systems.

In Experiment 2, the digestion time was the only significant factor regarding the inactivation of enterococci, which was in line with the results obtained in Experiment 1. It explained 92.9% of the variation in *Enterococcus* counts, with each additional day associated with a

0.165 log_{10} CFU/g reduction. The higher inactivation rate compared with Experiment 1 is explained by the higher initial *Enterococcus* counts. Free VFAs was the only significant factor regarding the inactivation of total coliforms and *E. coli*, with each mg/L associated with a 0.184 and 0.174 log_{10} CFU/g reduction in counts, respectively. It accounted for 97.5% and 96.1% of the variation in *E. coli* and total coliforms, respectively.

Enterococci are Gram-positive bacteria. The thick, multi-layered peptidoglycan and the presence of teichoic acids in the cell wall can act as a permeability barrier to prevent the passage of toxic factors from the environment into the cell (Dijkstra & Keck, 1996). Enterococci are known to be resistant to various stresses and hostile environmental conditions, such as extremes of temperatures (65 ^{o}C), pH (4.5-10.0) and high NaCl concentrations (6.5%) (Da Costa et al., 2013; Fisher & Phillips, 2009). This most likely explains their long survival time in the digesters. The decrease in enterococci with increasing digestion time may be caused by biological factors, such as competition with other bacteria and the depletion of nutrients or trace elements over time. The enterococci were inactivated much faster in the digestate inoculum systems, perhaps because the well-adapted microbial community out-competed the enterococci more effectively than in the sludge inoculum systems.

Apart from digestion time in the digesters, the only other factor that significantly impacted *Enterococcus* counts was free VFA concentrations. Compared with ionized VFA anions, free VFAs are usually considered more toxic to pathogens (Puchajda & Oleszkiewicz, 2006). They are lipophilic and can pass through the cell membrane and dissociate in the cell. The dissociated H^+ can acidify the cytoplasm, so the cell needs to export H^+ via a proton ATPase pump mechanism, which is energy demanding and may result in energy depletion (Puchajda & Oleszkiewicz, 2006). If H^+ continues to accumulate and reduce the intracellular pH to below the minimum value at which growth can occur, growth of the cell ceases (Russell & Diez-Gonzalez, 1997). In contrast to enterococci, total coliforms, including *E. coli*, are Gram-negative bacteria. Gram-negative bacteria have a much thinner cell wall, containing only a monolayer of peptidoglycan and a lipid-rich outer membrane (Beveridge, 1999). This thinner cell wall offers little protection as a permeability barrier, enabling free VFAs to easily pass through the cell membrane. This likely resulted in the earlier inactivation of *E. coli* and total coliforms in comparison to enterococci. It would also help to explain the greater impact of free VFAs on these indicator bacteria compared to enterococci.

The variation of free VFAs in the first 31 days was shown in Figure 6-3. Free VFAs had significant negative effect on enterococci only in sludge inoculum at the FW/PM ratio of 75:25, where the free VFA concentrations reached as high as 895 mg/L. It was likely too high for the enterococci defence mechanism, thereby contributing to its inactivation. Free VFA concentrations were much lower at the FW/PM ratio of 50:50 compared with those at 75:25. In experiment 1, when *E. coli* and total coliforms decreased below the LOD on Day 4, the free VFAs were 35 mg/L and 88 mg/L in digestate and sludge inoculum systems, respectively. And in experiment 2, the free VFA concentrations were 42 mg/L on day 7 when *E. coli* and total coliforms were below the LOD. Therefore the free VFA concentrations of 35-88 mg/L might be enough for inactivation of *E. coli* and total coliforms. Watcharasukarn et al. (2009) found *E. coli* decreased from 6×10^4 CFU/mL to below LOD within 5 days, the observed free VFA concentrations of 56-78 mg/L were considered to be responsible for this, which corresponded to this study. The different inactivation concentrations of free VFAs on *E. coli*, total coliforms and enterococci indicated their different resistance to toxic environment.

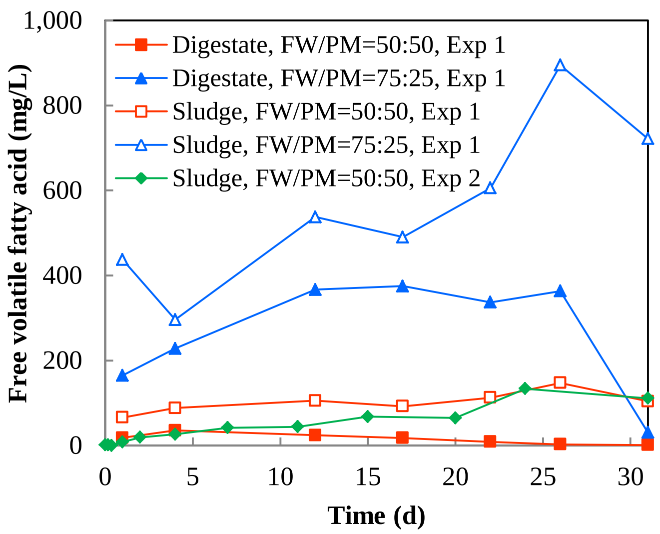


Figure 6-3: Variation of free volatile fatty acid during dry co-digestion of food waste and pig manure

6.3.3 Comparison with wet digestion

The inactivation of enteric indicator bacteria during dry co-digestion of FW and PM was compared with that in the more commonly used wet mesophilic digestion systems. As shown in Table 6-2, the results from the literature varied greatly. For *E. coli*, Pandey and Soupir (2011) reported a 6 $\log_{10}$ reduction in 41 days during wet digestion of dairy manure. Manyi-Loh et al. (2014) found that it took 62 days to decrease *E. coli* from 3.6×10^5 CFU/g to below the LOD in cattle manure wet digestion systems. On the other hand, Kearney et al. (1994) reported a 4 $\log_{10}$ reduction in 12 days in a cattle manure wet digestion system, but the bacteria were still detectable (at 10^3 CFU/mL) at the end of this period. The inactivation mechanism was not clearly described in these papers, with VFA and ammonia concentrations unknown (Table 6-2). In the present dry digestion study, it took only 4 - 7 days for *E. coli* counts to decrease below LOD and a > 7 $\log_{10}$ reduction was achieved, which was much more effective compared with the results reported for wet digestion.

For total coliforms, Varel et al. (2012) reported that it took 3 days and 14 days for the counts to decrease from 10^5-10^6 CFU/g to below the LOD in anaerobic digestion of swine manure and cow manure, respectively. The result for swine manure is comparable with the present study (4 days to decrease below the LOD from 10^4 - 10^6 CFU/g in Experiment 1). The total VFA concentrations in swine and cow manures were estimated to be about 760 - 2,260 mmol/kg DM and 0-1,120 mmol/kg DM, corresponding to about 7,410 - 22,510 mg/L and 0 - 5,100 mg/L, respectively. The maximum free VFA concentrations were calculated to be about 585 mg/L and 20 mg/L, respectively. It supported the results obtained in this study that free VFAs were main inactivation factors for total coliforms.

For enterococci, Watcharasukarn et al. (2009) reported a 3.13 $\log_{10}$ reduction in 15 days during wet digestion of cow manure. Free VFAs and exposure time were considered as the possible inactivation reasons, which were in line with this study. Lund et al. (1996) found a 4 $\log_{10}$ reduction in 12.5 days during co-digestion of pig and cattle slurry, with the inactivation factors not clear. In the present study, the 3 and 4 $\log_{10}$ reductions were observed within 7 and 11 days, respectively, which were shorter than those reported in the literature. Ottoson et al. (2008) observed a 5 $\log_{10}$ reduction in 4.2 days and 19 days in high FAN concentration (equivalent to 644 mg/L) and control (equivalent to 22.4 mg/L) systems. However, the even higher FAN concentration of 998 mg/L was observed in Experiment 2 in this study, and a 5 $\log_{10}$ reduction was not obtained until about day 18. It indicated that FAN should not be the only inactivation factor for enterococci, the different substrates and competitive bacteria in the system might be responsible for the inactivation.

Table 6-2 Data on inactivation of enteric indicator bacteria from previous studies on wet mesophilic anaerobic digestion of livestock manure and municipal solid waste in comparison to data from dry mesophilic co-digestion of food waste and pig manure from this study

Reference	Indicator	Feedstock	TS [a] (%)	T (^{o}C)	pH	TAN [b] (mg/L)	FAN [c] (mg/L)	Total VFA [d] (mg/L)	Free VFA (mg/L)	Initial count (CFU/g or mL)	Final result	T_c [e] (d)	DRT [f] (d)	Scale
(Pandey & Soupir, 2011)	*E. coli*	Dairy manure	2.05	37	6.5-7.4	-	-	-	-	1.85×10^7	6 $\log_{10}$ reduction	41	7-8	Lab, batch
(Kearney et al., 1994)	*E. coli*	Cattle slurry	5-10	37	7.0-7.4	-	-	-	-	10^7	10^3 CFU/mL	12	1.2	Lab, HRT=25d
(Manyi-Loh et al., 2014)	*E. coli*	Cattle manure	-	37	6.5-7.0	-	-	-	-	3.6×10^5	Below LOD [g]	62	-	8 m^3
(Watcharasukarn et al., 2009)	*E. coli*	Cow Manure	6.6	35	6.8-7.5	1,590-1,670	12.7-63.6	5,022-8,712	11-78	6×10^4	Below LOD	5	0.92	Lab, batch
	Enterococcus faecalis									6.6×10^5	3.13 $\log_{10}$ reduction	15	1.15	
(Olsen & Larsen, 1987)	*E. coli* Coliforms	Pig & cattle slurry	0.5-12	35	7.0-8.0	Adding 0, 2, 5 g NH_3-N/kg slurry	-	-	-	10^6	-	-	1.4-2.4 / 0.9-3.7	Lab, batch/ continuous
(Varel et al., 2012)	Coliforms	Swine manure	12.5-13.5	38	6.1-6.4	10-30 g/kg DM (1,300-3,900 mg/L)	-	760-2,260 mmol/kg DM (7,410-22,510 mg/L)	<585	10^5-10^6	Below LOD	3	-	Lab, batch
		Cattle manure	6.0-6.5		6.4-7.8	5-15 g/kg DM (312-938 mg/L)	-	0-1,120 mmol/kg DM (0-5,100 mg/L)	<20			14	-	
(Lund et al., 1996)	*Faecal enterococci*	Pig & cow manure	-	35	-	-	-	-	-	-	4 $\log_{10}$ reduction	12.5	-	Lab, batch

Reference	Indicator	Feedstock	TS [a] (%)	T (°C)	pH	TAN [b] (mg/L)	FAN [c] (mg/L)	Total VFA [d] (mg/L)	Free VFA (mg/L)	Initial count (CFU/g or mL)	Final result	T_c [e] (d)	DRT [f] (d)	Scale
(Ottoson et al., 2008)	*Enterococcus faecalis*	SS-OFMSW+egg albumin powder	-	37	7.9	6,900	46 mmol/L (644 mg/L)	30,000	<20	10^6–10^8	5 $\log_{10}$ reduction	4.2	0.8	Lab, HRT=30d
		SS-OFMSW [h]			7.3	900	1.6 mmol/L (22.4 mg/L)	100	~0		5 $\log_{10}$ reduction	19	3.7	
This study (Experiment1)	*E. coli*	Food waste & pig manure	20	37	6.0-8.8	3,880-7,261	5-3,317	0-48,809	0-895	10^4-10^5	Below LOD	4	-	Lab, batch
	Total coliforms									10^5-10^6	Below LOD	4	-	
	Enterococci									10^4-10^5	Below LOD	12-31	-	
This study (Experiment 2)	*E. coli*	Food waste & pig manure	20	37	7.0-8.9	1,576-4,689	96-998	0-31,033	0-134	6.20×10^8	Below LOD	7	1.1	Lab, batch
	Total coliforms									1.56×10^9	Below LOD	7	1.0	
	Enterococci									6.10×10^8	Below LOD	31	2.4	

[a] TS: Total solids

[b] TAN: Total ammonia nitrogen

[c] FAN: Free ammonia nitrogen

[d] VFA: Volatile fatty acid

[e] T_c: Time required to reach final result

[f] DRT: Decimal reduction time

[g] LOD: Limit of detection

[h] SS-OFMSW: source-sorted organic fraction of municipal solid waste

The inactivation kinetics of the enteric indicator bacteria were simulated using a modified Gompertz model (Eq. 6-1), with the results shown in Figure 6-4 and Table 6-3. The lower SSE (≤ 0.20) and higher R^2 (≥ 0.9918) indicated that the modified Gompertz model can simulate the inactivation kinetics well. The μ_{max} values for total coliforms and *E. coli* (1.65 and 1.67 $\log_{10}$ CFU/g d^{-1}) were much higher than that of enterococci (0.43 $\log_{10}$ CFU/g d^{-1}), and the DRT for total coliforms and *E. coli* (1.0 and 1.1 days) were shorter than that of enterococci (2.4 d). This again indicates the greater resistance of enterococci to environmental conditions compared to coliforms.

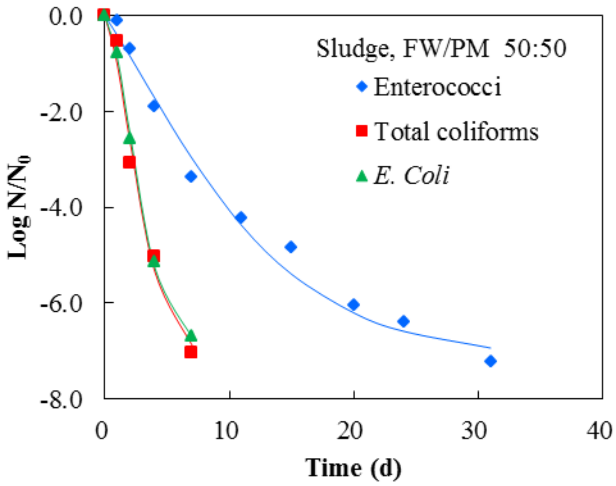


Figure 6-4: Simulation of pathogen inactivation during dry digestion of food waste and pig manure over time. The scatter plots are the experimental data, the solid lines are the responses of the model under the obtained parameters.)

Table 6-3 Summary of kinetic parameters according to Eq. 6-1 & 6-2

	Enterococci	Total coliforms	*E. coli*
S [a] (log_{10} CFU/g)	9.2	7.6	7.2
B [b] (d^{-1})	0.13	0.59	0.64
M [c] (d)	3.2	1.9	2.0
μ_{max} [d] (log_{10} CFU/g d^{-1})	0.44	1.65	1.67
DRT [e] (d)	2.4	1.0	1.1
SSE [f]	0.10	0.20	0.01
R^2 [g]	0.9947	0.9918	0.9995

[a] S: The difference between the upper and lower asymptote values, log_{10} CFU/g.

[b] B: The relative inactivation rate at time M, d^{-1}.

[c] M: The time required to reach the maximal absolute inactivation rate, d.

[d] μ_{max}: The maximum inactivation rate, log_{10} CFU/d.

[e] DRT: Decimal reduction time.

[f] SSE: Sum of square errors.

[g] R^2: The square of Pearson correlation coefficient.

Olsen and Larsen (1987) studied the DRT of different bacteria in anaerobic digestion of animal slurries, the results showed that the DRT was only significantly correlated with bacterial species and temperature (35^{o}C and 53 ^{o}C), but not influenced by slurry type (pig or cattle), operation process (batch or continuous), pH (7.0 or 8.0), TS content (0.5% - 12%) and concentration of NH_3-N added (0, 2 and 5 g/kg slurry). The DRT of 1.1 days for *E. coli* in this study was comparable to the 0.92 and 1.2 days observed by Watcharasukarn et al. (2009) and Kearney et al. (1994), but much shorter than the DRT of 7 - 8 days and 1.4 - 2.4 days reported by Pandey and Soupir (2011) and Olsen and Larsen (1987). The DRT of 1.0 day for coliform in this study lies in the range of 0.9 - 3.7 days reported by Olsen and Larsen (1987), but was in the lower level. The difference may be because some parameters in this study, e.g. pH of 8.8 and TS content of 20%, beyond Olsen and Larsen's (1987) research range. Besides, the effect of free VFAs was not discussed in Olsen & Larsen's (1987) study, which might be the main contributor to the inactivation of *E. coli* and total coliforms. The enterococci DRT of 2.4 days was longer than the 1.15 days reported by Watcharasukarn et al. (2009), and between the 0.8 - 3.7 days reported by Ottoson et al. (2008). It means the inactivation of enterococci varied in different anaerobic digestion systems and should be discussed according to the specific circumstances.

Overall, dry co-digestion of FW and PM can inactivate enteric indicator bacteria effectively. The inactivation of *E. coli* and total coliforms in dry co-digestion systems was more efficient than wet digestion because of free VFAs accumulation. The enterococci inactivation is more likely caused by biological factors and should be discussed in accordance with specific conditions.

The inactivation of enteric indicator bacteria asked for high free VFAs (35 - 88 mg/L), while the total VFAs need to be controlled below 20 g/L to prevent inhibition on methane production. An optimization and balance must be achieved during dry co-digestion of FW and PM to realize preferable methane production and digestate biosafety simultaneously. On the one hand, pH might be adjusted during operation to maintain proper total VFA and free VFA concentrations; and on the other hand, trace elements might be added to prevent the over accumulation of VFAs to get a stable and balanced dry co-digestion environment (Zhang et al., 2011a). Further studies will be conducted to investigate the optimization of FW and PM dry co-digestion.

6.4 Summary

The results showed that digestate inoculum systems were more effective with regard to predicted pathogen inactivation than the sludge inoculum systems. Under the preferable operating conditions (digestate as the inoculum and a FW/PM ratio of 50:50), *E. coli*, total coliforms and enterococci were decreased to below the detection limit within 4, 4 and 12 days, respectively, and counts in the digestate were within the limits required by the relevant standard. Free VFAs was the significant inactivation factor for total coliforms and *E. coli*. Digestion time was the most significant factor affecting the inactivation of enterococci, while free VFAs played a secondary role in the digesters with a high FW/PM ratio.

Chapter 7

Effects of pH, volatile fatty acid and ammonia on inactivation of *Salmonella* during dry co-digestion of food waste and pig manure

7.1 Introduction

The research objectives of this chapter were to (1) study the inactivation of *Salmonella* in dry co-digestion of FW and PM; and (2) study the main factors for *Salmonella* inactivation, including *Salmonella* serotype, pH, VFA type, VFA and ammonia concentrations.

7.2 Materials and Methods

7.2.1 Preparation of Salmonella strains

Three *Salmonella* serotypes were selected for use in this experiment as they are the ones most commonly found in PM in Ireland (Burns et al., 2016; McCarthy et al., 2013). They were *Salmonella Derby* (WIT 413), *Salmonella Typhimurium* DT104 (WIT 384) and a monophasic variant of *Salmonella Typhimurium* 4,[5],12:i:- (Ashtown No 2040). The preparation, spiking and analysis of *Salmonella* were described in detail by McCarthy et al. (2015). Briefly, each *Salmonella* serotype was streaked from -80 ^{o}C stock solution onto nutrient agar (Oxoid, Hampshire, UK) and incubated overnight at 37 ℃. A single colony from each culture plate was inoculated into separate 5 ml brain heart infusion (BHI) broth (Oxoid, Hampshire, UK) and incubated overnight at 37 ℃. The *Salmonella* broth cultures were centrifuged at 18,620 × g for 2 minutes, and the cell pellets were washed twice and then re-suspended in MRD (Oxoid, Hampshire, UK). The concentration of each *Salmonella* culture in CFU/mL was calculated using the standard curves of optical density at 600 nm (OD_{600}) versus CFU/mL (Appendix B).

7.2.2 Minimum inhibitory concentration experiment

The MIC experiment was conducted with 96-well microtiter plates (Sarstedt, Hildesheim, Germany) according to the standard protocol described by Wiegand et al. (2008). The antimicrobial activities of VFA, ammonia and pH against each *Salmonella* serotypes were assessed. The tested VFA included acetic acid (HAC), propionic acid (HPR), butyric acid (HB), isobutyric acid (HIB), valeric acid (HV) and isovaleric acid (HIV) and the equimolar mixture of the above individual VFA (MVFA) (Sigma-Aldrich, St. Louis, MO, USA). The ammonia was diluted from 35% ammonia solution (Fisher Scientific, Ballycoolen, Dublin, Ireland). The stock solutions of VFA and ammonia were prepared at 100 - 1,000 mmol/L (with a step of 100 mmol/L) using sterile distilled water and were adjusted to the pH of 6.0, 7.0, 8.0 and 9.0 with 2 mol/L HCl or NaOH controlled with a pH meter (pH 3210, WTW, Weilheim, Germany). The stock solutions were then filter-sterilised through 0.45 μm cellulose nitrate membrane filter paper (Whatman, Maidstone, UK) and stored at 4 ^{o}C until use.

The BHI broth was adjusted to pH of 6.0, 7.0, 8.0 and 9.0 using the same method. The pH probe was sterilized with 70% ethanol solution and Rely + On Virkon disinfectant (VWR, Lutterworth, Leicestershire, UK) and rinsed thoroughly with sterile distilled water between samples. Each *Salmonella* culture was diluted using BHI broth to obtain a suspension of about 1×10^6 CFU/mL according to the concentration calculated from the standard curves, and it was used within 30 mins. The sample wells contained 100 μL *Salmonella* suspension and 100 μL VFA or ammonia solution, resulting in a *Salmonella* concentration of about 5×10^5 CFU/mL and the VFA or ammonia concentrations of 50 - 500 mmol/L (in increments of 50 mmol/L). These VFA or ammonia concentrations were selected based on the maximum VFA or ammonia concentration observed in previous FW/PM dry co-digestion systems (Jiang et al., 2018). Three control wells were maintained for each test: sterility control (SC; 200 μL BHI broth), growth control (GC; 100 μL *Salmonella* suspension and 100 μL BHI broth) and antimicrobial agent control (AC; 100 μL VFA/ammonia solution and 100 μL BHI broth). As a result, totally 960 conditions (3 *Salmonella* serotypes $\times$ 8 VFA or ammonia solutions $\times$ 10 concentrations ×4 pHs) were assessed. All the samples and controls were pipetted to the mix wells and conducted in triplicate. The OD_{600} of each well was recorded at time 0 h using EL × 808 Ultra Microplate Reader (Bio Tek, Bad Friedrichshall, Germany), and then the microtiter plates were covered with lids and incubated at 37 ^{o}C. The OD_{600} of each well was read hourly in the first 7 hours and then the last value was read after 20 hours the next day. At each time point, the triplicate readings for each well were averaged.

The MIC is defined as the lowest concentration of the antimicrobial agent that visibly inhibits the growth of the bacteria as observed with the unaided eye (Wiegand et al., 2008). In this study, no visual growth was observed with unaided eye when the total *Salmonella* reduction (R_T) was ≥ 80%, i.e., a clear solution. To quantify it, the MIC was determined as the total *Salmonella* reduction (R_T) of ≥ 80% as described in Eq. 7-1, which is more accurate and less prone to experimental or subjective error than the unaided eye observation method.

$$R_T\ (\%) = \frac{(OD_{GC} - OD_{SC})_{pH=7.0} - (OD_{Sample} - OD_{AC})_{pH=\mathrm{i}}}{(OD_{GC} - OD_{SC})_{pH=7.0}} \qquad 7\text{-}1$$

where: R_T is the total *Salmonella* reduction (%); OD_{GC}, OD_{SC}, OD_{Sample} and OD_{AC} are the OD_{600} reading of GC, SC, sample and AC wells after 20 hours, respectively; *i* represents the assessed pH value (6.0, 7.0, 8.0 and 9.0).

It has been reported that pH plays an indirect role in bacteria inactivation by affecting the concentrations of free VFA and free ammonia (Himathongkham et al., 2000; Sahlström, 2003), but the effect of pH itself on *Salmonella* is not clear. Therefore the effect of pH was assessed and the *Salmonella* reductions caused by pH (R_{pH}) were calculated by Eq. 7-2.

$$R_{pH}\ (\%) = \frac{(OD_{GC} - OD_{SC})_{pH=7.0} - (OD_{GC} - OD_{SC})_{pH=\mathrm{i}}}{(OD_{GC} - OD_{SC})_{pH=7.0}} \qquad 7\text{-}2$$

where: R_{pH} is the *Salmonella* reductions only caused by pH (%).

7.2.3 Models

Five types of curves (Figure 7-1 A-E) were observed in the MIC experiment when describing the effects of VFA and ammonia concentrations on *Salmonella* reduction.

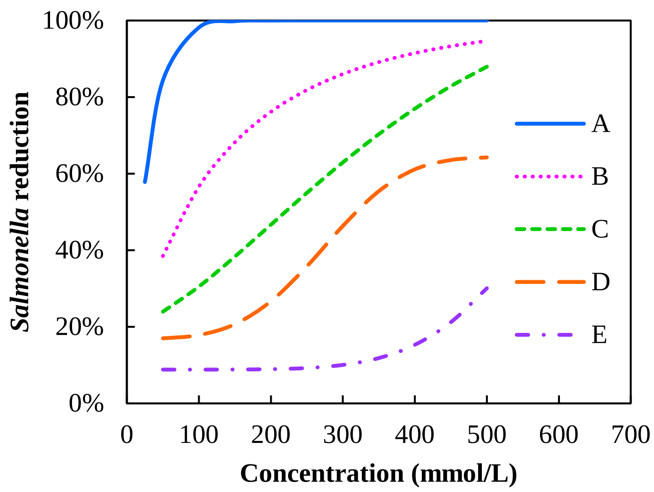


Figure 7-1 Graphic representations of five types of curves indicating the effects of VFA and ammonia concentrations on *Salmonella* reduction

Weibull model was selected to simulate these curves because of its diversified patterns, which can indicate concave, linear and convex curves (Boekel, 2002). Considering the effect of VFA/ammonia concentration on *Salmonella* reduction, the traditional Weibull model can be described as Eq. 7-3 and 7-4.

$$\frac{dN/dc}{N_0} = -f(c) = -\frac{\alpha}{\beta}(\frac{c}{\alpha})^{\beta-1}e^{-(\frac{c}{\alpha})^{\beta}} \qquad 7\text{-}3$$

$$R = -\frac{N-N_0}{N_0} = 1 - e^{-(\frac{c}{\alpha})^{\beta}} \qquad 7\text{-}4$$

where: N_0 and N are the *Salmonella* concentrations at VFA/ammonia concentration of zero and c, respectively, which was described with OD_{600} in this study; c is the VFA/ammonia concentration (mmol/L); α is the scale parameter; and β is the shape parameter.

However, the traditional Weibull model can only simulate curve types A, B and C, but not curves D and E. Both curves D and E have a lag *Salmonella* reduction (R_0). It means there exists a non-inhibition concentration (NIC) (Lambert & Pearson, 2000), below which the growth of the *Salmonella* was not affected by VFA/ammonia. Only when the concentration was above NIC, the VFA/ammonia inhibited the growth of *Salmonella*. Apart from R_0, curve D also has a stationary *Salmonella* reduction (R_f), indicating that after a threshold concentration (within the tested condition of 500 mmol/L in this study), the increase of VFA/ammonia did not result in higher *Salmonella* reduction. It may be because some *Salmonella* cells were intrinsically resistant to VFA/ammonia, or during this period some cells can resynthesize some vital components to protect them from the toxicity of VFA/ammonia (Xiong et al., 1999). R_0 and R_f were introduced into the traditional Weibull model with the modified model described in Eq. 7-5. The modified Weibull model was used to simulate the reduction of *Salmonella* in this study (curves A-E).

$$\mathrm{R} = R_0 + (R_f - R_0)(1 - e^{-\left(\frac{c}{\alpha}\right)^{\beta}}) \qquad 7\text{-}5$$

where: R_0 is the lag *Salmonella* reduction at low VFA/ammonia concentrations (%); and R_f is the stationary *Salmonella* reduction at high VFA/ammonia concentrations (%).

7.2.4 Dry co-digestion of FW and PM

To assess the inactivation of *Salmonella* during dry co-digestion of FW and PM, a laboratory scale batch experiment was conducted. PM was collected from a local category 1 farm, i.e. low *Salmonella* status, in Galway and centrifuged at 1,500 ×g for 5 minutes to get the solid fraction for digestion (MSE super minor centrifuge, London, UK). Fresh FW was collected from 10 local residences in Galway City, Ireland, and was mixed and ground to less than 2 mm particles prior to use. The inoculum was dewatered anaerobic sludge taken from a local wastewater treatment plant in Galway. The TS contents of FW, PM and sludge were 24.0%, 19.5% and 22.1%, respectively. Six 1 L glass digesters were applied in this experiment. In each digester, 130 g FW, 165 g PM and 505 g sludge (wet weight) were added to obtain the sludge rate of 50% and the FW/PM ratio of 50:50 based on VS according to the previous study (Jiang et al., 2018). The *Salmonella* cocktail was made up of ~1/3 of each *Salmonella* serotype and diluted in MRD so as to obtain high (~1×10^6 CFU/mL) and low (~1×10^4 CFU/mL) *Salmonella* concentrations in the suspensions. Unexpectedly, a high *Salmonella* concentration of 4.4×10^4 CFU/mL was measured in the sludge taken from a wastewater treatment plant, so the final concentrations of *Salmonella* detected were 8.2×10^5 and 5.9×10^3 CFU/mL, respectively, in high and low inoculated reactors. The TS content was adjusted to 20% by adding tap water. Then, the reactors were mixed well using a sterile wooden spatula, incubated at 37 ^{o}C and shaken by hand every day before sampling. About 2 g samples were taken every day for analysis of pH, TS, VS, VFA and TAN. The analysis methods and calculation of free VFA and free ammonia were detailed in Chapter 3. Each *Salmonella* concentration was assessed in triplicate.

7.2.5 Enumeration and presence/absence analysis of *Salmonella*

A 25 g sample was taken from each digester and homogenized in 225 mL BPW (Oxoid, Hampshire, UK) to get a 1 in 10 dilution. Then 1:10 serial dilution was performed using MRD. Enumeration of *Salmonella* was detailed by McCarthy et al. (2015). Briefly, 100 µL of the dilutions were spread in duplicate onto tryptone soy agar (TSA, Oxoid, Hampshire, UK) and incubated at 30 ^{o}C for 2 h. Then the plates were overlaid by xylose lysine deoxycholate (XLD) agar (Oxoid, Hampshire, UK) and further incubated at 37 ^{o}C for 24 h. Colonies between 30 and 300 on the plates were counted after the second incubation period. The counts were averaged and presented as CFU/mL. The LOD was obtained when the number of colonies on 10^{-1} (1:10) dilution plate was less than 10, i.e. 100 CFU/g. If the colonies on the plates were less than 10, the presence/absence of *Salmonella* was determined using standard method (ISO, 2007) with some modifications described by Mc Carthy et al. (2011). Apart from XLD agar, the modified Brilliant Green agar (BGA, Oxoid, Hampshire, UK) was used for an additional selective plating. Five suspect *Salmonella* colonies were taken from each XLD and BGA plate (totally 10 colonies) and streaked twice onto Nutrient Agar (NA, Oxoid, Hampshire, UK) to purify. Colonies from the NA plates were then inoculated into Urea Agar slants (Oxoid, Hampshire, UK), Triple Sugar Iron Agar Slants (Oxoid, Hampshire, UK) and Lysine Decarboxylase Broth (Oxoid, Hampshire, UK), and incubated at 37 ^{o}C for 24 h. The colonies with positive reactions were tested using the *Salmonella* Latex Agglutination kit (Oxoid, Hampshire, UK) to confirm identity. The enumeration method was continued in conjunction with the presence/absence method for further 3 days in case of uneven distribution or revival of *Salmonella*. Reactors were considered negative for *Salmonella* when 3 consecutive time point tests were negative.

7.2.6 Statistical analysis

Statistical analysis was performed using SPSS 22.0 (IBM, USA). *Salmonella* reduction across different pH values and *Salmonella* serotypes were compared using One-way ANOVA followed by Bonferroni post hoc test for pairwise comparison. The MANOVA was performed to determine the effect of multi-factors (*Salmonella* serotype, pH, VFA type, VFA and ammonia concentrations) and their interactions on *Salmonella* reduction and MIC. The range analysis was performed to assess the effect sequence of the above factors. Post hoc multiple comparisons were conducted using Bonferroni method to explore the effect sequence of different levels in each factor. A P value of less than 0.05 was considered statistically significant.

7.3 Results and discussion

7.3.1 Results of *Salmonella* inactivation during dry co-digestion of FW/PM

The laboratory scale dry co-digestion experiment was conducted to assess the inactivation of *Salmonella* during dry co-digestion of FW and PM. The *Salmonella* inactivation results and variations of pH/TAN/VFA in the reactors are shown in Figure 7-2. No significant difference was observed between the pH/TAN/VFA values in high and low inoculation reactors ($P > 0.05$). In both high (8.2×10^5 CFU/mL) and low (5.9×10^3 CFU/mL) inoculation reactors, the concentration of *Salmonella* increased in the first day (Figure 7-2 a). It may be because the low initial VFA (237 - 402 mg/L) and TAN (1,423 - 1,627 mg/L) concentrations in the reactors provided favorable conditions for the growth of *Salmonella* (Manyi-Loh et al., 2014). The VFA and TAN concentrations increased sharply after the first day and were steady at 7077 ± 593 mg/L (118 ± 10 mmol/L) and 3241 ± 386 mg/L (232 ± 28 mmol/L), respectively, with pH remaining 7.1 ± 0.2 (Figure 7-2 b - d). *Salmonella* concentrations in both high and low inoculation reactors started to decrease on the second day and were completely eliminated within 6 - 7 days. The enumeration and the presence/absence of *Salmonella* were continued for 3 days and the results were all negative, so the experiments finished after 9 days. High initial *Salmonella* concentration didn't prolong the inactivation time. The HAC, HPR and HIB were main VFA compositions, accounting for about 53.4%, 25.0% and 21.6% of the total VFA concentrations, respectively (Figure 7-2 c, d).

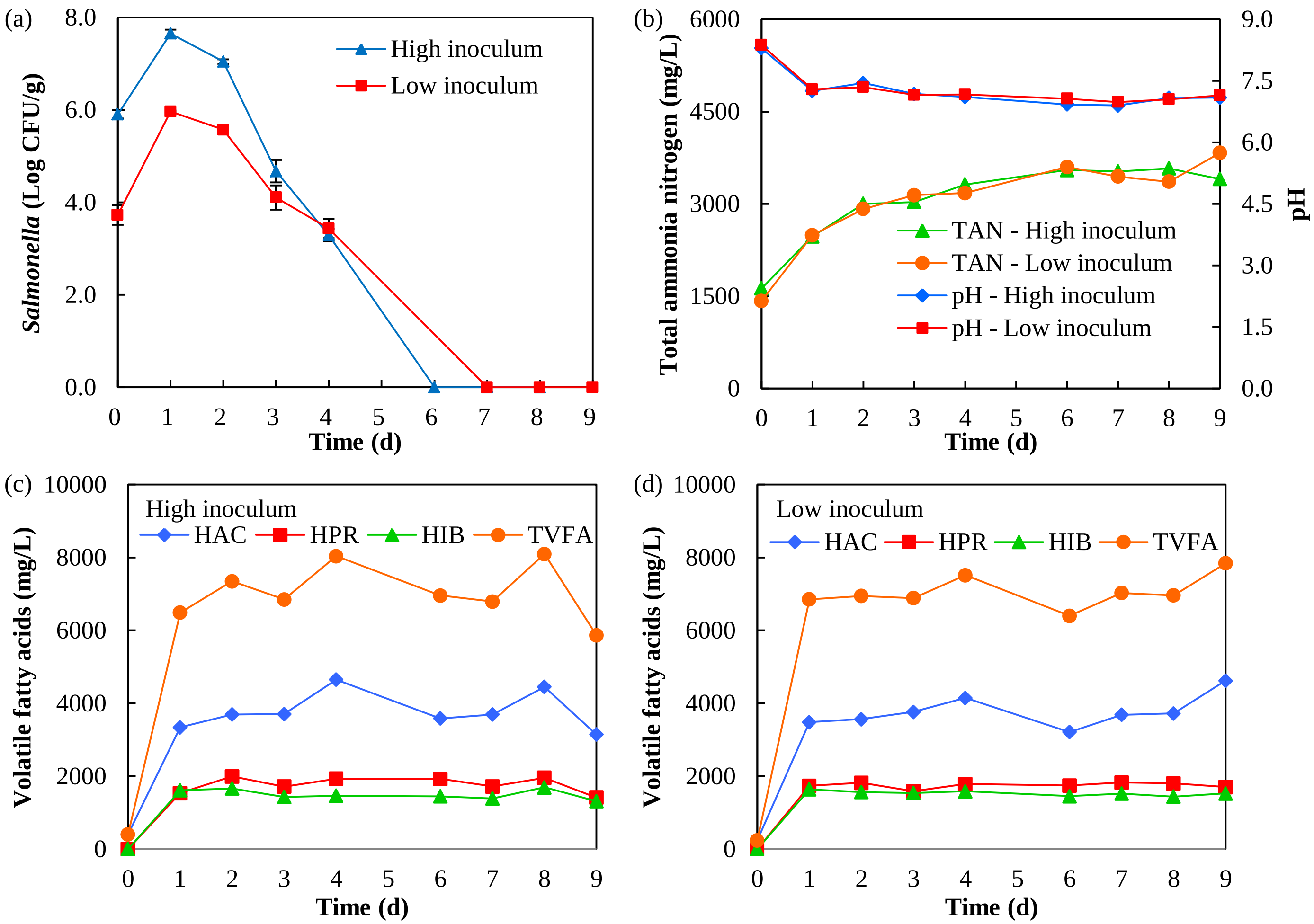


Figure 7-2 Inactivation of *Salmonella* (a), pH and total ammonia nitrogen variations (b) and volatile fatty acids compositions (c, d) during dry co-digestion of food waste and pig manure.

To further investigate the inactivation mechanisms of *Salmonella* in FW/PM dry co-digestion systems, the MIC experiments were conducted to explicate the effects of pH, VFA and ammonia.

7.3.2 Effects of pH on *Salmonella* inactivation

The effects of pH on *Salmonella* growth for the three *Salmonella* serotypes were determined using the OD_{600} readings obtained from GC wells without VFA/ammonia addition. As shown in Figure 7-3 a, for all the serotypes, the highest growth was observed at pH 7.0, indicating neutral pH was the optimal growing condition for *Salmonella*. This is in consistent with the results reported by Salsali et al. (2006). No significant difference was observed between pH 7.0 and 8.0, indicating slightly alkaline conditions didn't significantly inhibit the growth of *Salmonella*. The growth values at pH 9.0 ($P < 0.05$) and 6.0 ($P < 0.01$) were significant lower than those at pH 7.0 and 8.0. It means both acidic and alkaline conditions inhibited the growth of *Salmonella*. *Salmonella* reductions caused by pH were calculated according to Eq. 7-2, and the reductions at pH 6.0 and 9.0 ranged 50.6% - 60.3% and 12.4% - 20.4%, respectively. Therefore, the direct inhibitory effect of pH on *Salmonella* inactivation was demonstrated. It is reasonably hypothesized that the inhibitory effect of H^+ (10^{-6} mol/L at pH = 6.0) is much higher than OH^- (10^{-5} mol/L at pH = 9.0) on *Salmonella*.

The growth of different *Salmonella* serotypes varied at different pH (Figure 7-3 b). At pH 6.0, no significant differences were observed among serotypes. At pH 7.0 - 9.0, the growth of *S. Typhimurium DT104* was significantly higher than the others ($P < 0.01$). But the growth of *S. Derby* and *S. Typhimurium 4,[5],12:i:-* varied at different pH: no significant difference was observed at pH 7.0, while the growth of *S. Derby* > *S. Typhimurium 4,[5],12:i:-* at pH 8.0 ($P < 0.01$) and *S. Typhimurium 4,[5],12:i:-* > *S. Derby* at pH 9.0 ($P < 0.01$). *S. Typhimurium DT104* seemed to be the most robust in all pH conditions.

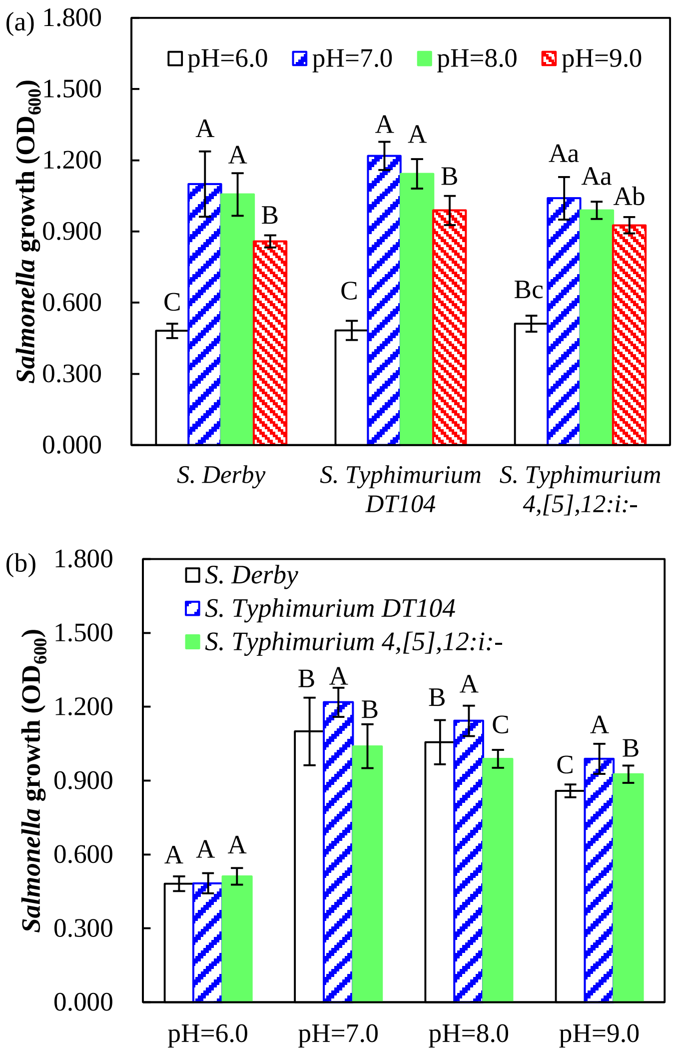
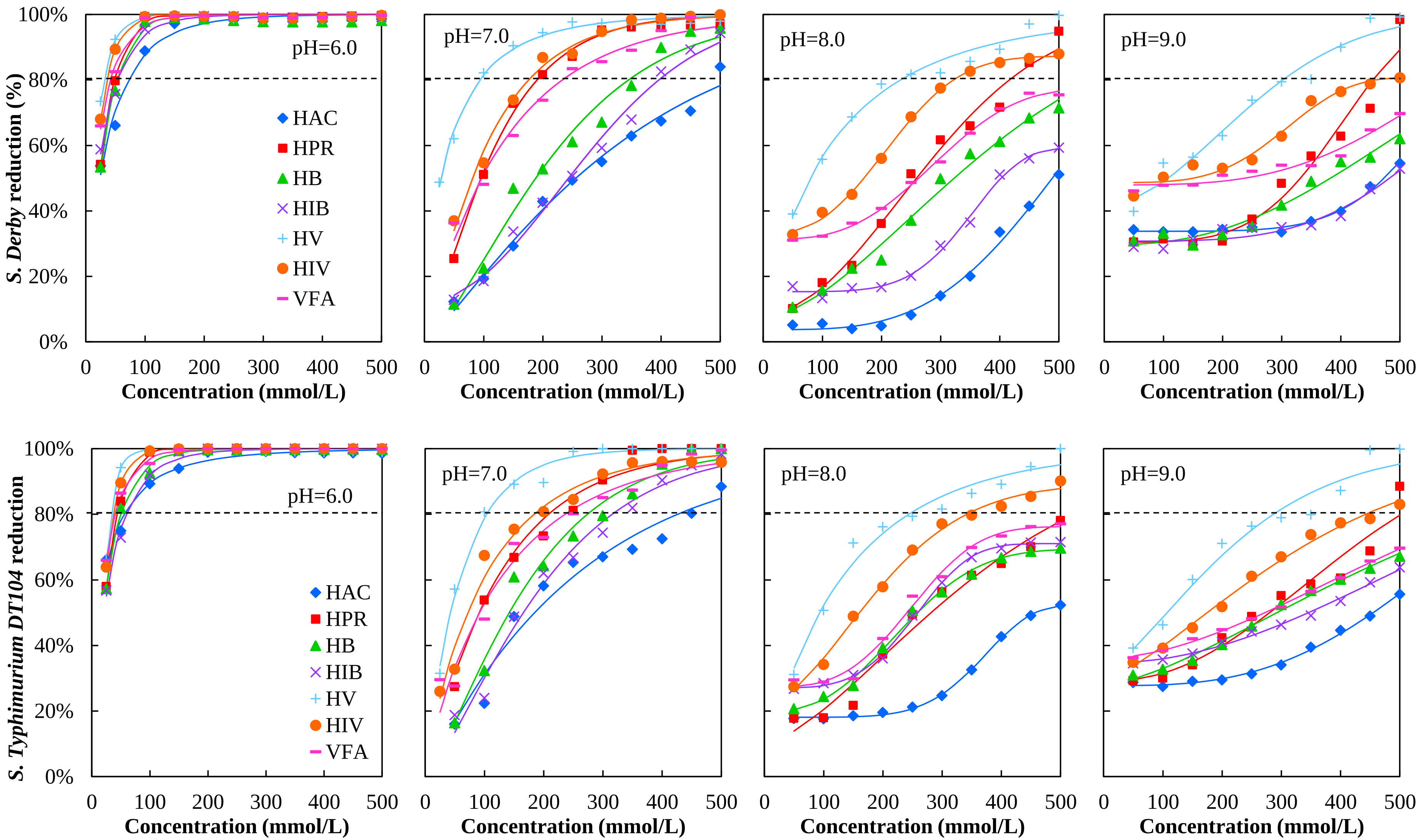


Figure 7-3 Effects of (a) pH and (b) *Salmonella* serotype on *Salmonella* growth

7.3.3 Effects of VFA on *Salmonella*

The reductions of *Salmonella* under various VFA concentrations were simulated by the modified Weibull model (Eq. 7-5), with the results shown in Figure 7-4. The SSEs were ≤ 0.023 and R^2 were ≥ 0.942, indicating the models can simulate the *Salmonella* reductions very well. The MIC values were calculated from the modified Weibull model at the *Salmonella* reduction of 80%, with the results shown in Table 7-1. Compared with being observed by unaided eyes, the calculation of MIC from the modified Weibull model can obtain more accurate values, and can predict the values beyond the experiment concentrations in terms of the available data. Some MIC values were not available due to the existence of stationary reduction phases, where the increase of VFA didn't result in more *Salmonella* reduction (Curve D in Figure 7-1), possibly because some *Salmonella* are mutated or intrinsically resistant to tress conditions. Wood et al. (2001) reported when exposed to high medium osmolality, *Salmonella typhimurium* can accumulate compatible solutes, e.g. trehalose, through biosynthetic or transport systems to protect itself from high osmotic pressure. Besides, *S. typhimurium* can change its membrane fatty acid composition under organic acid conditions so as to be resistant to stress. Alvarez-Ordonez et al. (2008) reported at 37 ^{o}C, the unsaturated fatty acids /saturated fatty acids ratio of acid-adapted *S. typhimurium* was very low (0.10-0.22) compared with the non-acid adapted cells (0.41-0.48), indicating it had a low fluidity of the membrane and was more resistant to stress environment.



100%
80%
60%
40%
20%
0%
S. Derby reduction (%)
pH=6.0
pH=7.0
pH=8.0
pH=9.0
HAC
HPR
HB
HIB
HV
HIV
VFA
0 100 200 300 400 500
Concentration (mmol/L)
S. Typhimurium DT104 reduction

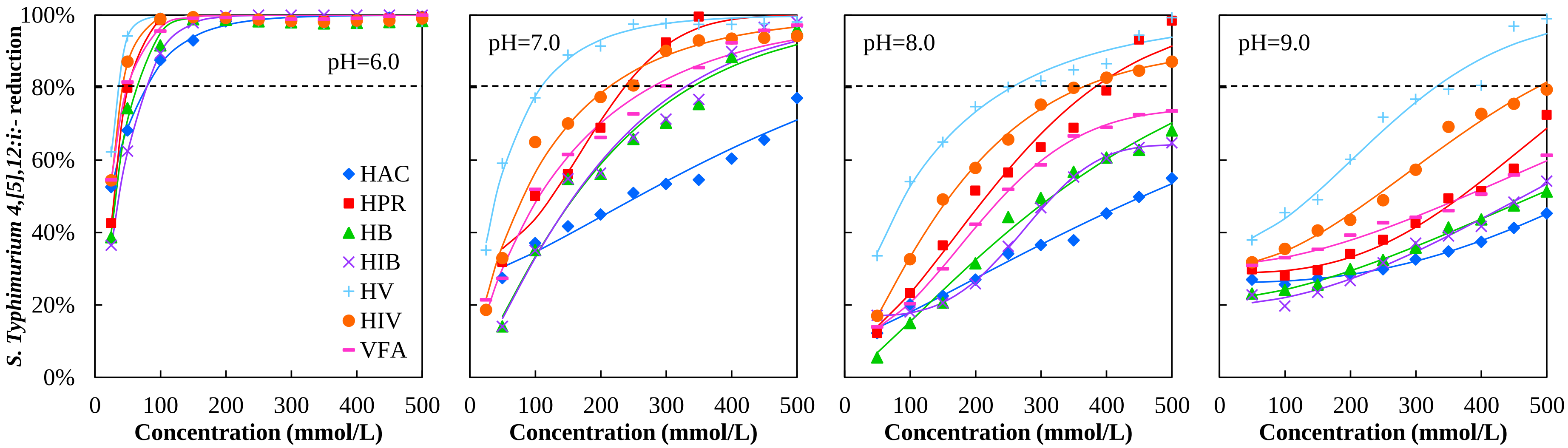


Figure 7-4 Simulation of *Salmonella* reduction caused by various VFAs. The scatter plots are the experiment data, and the solid lines are the responses of the modified Weibull model.

Table 7-1 Minimum inhibitory concentration of VFA on *Salmonella* (mmol/L)

MIC (mmol/L)		HAC	HPR	HB	HIB	HV	HIV	MVFA
pH=6	*S. Derby*	71	49	53	53	31	35	42
	S. Typhimurium DT104	55	44	50	59	33	37	40
	S. Typhimurium 4,[5],12:i:-	76	50	61	76	35	42	49
pH=7	*S. Derby*	522	190	345	396	93	176	233
	S. Typhimurium DT104	426	208	274	316	104	184	236
	S. Typhimurium 4,[5],12:i:-	640	236	338	327	109	211	276
pH=8	*S. Derby*	627	416	559	NA	232	322	NA
	S. Typhimurium DT104	NA	523	NA	NA	244	341	NA
	S. Typhimurium 4,[5],12:i:-	973	381	629	NA	254	362	NA
pH=9	*S. Derby*	605	454	653	676	302	458	596
	S. Typhimurium DT104	685	502	675	711	287	442	642
	S. Typhimurium 4,[5],12:i:-	908	584	934	815	329	484	792

NA: not available.

Four-way ANOVA was conducted to assess the effects of the following factors on *Salmonella* reduction: *Salmonella* serotype (*S. Derby*, *S. Typhimurium DT104* and *S. Typhimurium 4, [5], 12: i:-*), pH (6.0, 7.0, 8.0 and 9.0), VFA type (HAC, HPR, HB, HIB, HV, HIV and MVFA) and VFA concentration (50 - 500 mmol/L in increments of 50 mmol/L). The effects of factors *Salmonella* serotype, pH and VFA type on MIC were assessed by three-way ANOVA. The MANOVA results are shown in Table 7-2 Part A. Range analysis was conducted to analyze the effect sequence of these factors on each dependent variable, and the Bonferroni post hoc test was performed to assess the effect sequence of the levels for each factor, with the results shown in Table 7-2 Parts B and C.

Table 7-2 MANOVA, range analysis and Bonferroni post hoc test of VFA on *Salmonella* reduction (%) and MIC (mmol/L)

Part A: Multivariate analysis of variance (MANOVA)

Dependent variable	Main effect	Interaction effect	Non-analized interaction [e]
Salmonella reduction	T_{Salm}^{**} [a], pH^{**}, T_{VFA}^{**} [b], C_{VFA}^{**} [c]	$pH \times C_{VFA}^{**}$, $pH \times T_{VFA}^{**}$, $pH \times T_{Salm}^{**}$, $T_{VFA} \times T_{Salm}^{**}$, $T_{VFA} \times C_{VFA}^{**}$, $T_{Salm} \times C_{VFA}^{**}$ $T_{VFA} \times pH \times C_{VFA}^{**}$, $T_{VFA} \times T_{Salm} \times pH^{**}$, $T_{Salm} \times pH \times C_{VFA}^{**}$	$pH \times C_{VFA} \times T_{VFA} \times T_{Salm}$
MIC [d]	T_{Salm}^{**}, pH^{**}, T_{VFA}^{**}	$pH \times T_{Salm}^{**}$, $T_{Salm} \times T_{VFA}^{*}$, $pH \times T_{VFA}^{**}$	$pH \times T_{VFA} \times T_{Salm}$

** P < 0.01; * P < 0.05

a. T_{Salm}: *Salmonella* serotype

b. T_{VFA}: Volatile fatty acid (VFA) type

c. C_{VFA}: VFA concentration

d. MIC: minimum inhibitory concentration

e. Non-analized interaction: Some interactions were not analyzed because if they were included in the model, the error term would have zero degree of freedom.

Part B: Range analysis and Bonferroni post hoc test

T_{Salm}	Reduction	MIC	pH	Reduction	MIC	T_{VFA}	Reduction	MIC	C_{VFA}	Reduction
S. Typhimurium DT104	69.61 ^{A}	326 ^{A}	6.0	96.86 ^{A}	50 ^{A}	HV	84.52 ^{A}	171 $^{A\ a}$	0	
S. Derby	66.97 ^{B}	315 ^{A}	7.0	72.60 ^{B}	278 ^{B}	HIV	76.06 ^{B}	258 $^{B\ b}$	50	40.63 ^{A}
S. Typhimurium 4,[5],12:i:-	66.20 ^{C}	384 ^{B}	8.0	51.24 ^{C}	481 ^{C}	HPR	69.25 ^{C}	303 $^{B\ bc}$	100	51.05 ^{B}
			9.0	49.67 ^{D}	597 ^{D}	MVFA	68.87 ^{C}	323 $^{BC\ c}$	150	58.04 ^{C}
						HB	62.06 ^{D}	429 $^{C\ d}$	200	62.87 ^{D}
						HIB	59.65 ^{E}	381 $^{C\ d}$	250	67.82 ^{E}
						HAC	52.74 ^{F}	530 $^{D\ e}$	300	71.91 ^{F}
									350	75.53 ^{G}
									400	79.18 ^{H}
									450	82.85 ^{I}
									500	86.06 ^{G}
Range	3.41	69		47.19	547		31.78	359		45.43

HAC: acetic acid; HPR: propionic acid; HB: butyric acid; HIB: isobutyric acid; HV: valeric acid; HIV: isovaleric acid; MVFA: mixed volatile fatty acid.

Different capital letters indicated the significant difference at $P < 0.01$.

Different lowercase letters indicated the significant difference at $P < 0.05$.

Data with same letters indicated no significant difference ($P > 0.05$).

Part C: Inhibitory effect sequence		
Dependent variable	Reduction	MIC
Factor	pH > C_{VFA} > T_{VFA} > T_{Salm}	pH > T_{VFA} > T_{Salm}
pH	6.0 > 7.0 > 8.0 > 9.0	6.0 > 7.0 > 8.0 > 9.0
C_{VFA}	500 > 450 > 400 > 350 > 300 > 250 > 200 > 150 > 100 > 50	
T_{VFA}	HV > HIV > (HPR & MVFA) > HB > HIB > HAC	HV > (HIV & HPR) > (HPR & MVFA) > (HB & HIB) > HAC
T_{Salm}	*S. Typhimurium DT104* > *S. Derby* > *S. Typhimurium 4,[5],12:i:-*	(*S. Derby* & *S. Typhimurium DT104*) > *S. Typhimurium 4,[5],12:i:-*

Values separated by “>” indicated significant difference at P < 0.05.

Values separated by “&” in brackets indicated no significant difference (P > 0.05).

As shown in Table 7-2 A, all the factors analyzed had significant effects on *Salmonella* reduction and MIC ($P < 0.01$). Significant interaction effects were observed among different factors. Some interactions were not analyzed because if they were included in the model, the error term would have zero degree of freedom. The higher inhibitory effect of VFA can be indicated by higher *Salmonella* reduction and lower MIC. Range analysis results showed that the inhibitory effect sequence of these factors was: pH > VFA concentration > VFA type > *Salmonella* serotype (Table 7-2 B, C). However, the ranges of the first three factors were almost one order of magnitude larger than that of *Salmonella* serotype, therefore the effects of pH, VFA and their interactions will be highlighted in the following analysis.

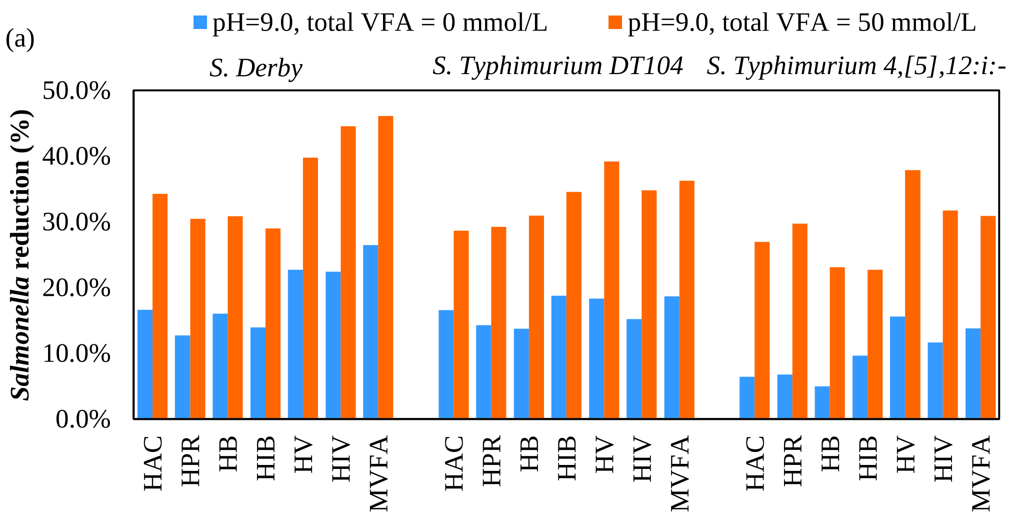
The inhibitory effect sequence of pH was 6.0 > 7.0 > 8.0 > 9.0 ($P < 0.01$), which differed from that of pure pH (6.0 > 9.0 > 8.0 & 7.0), indicating the interactions between pH and VFA (Table 7-2 C). The inhibitory effect of VFA was pH-dependent because pH affected the conversion of ionized VFA to free VFA (Sahlström, 2003). Free VFA was usually considered as the real toxic factor for bacteria inactivation, as it can pass through the cell membrane freely, dissociate in the cell and form a lower intracellular pH (Puchajda & Oleszkiewicz, 2006). It thereby decreases the PMF across the cell membrane, which inhibits the bacteria by impacting the metabolism and electrophysiology of the bacteria, acidifying the cytoplasm and causing osmotic problems (Kashket, 1987; Kashket, 1985; Roe et al., 1998; Sheu & Freese, 1972). Even though the pH values of 7.0 and 8.0 themselves didn’t cause significant *Salmonella* reduction (Section 7.3.2), higher free VFA concentrations were induced compared with at pH 9.0. Besides, a significant positive correlation was observed between VFA concentration and *Salmonella* reduction ($P < 0.01$). High VFA concentration meant high available free acid molecules and high osmotic gradient across cell membrane, resulting in high toxicity to *Salmonella*.

For VFA type, the inhibitory effect sequence observed in this study was: HV > HIV ≥ (HPR & MVFA) > HB ≥ HIB > HAC ($P < 0.05$, Table 7-2 C), and it generally accorded with the law that the inhibitory effect of VFA increased with the increase of the chain length. Sheu and Freese (1972) reported the inhibition of n-hexanoic acid > n-pentanoic acid > HB > HPR > HAC > formic acid. Back et al. (2009) also indicated the inhibitory effect of HPR > HAC > formic acid. But Salsali et al. (2006) reported the inhibitory effect of HAC > HPR > HB during *Salmonella* inactivation, which disagreed with the results observed in this study. However, Salsali et al. (2006) compared the effect of different VFAs at the same mass concentration; for instance, at 6,000 mg/L, the molar concentration of HAC (100 mmol/L) > HPR (81 mmol/L) > HB (68 mmol/L). Thus the high inhibitory effect of HAC on *Salmonella* may be not simply because of its short chain length as explained in the paper. As to VFA structure, it was noticed that the inhibitory effect of linear chain VFA was higher than branched chain VFA (e.g. HV > HIV and HB ≥ HIB), probably because the linear chain VFA was much easier to enter the cell.

Ionized VFA also affects the inactivation of pathogen because it may change the osmotic pressure of the cell (Roe et al., 1998), however, the detailed effect of ionized VFA has been rarely reported. As shown Table 7-3, the free VFA concentrations at pH 9.0 and total VFA of 50 mmol/L were negligible (0.003 - 0.004 mmol/L), which can be considered similar with those at pH 9.0 with no addition of VFA. As shown in Figure 7-5 a, *Salmonella* reductions at pH 9.0 and total VFA of 50 mmol/L were 12.1% - 23.0% higher than those at pH 9.0 and total VFA of 0 mmol/L, which could be attributed to ~ 50 mmol/L ionized VFA. On the other side, the free VFA concentrations at pH 7.0 and total VFA of 50 mmol/L were similar with those at pH 8.0 and total VFA of 500 mmol/L. However, the ionized VFA concentrations at pH 8.0 were ~ 450 mmol/L higher than those at pH 7.0 (Table 7-3). As indicated in Section 7.3.2, pH 7.0 and 8.0 themselves didn’t cause *Salmonella* reduction, thereby the higher *Salmonella* reductions of 23.0% - 82.3% at pH 8.0 than pH 7.0 could be considered the contribution of ~ 450 mmol/L ionized VFA (Figure 7-5 b). All these demonstrated the effects of ionized VFA on *Salmonella* reduction. However, the detailed inactivation mechanism is not clear and need further study.

Table 7-3 Free VFA and ionized VFA compositions at pH=9.0, VFA=0 mmol; pH=9.0, VFA=50 mmol/L; and pH=7.0, VFA=50 mmol/L; pH=8.0, VFA= 500 mmol/L

pH		9.0	9.0	7.0	8.0
Total VFA (mmol/L)		0	50	50	500
Free VFA (mmol/L)	HAC	0	0.003	0.28	0.29
	HPR	0	0.004	0.37	0.37
	HB	0	0.004	0.35	0.35
	HIB	0	0.003	0.33	0.33
	HV	0	0.003	0.30	0.30
	HIV	0	0.004	0.35	0.36
	MVFA	0	0.003	0.33	0.33
Ionized VFA (mmol/L)	HAC	0	49.997	49.72	499.71
	HPR	0	49.996	49.63	499.63
	HB	0	49.996	49.65	499.65
	HIB	0	49.997	49.67	499.67
	HV	0	49.997	49.70	499.70
	HIV	0	49.996	49.65	499.64
	MVFA	0	49.997	49.67	499.67



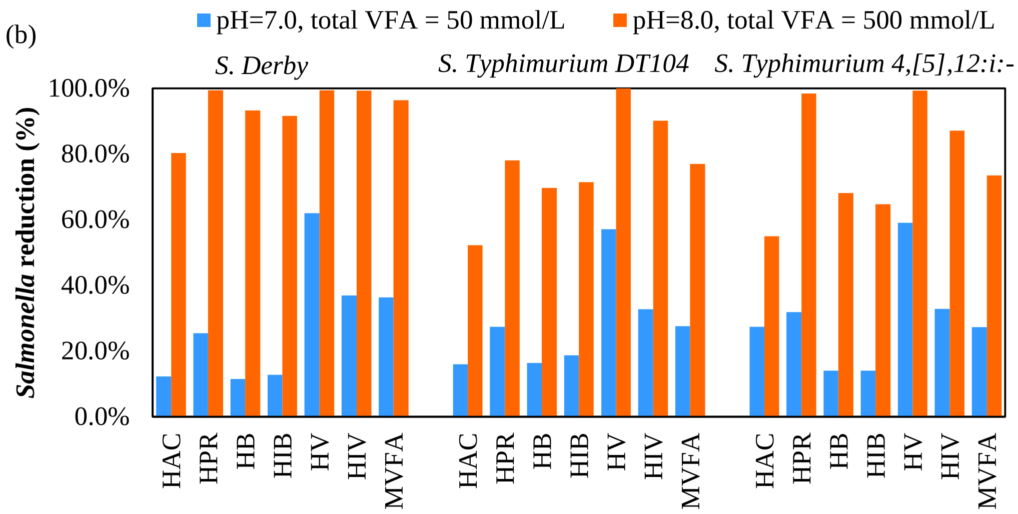


Figure 7-5 *Salmonella* reductions at (a) pH=9.0, VFA=0 mmol and pH=9.0, VFA=50 mmol/L; and (b) pH=7.0, VFA=50 mmol/L and pH=8.0, VFA= 500 mmol/L

7.3.4 Effects of ammonia on *Salmonella*

The effect of ammonia on *Salmonella* was also simulated by the modified Weibull model (Figure 7-6). The MIC of total ammonia on *Salmonella* was calculated from the modified Weibull model at the reduction of 80%, with the results shown in Table 7-4.

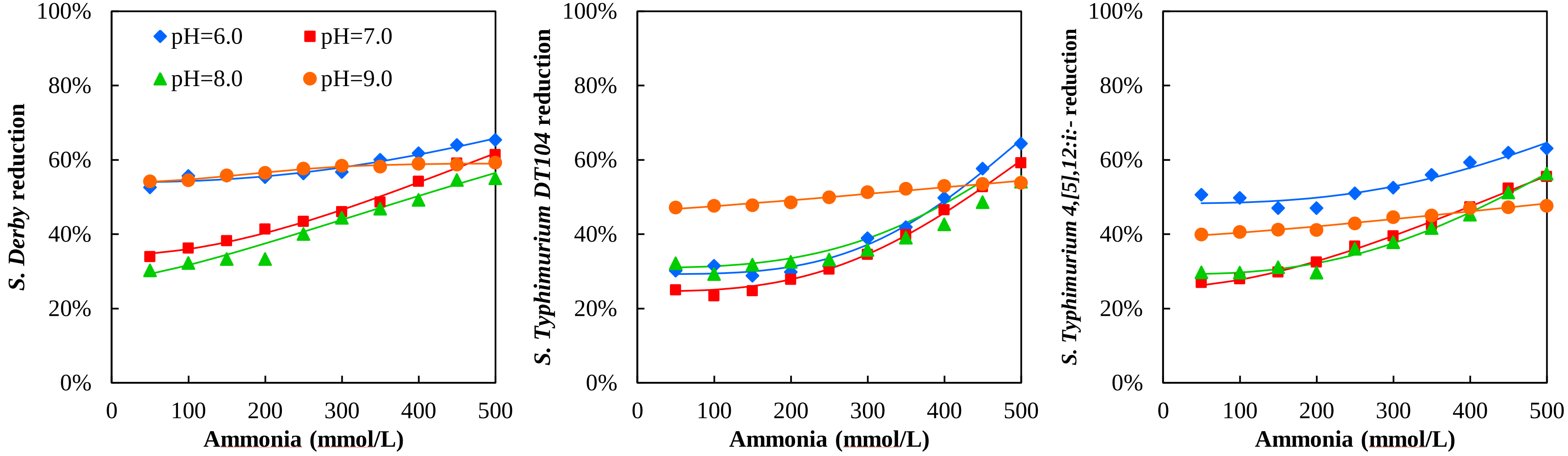


Figure 7-6 Simulation of *Salmonella* reduction caused by ammonia. The scatter plots are the experiment data, and the solid lines are the responses of the modified Weibull model.

Table 7-4 Minimum inhibitory concentration of total ammonia on *Salmonella* (mmol/L)

MIC(mmol/L)	pH=6.0	pH=7.0	pH=8.0	pH=9.0
S. Derby	781	758	988	NA
S. Typhimurium DT104	588	646	690	2284
S. Typhimurium 4,[5],12:i:-	697	841	720	2181

NA: Non-available

Three-way ANOVA was conducted to assess the effects of *Salmonella* serotype, pH, and total ammonia concentrations on *Salmonella* reduction, and two-way ANOVA was performed to assess the effects of *Salmonella* serotype and pH on MIC. Range analysis and Bonferroni post hoc test were used to analyze the effect sequence of the factors and their respective levels on each dependent variable. The results are shown in Table 7-5.

Table 7-5 MANOVA, range analysis and Bonferroni post hoc test of ammonia on *Salmonella* reduction (%) and MIC (mmol/L)

Part A: Multivariate analysis of variance (MANOVA)

Dependent variable	Main effect	Interaction effect	Non-analized interaction [d]
Salmonella reduction	T_{Salm}** [a], pH**, C_{Am}** [b]	pH × C_{Am}**, pH × T_{Salm}**, T_{Salm} × C_{Am}*	pH × C_{Am} × T_{Salm}
MIC [c]	pH**		pH × T_{Salm}

** $P < 0.01$; * $P < 0.05$

a. T_{Salm}: *Salmonella* serotype

b. C_{Am}: Total mmonia concentration

c. MIC: minimum inhibitory concentration

d. Non-analized interaction: Some interactions were not analyzed because if they were included in the model, the error term would have zero degree of freedom.

Part B: Range analysis and Bonferroni post hoc test

T_{Salm}	Reduction	MIC	pH	Reduction	MIC	C_{Am}	Reduction
S. Derby	50.93 ^{A}	1226 ^{A}	6.0	50.86 ^{A}	689 ^{A}	0	
S. Typhimurium 4,[5],12:i:-	43.87 ^{B}	1110 ^{A}	7.0	40.59 ^{B}	748 ^{A}	50	37.75 ^{Aa}
S. Typhimurium DT104	41.31 ^{C}	1052 ^{A}	8.0	39.55 ^{B}	799 ^{A}	100	38.18 ^{Aa}
			9.0	50.47 ^{A}	2281 ^{B}	150	38.76 ^{ABa}
						200	39.63 ^{ABab}
						250	42.40 ^{Bb}
						300	45.03 ^{BCbc}
						350	47.65 ^{Cc}
						400	51.23 ^{CDd}
						450	55.14 ^{De}
						500	57.91 ^{De}
Range	9.63	174		11.31	1592		20.16

Different capital letters indicated the significant difference at $P < 0.01$.

Different lowercase letters indicated the significant difference at $P < 0.05$.

Data with same letters indicated no significant difference ($P > 0.05$).

Part C: Inhibitory effect sequence		
Dependent variable	Reduction	MIC
Factor	C_{Am} > pH > T_{Salm}	pH > T_{Salm}
C_{Am}	(500 & 450) > 400 > (350 & 300) > (300 & 250 & 200) > (200& 150 & 100 & 50)	
pH	(6.0 & 9.0) > (7.0 & 8.0)	(6.0 & 7.0 & 8.0) > 9.0
T_{Salm}	*S. Derby* > *S. Typhimurium 4,[5],12:i:-* > *S. Typhimurium DT104*	*S. Derby* & *S. Typhimurium 4,[5],12:i:-* & *S. Typhimurium DT104*

Values separated by “>” indicated significant difference at P < 0.05.

Values separated by “&” in brackets indicated no significant difference (P > 0.05).

The results showed that *Salmonella* serotype, pH and total ammonia concentration were all significant factors for *Salmonella* reduction ($P < 0.01$), while pH was the only significant factor for MIC. Interaction effects were also significant among different factors ($P < 0.05$). Generally, the inhibitory effect sequence of the factors was: total ammonia concentration > pH > *Salmonella* serotype. For *Salmonella* serotype, *S. Derby* was most sensitive to ammonia environment and *S. Typhimurium DT104* was least sensitive. Hence, the sensitivity of *Salmonella* serotypes to VFA and ammonia environments was different. For pH, the inhibitory effects of pH 9.0 and 6.0 were significantly higher than those of pH 7.0 and 8.0. Higher free ammonia concentrations were induced at pH 9.0, resulting in higher *Salmonella* reduction compared with pH 7.0 and 8.0. The reduction at pH 6.0 should be mainly caused by H^+. The effect of ammonia on bacteria inactivation is reported pH-dependent, as pH affects the conversion of free ammonia and ammonium (Himathongkham et al., 2000). Free ammonia is considered more toxic because it can cross the cell membrane by simple diffusion, and dissociate to alkalinize the cytoplasm (Ottoson et al., 2008). Park and Diez-Gonzalez (2003) reported free ammonia concentration of 5 mmol/L was the threshold inhibitory concentration for *salmonella*, as *Salmonella* grew under this concentration, and more than 40 mmol/L free ammonia caused a complete *Salmonella* reduction. Ottoson et al. (2008) found it took 1.6 days to have 5 $\log_{10}$ reductions for *Salmonella* at the free ammonia concentration of 46 mmol/L. In this experiment, the free ammonia concentrations of 0.06 - 0.64 mmol/L at pH 6.0 were much lower than the threshold inhibitory concentration. The average *Salmonella* reduction was 50.9%, equivalent to *Salmonella* reduction of 50.6% - 60.3% at pH 6.0 with no addition of ammonia (GC tests; Section 7.3.2), so H^+ should be the main inhibitory factor at pH 6.0. At pH 7.0, the free ammonia concentration ranged 0.6-4.4 mmol/L (< 5 mmol/L) at the total ammonia concentration of 50 - 350 mmol/L, but a 25.0% - 43.4% *Salmonella* reduction was observed. As pH 7.0 didn't cause *Salmonella* reduction itself (Section 7.3.2), it was reasonably induced that ammonium contributed to the reduction. The free ammonia concentration at pH 8.0 ranged 5.7 - 56.8 mmol/L and caused the *Salmonella* reductions of 29.8% - 56.2%. At pH 9.0, the free ammonia concentration ranged 28.1 - 281 mmol/L, which increased *Salmonella* reduction to 39.9% - 59.3%. But the increase of *Salmonella* reduction rate at pH 9.0 was very low with the increase of total ammonia, and thereby led to the significantly higher MIC. It may be because the remaining alive *Salmonella* was adapted to high free ammonia environment. A similar phenomenon happened to VFA tests: the increase of VFA did not always promote the inactivation of *Salmonella*, and Curve D in Figure 7-1 indicated the adaptation and resistance of bacteria to stress environment. It's a non-negligible phenomenon, together with the complex inactivation mechanism of *Salmonella*, needs to be further studied.

Generally speaking, the *Salmonella* reduction increased with the increase of total ammonia concentration. But the range (20.2%) and reductions (37.8% - 57.9%) caused by ammonia concentrations were much lower than those caused by the same VFA concentrations (the range of 45.43% and the reduction of 40.63% - 86.06%, respectively). It indicated the inhibitory effect of VFA on *Salmonella* was much more significant than ammonia.

7.3.5 Inhibition of VFA/ammonia on *Salmonella* in the reactor

As shown in Figure 7-2, after the first day, the concentration of HAC, HPR and HIB remained at 63±6 mmol/L, 29±2 mmol/L and 25±1 mmol/L, respectively, with the pH of 7.1±0.2. These values were far below the MIC obtained at pH 7.0, but the *Salmonella* reductions caused by these VFA concentrations within 20 hours can be calculated from the modified Weibull model. The average *Salmonella* reductions caused by HAC, HPR and HIB within 20 hours were 21.3%, 16.7% and 8.7%, respectively (totally 46.7%). The average total ammonia concentration was 232 ±28 mmol/L and the average *Salmonella* reduction calculated from the modified Weibull model was 32.8%. If assuming antagonistic effects existed between VFA and ammonia due to their buffering effect, the total *Salmonella* reduction in 20 h might be ~13.9% (46.7% – 32.8%), and a complete *Salmonella* inactivation could be expected within 6 days (100%/13.9%*20/24). This is consistent with the experimental data of 6-7 days, thereby proving the assumption. Thus, the concentrations of VFA and ammonia should be properly controlled in order to inactivate pathogens effectively in dry co-digestion systems.

7.4 Summary

The inactivation of *Salmonella* during dry co-digestion of FW and PM was studied. Statistical analysis was conducted to assess the effects of pH, VFA, ammonia and their interactions on three *Salmonella* serotypes. The results showed that *Salmonella* was completely eliminated within 6-7 days in dry co-digestion systems due to the high VFA and ammonia concentrations. A modified Weibull model was established to simulate *Salmonella* reduction, and calculate or predict the MIC. The pH, VFA type, VFA/ammonia concentration and *Salmonella* serotype all had significant effect on *Salmonella* inactivation ($P < 0.01$). Antagonistic effects existed between VFA and ammonia against *Salmonella* inactivation. The toxicity of VFA was much greater than that of ammonia.

Chapter 8
Conclusions and Recommendations

8.1 Overview

Dry co-digestion of FW and PM was conducted to investigate methane production with the two types of waste as substrates. The optimal operation conditions, VFA inhibition, biosafety of digestate, and the microbial community structure in dry co-digestion systems were assessed.

8.2 Main conclusions

8.2.1 Digestion performance and system stability during dry co-digestion of FW and PM

The effects of different inoculum rates, inoculum types and FW/PM ratios on methane production kinetics and system stability were assessed in Chapters 3 and 4.

(1) The preferable operation conditions were obtained at a digestate inoculum rate of 50% and a FW/PM ratio of 50:50 based on VS, with a SMY of 252 mL/g VS_{added}.
(2) The use of digestate as an inoculum didn't affect the total SMY but decreased the lag phase from 28 days to 13 days compared with the sludge inoculum system.
(3) Total VFAs were the main factor inhibiting methane production, and the VFA concentration in the digester was suggested being controlled at less than 20.0 g/L to avoid complete inhibition and to shorten the lag phase.

8.2.2 Study of the microbial communities during dry co-digestion of FW and PM

The development of the microbial community structure during dry co-digestion of FW and PM was analysed in Chapter 3 and Chapter 5.

(1) Both inoculum and FW/PM ratio played important roles in determining microbial community structure during dry co-digestion of FW and PM.
(2) Syntrophic oxidation of VFAs with hydrogenotrophic methanogenesis was the dominant methane production pathway.
(3) *Methanoculleus* was the most abundant methanogen.
(4) Correlation analysis between the relative abundance of dominant bacterial/archeal taxa and physicochemical parameters provided a simple and effective tool for the exploration of microbial functions. The anaerobic digestion-associated functionalities of some bacterial taxa were predicted for the first time.

8.2.3 Biosafety analysis during dry co-digestion of food waste and pig manure

The effects of inoculum types and FW/PM ratios on the inactivation of enteric indicator bacteria (*E. coli*, total coliforms, *Salmonella* and enterococci) during FW and PM dry anaerobic co-digestion systems were assessed in Chapters 6 and Chapter 7.

(1) Digestate inoculum systems were more effective in pathogen inactivation than the sludge inoculum systems.
(2) Under the preferable operating conditions, *E. coli*, total coliforms and enterococci were decreased to below the LOD within 4, 4 and 12 days, respectively. Free VFAs was the significant inactivation factor for total coliforms and *E. coli*, and digestion time was the most significant inactivation factor for enterococci.
(3) *Salmonella* was completely eliminated within 6-7 days. Statistical analysis showed that pH, VFA type, VFA/ammonia concentration and *Salmonella* serotype all had significant effect on *Salmonella* inactivation ($P < 0.01$).

(4) Antagonistic effects existed between VFA and ammonia against *Salmonella* inactivation.
(5) For *Salmonella*, the toxicity of VFA was much greater than that of ammonia.

8.2.4 Summary

Dry co-digestion of FW and PM was proved to be an effective treatment method for both waste. The preferable operation conditions were recommended at the digestate rate of 50% and the FW/PM ratio of 50:50 based on VS, and the total VFAs should be controlled lower than 20.0 g/L to prevent system inhibition. Dry co-digestion system was effective in the inactivation of enteric indicator bacteria, including *E. coli*, total coliforms, enterococci and *Salmonella*, which would ensure the biosafety of digestate for land spreading. Therefore, dry co-digestion of FW and PM is a good technology to be applied on Irish farms so as to build the "green farm" by providing methane-rich biogas and providing nutrient-rich fertiliser with high biosafety.

8.3 Recommendations for future research

Based on the research results obtained in this study, several recommendations for future research directions are made, as follows.

(1) *Pilot scale semi-continuous dry co-digestion of FW and PM.* Pilot scale semi-continuous experiment should be conducted to assess the performance and stability of dry co-digestion systems, so as to provide reference for on-farm engineering application.

(2) *Cost-benefit analysis and life cycle assessment on dry co-digestion of FW and PM.* According to the semi-continuous experiment results, a cost-benefit analysis of dry co-digestion systems based on a typical Irish farm should be carried out, so as to

present a comprehensive picture for the adoption of the technology. Environmental sustainability of dry digestion will also be assessed with life cycle assessment.

(3) *Improvement of methane production from dry co-digestion of FW and PM.* Chapter 3 and Chapter 4 reported the SMY of 252 - 263 mL/g VS_{added} at the preferable operations of dry co-digestion systems. Improvement of SMY should be investigated to increase energy conversion efficiency, e.g. pre-treatment of substrates, addition of nano-graphene, carbon nanotube, trace elements or electrolyte to enhance the bacterial/archaeal activity and electron transfer.

(4) *Relief of VFA inhibition during dry co-digestion of FW and PM.* As indicated in Chapter 3 and Chapter 4, total VFAs are dominant inhibition factor on methane production. The relief of inhibition should be further studied, e.g. exporting of hydrogen to decrease the partial pressure in the digesters.

(5) *Pathogen inactivation and mechanisms during dry co-digestion of FW and PM.* Chapter 5 and Chapter 7 indicated dry co-digestion of FW and PM was effective in the inactivation of enteric indicator bacteria, including *E. coli*, total coliforms, enterococci and *Salmonella*. However, the detailed inactivation mechanisms are still not clear, e.g. the roles and quantitative contributions of free VFA/ammonia, ionized VFA/ammonia and pH. Besides, the inactivation of spore-forming bacteria, protozoa and viruses during dry co-digestion systems should be further studied.

(6) *Microbial community structure analysis.* The microbial community structure during dry co-digestion of FW and PM in semi-continuous systems should be studied, especially the interactions between hydrogen-producing bacteria and the syntrophic oxidizing bacteria.

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Appendix A
Microbial analysis by 16S rRNA gene sequencing

A.2 DNA extraction

Digestate samples (2 mL) were taken from the reactors, snap-frozen in liquid nitrogen and stored at -80 ^oC. Frozen digestate (1-2 grams) was crushed to a fine powder under liquid nitrogen using a pestle and mortar. Three hundred milligrams of this frozen powder was then weighed into a frozen (liquid nitrogen) 2 mL cryotube containing Zirconia beads (0.3g of 0.1 mm and 0.1 g of 0.5 mm, Biospec Products Inc. Bartlesville, OK, USA). Heated extraction buffer (70 ^oC) was then added to the powder and DNA was extracted using a repeat bead beating method (Yu & Morrison, 2004).

A.3 Library preparation

Modified 16S rRNA gene Illumina adapter fusion primers were used to generate amplicon libraries. The primers were CaporasoNexF 5'TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG[GTGCCAGCMGCCGCGGTAA]3' and CaporasoNexR 5'GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG[GGACTACHVGGGTWTCTAAT]3'. The primer sequences outside the square brackets are partial Illumina adapters. The primer sequences inside the square brackets bind to the hypervariable (V4) region of the 16S rRNA gene in bacteria and archaea and are derived from the 16S binding sites of primers previously described by Caporaso et al. (2012). PCR was conducted using 20 ng of digestate DNA as a template and Kapa HiFi Hotstart ReadyMix (Kapa Biosystems, London, UK) according to the manufacturer's instructions. PCR conditions were: one cycle of 95 ℃ for 3 minutes, then 26 cycles of 95 ℃ for 30 seconds, 55 ℃ for 30 seconds, 72 ℃ for 30 seconds, followed by one cycle of 72 ℃ for 5 minutes. Amplicons were purified using the QIAquick PCR Purification Kit (Qiagen, Manchester, UK), eluted in 30 µL of buffer EB, and then measured for purity and quantity on a Nanodrop 1000. Two unique 8 bp indices were then added (one index at the 5' end of the amplicon and the other at the 3' end) to each amplicon in a second round of PCR using primers from the Illumina Nextera XT indexing kit. PCR was performed with 5 µL of each amplicon as a template and Kapa HiFi Hotstart ReadyMix. PCR conditions for this second round of PCR were: one cycle of 95 ℃ for 3 minutes, then 8 cycles of 95 ℃ for 30 seconds, 55 ℃ for 30 seconds, 72 ℃ for 30 seconds, followed by one cycle of 72 ℃ for 5 minutes. Indexed libraries were then purified using the Qiagen MinElute PCR Purification Kit (Qiagen, Manchester, UK), eluted in 18 µL of buffer EB, quantified on a Nanodrop1000, then combined in equal concentrations into 2 pools. Each pool was agarose gel-purified to remove primer/adapter dimers using the QIAquick Gel Extraction Kit (Qiagen, Manchester, UK), with an extra purification step used to remove residual agarose. The two pools of gel-purified libraries were then measured for purity and quantity on the Nanodrop 1000 and further quantified using the KAPA SYBR FAST Universal qPCR kit with Illumina Primer Premix (Kapa Biosystems, London, UK). The library pools were then diluted to 2 nM and denatured according to the Illumina MiSeq library preparation guide. 6 pM amplicon library was spiked with 30% denatured and diluted PhiX Illumina control library version 3 (12.5 pM). Two sequencing runs (one library pool per run) were conducted on the Illumina MiSeq using 500 cycle (2x 250 bp) MiSeq reagent kits (version 2) (Illumina, San Diego, CA, USA).

Reads from all samples were assessed to identify and remove sequencing adaptors and contiguous low quality bases using the bbmap package (BBMap - Bushnell B. - sourceforge.net/projects/bbmap/). Overlapping reads for each sample were merged using bbmerge (BBMap - Bushnell B. - sourceforge.net/projects/bbmap/) and amplicons of 292 bp (+/- 1.sd) were retained. The open reference calling method, implemented within the Quantitative Insights Into Microb. Ecol. (QIIME) software package, was used to generate operational taxonomic units (OTUs) across all samples. Sequences were clustered at a default similarity level of 97% and a single representative sequence from each OTU was used to align to the Greengenes database (version: gg_13_8) (Caporaso et al., 2010). Taxonomic classification for each OTU was determined with the Ribosomal Database Project (RDP) Classifier using a minimum confidence cut-off of 0.8. OTUs with <100 sequences summed across all samples were removed from the analysis.

Appendix B
Bacterial Standard Curve (OD vs CFU/ml)

B.1 Objective

To construct standard curves of OD versus CFU/ml for *Salmonella* Typhimurium 4,[5],12:i:-, *Salmonella* Typhimurium DT104 and *Salmonella* Derby. These curves will then be used to read off the count of *Salmonella* contained in each washed *Salmonella* suspension for future experiments.

B.2 Procedures

1. Each *Salmonella* serotype (Typhimurium 4,[5],12:i:-, Derby &Typhimurium DT104) will be streaked from stock and grown overnight at 37 ^{o}C on nutrient agar. They will then be sub-cultured (i.e. re-streaked) onto nutrient agar and incubated overnight at 37 ℃
2. A single colony from each culture plate will be inoculated into separate 5ml volumes of BHI broth.
3. All 3 cultures of *Salmonella* will be incubated at 37 ^{o}C for 18 hours (together with a control of 5mL BHI broth).
4. Using a pipette, 1.5mL of each cultured bacteria is transferred into separate sterile eppendorfs and centrifuged at 13,000 rpm for 2 minutes.
5. The supernatant is discarded and the pellet washed with 1.5mL maximum recovery diluent (MRD) and vortex to re-suspend the pellets.
6. The re-suspended pellets will then be centrifuged at 13,000rpm for 2 minute.
7. Steps 5 and 6 will be repeated.
8. Pellets are again re-suspended in1.5mL MRD – known as NEAT (Sample A)
9. A series of dilutions will be made from Sample A:
    (1) 1/2 dilution – sample B (500 µl of neat & 500 µl of MRD)
    (2) 1/5 dilution – sample C (200 µl of neat & 800 µl of MRD)
    (3) 1/10 dilution – sample D (100 µl of neat & 900 µl of MRD)
    (4) 1/15 dilution – sample E (100 µl of neat & 1.4 ml of MRD)
10. The optical density (OD 600) of samples A-E will be measured.
11. Serial dilutions (10^{-1} – 10^{-8}) for samples A-E will be performed induplicate.

12. Dilutions will be labelled appropriately: A1-A8, B1-B8, C1-C8, D1-D8, E1-E8; A1'-A8', B1'-B8', C1'-C8', D1'-D8', E1'-E8'.

13. Using 100 µl of the appropriate dilution, the following samples will be spread plated onto nutrient agar:

(1) Sample A: A4-A8, A4'-A8'
(2) Sample B: B3-B7, B3'-B7'
(3) Sample C: C2-C6, C2'-C6'
(4) Sample D: D1-D5, D1'-D5'
(5) Sample E: E0-E4 , E0'-E4'

14. All plates will be incubated at 37 ^{o}C for 18 hours.

15. After incubation, colonies formed in each plate will be counted (30-300 colonies).

16. A graph of OD versus CFU/ml will be constructed for each *Salmonella* serotype.

17. Each curve will be done in triplicate for each serotype.