**Biogas Production, Waste Stabilization, and Microbial Community
Physiological Changes during Anaerobic Co-digestion of Swine Manure and
Corn Stover**

by

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ABSTRACT

BIOGAS PRODUCTION, WASTE STABILIZATION, AND MICROBIAL COMMUNITY PHYSIOLOGICAL CHANGES DURING ANAEROBIC CO- DIGESTION OF SWINE MANURE AND CORN STOVER

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This thesis is an investigation of the use of anaerobic co-digestion (AcoD) of swine manure (SM) and corn stover (CS) to produce biogas and reduce the environmental health risk of manure. Laboratory-scale mesophilic (38 °C) batch AcoD of different ratios of SM and CS were conducted to measure cumulative methane potential, organic matter reduction, hygienization efficiency, and microbial metabolic adaptation in response to increasing total solids (TS). The results revealed the highest methane potential and greatest organic matter reduction for SM mono-digestion. These same parameters were measured during long-term mesophilic AcoD in a semi-continuously fed auger plug-flow reactor (PFR), with a step-wise increase of organic loading rate (OLR) from 0.25-4.7 kg volatile solids fed (VS) m⁻³ d⁻¹. The results revealed the highest biogas potential and organic matter reduction at the lowest OLR. Hygienization efficiency was low in both experiments and lignocellulosic degradation increased in response to increased TS content.

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List of Abbreviations and Nomenclature

Abbreviations

AD	Anaerobic digestion
AcoD	Anaerobic co-digestion
TS	Total solids
VS	Volatile solids
BMP	Biochemical methane potential
COVID-19	Coronavirus disease 2019
USDA	United States Department of Agriculture
EU28	European Union (28 countries)
ASF	African Swine Fever
PEDV	Porcine Epidemic Diarrhea Virus
TGEV	Transmissible Gastroenteritis Virus
SADS-CoV	Swine Acute Diarrhea Syndrome Coronavirus
PDCoV	Porcine Deltacoronavirus
SAF	Saskatchewan Agriculture and Food
AAFC	Agriculture and Agri-Food Canada
FEMS	Farm Environmental Management Survey
EFP	Environmental Farm Plan
GHG	Greenhouse Gases
HRT	Hydraulic Retention Time
VFA	Volatile Fatty Acids
PFR	Plug Flow Reactor
TAN	Total Ammonia Nitrogen
CSTR	Continuously Stirred Tank Reactor
LBR	Leach Bed Reactor
ASBR	Anaerobic Sequencing Batch Reactor
SaBR	Self-Agitated Anaerobic Reactor
MBR	Membrane Bioreactor
TOC	Total Organic Carbon

TN	Total Nitrogen
OLR	Organic Loading Rate
TBP	Total Biogas Potential
TMP	Total Methane Potential
COD	Chemical Oxygen Demand
sCOD	Soluble Chemical Oxygen Demand
S/I	Substrate to Inoculum Ratio
CPE	Chlorinated Polyethylene
SM	Swine Manure
PS	Pig Slurry
NBW	Nanobubble Water
AAS	Aqueous Ammonia Soaking
w/w	% by weight
v/v	% by volume
MEC	Microbial Electrolysis Cell
MW-A	Microwave-Alkaline
WW	Waste Water
LMC	Lignocellulolytic Microbial Consortium
LFD	Liquid Fraction of Digestate
RS	Rice Straw
CS	Corn Stover
DS	Durian Shell
ABP	Animal By-Product waste
CF	Chicken Feathers
CR	Cucumber Residues
RG	Residual Glycerin
ESBP	Exhausted Sugar Beet Pulp
FW	Food Waste
DGGE	Denaturing Gradient Gel Electrophoresis
qPCR	Real-Time Polymerase Chain Reaction
SAO	Synrophic Acetate Oxidizing

OMAFRA	Ontario Ministry of Agriculture, Food, and Rural Affairs	
w.w	Wet Weight	
d.w	Dry Weight	
CFU	Colony Forming Unit	
MPN	Most Probable Number	
FIB	Fecal Indicator Bacteria	
AW	Agricultural Waste	
CLPP	Community Level Physiological Profiling	
AN	Anaerobe	
DNS	3,5-Dinitrosalicylic Acid	
EC	Electric Conductivity	
PBS	Phosphate Buffered Saline	
PVDF	Polyvinylidene Fluoride	
FOS/TAC	Volatile Fatty Acid to Alkaline Buffering Capacity Ratio	
TBX	Tryptone Bile X-glucuronide	
VRBA	Violet Red Bile Agar	
KAA	Kanamycin Aesculin Agar	
TSC	Tryptose Sulfite Cycloserine	
AWCD	Average Well Colour Development	
CMC	Carboxymethyl Cellulose	
FP	Filter Paper	
PCA	Principal Component Analysis	
VBNC	Viable but Non-Culturable	
spp.	Species	
PICRUSt	Phylogenetic Investigation of Communities by Reconstruction of States	Unobserved
ANOVA	Analysis of Variance	
PC	Principal Component	
MF	Membrane Filtration	
n.d	Not Determined	
n.a	Not Applicable	

Nomenclature

CaCO ₃	Calcium carbonate
C	Carbon
N	Nitrogen
C/N	Carbon to nitrogen ratio
P	Phosphorous
Mn	Manganese
Fe	Iron
Co	Cobalt
Cu	Copper
Mo	Molybdenum
Ni	Nickel
Se	Selenium
W	Tungsten
Ti	Titanium
CH ₄	Methane
CO ₂	Carbon dioxide
H ₂ S	Hydrogen sulfide
H ₂	Hydrogen
NH ₃	Ammonia
NH ₄ ⁺	Ammonium
NH ₄ -N	Ammonium nitrogen
NaOH	Sodium Hydroxide
KOH	Potassium Hydroxide
CaO	Calcium Oxide
Ca(OH) ₂	Calcium Hydroxide
MgO	Magnesium Oxide
Fe ₂ O ₃	Iron (III) Oxide
R	Substrate Richness
C _i	Absorbance of Carbon Substrate

n	Number of Carbon Substrates
H'	Shannon-Weiner Index (Shannon Diversity)
E	Shannon Evenness
P _i	Absorbance Ratio of Carbon Substrate to all Carbon Substrates
S	Number of Oxidized Carbon Substrates

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Chapter 1

Anaerobic Digestion of Swine Manure: Current Status and Perspective

1.1 Introduction

The increase in worldwide demand for meat over the past two decades has resulted in increased environmental pressure due intensified livestock farming and production operations. In particular, global pork production has increased by more than 31 % in the last 20 years from 83.6 million tonnes in 1998 to 109.8 million tonnes estimated for 2019 alone (Cameron, 2000; FAO, 2020). According to the Foreign Agricultural Service of the United States Department of Agriculture (USDA), global pork exports as of April 2020 increased by approximately 14% compared to 2019 (USDA, 2020). From the same report, China and the European Union have produced almost 34 million and 24 million metric tons of pork, respectively, which exceeds the total production of the next 4 leading pork producers combined. In light of the emergence of African Swine Fever (ASF) particularly in Asian pig populations and the ongoing COVID-19 pandemic, global pork production is forecasted to decrease by 8 % compared to 2019 (FAO, 2020). Despite the economic and public health setbacks, global trade is expected to increase by 11.2 % due to increased Chinese imports which has spurred increased exports from other countries such as the USA, the European Union, and the United Kingdom of Great Britain and Northern Ireland (FAO, 2020).

The steady increase in demand for animal-based protein globally translates to larger and more concentrated livestock production operations. As a result of larger animal concentrations, these operations must handle substantial amounts of manure and other excreta. In the context of the Canadian pork industry, there are more than 12.63 million pigs reported on Canadian farms as of the beginning of 2020 and an annual estimation of 15.8 million tonnes of manure produced (Canadian Pork Council, 2020; Statistics Canada, 2008). Currently there are 7,650 Canadian pig farms averaging 1,814 hogs per farm (Canadian Pork Council, 2020), highlighting the trend of decreasing farm numbers while intensifying pig production (Fig. 1.1). According to Saskatchewan Agriculture and Food (SAF), a single sow from a farrow-to-finish operation can produce enough

manure to fertilize 1 ha of land annually (SAF, 2003). Using this general rule of thumb and available Canadian pig herd/farm statistics (Fig. 1.1), one can roughly calculate that 14 million ha of land could be fertilized with swine manure alone.

Fig. 1.1. Number of Canadian pig farms and pigs from 1921-2011. Number of Canadian pig farms (in thousands) reported alongside number of pigs (in millions) over the 90-year period. Adapted from Brisson (2014).

The inverse relationship between the number of reported pig farms and pig population numbers highlight the growth of larger farm operations, which in turn results in higher waste concentrations for individual farms. This abundance of waste puts a larger burden on farming operations since over application of manure can have severe environmental impacts. Environmental hazards include unpleasant odours, attraction of pests/nuisance animals, animal pathogen dissemination, excess nutrient runoff into surface and ground water sources, and greenhouse gas (GHG) emissions (Nasir et al., 2014; Sakar et al., 2009). With these concerns in mind, industry stakeholders must come up with on-farm strategies to manage swine and other livestock wastes. In a 2011 Farm Environmental Management Survey (FEMS) conducted by Statistics Canada and Agriculture and Agri-Food Canada (AAFC), only 35 % of Canadian farms had a formal Environmental Farm Plan (EFP) (Statistics Canada, 2013). This is a voluntary written assessment that addresses environmental/on farm risks in addition to an outlined action plan detailing beneficial management practices (Haak, 2016). The other two thirds of Canadian farms did not report having any EFP or beneficial management practices in place for several reasons (Fig. 1.2), which demonstrates the

urgent need to prepare a management plan to mitigate any potential environmental hazards arising from manure accumulation and handling.

Fig.1.2. Main reasons for not implementing best management practices on Canadian farms. Percentages may not sum up to 100 due to rounding. Adapted from Statistics Canada (2013).

Anaerobic digestion (AD) is a promising and advantageous technology that has been implemented in treating livestock manure as well as other organic waste streams such as wastewater, food waste, and agricultural crop residues (Mao et al., 2015). The process mitigates the various previously discussed environmental hazards associated with manure while producing biogas during microbial degradation of organic matter in non-oxygen conditions, which can be used as fuel or for heating and electricity (Mao et al., 2015; Nasir et al., 2012). In general, the main objectives of anaerobic digestion are the following: maximize volatile solids (VS) degradation while simultaneously maximizing biogas yields. Further, shorter hydraulic retention times (HRT) help minimize reactor volume and settling of solids (Nasir et al., 2012; Ward et al., 2008).

While AD has shown promise for waste reduction and energy production (Sawatdeenarunat et al., 2015), reluctance to implement these designs on-farm stem from high start-up and operational capital requirements (Massé et al., 2013). In Canada, the low rate of AD adoption is exemplified by the small number of biogas plants utilizing swine manure as a feedstock. For example, only two biogas plants in Ontario use swine manure as a substrate (Canadian Biogas Association, 2020). Although previous research has addressed AD optimization

and development to enhance stability, there is little research on studying multiple aspects (such as reactor design, operational parameters, etc.) affecting the process (Nasir et al., 2012; Neshat et al., 2017), particularly in the context of swine manure AD. With these research gaps in mind, it is necessary to conduct an overall review and assessment of current AD techniques for biogas production from swine manure in order to optimize biogas production performance. The objective of this paper is to provide a comprehensive overview of recent swine manure AD achievements which could serve as a basis for future research in AD optimization. Detailed information on swine manure AD operations, pretreatment/enhancement strategies, microbial community dynamics, and hygienization will be discussed to highlight the future outlook of swine manure AD as a means of both sustainable energy production and waste management.

1.2 Anaerobic digestion of swine manure

1.2.1 Swine manure characteristics

The physiochemical composition of swine manure depends on the farming operation, animal feed diet, water consumption, and seasonal variations (Boursier et al., 2005). In conventional North American swine production, manure is flushed from animal pens with water, which results in diluted organic matter and solids (Bourque and Koroluk, 2003; Key et al., 2011). The general swine manure characteristics are outlined in Table 1.1 and are from studies from selected regions of the world to reflect the compositional variations. Important chemical parameters such as pH, total solids (TS), volatile solids (VS), and the carbon: nitrogen ratio (C/N) strongly impact the biogas potential of swine manure (Neshat et al., 2017). From the selected studies, generally the TS content is 10 % or lower which would be considered as a substrate for wet AD (Qian et al., 2019). Total solids content of 7-10 % is generally optimal for biogas production due to the higher moisture content, which limits accumulation of volatile fatty acids (VFA) that can be detrimental to methanogenic activity (Chandra et al., 2012). The large water content also demonstrates the buffering capacity (measured as CaCO_3 equivalents) against both VFA and CO_2 accumulation which can otherwise increase acidity and decrease biogas production.

Table 1.1.Swine manure characterization from selected studies in different countries.

Country	TS (%)	VS (%)	pH	TN (%)	NH ₄ -N (g L ⁻¹)	TP (%)	C/N	Cellulose (%)	Hemicellulose (%)	Lignin (%)	Reference
Canada	6.20- 7.20	4.77- 5.63	6.88- 7.33	0.59- 0.66	4.1-5.1	-	-	-	-	-	Massé et al. (2011)
China	29.53	21.03	7.67	3.38	-	-	11.70	12.33	27.64	2.22	Wang et al. (2020b)
Denmark	2.14	1.54	8.03	2.17	1.63	-	5.21	-	-	-	Tsapekos et al. (2017)
USA	13.20	10.12	8.08	3.49	-	-	-	-	-	-	Gautam et al. (2017)
Italy	4.90	3.06	-	0.43	-	0.17	-	-	-	-	Mussoline et al. (2012)
Bolivia	30.8	17.6	9.20	0.77	-	0.68	-	-	-	-	Alvarez and Lidén (2008)
Thailand	35.78	26.26	8.43	1.59	7.97	-	-	6.37	9.28	5.43	Tuesorn et al. (2013)

TS: total solids, VS: volatile solids, TN: total nitrogen, NH₄-N: ammonia nitrogen, TP: total phosphorous, C/N: carbon to nitrogen ratio

The low C/N ratio of swine manure reflects the high nitrogen and crude protein content (~22 % as reported by Gautam et al. (2017)) which makes this substrate suitable for AD, as the methane potential of proteins results in higher biogas yields (Hagos et al., 2017). Although lignocellulosic content was not measured in most swine manure AD studies outlined in Table 1.1, it can be seen that its manure composition varied depending the growth stage of the animal, feed, and the amount and type of bedding material (Møller et al., 2004). Tuesorn et al. (2013) and Wang et al. (2020b) reported the cellulose, hemicellulose, and lignin content was in the range of 7-12, 9-28, and 2-5 %, respectively. The polymeric nature of cellulose, comprised of unbranched D-glucose units linked by β -1,4 covalent bonds, allows it to be a potential energy source for microbial populations and thus suitable for AD (Sawatdeenarunat et al., 2015). Hemicellulose, a branched heteropolysaccharide polymer consisting of different C-5 and C-6 sugars, also provides sources of fermentable sugars for microbial consumption and subsequent conversion to methane (Sawatdeenarunat et al., 2015). While the overall lignocellulosic content in swine manure is lower compared to lignocellulosic biomass such as agricultural crop residues (Sawatdeenarunat et al., 2015), its presence in manure contributes to methane production during AD.

Swine manure typically contains sufficient concentrations of micronutrients to support microbial growth and enzymatic activities (Cestonaro do Amaral et al., 2014). Trace metals essential for AD that are found in swine manure include Mn, Fe, Co, Cu, Mo, Ni, Se, and W which are important cofactors for enzyme complexes responsible for methanogenic activity (Thanh et al., 2016). Overall, swine manure can be used as a sole substrate for AD and has shown considerable performance in terms of biogas production, process stability, and waste stabilization (discussed in Section 1.2.3). It should be noted, however, that feedstock source/composition and process parameters such as temperature, organic loading rate (OLR), hydraulic retention time (HRT), and reactor configuration dictates process performance – highlighting the sensitivity and complexity of the four-stage biochemical process (Neshat et al., 2017; Xie et al., 2017).

1.2.2 Anaerobic reactor configurations

Over the past two decades, there has been increased research and attention committed towards reactor configuration development due to the complexity of the anaerobic digestion process relating to the feedstock source/composition (Kondusamy and Kalamdhad, 2014; Neshat

et al., 2017; Xie et al., 2017). In particular, the presence of complex polymeric substrates such as lignocellulosic material in swine manure and TS content can decrease the hydrolysis rate and greatly impacts overall reactor performance in terms of stability and operational costs (Kondusamy and Kalamdhad, 2014). In addition to anaerobic reactor design, OLR and HRT are independent parameters which must also be considered with respect to reactor design and are adjusted according to the feedstock (Castellano-Hinojosa et al., 2018). Recent literature regarding swine manure AD performance and associated operating conditions are outlined in Table 1.2 which will be discussed in this section with respect to design configuration. Anaerobic digestion performance in terms of biogas production and organic matter removal efficiency will be discussed in section 1.2.2.

A commonly used reactor configuration in both household and commercial operations is the one-stage batch reactor which operates in a fill and draw manner with all stages of anaerobic digestion taking place in a single tank/basin (Castellano-Hinojosa et al., 2018; Mao et al., 2015). This design is popular due to its simplistic operation, handling of different wastewater concentrations, low-flow application, low energy input as a result of minimal mechanical requirements, and low capital investment (Mao et al., 2015). While one-stage batch reactors can handle different wastewater concentrations and are suitable for swine manure due to its lower TS content (Table 1.1), clogging becomes an issue as TS content is increased which results in biogas fluctuations and decreased yields therefore this reactor is more suitable for wet (i.e low TS) AD. To compensate for the low organic content of swine manure, larger reactor volumes are needed which can increase the capital cost of the system, increased energy consumption due to the high moisture content of swine manure, and poor biomass retention leading to washing out of microorganisms.

A better reactor design and AD operation compared to one-stage batch reactors is the continuously stirred tank reactor (CSTR) which can be operated as either batch or semi-continuously/ continuously fed. These systems are commonly operated as either single or two-stage reactors, although multi-stage reactors have been proposed and demonstrated higher digestion efficiency (Castellano-Hinojosa et al., 2018) although these systems have not been employed for swine manure AD. In general, CSTRs intermittently or continuously mix substrates and microorganisms which increases substrate-microorganism contact but requires higher energy inputs for both mixing and maintaining reactor temperature (Mao et al., 2015). Like batch reactors, they are suitable for handling low TS content wastes which is generally suitable for swine manure

but clogging and mass transfer issues are prevalent above 10% TS (Qian et al., 2019; Sawatdeenarunat et al., 2015). Single-stage CSTR is simplistic in design but reactor performance efficiency decreases in terms of effluent quality, whereas two-stage CSTRs can improve performance due to separation of anaerobic digestion into the acidogenic and methanogenic stages (Mao et al., 2015). Two-stage CSTR operation, however, is more sensitive to feedstock composition, require strict biochemical conditions to efficiently operate, and a higher tendency of microorganism washout of the system – similar to one-stage CSTR operation. To improve the organic matter reduction efficiency and biogas production performance of CSTRs, leach bed reactors (LBR) have been coupled with CSTRs due to their ability to retain microorganisms and promote mass transfer which allows for higher TS content processing (Yang et al., 2019b). While two-stage CSTR AD yields better process stability and overall reactor performance, one-stage CSTR is more commonly used for full-scale biogas plants due to the simpler operational design and lower operational requirements (Castellano-Hinojosa et al., 2018; Rapport et al., 2012).

Membrane bioreactors (MBR) and other anaerobic filter reactors were developed as a means for increasing solid mass and microorganism retention to enhance biodegradability and minimize microbial washout. The advantage of this system is decoupling of HRT and sludge retention time (SRT) which retains microbial biomass more effectively, resulting in better organic matter removal and higher energy recovery through increased biogas production and digestate reuse (Jiang et al., 2020a). Literature on MBR operation for swine manure is limited due to membrane fouling issues resulting from increase microbial colonization and organic pollutant accumulation (Jiang et al., 2020a). Membrane bioreactor operation is still underdeveloped compared to other reactor configurations and is sensitive to high TS and OLR which contributes to membrane fouling, membrane clogging, and decreased biogas production (Chen et al., 2016).

Anaerobic baffled reactors (ABR) are a type of sludge contact reactor consisting of separated compartments in one reactor which is baffled to force influent feedstock through blanketed sludge series (Mao et al., 2015). This design has been previously implemented for swine wastewater AD due to its non-requirement of mixing, longer microbial biomass retention, tolerance to OLR fluctuations, and lower risk of clogging and sludge bed expansion (Jiang et al., 2019b; Mao et al., 2015). Due to longer microbial biomass retention, HRT and SRT can be decoupled in a similar manner to MBRs which increases organic matter removal efficiency and potentially can increase TS loading into the system. Disadvantages to ABRs and their use at full-

scale include inadequate mixing and lower settle-ability of microbial biomass which results in some microbial washout (Mao et al., 2015).

Anaerobic plug flow reactors (PFR) are considered as conventional designs like CSTRs and one-stage batch reactors. Compared to these conventional designs, PFRs are advantageous due to simpler design configuration and minimal to no mixing requirements, owing to its relatively low energy input during operation (Lansing et al., 2010). Plug flow reactors are designed to separate the different anaerobic digestion steps without having multiple separated units like some CSTRs which results in shorter start-up and tolerance of TS content > 10 % which results in smaller reactor volumes (Dong et al., 2019; Patinvoh et al., 2017). Conversely, PFR performance is less efficient at lower TS loading due to thermal stratification, solid sedimentation, and floating issues which result in decreased biogas production and lower organic matter removal efficiency (Gómez et al., 2019). These issues are amplified due to no internal agitation or mixing built into the traditional PFR design (Mao et al., 2015), however, effluent recirculation using biogas injection (Gómez et al., 2019) and simple mixing components such as an auger conveying mechanism (Veluchamy et al., 2019) have demonstrated alleviation of these particular issues.

1.2.3 Biogas production from swine manure

Anaerobic digestion is a microbial driven process in which organic matter is degraded and converted into a biogas mixture comprising of mainly methane (CH_4), carbon dioxide (CO_2) and other trace gases like hydrogen sulfide (H_2S) and ammonia (NH_3) in the absence of oxygen. This process is advantageous and has gained popularity due to the wide range of feedstocks such as sewage sludge, food waste, and agricultural waste which can be used to produce biogas (Li et al., 2016; Mao et al., 2015; Neshat et al., 2017). Along with energy-rich biogas, the AD process produces nutrient-rich digestate which can be used a fertilizer for crop application and recycling of essential nutrients back to agricultural land (Iocoli et al., 2019; Ning et al., 2019).

The energy potential of biogas and its extraction from different organic waste streams has made this technology popular both on a small-scale (i.e. household) and large-scale such as for on-farm implementation (Bond et al., 2011; Li et al., 2016). In the US for example, there are 288 on-farm AD plants in use/under construction; however, swine manure AD plants only make up about 15% of on-farm plants either in use or under construction. In addition, a smaller proportion of

these plants (12.5%) use swine manure as the sole feedstock (EPA, 2020). In Sweden, the amount of on-farm biogas plants is significantly smaller compared to the US, however, around 11% of those plants use swine manure as the sole feedstock and about 44% use swine manure or swine manure digested with another feedstock (Ahlberg-Eliasson et al., 2017). In the European Union (EU28), it was estimated that around 16.1 billion m³ of biogas can be realistically extracted from livestock manure as of 2015 and depending on the age of the pig, methane yields per head per year are estimated to be 2.6-57.8 m³ (Scarlat et al., 2018). Swine manure AD has been conducted using different operational modes and optimized based on OLR, temperature, HRT, and TS content in influent feedstock (Kanani et al., 2020; Li et al., 2016; Nasir et al., 2012;). Since much research has been conducted in the area of mono-digestion, Table 1.2 displays the most recent literature that previous literature reviews did not cover. Specifically, research from 2019-2020 will be discussed in this section.

The total biogas potential and total methane potential (TBP and TMP, respectively) of swine manure depends on its chemical composition which is influenced by feedstock source and feeding diet of swine. For example, Kafle et al. (2016) reported the highest biochemical methane potential (BMP) from mesophilic AD of swine manure (323 mL g⁻¹ VS) compared to other livestock manures. Raw swine manure had a high level of crude protein compared to other manures, resulting in a higher total nitrogen (TN) content of 1116 mg L⁻¹. During the AD process, crude protein degradation resulted in an increase in total ammonia nitrogen (TAN) from 510 to 620 mg L⁻¹ which was well below inhibitory concentrations of around 1700-1800 mg L⁻¹ (Yenigün and Demirel, 2013). The study by Kafle et al. (2016) and most studies mentioned in Table 1.2 investigated mesophilic AD, which has better process stability in terms of lower TAN, VFA, and higher biogas production, compared to thermophilic AD (Yenigün and Demirel, 2013). The variability in effluent TAN concentrations reported by different researchers relates back to pork production operations such as growth stage of the animal, feed, and the amount and type of bedding material (Møller et al., 2004). Yenigün and Demirel (2013) suggested that most AD processes operating under mesophilic conditions (30-40 °C) with an acclimated inoculum can withstand elevated TAN concentrations up to 5000 mg L⁻¹, although reactor conditions and type dictates process tolerance (Hassan et al., 2017). Luján-Facundo et al. (2019) reported very low biogas potential despite good organic reduction efficiency during semi-continuous AD in a CSTR, highlighting the limited organic carbon available for biogas production (C/N of 3) which resulted

Table 1.2. Anaerobic digestion of swine manure with different operating conditions and reactor types.

Reactor Type	Temp. (°C)	OLR	Influent TS (%)	HRT (d)	VS/COD removal (%)	TBP	TMP	TAN (g L ⁻¹)	Reference
0.5 L (batch)	37	-	8.00	46	58.00	-	0.288	-	Lu et al. (2019)
20 L CSTR (semi- continuous)	35±1	1.89	5.00	22	66.22	-	0.438	~ 1.20	Duan et al. (2019)
235 L PFR (semi- continuous)	34	1.61	6.81	27	46.47	-	0.374	2.70	Gómez et al. (2019)
15 L LBR + 40 L CSTR (batch)	35 ± 1	-	21.68	17	90.88	0.259	0.167	0.61	Yang et al. (2019b)
2 L (semi- continuous)	35 ± 1	4.71 (TS basis)	28.00	60	47.00	-	-	3.18	Xiao et al. (2019)
2 L CSTR (semi- continuous)	37	-	0.80	28	59.87	0.086	-	~ 1.00	Luján-Facundo et al. (2019)

16 L SaBR (semi- continuous)	25	-	1.18	10	79	-	0.610	-	Jiang et al. (2019b)
1 L (batch)	37	-	10.30	120	70.72	-	0.470	~4.50	Cao et al. (2020)
1 L (batch)	50	-	8.00	23	31.76	0.562	0.325	3.01	Wu et al. (2020)
13 L MBR	35 ± 2	4.70	0.71	1	40	-	0.350	0.600	Jiang et al. (2020a)

OLR: organic loading rate ($\text{kg VS or COD m}^{-3} \text{ d}^{-1}$), TS: total solids, VS: volatile solids, COD: chemical oxygen demand, TBP: total biogas potential ($\text{m}^3 \text{ kg}^{-1} \text{ VS or COD}$), TMP: total methane potential ($\text{m}^3 \text{ kg}^{-1} \text{ VS or COD}$), TAN: total ammonia nitrogen, CSTR: continuously stirred reactor, PFR: plug flow reactor, LBR: leach bed reactor, MBR: membrane bioreactor, SaBR: self-agitated anaerobic reactor

in methane content of between 32-60%. These results and those reviewed by Mao et al. (2015) demonstrate that a balanced C/N (between 20-30) is necessary for stable process performance, where C is going towards methane (and CO₂) production while N supports microbial growth and metabolism. Cao et al. (2020) and Gomez et al. (2019), however, reported significantly higher nitrogen, in the form of NH₄⁺-N, even though the total methane potential (TMP) was significantly higher compared to the BMP study conducted by Kafle et al. (2016). In both studies, pH was slightly on the alkaline side (7.8-8.0) which could explain the higher TAN content since increased pH shifts the NH₄⁺-NH₃ equilibrium toward the toxic NH₃ form (Rajagopal et al., 2013).

Based on the select investigations into swine manure AD in Table 1.2 and reviewed by Nasir et al. (2012), most reactor designs are either CSTR or anaerobic sequencing batch reactors (ASBR) consisting of one or more tanks. Batch (including ASBR) and CSTRs, whether it be single or multi-tank reactors with (semi)-continuous operation, are popular for swine manure AD due to their relatively simplistic design, ease of use, and mixing capabilities (with respect to CSTR) which allow for better substrate-microorganism contact (Mao et al., 2015). Both reactor types are particularly common and popular for low TS waste (<10 %) treatment since mixing capabilities are not impeded and little mass transfer resistance occurs, leading to better biogas yields (Gómez et al., 2019; Mao et al., 2015). Although these systems are commonly used on small and large-scale operations, they are limited to only handling low TS content waste since mixing capabilities become impeded when semi or high solid AD is performed, resulting in process instability (Qian et al., 2019; Sawatdeenarunat et al., 2015). The high moisture content of swine manure also leads to increased energy consumption to maintain reactor temperature at mesophilic and especially thermophilic temperatures, while also requiring a larger reactor volume to compensate for the low solid content of manure in order to produce sufficient biogas quantities (Mao et al., 2015).

Dry fermentation (TS > 20 %) has gained increased attention due to decreased water usage, relatively easier operation, improved energy recovery, high biogas yields, and high slurry concentration (Chen et al., 2015; Hu et al., 2019). Xiao et al. (2019) and Yang et al. (2019b) worked in dry AD conditions, with considerable biogas yields reported in a combined leach bed LBR + CSTR system due to lower TAN concentrations and increased VFA consumption which imparted process stability. However, semi-continuous AD in a CSTR system at 28% TS led to total VFA accumulation up to around 3500 mg L⁻¹ (Xiao et al., 2019) which is well above the inhibitory threshold of 1500-2000 mg L⁻¹ for most AD studies concerning livestock manure and

other agricultural waste (Neshat et al., 2017). Stable biogas production was reported ($1.67 \text{ L L}^{-1} \text{ d}^{-1}$), however, as a result of higher resistance to VFA accumulation.

Despite demonstrated stable reactor performance in terms of biogas production and organic matter reduction efficiency during dry AD, the literature on this process is relatively scarce compared to wet AD (Patinvoh et al., 2017). Another knowledge gap is at large scale operations ($>700 \text{ m}^3$) since most research has been conducted in lab or small-scale batch and CSTR systems which is not always suitable for evaluating large scale operations (Dong et al., 2019a). In summary, optimization of the dry AD process for swine manure should be explored in different reactor types which are more suitable for handling higher TS content at small-scale. Increasing OLR and reducing HRT should be conducted in laboratory -scale reactors to maintain reactor prior to scaling up to larger operations.

1.3.1 Swine manure pretreatment strategies

An inherent issue of livestock manure is the high undigested fiber content which impacts anaerobic digestion efficiency due to limited utilization by anaerobic bacteria (Yu et al., 2017). In particular, lignocellulosic fibers can make up about 40 % of the dry matter content found in swine manure which highlights the necessity of employing treatment strategies to break down these materials (Carlsson et al., 2012). Another issue with mono-digestion of swine manure is the low C/N ratio which leads to low biogas production due to limited carbon availability for microbial consumption and increased NH_3 production which impedes microbial growth- both factors leading to process failure (Neshat et al., 2017). While there are different enhancement strategies to increase biogas production potential and in turn improve overall process stability, pretreatment strategies are a common practice for increasing the solubility of recalcitrant compounds such as cellulose and hemicellulose to be used for microbial consumption. Pretreatment strategies can be broadly categorized into three groups: chemical, physical, and biological pretreatment (Neshat et al., 2017). This section will cover recent literature in the context of swine manure pretreatment strategies to enhance AD process performance and will include literature from 2015 up to now not covered by previous reviews (Carrère et al., 2016; Li et al., 2019; Nasir and Gazi, 2015).

1.3.2 Chemical pretreatment

This pretreatment category utilizes acids, alkalis, and oxidants to improve degradation and extraction of organic compounds (Ariunbaatar et al., 2014; Jain et al., 2015). In particular, alkali pre-treatments are the preferred method since generally AD requires adjustment of pH towards the alkaline range (Li et al., 2012). Chemical reagents aim to increase solubilization and degradation of lignin which increases surface area and available substrates for microbial consumption owing to increased biogas production (Mao et al., 2015). Table 1.3 highlights recent investigations into different chemical pretreatment strategies for improving methane production and biodegradability of swine manure.

Other commonly used alkali reagents such as KOH, CaO, and MgO have also improved swine manure biodegradability (Mao et al., 2015; Yuan et al., 2017). In a study on enhancing nutrient availability during thermophilic dry AD, Yuan et al. (2017) utilized four different alkali reagents (NaOH, KOH, Ca(OH)_2 , and CaO). While Ca(OH)_2 exhibited the largest nitrogen (N) mineralization, VFA production, and soluble total organic carbon (TOC) increase, it was found that NaOH and KOH improved carbohydrate and protein solubilization the most. Overall, Ca(OH)_2 pretreatment had the largest positive impact on dry swine manure AD due to increased hydrolysis, however, biogas production performance was not investigated. The efficacy of NaOH pretreatment in the context of biogas production impact was investigated in mesophilic batch digesters in relation to the optimal substrate to inoculum (S/I) ratio for maximum process performance (Ortega-Martinez et al., 2016). Different temperatures (35 and 100 °C), treatment times (5, 18, and 30 min), and NaOH concentrations (0.45 and 2.25 % NaOH TS basis) had little impact on TMP compared to untreated pig slurry, although most treatments increased the maximum production rate. The results suggested that dilute sodium hydroxide concentrations led to improved maximum production rates which could in turn mean smaller reactor volumes and shorter retention times to obtain high TMP. It was noted though that the potential productivity during full-scale continuous AD may not change a lot due to the lower TMP associated with this pretreatment (Ortega-Martinez et al., 2016). Some disadvantages with using salt hydroxides include their corrosiveness and with NaOH loss of carbohydrates accumulation of sodium ions can lead to methanogen inhibition and subsequently lower TMP (Carrère et al., 2016).

Table 1.3.

Summary of different chemical pretreatments for enhanced anaerobic digestion of swine manure.

Feedstock	Reactor Type	Pretreatment type	Pretreatment Conditions	AD conditions	TMP	TMP (%) improvement	Outcome	Reference
SM	0.25 L (semi-continuous)	Zeolite addition	Oven drying of CPE bedding for 12 h, zeolite fixation to CPE	35 °C; 7 d HRT; 1.01 kg COD m ⁻³ d ⁻¹	0.212	180	83 %↑ CH ₄ content 37-43 % ↓ effluent NH ₄ ⁺	Zheng et al. (2015)
SM fibers + SM	3 L CSTR (semi-continuous)	Aqueous ammonia soaking (AAS)	32 % (w/w) NH ₃ for 3 d at 22 °C	38 °C; 25 d HRT	0.25	98	52 %↑ soluble COD; 10 % ↑ soluble glucose	Jurado et al. (2016)
PS	0.1 L (batch)	Weak thermal alkali (wTA)	100 °C for 30 min; 0.0045 g NaOH g ⁻¹ TS	37 °C; 35 d; S/I: 0.25; 5.54 % TS	-	-	57 % ↑CH ₄ production rate; ~10% ↓TMP	Ortega-Martinez et al. (2016)

SM	0.1 L (batch)	6 M Ca(OH) ₂	Stirred with SM	55 °C; 18d; 7.76 kg VS m ⁻³ d ⁻¹	-	-	88 %↑ soluble TOC; 71-105 %↑ soluble protein; 189 % ↑ soluble carbohydrates; 14 % ↑ N mineralization	Yuan et al. (2017)
SM	0.1 L (batch)	NH ₃ fermentation + stripping	fermentation for 7 d at 55 °C + 55 °C air stripping at for 24 h at 50 ml min ⁻¹	35 d; S/I of 6.66; 21.9 % TS	0.041	172	85 % ↓ TAN 36 % ↓ soluble TOC	Huang et al. (2019)
SM	0.5 L (batch)	Inorganic salts	75 mmol Fe ₂ O ₃	37 °C; 46 d; S/I of 3:1	0.288	11.04	13 %↑ soluble COD removal	Lu et al. (2019)
SM	0.25 L (batch)	H ₂ -NBW	Mixed with SM	35 °C; 28 d; 0.58 kg VS m ⁻³ d ⁻¹	0.225	39.3	10.8-17.2 % ↑ soluble TOC	Fan et al. (2020)

SM: swine manure, PS: pig slurry, NBW: nanobubble water, COD: chemical oxygen demand, AD: anaerobic digestion, VS: volatile solids, TS: total solids, TOC: total organic carbon, S/I: substrate to inoculum ratio, TAN: total ammonia nitrogen, CPE: chlorinated polyethylene, TMP: total methane potential (m³ kg⁻¹ VS or COD), N: nitrogen, HRT: hydraulic retention time

Another alkali chemical reagent that has shown promise in lignin degradation is aqueous ammonia soaking (AAS). Its advantages over hydroxide pretreatments are: 1) its high volatility, which allows for easier recovery and subsequent reuse; 2) it is a relatively weak base and less polluting, resulting in safer handling (Kim et al., 2003). In addition, AAS can be done at ambient temperatures (20-22 °C) which allows for a lower energy input into the process (Jurado et al., 2013). Jurado et al. (2016) found that AAS pretreatment conducted at 22 °C for 3 days at a 32 % (w/w) concentration almost doubled the TMP with respect to raw manure AD in semi-continuously operated CSTR systems. There was also increased solubilization of organic compounds- which freed up available fermentable sugars for microbial consumption and subsequent conversion to methane. It was found in a later study that AAS pretreatment can be applied at almost a fifth of the NH_3 concentration reported by Jurado et al. (2016) and still improved methane yields by 244 % in 17 d during mesophilic batch AD (Lymperatou et al., 2017). However, these select studies and also all of the studies listed in Table 1.3, were conducted in lab-scale batch reactors which does not fully reflect the applicability in semi-continuously or continuously operated large-scale biogas plants (Carrère et al., 2016), highlighting the need to implement these optimized AAS parameters in a (semi)-continuous system.

Although alkali reagent pretreatment is popular and has demonstrated efficacy, other processes and technologies have been explored which moved away from using toxic corrosive chemicals associated with hydroxide pretreatments. One of these processes is NH_3 fermentation coupled with NH_3 air stripping. The fermentation process involves microbial hydrolysis of proteins into $\text{NH}_4\text{-N}$ which enhances ammonia release from manure prior to subsequent NH_3 air stripping (Huang et al., 2016) This strategy is especially popular for nitrogen rich substrates such as swine manure and is attractive as a pretreatment strategy for dry AD due to inherent NH_3 inhibition at higher solids content. The process used by Huang et al. (2019) allowed for stable reactor performance when digesting alkaline swine manure (pH 8.8 and 12) while also having the added benefit of NH_3 recovery from the air stripping process and ultimately improving the volumetric biogas productivity compared to both untreated swine manure and swine manure AD at low TS. Both TBP and TMP, however, were quite low which highlights the mass transfer issues associated with dry AD (Ning et al., 2019) and a need to explore other reactor types that do not operate in batch mode in particular. While high TS content did not seem to exhibit the same mass transfer issues during swine manure AD in a zeolite fixed-bed reactor as seen by the total methane yield

and lower HRT (Zheng et al., 2015), other indicators such as organic matter reduction efficiency, VFA production, and alkalinity were not measured in that particular study. While the methane production capabilities were evident, the reactor performance on waste stabilization and nutrient content was not explored which makes it difficult to assess how efficient this reactor is for either lab- or industrial-scale operations. Other methods such as ferric oxide addition and nanobubble water (NBW) processes enhanced the AD process through hydrolysis/acidogenesis or methanogenesis stimulation (Fan et al., 2020; Lu et al., 2019). Both studies achieved improved performance without the use of harsh chemicals or long treatment times which could also be practical feasible strategies for large-scale biogas plants.

1.3.3 Physical pretreatment

This category can be divided into thermal, mechanical, ultrasound, high pressure, and electrohydrolysis pretreatments (Neshat et al., 2017; Paudel et al., 2017). Like chemical pretreatment, physical pretreatment achieves lignin disruption to free up cellulose and hemicellulose for subsequent hydrolysis into simple compounds to be utilized for the different stages of AD. The most common strategies within this category are thermal, steam explosion, and mechanical techniques whereas others such as irradiation, electrohydrolysis, and ultrasonic are infrequently reported (Neshat et al., 2017). Table 1.4 highlights recent studies on different physical pretreatment strategies for enhancing swine manure methane potential.

Processing organic waste within a wide temperature range from 50-250 °C increases biodegradability and pathogen reduction, resulting in its large-scale application for pretreatment (Paudel et al., 2017). Issues associated with thermal pretreatment include higher energy input and the formation of refractory and inhibitory compounds detrimental to biogas production (Paudel et al., 2017). Higher temperature and longer treatment times would be recommended for high lignocellulosic content biomass due to the recalcitrant nature of lignin. On the other hand, volatilization of substrates needed for methanogenesis can lead to decreased TMP when high temperature is applied to wastes with readily biodegradable organics. High temperature steam explosion substantially increased TMP compared to untreated manure during batch AD due to the breakdown of previously recalcitrant fibers (~7 %) that were used subsequently for methanogenesis (Ortega-Martinez et al., 2016). However, due to the high-water content this can make this process even more energy intensive. Huang et al. (2017) used lower temperatures which

increased TMP during dry AD and reported a net energy gain of $0.95 \text{ kJ g}^{-1} \text{ VS}$, which can make this treatment protocol attractive for large-scale application. Energy input can be further reduced by decreasing temperature and increasing reaction time while still enhancing digestion performance at high TS loadings and lower HRT (Hu et al., 2019).

Mechanical pretreatment aims to reduce solid particle size and ultimately increase surface area of smaller particulate matter to facilitate easier contact with microbes. While these strategies such as milling and grinding can decrease particle size, crystallinity, and polymerization, these processes are quite energy intensive (Panigrahi and Dubey, 2019). Yang et al. (2019a) reported better AD performance with pre-agitated swine manure and considered this process as a pretreatment. While agitation for the most part has been utilized during AD and has shown enhanced biogas production performance (Keanoi et al., 2014; Rusin et al., 2017), Yang et al. (2019) did not use agitation/stirring during AD. This process could be considered more as an operational parameter of AD rather than a mechanical pretreatment strategy such as grinding or milling. In that study, the higher TS content (18 %) did not seem to impede the agitation pretreatment or the batch AD process, however, problems associated with mixing at high TS content (Qian et al., 2019; Sawatdeenarunat et al., 2015) and foaming associated with mixing (Lindmark et al., 2014) have been reported. With these disadvantages in mind, either low TS manure or less frequent agitation/mixing of high TS manure should be used if agitation is to be practically implemented. Solid-liquid separation through gravitational settling and a mesh screen is another inexpensive and relatively energy efficient strategy for increasing biogas potential which can be performed both in batch and continuous operations (Cestonaro do Amaral et al., 2016; Ford and Fleming, 2012). Gravitational settling is advantageous over other manure separation pretreatment strategies such as screw presses and decanter centrifuges due to lower capital technological cost and investment (Sommer et al., 2015). While the supernatant fraction during batch AD demonstrated higher TBP and TMP compared to sludge retained on the mesh and settled solid fractions (Cestonaro do Amaral et al., 2016), this was due to the higher concentration of bioavailable carbon. It would be more appropriate to use the settled sludge or sludge retained on the mesh screen due to the higher organic content of these fractions which makes it feasible for dry AD operation, potentially making it less energy intensive due to smaller reactor volume requirements.

Table 1.4.

Summary of different physical pretreatments for enhanced anaerobic digestion of swine manure.

Feedstock	Reactor Type	Pretreatment type	Pretreatment Conditions	AD conditions	Outcome	TMP	TMP (%) improvement	Reference
PS	0.1 L (batch)	Steam explosion	170 °C for 30 min, 12 bar	37 °C; 35 d; S/I: 0.25; 5.54 % TS	↑ TMP	~550	46	Ortega-Martinez et al. (2016)
Separated SM	0.25 L (batch)	Solid-liquid separation	2mm mesh screen; settling of liquid fraction for 1 hr	37 °C; 17-24 d; 0.23-1.47 % VS	↑ TBP and TMP	0.201-0.737	1-47	Cestonaro do Amaral et al. (2016)
Filtered SM	0.5 L (batch)	Alkaline microwave	NaOH adjustment to pH 12.0; 300 W for 180 s	35 °C; 40d; 100 rpm stirring; 6.02% TS	200 %↑ soluble protein; 9 %↓ NH ₄ ⁺ ; 7 % ↑ CH ₄ content	~0.450	~38	Yu et al., (2017)

SM	0.5 L (batch)	Hydro-thermal	130 °C for 30 min, 0.18 MPa	35 °C; 70d; S/I: 0.5; 22.72% TS	62 %↑ soluble proteins; 220 %↑ soluble carbohydrates; 28 %↑ TAN; 18 %↓ bioavailable P	0.271	33	Huang et al. (2017)
SM	20 L CSTR (semi-continuous)	Thermal	70 °C for 3 d	35 °C; 29d HRT; 4.0 kg VS m ⁻³ d ⁻¹	32 %↓ NH ₃ ; 19 %↓ VFA	0.298	393	Hu et al. (2019)
SM	0.5 L (batch)	Agitation	propeller stirring for 22 h at 200 rpm and 25 °C	37 °C, 32 d, S/I :0.5	82.42 % ↑ soluble carbohydrates; 121.17 %↑ proteins; 158 %↑ soluble COD; 48 % ↓ VFA; 37.10 %↑ TAN	0.067	43.78	Yang et al. (2019a)

Raw SM	1 L (batch)	Graphene coupled with MEC	C anode and Ti cathode separated by 1 cm; 2.5V current 1 h d ⁻¹ during AD	38 °C, 24 d, 8 % TS	34 %↑ soluble COD removal; 26 %↑ effluent NH ₃ ; 25 %↑ coenzyme F ₄₂₀	0.269	60.89	Liu et al. (2020)
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SM: swine manure, PS: pig slurry, CSTR: continuously stirred tank reactor, COD: chemical oxygen demand, AD: anaerobic digestion, HRT: hydraulic retention time, TMP: total methane potential (m³ kg⁻¹ VS), TBP: total biogas potential (m³ kg⁻¹ VS), S/I: substrate to inoculum ratio, TS: total solids, VS: volatile solids, VFA: volatile fatty acids, TAN: total ammonia nitrogen, MEC: microbial electrolysis cell, P: phosphorous; Ti: titanium, C: carbon

Less common strategies such as electrohydrolysis and irradiation (e.g. microwave) pretreatment have recently gained popularity in the literature. While these technologies differ in terms of their operation, both achieve destruction of plant cell walls resulting in increased soluble COD and ultimately available nutrients for microorganisms (Carrère et al., 2016; Carlsson et al., 2012). Yu et al. (2017) and Liu et al. (2020) achieved higher biogas yields utilizing microwave-alkaline (MW-A) and electrohydrolysis, respectively, to achieve higher biodegradability. Despite the improved performance and relatively lower environmental impact of these strategies, the energy consumption was still considerable and high capital cost makes scaling-up these strategies impractical currently (Neshat et al., 2017).

1.3.4 Biological pretreatment

This pretreatment strategy utilizes living organisms, such as fungi and bacteria, and/or enzymes to break down the lignocellulosic structure of undigested fibres found within manure. The breaking of covalent links and non-covalent bonds between lignin and hemicellulose increases the particulate surface area allowing for enhanced microbial hydrolytic activity (Sun et al., 2015; Naran et al., 2016). Microbial and enzymatic consortiums are advantageous over chemical and physical pretreatment strategies due to lower capital cost and consumption of energy and chemicals, highlighting the relatively more environmentally sustainable nature of these agents (Tuesorn et al., 2013). Another added benefit of biological pretreatment is the reduction in inhibitory compound formation which can occur in other chemical and physical pretreatments (Li et al., 2019). At present, most investigations into biological pretreatment of swine manure for AD and AcoD have focused on microbial consortium isolation and inoculation (Table 1.5).

Biological pretreatment studies and implementation is less common compared to chemical and physical pretreatment. Some possible reasons for this could be the high costs associated with enzyme pretreatment, longer pretreatment times due to slower degradation rates, sensitivity to environmental factors, and competition for carbohydrates which can lead to lower biogas yields downstream (Carrère et al., 2016; Neshat et al., 2017; Zheng et al., 2014). Most studies which reported biological pretreatment conducted AD in batch reactors, which is not reflective of the full-scale AD process and therefore it is impractical to optimize the pretreatment in this operational mode. Also, these processes have been mostly applied to lignocellulosic biomass such as cereal residues due to the higher hemicellulose, cellulose, and lignin content compared to swine manure

(Paudel et al., 2017). Like other pretreatment categories, physical and chemical composition of swine manure dictates which pretreatment option is the most effective and efficient.

Two studies have reported (semi)-continuous digestion in CSTR systems (Table 1.5) and reported higher TMP compared to batch systems. Although in these studies, swine manure was co-digested with lignocellulosic wastes like crop residues and grass which could have enhanced TMP rather than the microbial consortium pretreatment itself (Jin et al., 2018; Shen et al., 2018). Jin et al. (2018) explained that longer pretreatment times (greater than what they used) led to increased hydrolysis and acidogenesis and proposed that the balanced nutrient profile of co-digestion might have had a more positive impact on process performance than pretreatment itself. It has been demonstrated by Hu et al. (2015) that the use of the liquid fraction of digestate (LFD) improved biogas production by 70% during corn stover AD and had a concentrated lignocellulose degrading microbial consortium, which can compensate for the low hydrolytic enzyme rates and make the process more cost-efficient. Future investigations into use of LFD and other biological pretreatments such as rumen microbial consortium addition (Yıldırım et al., 2017) should be conducted in the context of increasing swine manure biodegradability which can also elucidate the biological mechanisms of hydrolysis.

Table 1.5.

Summary of different biological pretreatments for enhanced anaerobic digestion of swine manure.

Feedstock	Reactor Type	Pretreatment type	Pretreatment Conditions	Outcome	AD conditions	TMP	TMP % improvement	Reference
SM	0.12 L (batch)	LMC addition	LMC inoculation during AD	18 %↑ NH ₄ -N; 1.87-fold↑ cellulose removal; 1.65-fold↑ hemicellulose removal	37 °C; 90 d; S/I: 1.5	0.180	55	Tuesorn et al. (2013)
Swine WW	1 L (batch)	Inoculum addition	AD of substrate and inoculum	56 %↑ VS removal; 84 %↑ VFA consumption	35 °C; 140 d; S/I :20	0.256	340	Córdoba et al. (2016)
SM + Napier grass	0.12 L (batch)	LMC addition	LMC inoculation during AD	43.6 %↑ VS removal; ↑ cellulase activity	55 °C; 35 d; S/I of 1:7	0.238	36.6	Wongwilaiwalin et al. (2018)

SM corn straw	+ 4 L CSTR (continuous)	Rumen culture	39 °C incubation for 3 d	26 %↑ VFA; 10 % ↑ VS removal	39 °C; 9 d HRT; 2 kg VS m ⁻³ d ⁻¹	0.258	-	Jin et al. (2018)
SM + RS	9 L CSTR (semi- continuous)	LMC addition	55 °C static incubation of 10 % (v/v) LMC with substrates for 30 h	12 % ↑ COD removal; 50.2 %↓ TAN; 15 % ↑ VFA	35 °C; 2.5 kg COD m ⁻³ d ⁻¹	0.456*	37.8	Shen et al. (2018)

SM: swine manure, WW: waste water, RS: rice straw, LMC: lignocellulolytic consortium, CSTR: continuously stirred tank reactor, COD: chemical oxygen demand, NH₄-N: ammonium nitrogen AD: anaerobic digestion, HRT: hydraulic retention time, TMP: total methane potential (m³ kg⁻¹ VS), S/I: substrate to inoculum ratio, TS: total solids, VS: volatile solids, VFA: volatile fatty acids, TAN: total ammonia nitrogen

*reported as m³ kg⁻¹ COD

1.4 Anaerobic co-digestion

Although AD of swine manure alone can yield significant biogas along with organic matter reduction, mono-digestion has drawbacks due to the chemical and compositional characteristics of swine manure. For example, in operations where manure is regularly flushed from the barn with water the organic matter is highly diluted (Bourque and Koroluk, 2003; Key et al., 2011). Further, the nutrient imbalance resulting from the low carbon to nitrogen ratio (C/N) of swine manure can lead to ammonia inhibition resulting in process instability and ultimately lower biogas yield (Mao et al., 2015; Nasir et al., 2012). To ameliorate biogas production and process stability, anaerobic co-digestion (AcoD) with carbon rich organic waste streams such as agricultural crop residues is an advantageous option since it balances out the C/N ratio. Other advantages of co-digesting different waste streams include dilution of inhibitory compounds, improving buffering capacity, and increasing microbial diversity, which all contribute to improved process performance (Neshat et al., 2017). Anaerobic co-digestion has advantages of pretreatment methods due to reduced water and energy requirements, making it more feasible to implement at industrial scale, though pretreatment methods can still be employed in conjunction with AcoD (Mussoline et al., 2012). The advantages of swine manure AcoD with different organic waste streams have been investigated and documented over the last several years (Hagos et al., 2017; Kanani et al., 2020; Neshat et al., 2017) and since Nasir et al. (2012) performed a comprehensive review of livestock manure AD progress. Due to the numerous studies published in this area, a review of research of swine manure AcoD not covered by Kanani et al. (2020) was conducted.

The most recent literature spanning from 2019 up to now concerning co-digestion of swine manure with other feedstocks is summarized in Table 1.6. Although AcoD has better overall performance in terms of stability and biogas compared to mono-digestion, this process is more prone to failure due to the complex biochemical and nutrient requirements for microbial performance in each stage of AD (Hagos et al., 2017). Due to the complexity of the process when co-digesting with chemically and compositionally different feedstocks, the process parameters and operational conditions to maximize reactor performance must be optimized according to feedstocks. For example, many of the studies optimizing AcoD have used lignocellulosic biomass, particularly agricultural crop residues, as a co-substrate (Table 1.6). Agricultural crop residues provide a substantial amount of lignocellulosic biomass annually around the globe, with over 5.35 billion tonnes produced from the world's top 4 agricultural crops (maize, rice, sugarcane,

wheat) (Chandra et al., 2012). The C/N ratio for crop residues vary, however, they generally fall well above the optimal range of 20-30 for AD, which can decrease digester performance when used as a sole substrate. However, they are suitable for AcoD with nitrogen-rich wastes like swine manure in order to bring the C/N ratio within optimal range (Sawatdeenarunat et al., 2015). Ning et al. (2019) reported a 15 % increase in TMP at a swine manure:corn straw (VS basis) of 1.25:1 and C/N ratio of 25 compared to mono-digestion of swine manure in semi-continuously operated mesophilic CSTRs. Another investigation into AcoD with durian shell (DS) and swine manure in batch experiments also demonstrated maximum TMP occurred at a SM:DS of 1:1 with a C/N ratio of 22.4, noted by a 17% increase in methane yield compared to swine manure AD alone (Shen et al., 2019).

In the context of co-digesting lignocellulosic biomass with swine manure, the optimal nutrient balance (reported as C/N) which yielded higher biogas and overall better digestion performance differed when co-digesting swine manure with other organic wastes. For example, a C/N of 10.3 was found to have the highest TMP during mesophilic AcoD of swine manure, animal by-product waste (ABP), and glycerine which was 40 % higher compared to swine manure AD (Rodríguez-Abalde et al., 2019). Although all 3 waste streams have relatively high protein and fat content (especially in the case of ABP and glycerin), ABP and glycerin contain readily biodegradable residues which provide the carbon necessary for gas production (Hagos et al., 2017) without leading to excessive VFA accumulation. In another study investigating thermophilic impact on AcoD of swine manure and sugar beet pulp waste, a C/N of 11.6 increased the methane yield more than 10-fold with respect to swine manure alone (Gómez-Quiroga et al., 2019). From these current studies and previous AcoD investigations in the context of balancing the C/N ratio to improve digestion performance (Hagos et al., 2017; Neshat et al., 2017), it is evident that nutrient requirements vary vastly depending on feedstock composition, source, and operational conditions. While the C/N ratio can give an estimation of carbon and nitrogen concentrations, it does not reflect actual bioavailable C or N within the substrates or the reactor and therefore should be used as preliminary guideline rather than as a standard for optimizing AcoD performance. The main point to take from these AcoD investigations is the delicate balance of C and N to ensure enough C for microbial consumption and enough N for supporting microbial growth and metabolism.

Some of the mentioned AcoD studies in Table 1.6 (Jiang et al., 2019; Ning et al., 2019;

Qian et al., 2019; Wang et al., 2020b) were also conducted at high solid (>10 % TS) conditions which has advantages over wet AD and AcoD (<10 % TS) due to smaller reactor size, lower energy consumption, and diminished digestate generation (Chen et al., 2015). For example, Qian et al. (2019) reported a 25 % increase in biogas production when co-digesting with alkaline and microwave pre-treated rice straw at greater than 20 % TS. Although biogas yields generally increased compared to mono AD, reactor performance (biogas production and solids removal) decreased as TS was increased during digestion depending on the feedstocks. Wang et al. (2020b) reported decreasing biogas yield and VS removal efficiency (below 20 %) trend as TS increased from 10 to 30 % when co-digesting swine manure with cucumber residues and corn stover in batch reactors. In another study working with corn straw and swine manure, CSTRs fed with mixtures ranging from 8-20 % TS revealed an increase in cumulative biogas yields from 0.499 – 0.550 m³ kg⁻¹ VS but dropped at 25 % TS and higher (Ning et al., 2019). In both studies, ammonia concentrations were well below the inhibitory threshold of 3,860 mg L⁻¹ (Li et al., 2015). While total VFA did not accumulate to inhibitory levels in the study by Ning et al. (2019), VFA levels in the batch AcoD study conducted by Wang et al. (2020b) fell outside the range of 1,500-2,000 mg L⁻¹ which lead to increased acidification and decreased biogas production. While high solids AcoD is an attractive and advantageous process for maximizing biogas production, more investigations into the impact of increasing TS and OLR on process performance are required to understand its implications on implementing the technology on a farm-scale operation.

All of the recent studies (Table 1.6) concerning AcoD of swine manure with other organic wastes have been conducted using CSTRs, both in batch and (semi) continuous operational modes. As mentioned in Section 2.2, reactor performance decreases when the feedstock contains high solids content (>10 % TS). At higher solids loading, mixing capabilities are reduced – in particular when co-digesting with lignocellulosic biomass (Qian et al., 2019; Sawatdeenarunat et al., 2015). Reduced mixing within a CSTR system leads to decreased substrate surface contact with microorganisms and settling of solids which results in lower biogas yields and increased energy input to overcome mixing issues (Gómez et al., 2019). While the operational mode combined with the reactor design has shown promise in lab and bench scale experiments, the impact of increasing TS and OLR on reactor performance parameters such as biogas production and organic matter removal efficiency is not as understood in (semi) continuous operations on a pilot and commercial scale (Dong et al., 2019a; Hagos et al., 2017). Reactor configuration should also be considered

when conducting investigations into dry AD and AcoD in particular, since certain feedstock mixtures can significantly impact mixing capabilities and operational parameters that ultimately dictate reactor efficiency. The issue of feedstock composition is particularly evident when co-digesting lignocellulosic biomass such as crop residues and pulp waste due to the rheological properties which increase feedstock viscosity at a certain TS level (Gómez-Quiroga et al., 2019; Wang et al., 2020a). Although pretreatment has inherent disadvantages compared to reactor design modification (Mussoline et al., 2012), a combination of pretreatment and a reactor able to handle high TS wastes would need to be considered in order to optimize performance both at lab and full-scale operation.

Table 1.6

Anaerobic co-digestion of swine manure with various organic waste streams under different operating conditions

Substrates	Reactor type	Temp. (°C)	OLR	SM:substrate ratio (VS basis)	HRT (d)	VS/COD removal (%)	TBP	TMP	Reference
SM + DS	0.5 L (batch)	37	-	1:1	30	-	-	0.225	Shen et al. (2019)
SM + pretreated CS	0.15 L (batch)	35	-	-	95	-	-	0.322	You et al. (2019)
SM + pretreated RS	1 L (batch)	35 ± 1	-	7:3 (w.w)	70	60.01	0.457	0.268	Qian et al. (2019)
SM + FW	1 L (batch)	37	-	1:1	120	-	-	0.252	Jiang et al. (2019a)
SM+ABP+RG	6 L (semi-continuous)	36 ± 1	1.6	35:74:1	33	55	0.535	0.380	Rodríguez-Abalde et al. (2019)
SM + ESBP	0.7 L (batch)	55	-	75:25 (w.w)	40	-	-	0.212	Gómez-Quiroga et al. (2019)

SM+ straw	corn	8 L (semi-continuous)	37 ± 1	2.0	4:1 (w.w)	21	-	0.515	0.320	Ning et al. (2019)
SM+ CF		0.5 L (batch)	35	-	3:2 (TS)	82	54.20	-	0.48	Schommer et al. (2020)
SM + CS		1.89 L (semi-continuous)	38 ± 1	3	2:1	21	57.46	0.097	0.073	Wang et al. (2020a)
SM + CS +CR		1 L (batch)	35	-	5:2:3 (w.w)	65	18.71	-	0.364	Wang et al. (2020b)

SM: swine manure, DS: durian shell, RS: rice straw, CS: corn stover, CF: chicken feathers, FW: food waste, RG: residual glycerin, ABP: animal by-product, CR: cucumber residue, ESBP: exhausted sugar beet pulp, OLR: organic loading rate ($\text{kg VS or COD m}^{-3} \text{ d}^{-1}$), TS: total solids, VS: volatile solids, w.w: wet weight, COD: chemical oxygen demand, TBP: total biogas potential ($\text{m}^3 \text{ kg}^{-1} \text{ VS or COD}$), TMP: total methane potential ($\text{m}^3 \text{ kg}^{-1} \text{ VS or COD}$), TAN: total ammonia nitrogen, CSTR: continuously stirred reactor, HRT: hydraulic retention time

1.5 Microbial community dynamics during anaerobic digestion

1.5.1 Microbial community changes in response to different AD operating parameters

Anaerobic digestion and co-digestion involves the breakdown of complex substrates into biogas through 4 microbial driven biochemical stages: hydrolysis, acidogenesis, acetogenesis, and methanogenesis (Neshat et al., 2017). The entire biochemical pathway is broken down into different steps which makes up the synergistic process driven by a diverse microbial consortium as seen in Fig. 1.3.

Fig. 1.3. Metabolic pathways during anaerobic digestion of swine manure. Adapted from Mao et al. (2015).

Anaerobic digestion can be performed at different temperatures, broadly classified as psychrophilic (10-20 °C), mesophilic (30-40 °C), and thermophilic (50-60 °C) (Massé et al., 2003; Wang et al., 2018b). Temperature is one of many factors which impacts the anaerobic digestion process, in addition to feedstock composition, source, and reactor design which was evident in the differences in process performances mentioned earlier in this paper (Section 1.2-1.4). Process performance is therefore related to the impacts of varying operational and physiochemical parameters on microbial community structure which highlights the sensitivity of the anaerobic digestion process. The four metabolic pathways which make up anaerobic digestion are governed

by complex interactions between two domains of microorganisms: Bacteria which are responsible for substrate hydrolysis and fermentation (first 3 steps) and Archaea which are responsible for methane production (Ren et al., 2018).

Wang et al. (2017) used Illumina MiSeq to sequence 16S rRNA gene amplicons and found that *Bacteroidetes* and *Firmicutes* were the two predominant phyla present over time during batch AD of corn straw, pig manure and AcoD of both substrates. Both phyla have been identified as acidogenic bacteria that are responsible for cellulose and protein degradation (Ntaikou et al., 2009; Whitehead et al., 2005). Another abundant phylum observed during swine manure AD and AcoD with corn straw was *Chloroflexi*, which plays an important role in the degradation of polysaccharides and monosaccharides and subsequent conversion to acetic acid (Yamada et al., 2005). Within Archaea, methanogens all belonged to the phylum *Euryarchaeota*, with the genus *Methanosaeta* being most abundant followed by *Methanosarcina*. *Methanosaeta* are strict acetoclastic methanogens, therefore, due to their high abundance the acetoclastic methanogenesis pathway was the main biochemical pathway of methanogenesis (Ferry, 1999). Although this pathway was the main process for methane production from acetate, hydrogenotrophic methanogenesis also occurred during swine manure AD as seen by the high prevalence of the genus *Methanobrevibacter* which has been implicated in this biochemical pathway in other animal manure AD studies (Sun et al., 2015). The findings from Wang et al. (2017) were similar to the findings of Zhou et al. (2016) who utilized denaturing gradient gel electrophoresis (DGGE) to determine that *Clostridia*, *Bacteroides*, and *Methanosarcina* were the most abundant genera during swine manure AD. Zhou et al. (2016) also investigated the impact acidic to alkaline (pH 6.0-8.0) conditions on microbial community dynamics in which bacterial diversity decreased and archaeal diversity changed slightly as a result. It was noted that at acidic and alkaline conditions, *Methanosarcina* was the dominant genus which contains acetoclastic methanogens whereas at the optimal pH (pH 7.0) *Methanocorpusculum* was the dominant genus which contains hydrogenotrophic methanogens.

Understanding microbial community shifts are an important aspect of AD due to the sensitivity of microorganisms to environmental changes, making it important to assess these microorganisms since they affect the function of the system. Cho et al. (2017) investigated the impacts of environmental perturbations (prolonged starvation, temperature fluctuations from

mesophilic to thermophilic conditions, and HRT) on the microbial community structure within a farm-scale digester treating swine wastewater. Using real-time polymerase chain reaction (qPCR) to identify methanogen orders and their abundances, it was found that the total methanogen population (based on gene copy numbers) did not change a lot throughout long-term operation or in response to environmental perturbations. After the prolonged starvation period, however, hydrogenotrophic methanogens outnumbered acetoclastic methanogens (>95% relative abundance) due to the inhibitory effect of ammonia (especially at thermophilic conditions) which favours the hydrogenotrophic pathway over the acetoclastic pathway (Schnürer and Nordberg, 2008). Using 454- pyrosequencing revealed that the only significant changes in bacterial community structure occurred at the order level, not in the phylum and class levels. The phylum *Firmicutes* remained relatively unchanged during all perturbations whereas the orders *Clostridiales* and *Bacteroidales* only became highly abundant after the transition from prolonged starvation to thermophilic AD and for the rest of the long-term operation. These orders become dominant during the period of hydrogenotrophic methanogen abundance, which suggested these orders may work with methanogens to produce hydrogen via the syntrophic acetate oxidizing (SAO) pathway. During this period of temperature and HRT changes, ammonia concentrations also increased which suggested that bacteria from these orders are tolerant to ammonia toxicity to a certain degree (Poirier et al., 2016). From these findings, it was found that microbial community structure (including Archaea and Bacteria) were only significantly shifted when temperature was increased or HRTs were prolonged but not when there were prolonged periods of no feeding into the digester.

From these select microbial community structure studies, high-throughput sequencing technologies such as Illumina MiSeq and 454-pyrosequencing have proven to be superior to other earlier molecular detection techniques such as qPCR and DGGE which are time-consuming and can only discern dominant microbes (Vanwonterghem et al., 2014). A limitation of molecular based techniques, however, is they are not able to identify metabolically active microorganisms since these genome sequencing base techniques can only discern the existence of all microorganisms (live, dead, or dormant) (Wang et al., 2018a). To overcome these limitations, metatranscriptomics and metaproteomics can better identify gene expression and provide a more

robust physiological profile of microbial communities throughout AD (Heyer et al., 2013; Lin et al., 2016a; Lin et al., 2016b). These approaches were able to identify acetoclastic methanogenesis as the main biochemical pathway dominated by the order *Methanosarcinales* (Heyer et al., 2013) and elevated temperatures reduced functional diversity but overall enhanced central functional pathways which resulted in increased reactor efficiency (Lin et al., 2016a; Lin et al., 2016b). Despite the successes of coupling metagenomics with metatranscriptomics and metaproteomics, there is limited availability of reference strains to compare unassigned sequences to their corresponding genomes (Maus et al., 2016). To better understand the overall microbial community structure from both a phylogenetic and physiological approach, a combination of -omics, traditional cultivation, and biochemical assays may be needed to overcome these limitations.

1.5.2 Pathogen inactivation and reduction

Anaerobic digestion and co-digestion have demonstrated feasible potential and success in converting swine manure into biogas while mitigating GHG emissions, recycling nutrients, and stabilizing waste via organic matter reduction. The resulting end by-product from AD and AcoD is digestate which can be used as a fertilizer for land application, however, swine manure is a large reservoir for pathogenic microorganisms which adds a biosafety aspect to proper utilization of digestate (Nag et al., 2019). The most commonly concerned pathogens found in swine manure include Gram-positive bacteria, Gram-negative bacteria, protozoa, nematodes, and viruses. While the prevalence and types of pathogens will vary depending on local climate and storage conditions (Avery et al., 2014), the most commonly encountered ones include *Salmonella*, *Escherichia coli*, *Listeria*, *Yersinia*, *Campylobacter*, *Enterococcus*, *Clostridium*, *Giardia*, *Ascaris*, *Toxoplasma*, norovirus, adenovirus, enterovirus, rotavirus, and hepatitis A/E (Jiang et al., 2020). Many of these pathogens are zoonotic, meaning they are able to cause disease in humans and have resulted in over 360 000 foodborne related illnesses and 156 deaths in 37 European countries as of 2016 (EFSA and ECDC, 2017). Proper handling of digestate must be considered, which ultimately stems from optimizing reactor performance for improved hygienization, just as many studies have looked at optimizing AD and AcoD performance in terms of enhanced biogas potential and waste stabilization (Mao et al., 2015; Neshat et al., 2017). A way of assessing the microbial quality of digestate, and in turn the hygienization efficiency of AD and AcoD, is the analysis of fecal indicator organisms. Biosafety regulations and standards for safe handling of digestate vary from

region to region (Jiang et al., 2020), therefore, the standards and regulations developed by the Ontario Ministry of Agriculture, Food, and Rural Affairs (OMAFRA) are outlined below (Table 1.7).

Table 1.7.

Threshold levels of indicator organisms and pathogens in digestate. Adapted from OMAFRA (2002)

Microorganism	Threshold in aqueous material (<1 % TS, w.w)	Threshold in non-aqueous material (>1 % TS, w.w)
<i>E. coli</i>	1000 CFU/100 mL	1,000 CFU/g TS, d.w
<i>Salmonella</i>	3 CFU or MPN/100 ml	3 CFU or MPN /4 g TS, d.w
<i>Giardia</i>	No detectable level in 100 ml	No detectable level in 4 g TS, d.w
<i>Cryptosporidium</i>	No detectable level in 100 ml	No detectable level in 4 g TS, d.w

TS: total solids, w.w: weight weight, d.w: dry weight, MPN: most probable number, CFU: colony forming unit

While human-specific or zoonotic pathogen inactivation in AD/AcoD systems have been the focus in the context of hygienization, swine-specific pathogen inactivation is limited (Fongaro et al., 2014). In particular, swine-specific and zoonotic enteric virus inactivation within these systems is limited (Jiang et al., 2020), which highlights the need for future investigations into directly assessing virus inactivation or indirectly using viral indicators such as adenovirus (Fongaro et al., 2014). The most concerning pathogen which threatens the majority of global swine populations is the ASF virus- a double stranded DNA enveloped virus with high environmental resistance and no available vaccine to counter it (De Lorenzi et al., 2020). Other pathogens of concern include porcine epidemic diarrhea virus (PEDV), transmissible gastroenteritis virus (TGEV), swine acute diarrhea syndrome coronavirus (SADS-CoV), and porcine deltacoronavirus (PDCoV), which cause highly contagious enteric diseases leading to significant morbidity, mortality, and economic losses as a result (Fu et al., 2020). The prevalence of swine-specific pathogens in manure and the reduction potential in biogas systems should be thoroughly investigated in order to develop more comprehensive biosafety regulations to protect both human and swine health.

Indicator microorganisms are typically used in environmental risk assessments to give an insight on pathogen presence and survival in various environmental matrices (Nag et al., 2019). Isolation and culturing of fecal indicator bacteria (FIB) such as fecal coliforms and enterococci is commonly performed because of their natural presence in the intestines of warm-blooded animals which makes detection of these microorganisms relatively easier and quicker compared to pathogen quantification (EPA, 2013). While presence of FIB in digestate and other environmental matrices can be associated with pathogen presence (Sahlström, 2003), there is not one appropriate indicator microorganism that can determine the overall microbial quality of digestate. Due to variations in physiology and structures of different bacterial pathogens and especially between other pathogen types, different indicators must be used (Avery et al., 2014). In a review on inactivation mechanisms of pathogens and indicator microorganisms in AD/AcoD systems conducted by Jiang et al. (2020), univariate analyses revealed that pathogen/microorganism type, temperature, and operational mode (batch vs. semi-continuous or continuous) significantly affected $\log_{10}$ reductions whereas HRT/residence time and substrate composition were insignificant factors. To understand which pathogen types were most resistant during AD/AcoD, Bonferroni post hoc multiple comparisons revealed a decreasing resistance order of spore forming Gram-positive bacteria > non-spore forming Gram-positive bacteria > nematode $\geq$ Gram-negative bacteria $\geq$ viruses. Gram-positive bacteria have the most resistance due to the thick peptidoglycan cell wall which also acts a permeable barrier to block out toxic chemicals from entering the cell, owing to its survival over a larger temperature, pH, and osmotic pressure range (Fisher and Philips, 2009; Jiang et al., 2018). Nematodes, represented by the indicator microorganism *Ascaris*, derive their resistance specifically as eggs due to the 4-layer structure of the eggshell composed of lipoprotein, chitin/protein, lipoprotein vitelline, and an acidic outer mucopolysaccharide/protein layer (Jiang et al., 2020). Gram-negative bacteria are fairly sensitive to AD conditions due to the thin peptidoglycan layer which offers little resistance to environmental conditions such as acidity and ammonia while viruses (in particular non-enveloped viruses) are sensitive to temperature which results in capsid denaturation and RNA cleavage (Jiang et al., 2020).

Select studies on swine manure AD and AcoD with other substrates are summarized in Table 1.8. From these studies and as noted by other researchers reviewing inactivation of pathogens in biogas systems (Avery et al., 2014; Jiang et al., 2020; Nag et al., 2019), pathogens and indicator microorganisms displayed different reduction profiles between pathogen types and

also within the same types of different operational designs. Generally, batch mode operation of AD and AcoD systems yielded better reduction and subsequent hygienization compared to continuous operation due to the continuous introduction of pathogens in continuously fed systems (Sahlström, 2003). Although temperature is a significant factor in pathogen reduction, most studies have reported mesophilic conditions during AD/AcoD which is optimal for stable biogas production but does not exert a direct impact on reduction like thermophilic conditions (Jiang et al., 2020). Rather, production and accumulation of intermediary metabolites such as VFAs and ammonia are the major inactivation mechanisms. In particular, free VFAs are detrimental to microorganisms due to the ability to permeate across the cell membrane which can result in acidification of the cytoplasm, increase in osmotic pressure, and electrophysiologic variation of the cell (Jiang et al., 2020). Free ammonia can permeate across the cell membrane and induce alkaline conditions and can cause RNA cleavage in non-encapsulated viruses, although the exact mechanisms are still unclear (Jiang et al., 2020). Despite the extensive research in hygienization potential of swine manure AD and AcoD with other waste sources, there are still knowledge gaps in terms of measuring potential in systems other than CSTRs and in particular at high TS loadings. More investigations into reduction of other pathogenic microorganisms such as viruses, protozoa, and nematodes should be conducted in the future to understand how operational parameters and the subsequent biochemical conditions arising from these operational modes impact the overall hygienization potential.

Table 1.8. Summary of studies evaluating pathogen and indicator microorganism inactivation during anaerobic digestion of swine manure and co-substrates.

Feedstock	Microorganism	Operational Mode	Temperature (°C)	Log ₁₀ Reduction	Reference
SM + FW	<i>E. coli</i>	Batch	37	2.8-6.9	Jiang et al. (2018b)
	Coliforms			3.5-7.0	
	Enterococci			~3-7.2	
SM + FW	<i>E. coli</i>	Continuous	39	1.5-2.5	Dennehy et al. (2018)
	Coliforms			1-4	
	Enterococci			2-3	
SM+FW	<i>Salmonella</i>	Batch	37	4-6	Jiang et al. (2018a)
SM	<i>E. coli</i>	Batch and	24	0.9-2.9	Massé et al. (2011)
	Coliforms	Continuous		1.0-2.9	
	<i>Salmonella</i>			0.9-1.4	
	Enterococci			0.6-1.0	
	<i>Clostridia</i>			0-0.2	
	<i>Campylobacter</i>			1.1-1.5	
	<i>Yersinia</i>			1.1-2.4	
SM + AW	<i>E. coli</i>	Continuous	39-42	0-5.7	Orzi et al. (2015)
	Coliforms			0.9-4.3	
	<i>Clostridia</i>			-1.3-4.63	

SM: swine manure, FW: food waste, AW: agricultural waste

1.6 Conclusion

Environmentally sustainable management of swine manure must be considered with high importance in the overall pork production system. Swine manure possesses the potential for energy production in the form of biogas generation using the anaerobic digestion process which has the added benefits of waste stabilization and hygienization efficiency. Due to the increased global demand for pork, farm-scale operations have become larger to meet this demand which results in larger quantities of manure that can be used as a readily available feedstock for biogas generation. While AD of swine manure alone has yielded biogas production and waste stabilization in small and large-scale operations, recent research has been geared toward improving AD performance efficiency by enhancing pretreatment strategies, co-digestion with high carbon feedstocks, and mitigating ammonia inhibition which is an inherent problem of swine manure. Microbial community shifts during stable AD operation and in response to environmental perturbations using different -omics approaches revealed both key microbial populations and an insight into their role throughout the entire process. Hygienization potential is evident in some AD operations although the variability of microorganism resistance and different biochemical conditions arising from different operational modes leaves a knowledge gap regarding the biosafety risk of the effluent digestate. In terms of enhancing biogas production, waste stabilization efficiency, and hygienization potential, utilizing different reactor designs and co-digesting with higher TS waste could alleviate issues associated with swine manure AD and incentivize farm operations to implement this technology more readily across Canada. Combined with a new reactor design and AcoD at higher TS, studying microbial community shifts using biochemical assays to assess metabolic activity or in combination with other -omics approaches should be implemented to provide a more robust profile of the microbial community.

Chapter 2

Hygienization and microbial metabolic adaptation during anaerobic co-digestion of swine manure and corn stover

Abstract

This study assessed the effect of different swine manure (SM)/corn stover (CS) mixtures based on total solids (TS) content with respect to hygienization, microbial community dynamics and methane yields on batch anaerobic co-digestion performance. Different ratios of SM and CS with TS content between 0.69 and 6 % digested at 75 d revealed SM had the greatest methane yield at 403.9 mL g⁻¹ volatile solids added (VS) and 86.31 % VS reduction. BIOLOG AN microplates and lignocellulolytic enzyme assays proved to be rapid tools for characterizing microbial community metabolism as noted by the different carbon source utilization patterns between TS loadings. Hygienization of fecal indicator bacteria groups was achieved with some (*E. coli*) but not all groups (*Clostridia* spp.). The results showed that colorimetric biochemical assays and culture-based techniques can rapidly assess microbial community dynamics during co-digestion, and that TS- in the form of lignocellulosic biomass- influences microbial metabolic activities.

Keywords

Agricultural biomass; biogas; community-level physiological profiling; fecal indicator bacteria; lignocellulolytic activity

2.1 Introduction

The growing middle class' demand for animal-based protein contributes significantly to the global pork industry, as seen by the 35 % proportion of global meat production and a quarter of total protein consumed worldwide attributed to pork (FAO, 2017). Livestock producers must process large amounts of manure, which can exceed 295 million t produced annually (Cameron, 2000), that places a serious strain on the environment. Improperly managed manure results in increased greenhouse gas (GHG) emissions, excess nutrient runoff into surface and groundwater

sources, and dissemination of clinically relevant pathogens into the surrounding environment (Nasir et al., 2014; Sakar et al., 2009).

Anaerobic digestion (AD) is a well-known and promising process that can reduce nutrient concentrations (Cuellar and Webber, 2008), pathogens (Dennehy et al., 2018; Orzi et al., 2015), and unpleasant odours (Orzi et al., 2015). Anaerobic digestion has the ability to valorize organic wastes and also provides an alternative pathway for biogas production from primary renewable resources as opposed to traditional fossil fuels (Chandra et al., 2012). While AD has shown promise for waste reduction and energy production (Sawatdeenarunat et al., 2015), reluctance to implement these designs on-farm stem from high start-up and operational capital requirements (Massé et al., 2013) as well as digester instability due to imbalance of feedstocks. Poor biogas yield can result from an imbalance of the carbon to nitrogen ratio (C/N), which leads to accumulation of volatile fatty acids (VFA), or excessive ammonia – both factors which inhibit methanogenesis (Ning et al., 2019). Extensive research into anaerobic co-digestion (AcoD) as a means to balance the C/N ratio and optimize biogas production has yielded better performance in efficiency and waste treatment compared to mono-digestion (Zhao et al., 2018). While most researchers have investigated the influence of physiochemical parameters such as total solids (TS) content on anaerobic mono-digestion, less research has focused on the impact of these same parameters on AcoD of manure and lignocellulosic biomass compared to manure mono-digestion.

AcoD studies have typically been conducted in batch systems and mainly focus on rate of substrate degradation via biogas volume measurement (Zhao et al., 2018) using biochemical methane potential (BMP) assays. There is, however, far less research investigating the microbial community within batch digesters. In particular, little is known about the impact of TS on pathogen reduction over time within BMP assays. Previous research has investigated the hygienization efficacy of different anaerobic digestion technologies with varying physiochemical parameters (Orzi et al., 2015; Scaglia et al., 2014). However, less research has addressed the influence of physiochemical parameters and operation conditions on hygienization during AcoD of swine manure and lignocellulosic biomass, compared to manure mono-digestion (Dennehy et al., 2018).

Microbial metabolic diversity is another microbial community parameter which has been investigated using community-level physiological profiling (CLPP), a biochemical technique that measures sole carbon substrate utilization patterns. A common tool used to perform CLPP are

BIOLOG plates (BIOLOG Inc., Hayward, CA, USA), particularly BIOLOG EcoPlates, because of their ability to characterize the functional ability of microbial communities (Tang et al., 2019). Other advantages of these plates are their relative ease of use and reduced cost compared to traditional cell and molecular based techniques (Lladó and Baldrian, 2017). While BIOLOG EcoPlates have been extensively used in microbial community characterization in various environments, including anaerobic digesters (Cardinali-Rezende et al., 2011; Dreher et al., 2012), there is no available research on impact of TS on metabolic diversity in AcoD of different feedstocks such as lignocellulosic biomass and livestock manure. Knowing the phylogenetic and physiological parameters of the microbial community can provide insight into maintaining specific physiochemical parameters during anaerobic digestion. Optimizing the parameters enhances these beneficial communities during start-up and operation which ultimately leads to improved digester performance.

In this study physiological, microbial, and physiochemical approaches were employed in order to analyze and compare microbial community profiles during AcoD of swine manure and corn stover. The effect of physiochemical parameters such as TS and volatile solids (VS) on methane production were investigated in BMP assays which operated as batch anaerobic digesters. Within this set-up fecal indicator bacteria were quantified to indirectly assess the degree of pathogen reduction during mesophilic anaerobic co-digestion in relation to the operational parameters of the system. To investigate the influence of operational parameters on CLPP, carbon substrate utilization and lignocellulolytic enzyme activity assays were used. Specifically, for carbon substrate utilization assays, a BIOLOG AN microplate was used to study CLPP in the digestates because it contains more ecologically relevant carbon substrates to anaerobes that can provide a deeper assessment of carbon source utilization patterns compared to BIOLOG Ecoplates.

2.2 Materials and Methods

2.2.1 Substrate characteristics

The physiochemical characteristics of the substrates are outlined in Table 2.1. Swine manure was collected from an in-ground open manure pit at a local pig farm in Ridgetown, Ontario, Canada early February 2019. Four subsamples of pig manure (~20 L) from the bottom of the pit were sampled in order to obtain a relatively homogenous sample of manure consisting of liquid

and solid material. Corn stover was collected from field plots at the University of Guelph Ridgetown campus in early February 2019. The stover was processed by using an electric chipper shredder (LawnMaster, USA), followed by blending (Waring Commercial, USA), and the blended material being separated through a 3 mm sieve.

Table 2.1

Initial characteristics of swine manure and corn stover.

Parameter	Swine Manure	Corn Stover
Total Solids (%)	0.69	74.06
Volatile Solids (% of Total Solids)	52.35	93.58
Organic Matter (%)	0.36	69.30
pH	7.25	n.a
Total Nitrogen (%)	0.10	1.22
NH ₄ ⁺ -N (mg L ⁻¹)	762	n.a
Phosphorous (Total) (%)	0.02	n.a
Carbon:Nitrogen Ratio (C/N)	2:1	35:1
Sulfur (mg L ⁻¹)	50.4	n.a
Conductivity (mS cm ⁻¹)	14.94±0.54	n.a
Cellulose (%)	n.a	38.82±0.49
Hemicellulose (%)	n.a	29.02±0.39
Lignin (%)	n.a	7.16±0.14

n.a: not available

2.2.2 Laboratory scale co-digestion of swine manure and corn stover in biochemical methane potential assays

The BMP of swine manure and corn stover was determined using an automated methane potential system (AMPTS II, BioProcess Control, Sweden). Within this system, 7 different feedstock combinations based on final % TS were prepared in duplicate 500mL Pyrex bottles with a final working volume of 350mL. The 6 feedstock combinations have been designated as the following treatment groups: 1 % TS, 2 % TS, 3 % TS, 4 % TS, 5 % TS, and 6 % TS (Table 2.2). A control consisting of only swine manure was incorporated in the BMP set-up in order to compare

the BMP of different feedstock mixtures with respect to mono digestion of swine manure. The bottles were incubated in a water bath at 38 °C and were mechanically stirred at 160 rpm for 20 s followed by no mixing for 60 s and the cycle being repeated. The digestion experiment was conducted for 75 d.

Table 2.2

Composition of feedstock used in the biochemical methane potential assay.

Solid content (%)	Mass of Substrate (g)		Total Mass (g)
	Corn Stover	Swine manure	
Control (0.69)	0	350	350.00
1	1.50	350	351.50
2	6.35	350	356.35
3	11.40	350	361.40
4	16.55	350	366.55
5	21.85	350	371.85
6	27.30	350	377.30

2.2.3 Physiochemical analyses

TS and VS of raw swine manure, corn stover, and digestates were determined using Standard Methods (APHA, 2017). The pH of swine manure and digestates was measured using pH/EC meter (Seven Excellence, Mettler Toledo, USA). Soluble chemical oxygen demand (sCOD) was determined colorimetrically (Photoflex, YSI, USA) using the potassium dichromate and sulfuric acid method according to manufacturer's instructions (YSI, USA). For sCOD, a subsample of initial feedstock mixtures and digestates were diluted 10-fold in 1X phosphate buffered saline (PBS) water (pH 7.2) and vortexed for 15 s to homogenize the samples. The homogenized samples were filtered through a Basix™ 0.45µm PVDF syringe filter (Fisher Scientific Canada) and concentrations were reported as mg L⁻¹ O₂. Volatile fatty acids (FOS), total alkalinity (TAC) and the FOS/TAC ratio were estimated according to the FOS/TAC protocol established by Hach Lange Laboratory in Germany (Lossie and Pütz, 2008).

2.2.4 Isolation and enumeration of fecal bacterial indicators

Subsamples of the treatments at different time points were serially diluted 10-fold in 1X PBS and appropriate 10-fold serial dilutions were prepared from the initial sample suspension for enumeration. The dilutions were directly plated on substrate-specific agar plates in order to isolate and enumerate the following fecal bacteria indicators: non-pathogenic *E. coli*, fecal enterococci, fecal (thermotolerant) coliforms, and *Clostridia* spp. Each sample was plated onto the following agar plates under specific conditions: Tryptone bile x-glucuronide agar (TBX, Sigma-Aldrich) at 44.5 °C for 24 h for *E. coli*, violet red bile agar (VRBA, Sigma-Aldrich) at 44.5 °C for 24 h for fecal (thermotolerant) coliforms, kanamycin aesculin azide agar (KAA, Oxoid) at 44.5 °C for 48 h for fecal enterococci, and tryptose sulphite cycloserine agar (TSC, Fisher Scientific) at 37 °C for 18-22 h for *Clostridia* spp. All TSC plates were incubated in an anaerobic environment using AnaeroPack®-Anaero (Mitsubishi Gas Chemical) (Bonetta et al., 2014). All results are reported as colony forming units (CFU) per mL of raw manure or digestate sample.

2.2.5 Community level physiological profiling

For bacterial community recovery from digestates, the following protocol was modified from previous research studying the microbial community of forest soils (Lladó and Baldrian, 2017). Briefly, 2 g of fresh sample was diluted in 20 mL of 1X PBS (pH 7.2) and was vortexed for 15 s followed by manual shaking for 2 min. After agitation, the samples were centrifuged at 500 x g for 4 min to remove large particulate matter and the resulting supernatant was centrifuged at 7000 x g for 25 min to pellet the bacterial cells. The supernatant was discarded and the pellet was resuspended in 10 mL of 1X PBS water to wash the cells and centrifuged again with the same parameters. The washed pellet was resuspended in fresh 1X PBS water and 150 µL was pipetted into each well of the 96 well BIOLOG AN microplate (BIOLOG Inc., Hayward, CA, USA) which contained 95 different carbon substrates and one well containing water (negative control). The plates were incubated at 38 °C for 24 h in an anaerobic environment and the degree of substrate oxidation was measured at 590 nm via the colour intensity of the tetrazolium dye which reacts with oxidized substrate during bacterial metabolism (Preston-Mafham et al., 2002). The A_{590} of each carbon substrate was corrected using the value obtained from the control well.

2.2.6 Determination of carbon substrate utilization parameters

Microbial activity within each sample was determined by calculating the following two parameters: substrate richness (R) and average well colour development (AWCD). Substrate richness was calculated as the number of oxidized C substrates with $A_{590} > 0.25$ (Cardinali-Rezende et al., 2011). The AWCD has been used previously to assess the degree of sole carbon substrate utilization (Garland, 1997). The formula for AWCD is as follows:

$$AWCD = \sum_{i=1}^n (C_i - R) / n \quad (1)$$

Where R is the absorbance value of the control well (water), C_i is the absorbance of each carbon substrate at 590nm, and n is the number of carbon substrates (95 substrates). The threshold for detectable carbon substrate oxidation was defined as ≥ 0.06 and any A_{590} less than the defined threshold was calculated as zero (Classen et al., 2003). In addition to calculating AWCD and substrate richness for the microplate, the carbon substrates were divided into six biochemical guilds: amino acids, amines/amides, carboxylic acids, carbohydrates, polymers, or miscellaneous (Ge et al., 2018) and the same parameters were calculated for each.

2.2.7 Calculation of metabolic functional diversity of microbial communities

Using substrate richness (R), the microbial diversity based on biochemical patterns of the BIOLOG AN microplate can be determined and expressed using the Shannon-Weiner Index (H') (Zak et al., 1994):

$$H' = - \sum P_i \ln P_i \quad (2)$$

$$P_i = (C_i - R) / \sum (C_i - R) \quad (3)$$

Where P_i represents the ratio of the absorbance value of the i^{th} (1 to 95) carbon substrate to the total absorbance of all the carbon substrates. Another measure of functional diversity that accounts for distribution of carbon substrate utilization levels and patterns is Shannon evenness (E) (Keylock, 2005) which was calculated as follows:

$$E = H' / \ln S \quad (4)$$

Where S represents the number of oxidized carbon substrate substrates based on the criteria of $A_{590} > 0.25$ constituting a positive reaction.

2.2.8 Lignocellulolytic activity assays

Aliquots of digestate samples were centrifuged at 7000 x g for 20 min in order to separate residual biomass and cells from the extracellular enzymes within the supernatant. To measure lignocellulolytic activity over time and between treatments, the measurement of liberated reducing sugars served as an indirect estimation of lignocellulolytic activity which was determined using the 3,5-dinitrosalicylic acid (DNS) method as previously described (Wongwilaiwalin et al., 2018). Briefly, an aliquot of supernatant from each treatment was incubated at 38 °C with the following substrates: 1 % carboxymethyl cellulose (CMC, MP Biomedicals), 1 % corn core xylan (TCI America), and Whatman no.1 filter paper (GE Healthcare Life Sciences), in order to assess the degree of degradation of each substrate via the release of glucose from the cellulose substrates and xylose from xylan. The enzyme source and the substrate were diluted in 50mM citrate buffer (pH 4.8) and then incubated for 24 h to allow for a quantifiable degree of degradation. At the end of incubation, reducing sugar concentrations were estimated via measurement of absorbance at 540 nm relative to a standard curve of glucose and xylose for the corresponding substrates. The total amount of released reducing sugars was converted to the rate of sugar released per μL of sample per minute to represent enzyme activity (Yen and Brune, 2007).

2.2.9 Statistical analysis

Principal component analysis (PCA) was performed to evaluate the effect of TS content on the metabolic functional diversity of the microbial community. Using A_{590} of each carbon substrate in the BIOLOG AN microplate, PCA allowed for discrimination of carbon substrate utilization according to %TS over time. In order to avoid the influence on statistical analyses, A_{590} values were first divided by the AWCD and the corrected values were then used for PCA. Results from one and two-way analysis of variance (ANOVA) over the entire digestion period were considered significant when the p-value was less than 0.05. A Pearson correlation matrix was constructed in order to evaluate correlation between fecal indicator bacteria content with different process parameters. All statistical analyses were performed using OriginPro 9.

2.3 Results and Discussion

2.3.1 pH, volatile fatty acids (VFA as FOS) and buffering capacity (TAC) profiles

The pH profiles for the different % TS treatments of the BMP assays were measured to observe for influence of % TS content (Fig. 2.1a). For the control, 1 %, and 2 % TS the pH fluctuated between pH 7.25 and 8.16 throughout digestion which is within the optimal range for methanogenesis (Ferrer et al., 2010). As TS increased past 2 %, pH dropped to below 6 after 10 days due to higher organic content being converted into volatile organic acids, carbon dioxide and hydrogen (Meng et al., 2018; Veluchamy et al., 2019). The 6 % TS treatment remained acidic for the rest of the digestion period whereas 3 % to 5 % TS reached the optimal pH range starting from day 30 onwards. The pH of 1 % to 3 % TS did not differ from the control ($p>0.05$), whereas 4%-6 % TS were significantly different compared to the control ($p<0.05$). Total VFA concentration (expressed as FOS, i.e. acetic acid equivalents) and alkalinity (expressed as TAC, i.e calcium carbonate equivalents) were represented as a FOS/TAC ratio (Fig 2.1b) to indicate anaerobic digestion stability and measure the balance between acidity and buffering capacity (Lossie and Putz, 2008; Riaud et al., 2010). The 1 %-4 % TS treatments did not differ from the control or within treatments ($p>0.05$), whereas the higher % TS treatments were significantly different from the control and the lower % TS treatments ($p<0.05$). As seen in Figure 2.1a, b, acidity and FOS/TAC generally increased with TS, which was previously observed (Veluchamy et al., 2019), although the mean levels stabilized over time within the optimum range in all but the 6% TS treatment.

2.3.2 BMP of SM-CS mixtures at different SM:CS ratios

Cumulative methane production profiles of the BMP assays were recorded over the 75 d digestion period (Fig. 2.1c). Within the first 10 days there was a spike in cumulative methane production for all treatments due to consumption of free soluble organic material found within the feedstocks. After 10 days, methane production increased the most for the 2 % TS treatment followed by 1% TS due to slightly higher sCOD concentration at 820 mg L⁻¹ vs 800 mg L⁻¹ for 2 % and 1 % TS, respectively, owing to more readily available free soluble organic matter for methane production. The 3 % TS treatment demonstrated greater methane production and surpassed all treatments after 30 days, which coincided with pH returning to around neutral due to

increased consumption of VFA, also noted by the lower FOS/TAC ratio (Fig. 2.1b). Methane production increased in 4 % TS from day 30 onwards and coincided with a decrease in FOS/TAC and pH increase to neutrality, suggesting continuous consumption and conversion of VFA. Although 4% TS produced the most methane within 75 days (Fig. 2.1b), the specific methane yields for each mixture varied-with SM demonstrating the highest yield at 403.1 mL g⁻¹ VS. For 1 %, 2 %, 3 %, 4 %, 5 %, and 6 % TS mixtures the specific methane yields were 384.94, 370.94, 302.82, 263.52, 53.94, and 15.14 mL g⁻¹ VS, respectively. You et al. (2019) investigated co-digestion of swine manure with NaOH and Cao pre-treated corn stover in mesophilic batch assays which reported methane yields of 237 to 328 mL g⁻¹ VS which is comparable to the results reported in this study. In this BMP assay, raw swine manure was used as the inoculum source instead of anaerobic digestate yet methane yields were comparable to digesters utilizing digestate as the inoculum. These results demonstrate that the native microflora in swine manure acclimated within the anaerobic conditions and were able to produce methane.

2.3.3 Organic removal profile

VS content was only measured before and after digestion, however, VS decreased in all treatments ranging from 17.36 % to up 86.31 % reduction. The magnitude of VS reduction was inversely proportional to influent % TS content. In previous studies, VS reduction for co-digested livestock manure was estimated to be between 37-60 % (Quiroga et al., 2014), therefore, it was evident that all but 6% TS had substantial VS reduction. The low VS reduction seen in the 6 % TS treatment could be attributed to the slow acclimation of the microbial community after acidogenesis, as seen by the constant low pH throughout digestion caused by the accumulation of VFA (Fig. 2.1b). Within this experimental design the inoculum was derived from raw swine manure instead of from an operational anaerobic digester, therefore, the microbial community needed to acclimate to their environment which could also explain the lower VS removal rate. The sCOD concentrations for the control and % TS feed mixtures varied over time and with respect to initial % TS loading (Fig. 2.1d). The overall profile revealed greater mean sCOD concentration as the % TS content increased which was expected since more solids means more oxidizable material (Fig. 1d). The 1 % and 2 % TS treatments had comparable mean sCOD concentrations compared to the control ($p>0.05$) whereas the other % TS treatments were different ($p<0.05$). Although there was detectable cumulative methane production (Fig. 2.1c) and % VS reduction for

all treatments, the effluent sCOD increased between ~200-300 % for all treatments with respect to the influent sCOD. Since corn stover was co-digested with manure, there was an increase in solubilization within the first 10-20 days due to readily available volatile solids, which has been observed previously in pulp paper mill sludge batch digestion (Veluchamy and Kalamdhad, 2017a; Wang et al., 2005). Over time the microbial community adapted to process conditions, leading to a second spike of solubilization after 50 days likely due to further hydrolysis of the insoluble organic fraction of corn stover.

Fig. 2.1. Variations of a) pH, b) FOS/TAC ratio, c) cumulative methane production, and d) soluble chemical oxygen demand (sCOD) concentration with increasing total solids (TS) in a biochemical methane potential assay. SM: swine manure.

2.3.4 Hygienization effect of the mesophilic batch anaerobic digestion

The initial and subsequent bacterial concentrations over the digestion period for each % TS treatment can be seen in Figures 2.2a-d, with each treatment displaying different bacterial

reduction patterns. For all four fecal indicator bacteria groups, 2-way ANOVA revealed significant impacts of % TS, digestion time, and the interaction of both factors on bacterial concentrations ($p < 0.05$). Overall, *E. coli* was reduced with respect to the influent concentration and undetectable in all treatments by the end of digestion ($\sim 4 \log_{10}$ reduction), with 2 % to 4 % TS exhibiting significantly different *E. coli* concentrations over time with respect to the control ($p < 0.05$). *E. coli* reduction in this study was greater than previously reported in manure co-digested with crop biomass and raw sewage sludge digestion studies (Borowski et al., 2014; Orzi et al., 2015), highlighting the significance of % TS content, digestion time, and the interaction of both parameters in reduction ($p < 0.05$). Fecal coliforms were generally reduced between days 20-40 of digestion (Fig. 2b); however, regrowth occurred after 40 days of digestion. Despite this regrowth, final concentrations in most treatments were below the initial influent concentration. Only 4 % TS exhibited an increase of $\sim 1 \log_{10}$ while only 6 % TS had complete coliform removal ($\sim 4 \log_{10}$ reduction). The control and remaining treatments had a reduction between 0.1 to less than $1 \log_{10}$ units, which was much lower than in other anaerobic digesters processing swine manure (Dennehy et al., 2018; Orzi et al., 2015). These differences could be attributed to reactivation of viable but non-culturable (VBNC) cells under favourable conditions. Figure 2.2c reveals the fecal enterococci profile for all treatments with no clear reduction trend and only the 5 % TS treatment differing from the control ($p < 0.05$). For all but 4 % TS, the reductions were less than $1 \log_{10}$ unit, which is similar to previously reported studies studying fecal enterococci reduction during AD of swine manure (Avery et al., 2014). Fecal enterococci are facultative anaerobes and able to survive under a wide range of physiochemical conditions such as temperature (5–65 °C) and pH (4.5-10), explaining the survival patterns observed in this study (Fisher and Phillips, 2009). *Clostridia* spp. remained between ~ 3 to $\sim 6 \log_{10}$ CFU mL⁻¹ throughout the digestion process in all co-digestion mixtures and the control (Fig. 2.2d). Many *Clostridia* spp. are normally found in the anaerobic digester environment due to their role in acidogenesis (Chen et al., 2005) and generally are resistant to anaerobic digestion, especially in mesophilic environments (Orzi et al., 2015). With the exception of 2 % TS, % TS had a significant effect on *Clostridia* spp. concentrations compared to the control ($p < 0.05$). All treatments except 1 % TS ($0.7 \log_{10}$ reduction) demonstrated increases of ~ 0.1 to $\sim 2.6 \log_{10}$ CFU mL⁻¹. While the hygienization efficiency of the BMP assays was generally low, pathogens were not investigated in this study. Some prevalent pathogens in manure such as *Salmonella*, *Yersinia*, *Campylobacter*,

Giardia and *Cryptosporidium* have been shown to be significantly reduced during AcoD (Orzi et al., 2015; Scaglia et al., 2014) but correlation between fecal indicator reduction and prevalence of these organisms were not investigated in this study.

Fig 2.2. Variations in a) *E. coli*, b) fecal coliforms, c) fecal enterococci, and d) *Clostridia* spp. concentrations with different total solids (TS) content during anaerobic codigestion. SM: swine manure.

2.3.5 Inactivation mechanism of fecal indicator bacteria

The % TS loading and digestion time had an impact on bacterial counts for each indicator group examined in this study, but physiochemical factors such as pH, sCOD, methane production, and FOS/TAC also contributed to reduction of fecal indicator bacteria. A Pearson correlation matrix was constructed to map significant correlations between pH, sCOD, daily methane production, and FOS/TAC to elucidate potential inactivation factors. The pH correlated with significant reduction in *E. coli* only for the control and 2 % TS, in coliform concentration for 4 %

TS, and in *Clostridia* spp. concentration for 6 % TS. The FOS/TAC correlated with significant reduction in *E. coli* only for the control, and significant positive correlation in *Clostridia* spp. for 6 % TS only. Daily methane production correlated with significant reduction in 4 % TS only with respect to fecal enterococci. The last physiochemical parameter, sCOD, only correlated with significant reduction in 2 % and 6 % TS with respect to fecal enterococci but no other % TS loadings or fecal indicator bacteria. Orzi et al. (2015) reported significant negative correlation in *E. coli* and fecal coliforms with respect to pH and significant negative correlation in fecal enterococci and *Clostridia* spp. with respect to sCOD and methane production. In this study the effect of pH on *E. coli* and coliform reduction and the effect of methane production and sCOD concentration on fecal enterococci were in accordance with the full-scale anaerobic digesters study (Orzi et al., 2015); however, these effects were only evident in a few of the % TS treatments. The results suggest that pH and substrate competition (via methane production and sCOD concentration) were the main correlated factors in fecal indicator bacteria reductions. *Clostridia* spp. was the only exception in which no significant reduction resulting from substrate competition was observed, which has been previously noted (Skillman et al., 2009). In this study ammonia concentrations were not estimated, which has demonstrated sanitization effects (Scaglia et al., 2014) and could have contributed to the hygienization trends observed in this study. Another potential inactivation mechanism relating to digester operation is batch feeding, which has been shown to significantly reduce pathogen and indicator bacteria compared to continuous feeding. Continuous feeding and removal between 1-2 days in mesophilic digesters has resulted in poor hygienization previously (Sahlström, 2003). Hygienization during AcoD of swine manure and corn stover should be investigated in (semi)-continuously fed digesters of different designs in order to determine the influence of feeding regime on bacterial reduction.

2.3.6 Lignocellulosic substrate degradation efficiency

The activities of key lignocellulolytic enzymes (FPase, CMCase, and xylanase) were compared between treatments (Fig. 2.3). The first 10 days showed an immediate drop in other enzymatic activities for all treatments followed by an increase in activities, with each enzyme activity peaking after 30 days of digestion. The peak CMCase activity on average was $1.5 \times 10^{-3} \mu\text{g } \mu\text{L}^{-1} \text{ min}^{-1}$, which was about 33 % higher than the peak FPase activity on the same day. This observation could be due to the availability of cellulose and xylan within corn stover, therefore

leading to increased enzyme production in response. Since FP contains crystalline cellulose it was not as easily hydrolysable as CMC, which has been observed previously in a study that used lignocellulosic microbial consortium for digesting swine manure and Napier grass (Wongwilaiwalin et al., 2018). The relatively higher xylanase activity could be attributed to the structural barrier of hemicellulose within corn stover, limiting cellulase access to cellulose and inducing increased xylanase production to overcome this barrier (Gu et al., 2014). Xylanase reached peak activity on the same day as CMCase and FPase, revealing the synergistic effect of cellulase and hemicellulase (represented as xylanase) in lignocellulose degradation (Gu et al., 2014). Although enzyme activities fluctuated over time and between treatments, 4 % TS demonstrated maximum CMCase with a peak of $1.5 \times 10^{-3} \mu\text{g } \mu\text{L}^{-1} \text{min}^{-1}$ and FPase and xylanase activity levels higher than most treatments. The peak in all enzyme activities corresponded with the cumulative methane production profile for 4 % TS (Fig. 2.1c), which showed an upward trend in methane production starting around the same day of the maximum enzyme activities. TS content had a significant impact on xylanase activity ($p < 0.05$) while for CMCase activity, only 4 % to 6 % TS showed significant differences compared to the control ($p < 0.05$). The influence of lignocellulosic biomass on enzymatic activity was also observed in the co-digestion of algal sludge and paper waste feedstocks (Yen et al., 2007). Overall, all the treatments demonstrated higher xylanase, CMCase, and FPase activities compared to mono-digestion of swine manure. Though no studies have investigated the impact of lignocellulosic waste co-digestion with swine manure on lignocellulolytic activity, Zhao et al. (2014) previously reported higher lignocellulolytic activity during co-digestion of rice straw and municipal sewage sludge vs mono-digestion. The CMCase and FPase activities, which represent cellulase activity, were much higher than measured cellulase activities in algal sludge digestion ($1.25 \times 10^{-6} - 3 \times 10^{-6} \mu\text{g } \mu\text{L}^{-1} \text{min}^{-1}$) (Yen et al., 2007) and similar to the activities measured during AcoD of food waste and wheat straw in BMP assays ($3 \times 10^{-4} - 3.75 \times 10^{-4} \mu\text{g } \mu\text{L}^{-1} \text{min}^{-1}$) (Yong et al., 2015). AcoD trials demonstrated microbial community acclimation to degrading lignocellulosic biomass even though inoculum from an operational digester was not used in the BMP assays. Although swine manure contains fewer hydrolytic microbes owing to the high content of undigested lignocellulosic fibres (Wongwilaiwalin et al., 2018), this study suggested that the native microflora can be acclimated to lignocellulose-rich substrate given an appropriate retention time and % TS loading rate.

Fig. 2.3. Lignocellulolytic activity profiles of a) CMCCase, b) FPase, and c) xylanase in crude supernatants obtained from digestates taken over time during a biochemical methane potential assay. SM: swine manure.

2.3.7 Carbon substrate metabolism

BIOLOG AN microplates contain 95 different carbon substrates and were divided accordingly into 6 distinct biochemical categories and the carbon substrate utilization profiles related to % TS were studied over the course of digestion (Fig. 2.4). One-way ANOVA revealed significant impacts of % TS on carbohydrate and polymer utilization ($p < 0.05$), however, there were no significant impacts on the other biochemical categories ($p > 0.05$). There were significant differences in carbohydrate and polymer utilization in 3 % TS with respect to the control ($p < 0.05$) and differences between 1 % and 3 % TS in terms of polymer utilization ($p < 0.05$). The 3 % TS

treatment demonstrated the highest mean consumption of carbohydrates, miscellaneous, and polymers (AWCD= 0.323, 0.220, and 0.170, respectively). For the remaining categories, the control demonstrated the highest mean consumption of amides/amines and amino acids (AWCD=0.0705 and 0.0675, respectively), while 2 % TS had the highest mean consumption of carboxylic acids (AWCD=0.148). Since animal manure is rich in proteins (Ning et al., 2019), as the % TS increased the metabolic profile changed to utilization of carbon-rich substrates due to a favourable C/N ratio, allowing for microbial growth and production of enzymes. Overall, microbial communities favoured carbohydrates the most while amino acids and amides/amines were the least consumed carbon source, which has been previously observed in other CLPP studies (Ge et al., 2018). The observed results suggest that the microbial communities preferred carbohydrates as their main carbon source during anaerobic co-digestion and provide additional evidence of microbial adaptation to lignocellulose metabolism.

Fig. 2.4. Changes in carbon source utilization over time for six different groups of carbon sources: a) carbohydrates, b) carboxylic acids, c) amino acids, d) polymers, e) amides/amines, f) miscellaneous. Values on the y-axis are based on average well colour development (AWCD) in Biolog AN microplates. SM: swine manure.

2.3.8 Differences in metabolic functional diversity indices between % TS treatments

To assess the microbial functional diversity with respect to % TS, Shannon diversity (H') and Shannon evenness (E) based on carbon substrate utilization within BIOLOG AN microplates were calculated (Table 2.3). The Shannon diversity index is highly influenced by species diversity and a higher index reveals a higher functional diversity of the microbial community (Ge et al., 2018). The Shannon evenness index reflects distribution of species as a function of metabolic diversity; therefore, a higher evenness signifies an even distribution of species within the microbial community (Keylock et al., 2005). Two-way ANOVA revealed no significant differences between % TS or digestion time and Shannon diversity, and no significant differences amongst % TS ($p > 0.05$). No significant differences between % TS and Shannon evenness or between treatments ($p > 0.05$) were observed, although there was a significant difference between digestion time and evenness ($p < 0.05$). For both indices, there were noticeable differences among % TS regardless of statistical significance. The 4 % TS treatment showed the highest mean microbial functional diversity ($H' = 3.66$) which coincided with the highest cumulative methane production whereas the 2 % TS treatment showed the highest mean microbial species evenness ($E = 3.23$). Although there was no clear influence of time or % TS on metabolic diversity, evenness generally decreased over time which could suggest that AcoD selected for microbial species with specific metabolic functions responsible for methane production.

This study reported the use of BIOLOG AN microplates for the first time as both a CLPP tool and as a tool for specifically characterizing microbial metabolic functionality during AcoD. BIOLOG AN microplates contain more carbon sources compared to BIOLOG Ecoplates which allows for a more in-depth metabolic fingerprint of both AD and AcoD microbial communities. Overall, the BIOLOG method is advantageous because preparation and analysis of these plates requires less hands-on time, has a simpler protocol compared to DNA/RNA amplification (Lladó and Baldrian, 2017), has the ability to capture metabolic functionality of non-culturable microorganisms (Cardinali-Rezende et al., 2011), and temporal evaluation of microbial community metabolic diversity can be performed due to the simplified protocol and cheaper per sample cost. While the BIOLOG method is a relatively cost-effective and user-friendly CLPP tool, its culture-based approach favours less fastidious microorganisms and could discriminate against

fastidious and VBNC organisms, thus not accounting for the full metabolic functionality of the anaerobic digester community (Garland et al., 1997). Another disadvantage to this method is it can't distinguish the phylogeny of the microbial community and doesn't provide information on genetic parameters such as energy metabolism and secondary metabolism (Wang et al., 2018). A molecular approach, such as 16S rRNA sequencing could provide a more clear and robust profile of the microbial community composition since it can detect with more sensitivity the culturable and non-culturable microorganisms (i.e. methanogens) which interact with one another during methanogenesis (Cardinali-Rezende et al., 2011). Phylogenetic Investigation of Communities by Reconstruction of Unobserved States (PICRUSt) is a computational approach for predicting microbial community functionality based on high-throughout molecular sequencing which is been employed in understanding organics degradation during anaerobic digestion (Buhlmann et al., 2019). The disadvantages of molecular analyses and PICRUSt, however, is the inability to completely reflect the actual microbial community functionality due to the impact of functional gene expression on microbial activities (Wang et al., 2018). Despite some of the shortcomings of BIOLOG assays, BIOLOG AN assays have clearly discriminated distinct metabolic functional profiles, revealing the impact of TS on microbial community structure and demonstrating its effectiveness as a rapid CLPP tool. Because of the lower cost and technological requirements, the microplate method could be used as a stand-alone CLPP tool or can be coupled with metagenomic analyses such as PICRUSt to assign observed microbial functional traits with genetic information and microbial taxa.

2.3.9 PCA analysis of carbon substrate utilization

PCA analysis has been used to evaluate carbon substrate utilization of microbial communities in various environments such as rice storage and soils amended with digestate (García-Sánchez et al., 2015; Ge et al., 2018). The analysis was performed on overall carbon substrate utilization in each % TS treatment with respect to digestion time using two uncorrelated components in order to completely differentiate the treatments (Fig. 2.5). The variances explaining PC1 and PC2 components for each treatment were different, ranging between 25.25 % to 84.10 %, yet still revealed distinct carbon substrate utilization profiles with respect to % TS over time. One trend in particular was the clustering of 3 % and 4 % TS in almost identical positions on the plots from day 40 onwards- demonstrating similar carbon substrate utilization patterns between

Table 2.3

Changes in microbial functional diversity indices: a) Shannon Diversity (H'), b) Shannon Evenness (E) over time with different total solids (TS) content during anaerobic co-digestion. SM: swine manure.

Solid Content (%)	Index	Time (d)							
		0	10	20	30	40	50	60	75
Control (0.69)	Diversity (H')	3.958	3.271	2.842	3.436	0.692	3.595	3.707	2.795
	Evenness (E)	2.607	2.717	0	0	0	0	0	0
1	Diversity (H')	2.036	3.662	2.475	3.252	3.354	3.714	3.988	3.256
	Evenness (E)	6.765	3.041	5.187	0	5.571	3.240	0	0
2	Diversity (H')	3.686	4.161	4.063	4.028	3.873	3.047	3.580	2.472
	Evenness (E)	3.416	2.402	2.984	2.588	2.573	0	11.892	0
3	Diversity (H')	3.410	3.970	3.899	2.252	3.916	3.914	3.934	3.665
	Evenness (E)	2.667	2.571	2.864	3.740	2.479	2.368	2.591	2.622
4	Diversity (H')	3.217	3.792	2.883	3.722	3.732	4.192	3.907	3.835
	Evenness (E)	2.473	3.224	0	0	2.398	2.507	3.112	2.394
5	Diversity (H')	3.517	3.966	3.411	3.442	3.281	3.483	3.8189	3.657
	Evenness (E)	2.548	3.808	0	0	2.522	2.893	5.463	0
6	Diversity (H')	3.566	4.339	3.916	0	3.655	3.009	3.364	0.663
	Evenness (E)	2.551	3.104	0	0	2.526	4.999	0	0

microbial communities. Cumulative methane production, lignocellulolytic activities, and carbon substrate utilization based on biochemical groups (Fig. 2.1, 2.2, 2.4) were similar between treatments starting from day 40 onwards which further reinforces the results from PCA analysis. Although no previous research or PCA analysis has investigated the impact of TS on CLPP during AcoD of swine manure and corn stover, researchers noted different carbon substrate utilization patterns in soil microbial communities amended with digestate (consisting of beet pulp, maize

silage, and fruit marc) compared to non-amended soils (García-Sánchez et al., 2015). The carbon substrate utilization patterns in amended soils noted by García-Sánchez et al. (2015) utilized amides and amines, whereas carbohydrate utilization decreased. However, the introduction of organic matter in the form of anaerobic digestate clearly influenced CLPP which is in agreement with the results of this study. CLPP analysis using BIOLOG AN microplates provided novel insight into microbial metabolic functionality of co-digesting manure with lignocellulosic biomass and outlined influence of TS on microbial metabolism which has not been reported previously in AcoD using these particular assays.

Fig. 2.5. Principal component analysis (PCA) of the microbial carbon source utilization patterns in digestates from a biochemical methane potential assay. a) day 0, b) day 10, c) day 20, d) day 30, e) day 40, f) day 50, g) day 60, h) day 75. The position of each % total solids (TS) treatment indicated the load on the PC1 (x-axis) and PC2 (y-axis).

2.4 Conclusion

The impact of TS during AcoD of swine manure and corn stover revealed different fecal indicator bacteria reduction rates. BIOLOG AN microplates discriminated microbial carbon substrate utilization according to TS, a pattern which was also observed in lignocellulolytic activity profiles. The results suggest the native microflora within these wastes possess the ability to degrade lignocellulose and can adapt to utilizing this feedstock during AcoD. This study outlines a novel CLPP approach for determining the relationship between the microbial community and digester operation performance. This information can provide a basis for optimizing co-digestion of higher TS loadings in other digester systems.

Chapter 3

Biogas production, waste stabilization efficiency, and hygienization potential of a mesophilic anaerobic plug flow reactor processing swine manure and corn stover

Abstract

Anaerobic co-digestion of swine manure and corn stover was investigated during semi-continuous operation of a plug flow reactor (PFR) at mesophilic temperature. Long-term PFR operation was studied during a step-wise increase in organic loading rate (OLR) over the range of 0.25-4.7 kg volatile solids added (VS) $\text{m}^{-3} \text{d}^{-1}$, which corresponded to total solids content (TS) of 1.5-9.0 %. Process stability was observed at all OLR, with the highest total biogas yield and methane content of 0.738 $\text{m}^3 \text{kg}^{-1}$ and 62 %, respectively, at 0.25 $\text{kg m}^{-3} \text{d}^{-1}$. As OLR and TS increased, VS reduction decreased and volatile fatty acids (VFA) increased due to shorter hydraulic retention times (HRT). Hygienization potential was assayed using fecal indicator bacteria (FIB), with some groups being reduced (*E. coli*, fecal coliforms) and others not (*Clostridia* spp., fecal enterococci). Lignocellulolytic enzyme activity trended upward as OLR was increased, highlighting changes in microbial activity in response to feeding rate.

Keywords

Co-digestion; biogas production; hygienization potential; lignocellulolytic activity; plug flow reactor

3.1 Introduction

Bioenergy is an attractive alternative to fossil fuel consumption and accounts for 14 % of total energy consumption, making it the fourth largest global energy source (Chen and Lee, 2014). In particular, anaerobic digestion (AD) technology has shown promise in bioenergy generation from different organic waste streams (Neshat et al., 2017) but also as a means of nutrient recycling to limit terrestrial eutrophication and reduction of greenhouse gas (GHG) emissions (Muhammad et al., 2016; Nasir et al., 2014). This technological process has been popular for animal manure

management due to increased livestock farming operations generating one of the most abundant organic waste streams worldwide (Dong et al., 2019b).

However, despite abundant availability and proven methane production potential in AD systems (Nasir et al., 2012), manure can pose challenges as a substrate. In North American swine production, manure is typically flushed from barns with water, resulting in dilute organic matter that typically has <5 % solids content (Bourque and Koroluk, 2003; Key et al., 2011). Further, swine manure exhibits a nutrient imbalance due to a low carbon to nitrogen (C/N) ratio, which leads to decreased process stability and biogas production during AD (Mao et al., 2015; Nasir et al., 2012). To compensate for the nutrient imbalance and high-water content of swine manure, anaerobic co-digestion (AcoD) is an established process which utilizes carbon-rich substrates such as lignocellulosic biomass for feedstock in combination with the nitrogen-rich swine manure. The AcoD process proved to yield better process performance in terms of higher biogas production and reactor stability compared to mono-digestion of manure (Ning et al., 2019; Qian et al., 2019). Despite its successes and advantages, the recalcitrant nature of lignin and tight linkage to cellulose somewhat inhibits microbial degradation leading to decreased biogas production and solids removal efficiency (Neshat et al., 2017).

Reactor configuration is one important aspect of improving AcoD because operational conditions can be optimized and modified in order to improve the biogas potential depending on the feedstock characteristics. On a pilot and farm-scale operation, reactor configuration is advantageous over pre-treatment methods due to lower energy inputs and water usage associated with chemical and physical pretreatment (Mussoline et al., 2012). Most research and commercial installations focused on AcoD of manure and lignocellulosic biomass utilize continuously stirred tank reactor (CSTR) designs. These designs are popular due to their ability to handle low total solids (TS) content waste such as manure, relative ease of use, and simplistic design. However, as TS content increases to above 10 % the mixing capabilities become reduced which leads to process instability (Qian et al., 2019; Sawatdeenarunat et al., 2015).

Plug flow reactors (PFR) have been reported to work well at high TS content (>10 % TS) and are considered low-cost digesters compared to CSTRs because of simpler construction and mixing mechanisms (Lansing et al., 2010). The inherent operational design allows for phasing of the different anaerobic digestion stages, shorter start-up, and a relatively smaller reactor volume

due to its ability to accommodate high solid waste (Dong et al., 2019b; Patinvoh et al., 2017). Veluchamy et al. (2019) reported mesophilic corn silage digestion in an auger fitted PFR during long operation could successfully handle 12 % TS and was stable at organic loading rates (OLR) of 3.5-6.0 kg VS m⁻³ d⁻¹, highlighting the effectiveness of the auger conveying mechanism to slowly move material along the digester. Although there is previous research on long-term PFR operation with different PFR designs, there is no available literature on long-term AcoD of swine manure and corn stover in a semi-continuously fed PFR nor has the impact of increasing OLR on AcoD of these feedstocks been extensively studied.

While research on AcoD systems using agricultural wastes has mainly focused on optimization of feedstock ratios, operational conditions, and reactor designs for enhanced biogas production, little research has been conducted investigating the impact of these same parameters on waste hygienization (Dennehy et al., 2018). Dennehy et al. (2018) reported no significant impacts of swine manure (SM): food waste (FW) ratios and hydraulic retention time (HRT) on fecal indicator bacterial (FIB) removal, whereas Arias et al. (2020) reported the impact of increasing %TS on removal of some FIB groups such as non-pathogenic *E. coli* during batch AcoD of swine manure and corn stover (CS). Extensive research has been conducted on hygienization of different waste streams during AD and AcoD in different reactor designs (Jiang et al., 2020), however, the impact of increasing solids content and OLR on hygienization during AcoD in a PFR system has not been investigated.

The main objective of this study was to investigate AcoD of swine manure and corn stover in a semi-continuously fed auger PFR system during step-wise OLR increase. Over the course of PFR operation the impacts of OLR, % TS, and digestion time were varied in order to assess the methane production potential, organic matter reduction efficiency, and hygienization potential. In addition to understanding the physiochemical and biological dynamics during PFR digestion, the metabolic adaptation of the microbial community to increasing OLR was also investigated by measuring key enzymatic activities in relation to lignocellulosic content of the feeding regimes. The overall approach and methodology provided insight on increasing organic loading in the form of lignocellulosic biomass in terms of overall PFR performance and microbial metabolic adaptation.

3.2 Materials and Methods

3.2.1 Substrate and inoculum characteristics

The physiochemical characteristics of the substrates and inoculum are outlined in Table 3.1. Swine manure was collected from an open in-ground manure pit located on a local pig farm in Ridgetown, Ontario, Canada in February, March, August, and November 2019. Corn stover was collected from the field plots at the University of Guelph Ridgetown campus around the same time as swine manure collection. Both substrates and the inoculum in this study were collected from the same sources and processed according to a previous study (Arias et al., 2020). The inoculum used within this study was prepared from swine manure used for the feeding trials and briefly described below in Section 3.2.2.

Table 3.1.

Physiochemical characteristics of feedstock components adapted from Arias et al. (2020).

Parameter	Units	Swine Manure	Corn Stover
Total Solids	%	0.92±0.31	74.06
Volatile Solids (of Total Solids)	%	52.35±3.1	93.58
Organic Matter	%	0.36	69.30
pH	n.a	7.25	n.d
Total Nitrogen	%	0.10	1.22
NH ₄ ⁺ N	mg L ⁻¹	762	n.d
Total phosphorous	%	0.02	n.d
Carbon:Nitrogen Ratio (C/N)	n.a	2:1	35:1
Sulfur	mg L ⁻¹	50.4	n.d
Conductivity	mS cm ⁻¹	14.94±0.54	n.d
Cellulose	%	n.d	38.82±0.49
Hemicellulose	%	n.d	29.02±0.39
Lignin	%	n.d	7.16±0.14

n.a: not applicable; n.d: not determined

3.2.2 Semi-continuous long-term digester operation

The PFR used in this study is the same one used in a previous study investigating biogas production and process performance of mesophilic semi-continuous AD of corn silage (Veluchamy et al., 2019). The 65 L reactor (working volume of 50 L) operated in a draw and fill manner, with daily morning feeding and an auger fitted within the reactor to convey the substrate plug along the length of the reactor (from inlet to outlet port). Stainless steel impeller paddles were spaced along the auger to improve axial and longitudinal mixing of substrate in order to convey the material towards the outlet port. The PFR was operated and maintained at mesophilic temperature (35 ± 2 °C) using a heating mat covering the outside of the reactor to reduce thermal loss. Prior to semi-continuous operation of the PFR, the reactor was fed with swine manure to promote an active microbial community and allowed to acclimate until biogas production stabilized after 15 days. After the acclimation period, long-term operation began on day 0 with initial feeding at $0.25 \text{ kg m}^{-3} \text{ d}^{-1}$ followed by a stepwise increase up to $4.7 \text{ kg m}^{-3} \text{ d}^{-1}$. The OLR and feedstock composition for this study are outlined in Table 3.2. The first two OLR steps (OLR 1 and 2) were maintained at 50 d, decreased to 40 d for OLR 3 and OLR 4, and finally to 15 d for OLR 5. Inlet feedstock and digestate sampling occurred at the four ports (inlet, port 1, port 2, outlet) spaced out along the length of the reactor (Veluchamy et al., 2019) every 5 days for physiochemical, microbiological, and biochemical analyses. Biogas production from the PFR was measured daily using a multi-chamber rotor gas meter (Integrated Exploration Inc., Guelph, Canada).

Table 3.2.

The organic loading rate and composition of the feedstock for PFR operation.

Solid content (%)	OLR (kg VS m ⁻³ d ⁻¹)	Designation	Dosage of each material (g)		Total dosage (kg d ⁻¹)
			Corn Stover	Swine manure	
1.5	0.25	OLR 1	11.20	1000	1.011
3	0.75	OLR 2	32.50	1000	1.032
6	3.0	OLR 3	78.75	1000	1.078
7.5	3.8	OLR 4	102.30	1000	1.102
9.0	4.7	OLR 5	128.80	1000	1.128

3.2.3 Physiochemical analysis

Total solids (TS) and volatile solids (VS) of raw swine manure, corn stover, and digestate were determined according to Standard Methods (APHA, 2017). The pH of influent feedstock mixtures and digestate was measured using a pH/EC meter (Seven Excellence, Mettler Toledo, USA) according to Standard Methods (APHA, 2017). Soluble chemical oxygen demand (sCOD) was determined colorimetrically (Photoflex, YSI, USA) as previously described by Arias et al. (2020). Volatile fatty acids (FOS), buffering capacity (TAC), and the FOS/TAC ratio values were estimated according to the Hach Lange Laboratory protocol for FOS and TAC measurements (Lossie and Pütz, 2008). Biogas composition (CH_4 , CO_2 , and H_2S) was measured every 3 days using an infrared multiple gas measuring device (Multitec 540, Sewerin, Germany). All physiochemical analyses, except biogas composition, were performed in duplicates.

3.2.4 Microbiological analysis

To determine the PFR hygienization potential, extraction and enumeration of FIB at different time points and reactor ports for each OLR was performed as an indirect measurement of pathogen reduction. Influent feedstock mixtures and digestates sampled every 10 days for OLR 1 and 2 and then every 5 days for OLR 3-5 were serially diluted 10-fold in 1X phosphate buffered saline (PBS) water (pH 7.2). To extract and enumerate bacteria, the membrane filtration (MF) technique was employed as described by Pandey and Soupir (2011). Briefly, triplicate membrane filtrations were conducted on each port sample using 1 mL of diluted sample which was washed with 50 mL PBS water to evenly distribute bacteria on the membrane filter. The following FIB were isolated and cultured on substrate-specific agar plates for quantification: non-pathogenic *E. coli*, fecal (thermotolerant) coliforms, fecal enterococci, and *Clostridia* spp. The specific culture conditions such as incubation length, temperature, and culture media for each FIB group were followed as in previous studies investigating biosafety of anaerobic digestate and hygienization during batch mesophilic AcoD of swine manure and corn stover (Arias et al., 2020). The results were reported as log₁₀ colony forming units (CFU) g⁻¹ of wet sample (Bonetta et al., 2014).

3.2.5 Lignocellulolytic enzyme activity assay

The lignocellulose degrading efficiency of the microbial community over time and between OLR was estimated by measuring reducing sugar concentrations from degradation of lignocellulosic substrates in the presence of digestate supernatant. Sampling frequency of inlet feedstock and digestate was performed the same as in Section 3.2.4. Aliquots of digestate were prepared according to Arias et al. (2020) and the enzyme activities estimated using the 3,5-dinitrosalicylic acid (DNS) method as previously described (Arias et al., 2020; Wongwilaiwalin et al., 2018). The following substrates were used in this assay in order to assess glucose and xylose release from lignocellulose degradation: 1 % carboxymethyl cellulose (CMC, MP Biomedicals), 1 % corn core xylan (TCI America), and Whatman no.1 filter paper (GE Healthcare Life Sciences). Degradation of CMC and filter paper (FP) represented cellulase activity (denoted as CMCase and FPase respectively) while xylan degradation represented hemicellulase activity, denoted as xylanase. All enzymes for each substrate were performed in triplicate assays. At the end of incubation, reducing sugars were quantified via spectrophotometer measurement at 540 nm relative to the appropriate reducing sugar standard curve and enzyme activities represented as amount of sugar released per μL of sample per minute.

3.2.6 Statistical analyses

One-way ANOVA with Tukey means comparison was used to compare the impact of OLR on all physiochemical parameters of the outlet digestate. Two-way ANOVA with Tukey means comparison was used to determine the impact of digestion time and OLR on FIB concentrations and enzymatic activities in the outlet digestate. In order to assess any differences in reactor performance spatially across the digester, two-way ANOVA was employed to compare the average measurements from each port and between OLR steps. Tukey means comparison was employed in conjunction with ANOVA to determine if dependent variables were impacted by OLR, time, and port. A Pearson correlation matrix was used in order to determine any significant correlations between FIB concentrations and different physiochemical parameters. Results were considered significant when $p < 0.05$. All analyses were performed using OriginPro 9.

3.3 Results and Discussion

3.3.1 Biogas production and methane yields at different OLR

In order to assess overall reactor performance, biogas production and cumulative methane yields were measured over time and in relation to the OLR regimes (Fig. 3.1a). The acclimation period in this study was shorter in duration (15 d) and was noted by stabilized biogas production rates (data not shown) compared to the start-up periods of 20 d and 40 d, respectively, reported by Dong et al. (2019b) and Patinvoh et al. (2017). After start-up, the long-term operation commenced at day 0 with the feeding of the PFR at OLR 1. Daily biogas production (L d^{-1}) increased in relation to OLR as a result of greater availability of organic matter for subsequent conversion into biogas (Fig. 3.1a). One-way ANOVA revealed significant differences in daily biogas production rates between all OLR regimes ($p < 0.01$) and within each OLR, production rates generally trended upwards. This was indicative of stable biogas performance due to microbial community acclimation to the OLR. For OLR 1 and 2, digester performance resulted in more stable biogas production over their respective retention time compared to higher OLR, with more biogas production fluctuation occurring at higher organic loadings. Patinvoh et al. (2017) also reported similar findings at OLR of 2.8 and 4.2 $\text{kg VS m}^{-3} \text{ d}^{-1}$ during digestion of cow manure with straw in a PFR system which suggests that the microbial community adapted to increased organic loading. In the current study, average daily biogas production at OLR 3 and 4 were higher than the reported values of 3.5 and 5.0 L d^{-1} for OLR 1 and 2 in the study conducted by Patinvoh et al. (2017). The differences in daily biogas production between both studies could be attributed to feedstock composition/origin which ultimately determines biogas potential (Xie et al., 2017). The digester used in this study was incrementally fed with higher OLR, but more gradually than the study conducted by Patinvoh et al. (2017), which gave sufficient time for microbial adaptation to higher organic content within the digester system (Ning et al., 2019). Total solids content greater than 20 % has been reported to result in decreased CH_4 production due to mass transfer hydrolysis inhibition, but below this threshold the production rate trends upward with TS content (Ning et al., 2019). One challenge with increased TS content is clogging during feeding and crust formation within the reactor, which increased the working reactor volume observed within this study as OLR and TS increased (0.25-4.7 $\text{kg m}^{-3} \text{ d}^{-1}$ and 1.5-9.0 %, respectively). Patinvoh et al. (2017) reported feeding difficulty when the TS content of cow manure exceeded 13 % which is higher than OLR

5 of this current study. Another issue, particularly with lignocellulosic biomass, is low bulk density which results in highly viscous slurry production which poses challenges to mixing and feeding, resulting in crust formation (Crawford et al., 2016; Klingenberg et al., 2017; Wang et al., 2016). This issue was also noted in this current study at higher OLR due to increased corn stover content. To increase biomass densification, pelleting has been employed not only as a means of reducing transportation and storage but also increases the biogas potential at higher TS loading during anaerobic digestion (Wang et al., 2016). This method could be applied to assess the impact of AcoD with manure and other organic fractions at higher OLR in both semi and continuously fed systems, which so far has not been investigated.

Table 3.3 shows the total biogas and methane yields based on VS fed over the entire retention time at each OLR. There is a downward trend in biogas yields from OLR 1 to OLR 3, demonstrating a decrease in reactor efficiency, possibly due to not enough time for acclimation to the increasing OLR and also the PFR being fed with a lower OLR than what is normally fed in a typical PFR system. By comparison, Veluchamy et al. (2019) utilized this PFR system for corn silage AD at an OLR of 3.5-8.5 kg VS m⁻³ d⁻¹. After OLR 3, the biogas yields started to increase again as a result of increased adaptation to the higher organic content. Although daily biogas production increased with OLR, production rates fluctuated more at higher OLR, particularly at OLR 5, which indicated some process instability. When loading cow manure bedded with straw at an OLR of 6.0 g VS L⁻¹ d⁻¹ in a PFR at 28 d HRT, biogas production slightly stabilized after 20 days but began to decrease near the end of the retention time with significant VFA accumulation (Patinvoh et al., 2017). Total VFA concentrations in this study (discussed further in Section 3.3.3) did not exceed inhibitory levels, although the concentrations were significantly higher in OLR 5 ($p < 0.05$), suggesting VFA accumulation was faster than VFA consumption and subsequent conversion into methane. The HRT for OLR 5 was also shorter than the HRT from the highest OLR investigated by Patinvoh et al. (2017) which is another indication that a longer HRT is needed for methanogenic community acclimation. The HRT for the last OLR was cut short due to crust formation associated with the low density of corn stover which limited mass transfer resulting in decreased PFR working volume and ultimately decreased biogas production. The use of pelleting to improve lignocellulosic biomass densification could alleviate the feeding issues associated with ground particulate matter, in turn increasing the HRT and allowing for higher OLR operation to see if the microbial community can adapt to increased total solids. While OLR 1 had the lowest

organic content, it had a relatively longer HRT (50 d) compared to OLR 3-5 (≤ 40 d) which allowed for increased consumption and conversion of metabolites to biogas coupled with available soluble material due to lower lignocellulosic content. The highest cumulative biogas and methane yields occurred during OLR 1, with reported values of 0.738 and 0.457 m³ kg⁻¹ VS, respectively. These reported biogas and methane yields were between 1.53-2.95 and 1.41-2.80 fold higher, respectively, than in other PFR systems co-digesting manure with lignocellulosic biomass (Adl et al., 2012; Patinvoh et al., 2017). When compared to mono-digestion of swine manure in other PFR systems, the cumulative methane yields were comparable or higher than reported by previous researchers. Gómez et al. (2019) reported values between 0.12-0.37 m³ kg⁻¹ VS whereas Adl et al. (2012) reported 0.202 m³ kg⁻¹ VS, which are within the range of this co-digestion study (0.173-0.457 m³ kg⁻¹ VS), highlighting the benefits of a more balanced biochemical profile during AcoD. Although OLR 1 had the highest cumulative methane yield within this system, other performance parameters such as organic matter reduction and hygienization must be considered since these parameters can differ from biogas production in terms of operational factors (Jiang et al., 2020; Nag et al., 2019). This will be discussed further in Sections 3.4 and 3.5.

3.3.2 Biogas composition during step-wise OLR increase

Biogas composition was reported over time during PFR operation in terms of methane (% CH₄), carbon dioxide (% CO₂), and hydrogen sulfide (ppm H₂S) (Fig. 1b) while average biogas composition at each OLR is reported in Table 3.3. From Fig. 3.1b, it is evident that methane content decreased as OLR and TS increased. The highest average methane content of 62 % was measured for OLR 1 and 2, which was significantly higher than methane content at higher OLR ($p < 0.01$). The lower proportion of corn stover to swine manure in OLR 1 and 2 resulted in relatively lower hydrolysis and acidogenesis rates since lignocellulosic biomass content influences the microbial activities driving these biochemical processes (Arias et al., 2020). The lower organic loading, combined with a longer retention time allowed for stable methanogen growth and acclimation within the digester allowing for increased conversion of CO₂ to CH₄ (Veluchamy et al., 2019). Conversely as organic loading increased, the retention time was incrementally reduced in addition to higher corn stover content which leads to increased hydrolysis and acidogenesis rates compared to methanogenesis. Production of VFA at higher OLR exceeded consumption and

into methane, which demonstrated the stabilization period was not long enough to allow for methanogen activity to fully acclimate to the increased organic content (Mao et al., 2015; Veluchamy et al., 2019). Concentration of H_2S differed significantly according to OLR ($p < 0.01$), except between OLR 4 and 5 ($p > 0.1$). The H_2S concentrations ranged from 4-390 ppm in all OLR which is similar to concentrations reported during corn silage digestion within this PFR system and ambient swine manure co-digestion with different amounts of grease (Lansing et al., 2010; Veluchamy et al., 2019). The production of H_2S is both toxic to human health and methanogenic populations which leads to decreased reactor performance and presence of foul odours (Lansing et al., 2010). Lansing et al. (2010) proposed in their study that elemental sulfur levels were higher in manure than the cooking grease co-substrate, leading to decreased H_2S production in treatments with higher grease content. It is likely that a similar phenomenon occurred in this study as increased corn stover feeding occurred at higher OLR, reducing H_2S production. Although H_2S concentration did not exceed inhibitory levels, its presence can negatively impact mechanical parts within an internal combustion engine resulting in decreased engine performance, therefore, the biogas should be scrubbed prior to engine combustion (Veluchamy et al., 2019).

Fig 3.1. Variations of a) biogas production and b) biogas composition and temperature at varied OLR during semi-continuous PFR digestion.

3.3.3 pH, volatile fatty acids (VFA), and buffering capacity (TAC) profiles

Overall process stability in terms of pH, FOS (total VFA, expressed as acetic acid equivalents), and TAC (buffering capacity, expressed as calcium carbonate equivalents) was estimated over the course of the experiment (Fig. 3.2a-c). One-way ANOVA and Tukey means comparison revealed significant differences in effluent pH at OLR 1 and 2 compared to the higher OLR regimes ($p < 0.05$). In terms of the pH profile across the digester, there were significant differences ($p < 0.05$) except between port 1 and 2 ($p > 0.05$). From the digestate ports spaced along the reactor (port 1, port 2, and outlet), pH values fluctuated between 7.2-8.2 which is similar to other long-term PFR operational studies (Gómez et al., 2019; Patinvoh et al., 2017; Veluchamy et al., 2019) and lower than the influent pH (Fig. 2a).

The concentration of total VFA generally increased with OLR (Fig. 3.2b) and ranged between 500 and 1500 mg L⁻¹ across all sampling ports, which was within optimal range for stable process performance (Neshat et al., 2017). Influent VFA concentration was much higher than in digestate from the different sampling ports, demonstrating consumption and conversion of VFA. Average effluent VFA concentrations of OLR 1 were significantly lower than in the higher OLR regimes ($p < 0.05$) whereas OLR 5 was only significantly different from OLR 1 ($p < 0.05$). Across the digester, there were no significant differences in VFA between port locations ($p > 0.05$). Previous investigation into dry mesophilic AD of cow manure mixed with straw (22 % TS) in a PFR system revealed no VFA accumulation at OLR 2.8 and 4.2 g VS L⁻¹ d⁻¹, a good indication of process stability (Patvinoh et al., 2017). The start-up period of the PFR investigated by Patvinoh et al. (2017) involved wastewater treatment AD inoculum mixed with cow manure and straw to acclimate the microbial community while this study utilized raw swine manure for start-up. Although the retention time was incrementally reduced as OLR increased, total VFA did not accumulate as seen in previous studies (Lee and Rittman, 2011; Li et al., 2017), showing that VS is not being degraded and consumed as quickly as total VFA. Individual VFA profiles were not measured in this study; however, it is possible that certain inhibitory VFA such as butyric and propionic acid were produced and accumulated with respect to acetic acid while still remaining below inhibitory levels for total VFA. Drosig (2013) reported that propionic acid concentrations of 250-1000 mg L⁻¹ leads to process instability. In addition to the low conversion rate of propionic acid to methane compared to acetic and butyric acid conversion (Wang et al., 2009) combined with shorter HRT, it is possible these inhibitory VFA accumulated and exceeded the rate of subsequent

VFA conversion to methane. In the future, individual VFA concentrations (particularly propionic and butyric acids) should be measured within this system at higher OLR to gain insight into fermentation dynamics and its impact on overall process performance.

Buffering capacity (Fig. 3.2c) was within the reported optimal range of 1000-5000 mg L⁻¹ CaCO₃ (Khanal, 2011; Veluchamy and Khalamdhad, 2017b) for OLR1-3, however it exceeded that range for OLR 4 and 5. Buffering capacity differed spatially along the digester except between port 1 and 2 ($p > 0.05$) with respect to influent feedstock. Despite buffering capacity increasing beyond the reported optimal range, the digester operated stably which is in agreement with previous research (Veluchamy et al., 2019). A good indicator of anaerobic digestion stability is the FOS/TAC ratio which can vary between 0.3-0.5 depending on digester operation and feedstock composition (Lossie and Pütz, 2008; Ning et al., 2019; Riahi et al., 2010). Influent FOS/TAC increased with OLR (Fig. 3.2d) and although the ratio went above 0.5 from OLR 3-5, within the digester the ratio was below 0.5.

3.3.4 Volatile solids (VS) and soluble chemical oxygen demand (sCOD) reduction

To assess organic matter reduction and mass loss during anaerobic co-digestion, VS and sCOD were measured over time and across the digester for each OLR (Fig. 3.2e-f). It was evident that as OLR increased, so too did VS and sCOD within the digester. However, within each OLR VS reduction efficiency significantly increased across the digester ($p < 0.05$) (inlet, port 1, port 2, outlet). Overall VS reductions between OLR also differed significantly ($p < 0.05$) except between OLR 3 and 4 ($p > 0.05$) and between OLR 4 and 5 ($p > 0.05$). Volatile solids removal (calculated from influent to effluent VS) ranged from 7.14 – 33.49 % with a decreasing removal rate as OLR was increased and HRT was reduced (Table 3.3). Volatile solids (VS) removal efficiency within this PFR system was lower than reported in some long-term PFR studies processing swine manure (Adl et al., 2012; Gómez et al., 2019; Patinvoh et al., 2017), while other PFR studies revealed similar values (Lansing et al., 2010; Veluchamy et al., 2019). Although influent % TS and substrate characteristics varied between studies, as % TS (and OLR) increased the removal efficiency decreased. This was likely due to increased organic content, particularly in the form of lignocellulose, which has comparatively lower biodegradability (Jabeen et al., 2015). Lansing et al. (2010) also reported organic matter accumulation (in the form of VS) during ambient AcoD of swine manure and used cooking grease, which could explain the lower VS removal capabilities

at higher OLR due to higher solids retention within a PFR system.

Soluble COD (sCOD) is another physiochemical parameter frequently used to assess both digestate stabilization and volume reduction during anaerobic digestion (Li et al., 2017). Compared to VS content, influent sCOD within OLR regimes was more variable (Fig. 3.2f), possibly due to prolonged storage of feedstock substrates prior to feeding which can impact quality of feedstock (Gómez et al., 2019). Despite the sCOD variations, the overall trend of increasing organic matter in influent and within the digester was observed as OLR increased, due to greater availability of oxidizable matter (Fig. 1f). There were only significant increases ($p < 0.05$) in effluent sCOD in OLR 5 with respect to the lower OLR steps (1 and 2) and are reflected in the removal efficiencies. Soluble COD removal, unlike VS removal, did not decrease with increasing OLR and although there were no significant differences in efficiencies along the digester ($p > 0.05$), the removal efficiency generally did increase from inlet to outlet. This provides additional evidence of solid/liquid phase separation due to the auger mechanism and in turn different physiochemical environments along the length of the PFR as reported previously (Veluchamy et al., 2019). Overall reduction profiles also significantly differed between some OLR steps ($p < 0.05$) as seen in Table 3. Previous reports on sCOD removal within (semi)-continuous PFR and CSTR operations reported varying results which were either lower (Massé et al., 2013; Xie et al., 2017) or higher (Lansing et al., 2010; Li et al., 2017) than the results of this study. Like VS content, sCOD accumulated and increased within PFR systems co-digesting used cooking grease and swine manure, most likely due to the nature of PFR operation which involves longer solids retention compared to other systems (Lansing et al., 2010). In addition to reactor configuration and operation, initial substrate properties and composition can influence organic matter removal and methane yields as previously reported (Xie et al., 2017). However, at all OLR levels examined here, there was an overall decrease in sCOD in the effluent with respect to influent feedstock. Like VS and total VFA, hydrolysis and acidogenesis by-products accumulated due to shorter retention times at higher OLR, resulting in less time for consumption and conversion of intermediate metabolites (Patinvoh et al., 2017).

Although the TS content within the PFR was below the typical PFR operating range of 11 to 14 % TS for manure AD (Gómez et al., 2019), this study co-digested with increasing lignocellulosic corn stover content which has lower bulk density. Despite the low bulk density of corn stover, the solids content is distinctly phased based on sampling port location along the PFR

(Fig. 3.2e). The pH, VFA, buffering capacity, and sCOD, on the other hand, did not significantly differ based on sampling port location, which could suggest that the solid portion of the feedstock mixture was moving as a true plug-flow while the measured aqueous chemicals demonstrated fluid movement. However, due to the low density of corn stover, there is potential for solid stratification and foaming associated with low TS (< 10 %) feeding into PFR systems which leads to lower mass transfer and biogas production performance. This could also explain the insignificant physiochemical phasing across sampling ports, whereas for the first two OLR in particular, the solids content is distinctly different based on sampling port location (Fig. 3.2). For OLR 1 and 2, TS content is low and combined with the low density of corn stover, solid stratification was more likely to occur, resulting in possible non-representative sampling from port locations across the PFR.

Fig. 3.2. Reactor performance in terms of a) pH, b) FOS (representing VFA), c) TAC (representing buffering capacity), d) FOS/TAC ratio, e) VS content, and f) sCOD at varied OLR during semi-continuous PFR digestion.

Table 3.3

PFR physiochemical performance during step-wise OLR increase.

OLR	Percent reduction (%)		Biogas production rate (L d ⁻¹)	Biogas composition			Total yield (m ³ kg ⁻¹ VS _{fed})	
	VS	sCOD		CH ₄ (%)	CO ₂ (%)	H ₂ S (ppm)	Biogas	CH ₄
OLR1 (0.25)*	33.49	15.35	8.22	61.97	39.88	338.65	0.738	0.457
OLR2 (0.73)*	22.46	-12.78	10.90	61.94	38.58	276.38	0.417	0.258
OLR 3 (3.0)*	10.69	33.57	23.02	56.42	43.82	216.00	0.307	0.173
OLR 4 (3.8)*	8.61	21.00	37.36	55.33	47.54	68.23	0.514	0.284
OLR 5 (4.7)*	7.14	51.36	48.03	54.12	47.17	17.83	0.621	0.336

*values in brackets represent OLR measured as kg VS_{fed} m⁻³ d⁻¹

3.3.5 Fecal indicator bacteria (FIB) reduction

The FIB concentrations of the influent feedstock and digestate sampled across the different reactor ports at different OLR during long-term PFR operation can be seen in Fig. 3.3a-d. Though the influent concentrations for each bacterial group varied significantly ($p < 0.05$) between OLR steps (Fig. 3.3a-d), two-way ANOVA also revealed a significant impact of time on influent concentrations ($p < 0.05$). This result suggests that the variation could be attributed to differences in the length of time manure was stored prior to feeding, which was on average a couple months at a time. The differences in bacterial concentrations could also be due to sampling heterogeneity when manually mixing both feedstocks before feeding or during sampling of raw swine manure from the outdoor manure pit.

The degree of bacterial reduction differed based on the FIB type and in terms of OLR over the digestion period. However, two-way ANOVA revealed no significant differences in effluent FIB concentration ($p > 0.05$) while digestion time had a significant impact instead ($p < 0.05$). However, Tukey HSD analysis did reveal significant differences between effluent concentrations between OLR regimes ($p < 0.05$), suggesting that digestion time as an independent variable solely accounted for significant differences. There were no significant differences in FIB reduction across the spatial profile of the digester ($p > 0.05$). Fig. 3.2a-b revealed lower *E. coli* and fecal coliform populations in the effluent and port digestates compared to the influent throughout the entire digestion experiment. By the end of the HRT for OLR 1 and 3, fecal coliform and *E. coli* were undetectable in the effluent digestate ($< 1 \log_{10} \text{CFU g}^{-1}$) (Table 3.4). At higher OLR, both bacterial groups were present in concentrations between 1 to $\sim 5 \log_{10} \text{CFU g}^{-1}$ in the effluent digestate, which could be attributed to lower HRT resulting in insufficient digestion time to reduce both organic matter and FIB. Overall, the $\log_{10}$ reductions for *E. coli* and fecal coliforms were comparable regardless of the OLR (Table 3.4) and were similar to previous studies where AcoD of swine manure and crop residues such as maize silage and triticale were investigated (Orzi et al., 2015). These results suggest that although the interaction of digestion time and OLR on bacterial reduction was not significant ($p > 0.05$), these two independent variables on their own have a significant impact on *E. coli* and fecal coliform removal ($p < 0.05$).

There was not a clear trend for reduction of fecal enterococci (Fig. 3.3c), however, as noted by the $\log_{10}$ reductions calculated for each OLR (Table 3.4). Throughout most of digestion, fecal

enterococci concentrations across the reactor generally increased with respect to the influent feedstock. Previous studies which reported fecal enterococci after AD of swine manure or AcoD of swine manure with crop residues vary greatly in their results, ranging from 0 to $\sim 5 \log_{10}$ reduction (Dennehy et al., 2018; Orzi et al., 2015). A potential explanation for the limited fecal enterococci reduction (or even growth) observed in this study is temperature (5-65 °C), pH (pH 4.5-10), and salt resistance (Fisher and Phillips, 2009).

Clostridia spp. also demonstrated either limited reduction or an increase during digestion depending on OLR (Table 3.4). This finding is in agreement with previous investigations into *Clostridia* spp. survival during (semi)- continuous AD and AcoD operation (Jiang et al., 2020). Overall *Clostridia* spp. concentrations fluctuated with respect to influent feedstock concentrations, with digestate samples from across the reactor showing little or no reduction (Fig. 3.3d, Table 3.4). The largest $\log_{10}$ reduction of ~ 0.85 was achieved at OLR 2, which is in agreement with other reported literature values (Jiang et al., 2020; Orzi et al., 2015). Overall, mesophilic AcoD does not effectively reduce *Clostridia* spp. numbers. Future investigations into characterization of *Clostridia* spp. in terms of whether they exist as spores or vegetative cells during digestion and in the influent feedstock should be conducted in order to verify if the bacteria are still viable.

Operational conditions and intermediate metabolite impact on pathogen reduction and its relation to other AD performance parameters such as biogas production and waste stabilization requires more investigation to develop better pathogen risk assessments (Nag et al., 2019). Little research into the cumulative effect of all AD parameters on pathogens, FIB and the relation between pathogens and FIB concentrations has been conducted in this area. It is important from both a biosafety perspective, since biological risk varies greatly between AD operations, and an operational stand point since biogas production has requirements different from hygienization (Jiang et al., 2020; Nag et al., 2019). For example, a longer HRT at high OLR can result in better biogas production and waste degradation but lower VFA accumulation is counter productive to pathogen reduction since VFA has shown to correlate with hygienization (Jiang et al., 2020). In this study, however, as HRT decreased and OLR increased hygienization efficiency did not improve substantially as seen by *Clostridia* spp. and fecal enterococci regrowth. The typical threshold TS content of 11-14 % for a PFR system was not achieved due to feeding issues associated with the low bulk density of corn stover mixed with swine manure, resulting in a shorter HRT for OLR 5. It is possible though that if the PFR was able to reach semi-solid or dry digestion

conditions, hygienization may have improved. This was noted by Orzi et al. (2015) who reported higher hygienization efficiency in dry AD plants processing manure and crop residues, no VFA accumulation above inhibitory concentrations, and biogas yields from 332 to 343 L g⁻¹ TS. Orzi et al. (2015) showed that mesophilic dry digestion can reduce both FIB and pathogen concentrations while maintaining stable performance and biogas production, although VS removal was comparable to this study. Further investigation into the relations between performance parameters with this PFR system at dry conditions need to be conducted to optimize overall reactor performance and efficiency.

Fig. 3.3. Average fecal indicator bacteria counts (log₁₀ CFU g⁻¹) of a) *E. coli*, b) fecal coliforms, c) fecal enterococci, and d) *Clostridia* spp. at varied OLR during semi-continuous PFR digestion. Detection limit was 1 log₁₀ CFU g⁻¹ for all bacteria.

Table 3.4

PFR hygienization performance during step-wise OLR increase. Negative values represent growth.

Parameter	Inlet concentration (log ₁₀ CFU g ⁻¹)	Log ₁₀ Reduction*				
		OLR 1	OLR 2	OLR 3	OLR 4	OLR 5
<i>E. coli</i>	2.00 – 5.77	2.06	1.33	1.62	1.20	1.28
Fecal coliforms	1.36 – 5.77	3.50	1.80	2.85	1.15	1.21
Fecal enterococci	2.63 – 4.46	0.21	-0.47	0.29	-0.72	-1.56
<i>Clostridia</i> spp.	3.03 – 5.31	-0.13	0.85	0.041	-0.24	0.15

* calculated with respect to outlet concentrations

3.3.6 Relationship between physiochemical parameters and hygienization efficiency

As previously reported by researchers investigating hygienization during AcoD of different agricultural wastes such as manure and crop residues, digestion time and solids content had a significant impact on outlet digestate FIB concentrations (Arias et al., 2020; Orzi et al., 2015). Important physiochemical parameters such as pH, VFA and buffering capacity (represented as FOS/TAC), and substrate consumption (represented as daily biogas production rate and sCOD) were analyzed in relation to final FIB concentrations. To analyze the relationship, a Pearson correlation matrix was created to elucidate the role of these parameters in bacterial reduction at each OLR step. For OLR 1, sCOD and pH were significantly correlated to fecal coliform concentrations ($p < 0.05$) although for *E. coli*, significant correlation was only evident with sCOD concentrations ($p < 0.05$). As OLR was increased, generally most of the physiochemical parameters did not significantly correlate with any FIB group. The only exceptions were a negative correlation between sCOD and fecal enterococci at OLR 2, positive correlation between daily biogas production and fecal enterococci at OLR 2, and negative correlation between pH and fecal enterococci at OLR 5 ($p < 0.05$). Orzi et al. (2015) determined that pH was significantly correlated to *E. coli* and fecal coliform concentrations whereas for fecal enterococci and *Clostridia* spp. substrate consumption was the main correlated factor. The pH of the effluent digestate sampled from different AD plants were in the range of 7.55 to 8.53 in the study conducted by Orzi et al. (2015) which is slightly higher compared to the current study (pH 7.46-7.94).

On the other hand, FOS/TAC was not significantly correlated to any FIB group compared to observations made by Arias et al. (2020). However, physiochemical parameters such as pH and FOS/TAC reported by Arias et al. (2020) exhibited a much wider range (pH 5.5 to 8.5 and FOS/TAC 0.091 to 6.8), which explained why some FIB were correlated to those parameters. Semi-continuous and continuously fed reactors introduce new microbial loads over time compared to a single initial loading in batch operation, resulting in different reduction efficiencies (Jiang et al., 2020). Another inactivation factor which was not measured during PFR operation is ammonia, which has demonstrated a hygienization effect due to the combined effect of temperature and operational parameters (Jiang et al., 2020). Dry anaerobic digestion and co-digestion revealed significant reductions in both Gram-negative and Gram-positive bacteria as a result of intermediate metabolite accumulation (Jiang et al., 2018; Orzi et al., 2015). However, the exact mechanisms are yet to be elucidated in dry AD and AcoD processes.

It is evident that batch CSTR and (semi) continuously fed reactors differ in hygienization efficiency, therefore, the focus of future research should be extensively shifted towards these (semi) continuously fed reactors which are more representative of real-world conditions. In particular, little research into hygienization during PFR operation has been conducted and no literature exists with regards to AcoD of manure and lignocellulosic biomass at different OLR (Chiapetta et al., 2019). Another aspect of PFR operation that was not incorporated in the current reactor design, nor has been studied with regards to hygienization, is effluent recirculation. Effluent recirculation has demonstrated enhanced PFR performance in terms of increased methane production from feedstocks such as manure and fruit waste due to dilution of inhibitory metabolites (Dong et al., 2019b; Gómez et al., 2019; Namsree et al., 2012). From a microbial community aspect, cellulolytic bacteria such as *Christensenellaceae*, *Ruminofilibacter* and *Fibrobacteres* were enriched during acidizing fluid reflux within a PFR digesting cow manure (Dong et al., 2019b). Li et al. (2014) reported the genus *Methanosaeta* as the dominant methanogen genus during cow manure AD within a mixed plug flow loop reactor. Literature is scarce in regards to understanding the effect of effluent recirculation on microbial community dynamics, therefore, more investigations into that aspect and especially pathogen reduction should be explored.

3.3.7 Lignocellulolytic enzyme activity

Lignocellulosic substrate degradation ability during digestion at each OLR was monitored by quantifying cellulase and hemicellulase activity as CMCase, FPase, and xylanase, respectively (Fig. 3.4).

For OLR 1 and 2, cellulase activity was generally undetectable in the outlet digestate except near the end of the retention time (Fig. 3.4a,b). At OLR 1, the highest CMCase and FPase activities were 4.51×10^{-4} and $1.61 \times 10^{-4} \mu\text{g } \mu\text{L}^{-1} \text{ min}^{-1}$, respectively, at day 50. At OLR 2 the maximum CMCase activity was reduced by half whereas maximum FPase activity doubled at day 80 compared to the enzyme activities of OLR 1 (Fig. 3.4a,b). For the rest of the digestion period, both enzyme activities were generally higher across the digester (port 1, port 2, outlet) to the activities of the OLR 1 and 2. At OLR 3-5, the outlet CMCase activities ranged from 3.17×10^{-5} – 8.56×10^{-4} , 1.11×10^{-4} – 6.97×10^{-3} , and 4.33×10^{-4} – $6.33 \times 10^{-4} \mu\text{g } \mu\text{L}^{-1} \text{ min}^{-1}$, respectively. The FPase activity also varied over time and were generally lower with respect to CMCase activity due to the crystalline cellulose structure of FP which limits hydrolysis compared to CMC (Arias et al., 2020; Wongwilaiwalin et al., 2018). The increased corn stover content, and consequently % TS, at higher OLR may have influenced the cellulase activities by increasing enzyme production in response to cellulose and hemicellulose availability (Arias et al., 2020; Gu et al., 2014). Digestion time had a significant impact on effluent cellulase activities ($p < 0.05$) whereas OLR had no significant impact ($p > 0.05$). However, CMCase activities at OLR 4 and 5 were significantly different compared to the activities from OLR 1-3 ($p < 0.05$).

Xylanase activity throughout the entire digestion trial and across the reactor were overall much higher compared to CMCase and FPase activities (Fig. 3.4c). Similar to the trend observed for cellulase activity, xylanase activity was lower in OLR 1 and 2 relative to the higher loading rates. Although peak xylanase activity for each OLR sometimes occurred on different days from that of cellulase activities (Fig. 3.4a-c), generally all enzymes reached maximum activity on the same days which highlights the synergistic activity of both enzymes in achieving lignocellulose degradation (Gu et al., 2014). Like CMCase and FPase, digestion time rather than OLR had a significant impact on outlet xylanase activity ($p < 0.05$) and overall activities between each OLR were generally similar to one another ($p > 0.05$). With the exception of OLR 1, OLR 3 was the only loading rate to demonstrate significantly different xylanase activity from the other OLR

regimes ($p < 0.05$). There is limited research on the influence of lignocellulosic biomass on xylanase activity during co-digestion with swine manure, however, researchers have reported higher xylanase activity during AcoD of lignocellulosic biomass with other organic waste sources compared to mono-digestion (Arias et al., 2020; Zhao et al., 2014) which is in accordance with this study's results.

There is little available literature on lignocellulolytic enzyme activity profiling within a semi or continuously-fed anaerobic digester. One study reported by Jin et al. (2018) used rumen cultures for enhanced methane production performance during AcoD of swine manure and corn straw in a continuously operated CSTR. Jin et al. (2018) reported higher FPase and CMCase activity during the first HRT followed by a decrease in the second HRT and then increased by the end of the digestion period. Their results slightly differed from this study such as high activity at the beginning of digestion. The higher activity can be attributed to the high cellulase and hemicellulase activity of endogenous rumen microorganisms found in cow manure (Gu et al., 2014). Otherwise, enzymatic assays revealed increased activity as TS content increased like in this current study – in particular with increased xylanase activity as a result of higher hemicellulose content in corn straw (Jin et al., 2018). In addition, other studies have been conducted in batch AcoD assays. Arias et al. (2020) reported higher lignocellulolytic activity as lignocellulosic biomass content increased during batch AcoD of swine manure and corn stover. It is possible that if the semi or continuously-fed anaerobic digester was to operate at dry conditions, the enzyme activity would increase since the lignocellulosic biomass content would be higher. However, the study conducted by Arias et al. (2020), Jin et al. (2018), and the current study were still operating below dry AD conditions which makes it difficult to assess how enzymatic activity will change in response to increased lignocellulosic content. While the impact of increasing TS content in the form of corn stover on the measured enzymatic activities for this study were evident, significant differences on xylanase activities with respect to OLR were not as evident as reported by Arias et al. (2020). The difference between patterns could be due to the operational mode since within this study, OLR was gradually increased which allowed for acclimation to the higher organic content and greater process stability (Fig. 1 and 2). In the batch AcoD study conducted previously by Arias et al. (2020), no active inoculum or acclimated manure was used, which resulted in different physiochemical profiles and ultimately xylanase activities as TS content increased. Overall, this study not only demonstrated that the acclimated microbial community in swine manure can

degrade lignocellulosic biomass but the lignocellulolytic activities stabilized at higher OLR. The general increase in VFA, % VS, and sCOD (Fig. 3.2b, e, f) were possibly due to shorter retention times and thus accumulation of hydrolysis and acidogenesis metabolites which can be seen by the upward trend in lignocellulolytic enzyme activity at higher OLR.

These results are the first reported for a semi-continuously fed AcoD system, although more research into lignocellulose degradation should be investigated in other semi-(continuous) systems digesting other organic wastes at higher OLR. As mentioned in section 3.3.6, the effects of effluent recirculation have a positive influence on methane production and maintaining hydrolytic capacity during AD (Dong et al., 2019b; Namsree et al., 2012) a reflection of microbial metabolic adaptation. The hydrolytic enzyme activities were not qualitatively or quantitatively assessed in either study; however, that research has been conducted in other reactor designs. To assess the influence of effluent recirculation on hydrolytic enzyme activity, Zuo et al. (2013) anaerobically digested vegetable waste in a two-stage reactor and reported increased amylase activity up to an OLR of 2.1 g VS L⁻¹ due to dilution effects and pH adjustment. Using a leach bed reactor digesting food waste, continuous leachate recirculation at a liquid to solid (L/S) ratio of 1:1 demonstrated 69.4 % VS removal and enhanced key enzymatic activities such as α -mannosidase, α -fucosidase, lipase and β -galactosidase resulting in better hydrolytic-acidogenic performance (Xu et al., 2014). From these studies it is evident that effluent recirculation improved biogas yields, organic matter reduction efficiency, and decreased HRT ranging from 10 to 21 days (Dong et al., 2019b; Namsree et al., 2012; Zuo et al., 2013). The combination of corn stover pelleting and effluent recirculation could achieve higher OLR in the current PFR to achieve dry AcoD conditions to improve biogas yields while maintaining reactor stability, organic matter reduction, and sufficient hygienization. Increasing OLR using pellets can minimize mass transfer issues associated with the low bulk density of corn stover while effluent recirculation can maintain hydrolytic microbes within the reactor, leading to increased biodegradation. With the benefits of both strategies in mind, pelleting and effluent recirculation strategies should be investigated within this system and other semi-continuously fed reactors.

Fig. 3.4. Average enzymatic activities of a) CMCase, b) FPase, and c) xylanase at varied OLR during semi-continuous PFR digestion.

3.4 Conclusion

The PFR operated stably up to $4.7 \text{ kg VS m}^{-3} \text{ d}^{-1}$, with the highest total methane yield of $0.457 \text{ m}^3 \text{ kg}^{-1} \text{ VS}_{\text{fed}}$ at OLR $0.25 \text{ kg m}^{-3} \text{ d}^{-1}$. Higher OLR and TS combined with lower HRT decreased organic removal, with a maximum VS reduction of 33.5 %, and hygienization efficiency. These results showed that operational parameters should be optimized depending on whether the desired outcome is enhanced biogas production or biowaste stabilization. Further investigation into increasing OLR, use of pellet feeding, and effluent recirculation to enhance dry AcoD should be conducted to understand its impact on biogas production and hygienization.

Chapter 4

Conclusions

4.1 Summary of research findings

The overarching objectives of this thesis project was to assess anaerobic co-digestion (AcoD) performance of swine manure and corn stover in terms of biogas production, process stability, organic matter reduction efficiency, and hygienization potential. In addition to process performance optimization, microbial CLPP was also investigated in terms of community shifts relating to different AcoD operational conditions. Both research chapters investigated process performance and microbial CLPP associated with AcoD of swine manure and corn stover with the key differences between both chapters being reactor types, operational mode, and microbial CLPP analyses.

Chapter 2 described batch mesophilic AcoD of swine manure and corn stover at 6 different TS loadings (1-6 %) in duplicate assays to be compared against AD of swine manure which was the control (0.69 % TS). The 75-day digestion study revealed that swine manure AD yielded the best process performance in terms of TMP and VS reduction, with values of 403.9 mL g⁻¹ VS and 86.31 %, respectively. As TS content increased, methane yields and VS reduction decreased while volatile fatty acid (VFA) accumulation increased due to greater availability of biodegradable organic content. The ratio of VFA: buffering capacity (TAC), which was represented as FOS/TAC, did not differ significantly between the control and 1-4 % TS AcoD treatments and remained within the optimal range of 0.3-0.5 for most of the digestion study. In terms of hygienization potential, non-pathogenic *E. coli* and fecal coliforms were reduced across all treatments ranging from 0.1 – 4.0 log₁₀ colony forming units (CFU) mL⁻¹ although some regrowth was evident from the middle to near the end of digestion (30 – 60 d). *Clostridia* spp. and fecal enterococci generally grew during the course of digestion, highlighting incomplete hygienization of fecal indicator bacteria (FIB) and potentially incomplete pathogen inactivation. Community level physiological profiling of the microbial community using BIOLOG AN microplates revealed a metabolic shift towards increased carbon substrate utilization and particularly as TS content increased. Enzyme activity assays revealed higher cellulase and hemicellulase activity throughout

digestion and as TS content increased. Although the methane potential and VS reduction efficiency was lower during AcoD compared to swine manure AD, co-digestion with corn stover created a balanced nutrient profile which stimulated microbial adaptation to lignocellulose metabolism and resulted in overall stable AcoD performance. These results demonstrated that colorimetric biochemical assays can rapidly assess microbial community dynamics and increased TS content (in the form of corn stover) influenced metabolic activities.

Chapter 3 described semi-continuous long-term AcoD of swine manure and corn stover in an auger fitted plug flow reactor (PFR) during step-wise organic loading rate (OLR) increase. The step-wise OLR increase also corresponded with an increase in TS loading, with OLRs and TS loadings of 0.25-4.7 kg volatile solids fed (VS) $\text{m}^{-3} \text{d}^{-1}$ and 1.5-9.0 %, respectively. Over the 190 d digestion period, biogas and methane yields decreased for the first 3 OLR (0.25, 0.75 and 3.0 kg $\text{m}^{-3} \text{d}^{-1}$) and increased for OLR 4 and 5 (3.8 and 4.7 kg $\text{m}^{-3} \text{d}^{-1}$, respectively). The highest biogas yield was 0.738 $\text{m}^3 \text{kg}^{-1}$ at OLR 1 (0.25 kg $\text{m}^{-3} \text{d}^{-1}$) with 62 % methane content at a 50 d hydraulic retention time (HRT). Volatile solids reduction decreased as TS and OLR were increased, with reduction efficiencies between 7.14 – 33.49 %. Process stability, indicated by FOS/TAC, was within the optimal range for all OLR/TS increases although VFA accumulation increased due to increased organic content and shorter HRT (40 d for OLR 3 and 4, 15 d for OLR 5). Throughout the entire digestion study, mass transfer issues were evident, especially at OLR 4 and 5, which resulted in crust formation, and shortening of HRT for OLR 5 due to reduced reactor working volume. Another factor which contributed to mass transfer issues was the low bulk density of corn stover which produced a viscous slurry when mixed with swine manure, causing clogging issues during feeding into the PFR. Hygienization potential was low in this system compared to Chapter 2 findings although it was generally found that non-pathogenic *E. coli* and fecal coliforms were more sensitive to AcoD compared to fecal enterococci and *Clostridia* spp. Lignocellulolytic enzyme activity assays also revealed an increasing cellulase and hemicellulase activities with an increase in OLR/TS, highlighting microbial adaptation to lignocellulosic substrates which is accordance with the findings from Chapter 2.

This study is the first published report to assess the impact of increasing TS, in the form of lignocellulosic biomass, on microbial community dynamics in terms of metabolic adaptation and

hygienization potential. Chapter 2 presented a novel CLPP tool for rapidly characterizing microbial community shifts in response to changes in AcoD operation and how those changes are related to observed physiochemical parameters. Chapter 3 presented a novel anaerobic digestion reactor for AcoD of swine manure and corn stover and in particular, for handling increased TS content in the form of lignocellulosic biomass. Like Chapter 2, the impact of increasing TS and OLR on microbial community dynamics such as hygienization potential and metabolic adaptation were investigated for the first time in a semi-continuously fed PFR system.

4.2 Recommendations and improvements for future research

Future research directions into AcoD of swine manure and corn stover should focus on semi-dry conditions (>10 % TS) and if possible, reaching dry conditions (> 20 % TS) within the current PFR design. To achieve (semi) dry conditions, corn stover pretreatment strategies should be employed in order to increase the bulk density which could alleviate viscous slurry production when mixed with swine manure, which would in turn increase mass transfer and result in greater biogas yields and organic matter reduction efficiency. One potential pretreatment strategy to overcome the low bulk density issue associated with corn stover and other lignocellulosic biomass feedstocks is pelleting, which densifies the material and facilitates feeding into anaerobic reactors in addition to facilitating transportation. Pelleting is an attractive mechanical pretreatment which could be implemented for farm-scale operations where corn stover can be processed more efficiently and at greater quantities compared to other chemical, biological, and physical pretreatments. Another research direction tied in with pelleting and using the current PFR system is effluent recirculation which can minimize washing out of necessary microbes and associated enzymes for the anaerobic digestion process. Other benefits of recycling digestate through digestion systems include increased buffering capacity, lowering the cost of digestate disposal, reduce pretreatment costs, and minimize environmental pollution. The combined effect of corn stover pelleting to achieve (semi) dry conditions and implementing effluent recirculation can potentially increase the biogas potential and organic matter reduction capabilities of this PFR system without sacrificing reactor stability which can occur as a result of excess VFA accumulation at high solids loading. From a biosafety standpoint, hygienization potential should be investigated at (semi) dry conditions since excess VFA accumulation and ammonia production

have demonstrated antimicrobial capabilities although this potential has not been explored at high solids loading in a PFR system. In addition to culturing FIB as a biosafety indicator of digestate, adenovirus and *Cryptosporidium* should be utilized as biosafety indicators for virus and protozoa, respectively. Human and swine related pathogens such as porcine coronaviruses, ASF virus and *Salmonella* should be quantified to better assess the sanitation capabilities of mesophilic AcoD in a PFR system at (semi) dry conditions. Finally, microbial community shift changes in response to increased solids loading should be investigated utilizing a combined approach of metagenomics, transcriptomics/proteomics and biochemical based assays such as BIOLOG AN microplate or Ecoplates. These techniques can assess which microorganisms are present (metagenomics), which microorganisms are carrying out certain functions (metatranscriptomics and proteomics), and the overall assessment of metabolic activities of an entire microbial community (BIOLOG assays). The suggested directions from an operational, biochemical, and microbiological standpoint can potentially improve AcoD efficiency and make it more feasible to be implemented for on-farm use as a means of sustainable renewable energy production and an environmentally friendly waste management approach. The directions for improving PFR AcoD performance should eventually be applied to understanding how performance is impacted under psychrophilic and below freezing conditions in order to increase its feasibility in Canada and other cold climate regions.

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Appendices

Table A.1.

Methane yield and percent reduction of co-digestion of swine manure and corn stover

Solid content (%)	Percent reduction (%)		CH ₄ yield (m ³ kg ⁻¹ VS)
	VS	TS	
Swine Manure(0.69)	86.31	22.86	403.10
1	70.60	40.00	384.94
2	72.02	54.00	370.94
3	71.07	56.67	302.82
4	67.00	54.75	263.52
5	54.66	44.40	53.94
6	17.36	12.00	15.14

Table A.2.

Correlation matrix for physiochemical parameters vs. average fecal bacterial indicator content during AcoD of swine manure and corn stover.

Total solids content (%)	Bacteria	pH	FOS/TAC	Daily CH ₄ Production	sCOD
Swine Manure (0.7)	<i>E. coli</i>	-0.769*	-0.087	-0.589	0.139
	Fecal coliforms	0.929 *	0.318	0.570	-0.446
	Fecal enterococci	-0.035	-0.335	0.245	0.454
	<i>Clostridia spp.</i>	0.258	0.522	0.055	-0.552
1	<i>E. coli</i>	-0.539	0.166	-0.668	-0.218
	Fecal coliforms	0.466	-0.128	0.550	0.529
	Fecal enterococci	0.093	-0.532	0.251	0.081
	<i>Clostridia spp.</i>	-0.180	-0.032	0.400	0.050
2	<i>E. coli</i>	-0.877*	-0.216	0.086	0.510
	Fecal coliforms	0.621	0.704	-0.492	-0.667
	Fecal enterococci	0.696	0.651	-0.623	-0.387
	<i>Clostridia spp.</i>	0.070	0.611	-0.857*	-0.466
3	<i>E. coli</i>	-0.496	0.206	0.265	-0.006
	Fecal coliforms	0.583	0.143	-0.311	0.110
	Fecal enterococci	-0.099	0.168	-0.100	0.191
	<i>Clostridia spp.</i>	0.414	0.586	-0.509	0.016
4	<i>E. coli</i>	0.145	0.794*	-0.340	-0.071
	Fecal coliforms	0.069	-0.476	0.421	0.134
	Fecal enterococci	-0.086	0.480	-0.771*	0.696
	<i>Clostridia spp.</i>	0.440	0.298	-0.513	0.492
5	<i>E. coli</i>	0.357	0.622	0.429	-0.565
	Fecal coliforms	-0.472	-0.590	-0.337	0.443
	Fecal enterococci	-0.287	0.354	-0.301	0.203
	<i>Clostridia spp.</i>	0.182	0.428	-0.143	0.228

6	<i>E. coli</i>	0.610	0.399	0.704	-0.979*
	Fecal coliforms	-0.354	-0.343	-0.427	0.857*
	Fecal enterococci	0.521	0.324	0.162	0.129
	<i>Clostridia spp.</i>	-0.547	-0.104	-0.713*	0.682

* Pearson correlation significant with $p < 0.05$

Table A.3.

Carbon substrate guilds groupings in BIOLOG AN MicroPlate™

Carbohydrates	Carboxylic Acids	Amino Acids
N-Acetyl-D-Galactosamine	D-Galacturonic Acid	L-Alanine
N-Acetyl-D-Glucosamine	D-Gluconic Acid	L-Alanyl-L-Glutamine
N-Acetyl-β-D-Mannosamine	D-Glucosaminic Acid	L-Alanyl-L-Histidine
Adonitol	Acetic Acid	L-Alanyl-L-Threonine
D-Arabitol	Formic Acid	L-Asparagine
Arbutin	Fumaric Acid	L-Glutamic Acid
D-Cellobiose	Glyoxylic Acid	L-Glutamine
Dulcitol	α-Hydroxybutyric Acid	Glycyl-L-Aspartic Acid
i-Erythritol	β-Hydroxybutyric Acid	Glycyl-L-Glutamine
D-Fructose	Itaconic Acid	Glycyl-L-Methionine
L-Fucose	α-Ketobutyric Acid	Glycyl-L-Proline
D-Galactose	α-Ketovaleric Acid	L-Methionine
Gentiobiose	D,L-Lactic Acid	L-Phenylalanine
α-D-Glucose	L-Lactic Acid	L-Serine
m-Inositol	D-Malic Acid	L-Threonine
α-D-Lactose	L-Malic Acid	L-Valine
Lactulose	Propionic Acid	L-Valine + L-Aspartic Acid
Maltose	Pyruvic Acid	
Maltotriose	D-Saccharic Acid	Miscellaneous
D-Mannitol	Succinic Acid	Amygdalin
D-Mannose	m-Tartaric Acid	α-D-Glucose ⁻¹ -Phosphate
D-Melezitose		D-Glucose-6-Phosphate
D-Melibiose	Polymers	Glycerol
3-Methyl-D-Glucose	α-Cyclodextrin	D,L-α-Glycerol Phosphate
α-Methyl-D-Galactoside	β-Cyclodextrin	Salicin
β-Methyl-D-Galactoside	Dextrin	D-Lactic Acid Methyl Ester
α-Methyl-D-Glucoside		Pyruvic Acid Methyl Ester
β-Methyl-D-Glucoside	Amides/Amines	Succinic Acid Mono-Methyl Ester
Palatinose		Urocanic Acid
D-Raffinose	Succinamic Acid	2'-Deoxy Adenosine
L-Rhamnose	Alaninamide	Inosine
D-Sorbitol		Thymidine
Stachyose		Uridine
Sucrose		Thymidine-5' Mono-phosphate
D-Trehalose		Uridine-5'- Monophosphate
Turanose		

Table A.4.

VS, sCOD, and FIB removal across PFR length during co-digestion of swine manure and corn stover

OLR	Port	Percent reduction (%)		Log ₁₀ reduction			
		VS	sCOD	<i>E. coli</i>	Fecal coliforms	Fecal enterococci	<i>Clostridia</i> spp.
OLR1	1	14.47	15.67	2.35	4.02	0.17	-0.35
	2	24.98	12.67	2.92	4.27	0.077	-0.039
OLR2	1	10.94	-31.59	1.33	1.60	-0.43	0.87
	2	16.78	-31.97	1.11	1.50	0.68	0.68
OLR 3	1	4.83	33.86	1.36	2.30	-0.12	0.037
	2	7.99	33.75	1.70	2.87	0.70	0.075
OLR 4	1	3.29	-13.25	1.32	1.64	-0.88	-0.18
	2	5.71	18.17	1.66	2.09	-1.07	-0.20
OLR 5	1	3.12	42.45	2.10	2.03	-0.77	0.19
	2	4.82	47.68	2.16	2.20	-1.29	0.13

Table A.5.

Pearson correlation coefficients of different physiochemical parameters in relation to average outlet FIB concentrations during PFR operation.

OLR	Bacteria	Daily Biogas			sCOD
		pH	FOS/TAC	Production	
OLR 1	<i>E. coli</i>	0.872	-0.733	-0.875	0.945*
	Fecal coliforms	0.888*	-0.754	-0.862	0.949*
	Fecal enterococci	0.095	-0.418	0.388	-0.244
	<i>Clostridia spp.</i>	0.666	-0.414	-0.596	0.769
OLR 2	<i>E. coli</i>	0.379	-0.934	-0.336	0.413
	Fecal coliforms	0.379	-0.934	-0.336	0.413
	Fecal enterococci	-0.704	0.662	0.971*	-0.990*
	<i>Clostridia spp.</i>	0.451	-0.249	-0.941	-0.900
OLR 3	<i>E. coli</i>	0.298	0.222	0.242	0.028
	Fecal coliforms	0.240	0.331	0.315	0.040
	Fecal enterococci	0.119	0.469	0.287	-0.316
	<i>Clostridia spp.</i>	-0.399	0.050	-0.051	-0.551
OLR 4	<i>E. coli</i>	-0.614	0.728	-0.262	-0.515
	Fecal coliforms	-0.530	0.677	0.356	-0.224
	Fecal enterococci	0.315	0.288	-0.006	-0.230
	<i>Clostridia spp.</i>	-0.425	0.259	0.341	-0.747
OLR 5	<i>E. coli</i>	-0.605	0.074	-0.144	-0.113
	Fecal coliforms	-0.658	0.147	-0.132	-0.114
	Fecal enterococci	-0.958*	0.917	0.276	-0.257
	<i>Clostridia spp.</i>	0.854	-0.548	0.108	0.042
	Fecal enterococci	0.521	0.324	0.162	0.129
	<i>Clostridia spp.</i>	-0.547	-0.104	-0.713*	0.682

*Pearson correlation significant with $p < 0.05$