Correlating physico-chemical process parameters with microbial community dynamics in co-digestion of cattle slurry and grass silage

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Abstract

Improvements in biogas and methane production from on-farm anaerobic digesters (AD) are limited by the inherent complexity of the microbial community (MC) dynamics. The aim of this research was to improve CH_4 production by investigating the impact of co-digesting of cattle slurry (CS) and grass silage (GS) on microbial community dynamics and the AD process. Batch and continuously stirred tank reactor (CSTR) laboratory experiments were conducted under mesophilic conditions (37°C) with three feedstock mixtures of CS:GS ratios 100:0, 80:20 and 60:40. The microbial community was characterised using high throughput next generation sequencing of the V3-V4 region of the 16S rRNA amplicons on an Illumina MiSeq platform along with a novel high resolution denoising bioinformatics algorithm called DADA2 and the Sylva 123 reference database. The research findings indicate that:

- The co-digested treatment with the highest level of GS had the highest specific CH_4 production. CH_4 production from the 60:40 treatment was approximately 30% higher than the CS-only treatment. The CS-only and co-digested treatments both had similar hydrolysis constants therefore on-farm AD operators can co-digest CS with GS to increase CH_4 production without having to alter the AD plant's hydraulic retention time.
- Increased levels of CH_4 production was associated with decreasing levels of total ammonia nitrogen (TAN), effluent volatile solids (VS), pH and total volatile fatty acids (TVFA). Over 80% of the phylogenetic differences observed were due to changes in these AD process parameters. Therefore TAN, %VS destroyed, pH and TVFA are important AD process parameters to monitor CH_4 production when co-digesting CS with GS.
- The GS co-substrate enhanced CH_4 production by influencing microbial community shifts. The relative abundance of the bacterial phylum *Bacteroidetes* was higher in the co-digested treatment (26%) than the CS-only treatment (19%) in the CSTR experiment. Novel bacteria from phylum *Bacteroidetes* namely *Fermentimonas caenicola* strain ING2-E5B and *Petrimonas mucosa* strain ING2-E5A, purported to have enhanced hydrolytic capabilities for complex carbohydrates, peaked in relative abundance in the co-digested treatment.

- Process instability occurred when the loading rate of the CSTRs treating the CS-only and the co-digested treatments increased to 1g VS/m^3 per day. This was likely due to increased levels of TAN during HRT 1 and 2 along with increases in the relative abundance of *Dysgonomonas spp.* which degrade carbohydrates without producing H_2/CO_2. Subsequent substrate limitation adversely affected the growth of hydrogenotrophic methanogens and resulted in propionate and butyrate accumulation.

CH_4 production increased by co-digesting cattle slurry (CS) with grass silage (GS). However, operators of on-farm AD plants should correlate microbial community changes with variations in the physico-chemical AD process parameters during reactor start-up and at regular intervals during normal operations to maximize digester performance.

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List of Abbreviations

60:40: Treatment of 60% cattle slurry and 40% grass silage

80:20: Treatment of 80% cattle slurry and 20% grass silage

AcoD: Anaerobic codigestion

AD: Anaerobic Digestion

ASBR: Anaerobic sequence batch reactors

B1-01: Batch Experiment one run 1

B1-02: Batch Experiment one run 2

B2-01: Batch Experiment two run 1

B2-02: Batch Experiment two run 2

BD: Biodegradability

BFTs: Batch fermentation tests

BMW: biodegradable municipal waste

BMP: Biochemical methane production

BP: Biogas Production

C1-01: CSTR Experiment one run 1

C1-02: CSTR Experiment one run 2

C2-01: CSTR Experiment two run 1

C2-02: CSTR Experiment two run 2

COD: Chemical oxygen demand

CS: Cattle Slurry

CSTR: Continuously stirred tank reactor

DADA2: Divisive Amplicon Denoising Algorithm

EA: Elemental Analysis

ED: Entner-Doudoroff

EMP: Emben-Mayerhof-Parnas

EU: European Union

FAN: Free Ammonia Nitrogen

FISH: Fluorescence in-situ hybridization

GHG: Greenhouse gases

GS: Grass Silage

GWh: Gigawatt hours

HRT: Hydraulic retention time

LAB: Lactic Acid Bacteria

LATS: Landfill Allowance Trading Scheme

LB: Lignocellulosic Biomass

MC: Microcrystalline Cellulose

MGM: Modified Gompertz Model

MW: Megawatts

NGS: Next generation sequencing

OFC: Organic fraction composition

OTUs: Operational taxonomic units

PCR: Polymerase chain reaction

RP: Raw protein

rRNA: Ribosomal ribonucleic acid

RSV: Ribosomal sequence variants

RT qPCR: Reverse transctiption polymerase chain reaction

SBP: Specific Biogas Production

SMP: Specific Methane Production

TAN: Total Ammonia Nitrogen

TKN: Total Kjeldahl Nitrogen

T-RFLP: Terminal restriction fragment length polymorphism

TS: Total Solids

UK: United Kingdom

VFA: Volatile Fatty Acids

VS: Volatile Solids

WLP: Wood-Ljungdahl pathway

Chapter 1 Introduction

The government of the twin island nation of Antigua and Barbuda is seeking to adopt the Renewable Energy Act, 2015 in order to achieve the United Nations (UN) millennium development goal (MDG) 7 which promotes environmentally sustainable energy production (GOAB, 2015; UN, 2015). In 2014 fuel imports totalled 193 kilo tonnes of oil equivalent, 40% of which was heavy fuel oil (HFO) which is used to produce electricity as well as water via seawater desalination (IRENA, 2016). Approximately 33,000 households, commercial and industrial installations annually consume 330GWh of electrical energy at a peak diurnal power demand of 52MW. Hunger and poverty eradication along with environmental sustainability as outlined in the MDGs 1 and 7 remain the primary drivers for the installation of low-carbon renewable energy sources in Antigua and Barbuda. The agricultural sector only comprise 2.3% of the country's GDP since most of the food consumed is imported (CIA, 2016). Global crude oil market forces have made energy costs relatively high and volatile. Renewables such as wind and solar photovoltaics (PV) provide a low-carbon alternative to electrical power generation using diesel engines. However wind and solar PV systems are considered as intermittent sources of renewable energy and small island developing states like Antigua and Barbuda limit their % contribution to the national grid system due to voltage regulation concerns (Taylor *et al.*, 2016).

The integration of low-carbon, efficient energy sources into the national grid is not unique to small island developing states within the Caribbean region. The United Kingdom (UK) has adopted a similar strategy as the country's energy demand continues to rise (Zglobisz *et al.*, 2010). One of the drivers escalating energy demand is population growth as the UK population is projected to increase annually by 0.7% from its level in 2014 and expand to 70M by 2027 (UK Office of National Statistics, 2015). The European Union (EU) Renewable Energy Directive (RED) requires that 15% of the energy used in the UK originate from renewable sources (EC, 2009; Jones and Salter, 2013) while the EU Revised Waste Framework Directive (RWFD) mandates the recycling of 50% of household waste by 2020.

The RWFD is also supported by the EU Landfill Directive (EU99/31/EC), which requires the amount of biodegradable municipal waste (BMW) sent to landfill be limited to 35% of its 1995 levels by 2020 (EC, 2008; Zglobisz *et al.*, 2010). The UK government, through the Landfill Allowance Trading Scheme (LATS), has therefore empowered local authorities to divert BMW away from landfill to be further processed using energy from waste technologies. The EU RED and RWFD are also supported by the recent adoption of the Paris Agreement where the UK ratified the international agreement to curb global greenhouse gas (GHG) emissions (UK.Gov, 2016).

In 2015 the UK contributed 507M tonnes of CO_2 equivalent GHG emissions, of which 16 kilo tonnes CO_2 equivalent were due to CH_4 emissions from manure management (EEA, 2017). Animal manure comprises the largest source of organic waste within the EU (Banks *et al.*, 2011). The EU Landfill and Renewable Energy Directives have facilitated the installation of biogas production plants that process waste streams (Evangelisti *et al.*, 2013). Improper treatment or disposal of farmyard manure may cause eutrophication of water receptors and ground water pollution due to its high organic matter, phosphorus and nitrogen concentrations as well as air pollution due to ammonia volatilization and other compounds and soil degradation if manure is applied in excess (Nasir *et al.*, 2012).

The agricultural sector within the UK and the wider EU have therefore embraced Anaerobic Digestion (AD) as a proven pathway for the waste management of agricultural residues in order to achieve its obligations under the current EU legislative framework. Anaerobic digestion is the degradation and stabilization of organic matter by microbial organisms within a closed reactor in the absence of oxygen under psychrophilic (<25°C), mesophilic (28 - 42°C) or thermophilic (55 - 72°C) temperatures (Chen *et al.*, 2008; Mir *et al.*, 2016).

The two main products of AD are biogas which consists of mainly methane (CH_4) (40-70%) and carbon dioxide (CO_2) (30-60%), and digestate -a liquid fraction of soluble organic compounds and insoluble organic matter (Angelidaki *et al.*, 1993; Wilkinson, 2011).

Banks *et al.* (2011) note that substrates used in AD vary widely and include:

- Agricultural wastes
- Energy crops
- Industrial waste and waste water
- Municipal biowaste

Small island developing states within the Caribbean such as Antigua and Barbuda can benefit from the socio-technical benefits realised from over two decades of successful AD installations throughout Europe. CPI (2017) identified agricultural waste, the organic fraction of municipal solid waste and sargassum as a potential waste streams for the implementation of AD within the Organization of Eastern Caribbean States (OECS) of which Antigua and Barbuda is a member. An AD plant can extend the economic lifespan of landfills in Antigua by diverting the organic fraction of municipal solid waste. CPI (2017) suggest, energy for transport and domestic cooking as well as trigeneration i.e. combined cooling, heating and electrical power generation (CCHP) as possible uses for the biogas and that the digestate from the AD process may be used as fertiliser. Firm power obtained from an AD plant can facilitate a higher % integration of renewables such as solar PV and wind and reduce Antigua and Barbuda's dependence on energy derived from fossil fuels. AD plants can act as a source of base load power on small grid systems similar to Antigua i.e. 52MW peak load, or as an energy storage system when the biogas is allowed to accumulate. There is also the viable prospect of utilising the CO_2 either from biogas or from the combustion of CH_4 for greenhouse crop production within the agricultural sector (Egigian-Nichols, 2013).

Within the UK, the adoption of AD within the agricultural sector is beneficial to farmers in that it reduces GHG emissions from their waste streams, provides a waste-to-energy resource of combined heat and power (CHP) to augment on-farm energy demand and produces digestate that can be applied on land as fertiliser (Zglobisz *et al.*, 2010). There were 366 operational AD plants in the UK as of December 2016 (BEIS, 2017). On the other hand, agricultural AD plants are well established in other EU countries such as Germany, Denmark, Sweden and Austria; with Germany leading with over 8700 plants in operation (Bacenetti *et al.*, 2016).

In the UK, the National Farmers Union of England and Wales (NFU) have set an ambitious target of 1000 on-farm AD plants by 2020. European Union countries adopt two main commercial AD models to process animal manure. Firstly there are large-scale centralized plants that co-digest animal manure from surrounding farms with organic waste from nearby industries and townships. Secondly, smaller farm-scale plants co-digest animal manure and energy crops from either one farm or two to three neighbouring farms.

The UK also produces approximately 100 million tonnes of animal slurry and manure every year. Cattle slurry (CS) has a relatively low specific biogas production (SBP) due its high lignin and water content and low Carbon:Nitrogen (C:N) ratio and high ammonia content (Banks *et al.*, 2011; Triolo *et al.*, 2013; Osborne Homeky *et al.*, 2016; Neshat *et al.*, 2017). The AD of low C:N ratio substrates are also prone to perform sub-optimally due to microbial inhibition caused by free ammonia (FA). On the other hand, AD systems processing substrates with high C:N ratios such as energy crops or grass silage (GS) can lead to low AD process efficiencies because the microbial growth is limited by low levels of organic Nitrogen (Risberg *et al.*, 2013). Ensiled grass is traditionally used to ensure that there is a year-round supply of feedstock for farm animals. Over the years, this biofuel resource has also become a useful co-substrate for manure-based AD systems because of its availability (Wall *et al.*, 2013; Allen *et al.*, 2016; Osborne Homeky *et al.*, 2016). Grass silage is the second most important feedstock in German biogas plants (Nizami and Murphy, 2010). The use of GS reduces the transport cost of AD feedstock over long distances for farmers which would adversely affect the net GHG emissions balance. Therefore co-digesting with GS makes on-farm AD systems more financially viable (Zglobisz *et al.*, 2010).

Many on-farm AD plants co-digesting manure and energy crops currently operate inefficiently with long retention times, low organic loading rates (OLR) and/or the inefficient use of the organic substrate. In addition to high levels of ammonia and VFA, the microbial community processing agricultural waste streams are inhibited by several environmental factors such as process pH, substrate lignin content, organic compounds, causing sub-optimal performance due to process disturbances or process failure (Chen *et al.*, 2008; Ward *et al.*, 2008).

Many on-farm AD installations also lack closed-loop control systems inclusive of sensory device actuators required to respond to process variable deviations and many existing monitoring systems provide a poor indication of process stability (Ward *et al.*, 2008; Nizami *et al.*, 2009; Madsen *et al.*, 2011). Human intuition and plant operator experience is relied upon more than classic control systems (Madsen *et al.*, 2011). Mason and Stuckey (2016) state typical organic loading rates (OLR) for commercial AD plants range between 5-6kg VS m^{-3} d^{-1} before experiencing process disturbances due to inhibition. Mason and Stuckey (2016) note that ruminants on the other hand process lignocellulosic biomass at an approximate rate of 165kg VS m^{-3} d^{-1} and suggest that there is little evidence that AD plant OLR levels will increase significantly as a function of research. The disparity between organic loading rates suggests there is room to improve current commercial AD plant efficiencies by obtaining a clearer understanding of the mechanisms which cause process disturbances. The performance and stability of AD systems are directly related to the function and interactions between the microbial consortia within them which are currently poorly understood (De Francisci *et al.*, 2015; Cardinali-Rezende *et al.*, 2016; Mosbaek *et al.*, 2016; Town and Dumonceaux, 2016). It remains unclear how the microbial community influences digester performance (Mata-Alvarez *et al.*, 2014).

This research examines the co-digestion of cattle slurry (CS) with grass silage (GS) and uses second generation NGS technology to characterise the microbial community and correlate shifts in community diversity and population with physico-chemical AD process parameters. The remainder of this chapter will review the literature on various pre-treatment methods and reactor designs that enhance biogas production from on-farm digesters with particular reference to the co-digestion of CS with GS. The AD process and the impact of process parameters on the microbial community will also be reviewed along with the impact molecular tools and fingerprinting techniques have had on AD process improvements.

1.1 Aims and Objectives

The previous research illustrated the complexities of the AD process and outlined physico-chemical parameters that limit increased efficiencies of a typical on-farm AD. Grass silage is widely available for farmers and is an affordable form of pre-treatment. However, the optimal amount of GS to use as a co-substrate is not prescriptive since silage quality is dependent on plant maturity, duration of ensiling, geographical location etc. and the AD of high quantities of GS is problematic due to lack of buffering capacity. The efficiency of an on-farm AD plants treating of animal slurries which contain high levels of TAN and are characteristically slow to degrade is often limited by ammonia and hydraulic inhibition. This results in on-farm AD plants operating with long HRT at sub-optimal steady state conditions to avoid AD process instability. The literature indicates that the diversity and richness of the microbial community mediating the AD co-digestion process is affected by changes in co-substrate as well as plant operational conditions and AD process parameters. The causes of process instability are generally related to the characteristics and complexities of the microbial community mediating AD process, which are still poorly understood. Research reporting the co-digestion of CS and GS at mesophilic temperatures, using current molecular techniques and multivariate statistical analysis to correlate biological and physico-chemical factors for AD process improvement is limited.

This research aims to reduce the complexity surrounding the biological processes involved in the anaerobic codigestion of cattle slurry (CS) and grass silage (GS) in order to improve methane production. This will be accomplished by achieving the following objectives:

- Conduct BMP tests of cattle slurry and grass silage with different ratios of feedstock to determine the level of co-substrate that had the highest specific methane production (SMP).
- Monitor the physico-chemical parameter changes under batch and CSTR operation in order to assess their influence on biogas production and quality.

- Assess the phylogenetic differences within and between treatments from samples taken from both batch and CSTR treatments and subsequently use high throughput NGS of the V3-V4 region of the 16S rRNA gene amplicons in order to determine putative functions of the dominant genera identified.
- Correlate shifts in the microbial community to changes in the physico-chemical process parameters in the CS and CS:GS treatment to determine:
    - The effect of changes in the physico-chemical parameters on microbial community dynamics
    - The effect of the GS co-substrate on microbial community dynamics and whether these community shifts resulted in improved biogas production and quality.

Chapter 2 Literature Review

2.1 Pretreatment Methods for Lignocellulosic Biomass

Co-digestion of manure-based feedstocks with grass silage (GS) or energy crops is the most cost effective, simple technique to reduce ammonia toxicity available to farmers operating on-farm AD plants (Frear *et al.*, 2011; Estevez *et al.*, 2012; Rajagopal *et al.*, 2013). Although co-digestion of cattle slurry (CS) with GS may achieve optimal C:N ratios ranging between 20 – 30 as reported in the literature, feedstocks with these C:N ratios must also be digestible to micro-organisms (Chen *et al.*, 2008; Mata-Alvarez *et al.*, 2014). Lignocellulose in grasses comprise cellulose (40-50%), hemicellulose (25-30%) and lignin (15-20%) that originate from plant biomass which also includes pectin, protein extractives, inorganic ingredients and ash (Jørgensen *et al.*, 2007; Koch *et al.*, 2010; Chen *et al.*, 2017). Cellulose, a component of the plant cell wall, is a linear syndiotactic polymer of glucose connected via β-(1-4) glycosidic bonds. Cellulose is insoluble in water, unless at high temperatures or in the presence of a catalyst and has a highly crystalline structure which is resistant to microbial attack (Jørgensen *et al.*, 2007; Chen *et al.*, 2017).

Hemicellulose does not have a stable crystalline structure and is therefore easily degraded and hydrolysed by diluted alkali, acid or enzymes under mild conditions (Lee *et al.*, 2014). Hemicelluloses are classified by the predominant sugar backbone of the polymer e.g. xylan or mannan. Jørgensen *et al.* (2007) note that plants belonging to the grass family typically contain hemicelluloses consisting of mainly glucuronoarabinoxylans and are rich in the pentose sugar xylose. The extraction of hemicellulose improves the formation of pentosans and increases subsequent enzymatic hydrolysis of cellulose (Mohapatra *et al.*, 2017).

Lignin is the most abundant non-polysaccharide fraction of lignocellulose and is an aromatic tri-dimensional polymer consisting of three monomers namely p-coumaryl, coniferyl and sinapyl alcohol that are joined through alkyl–alkyl, alkyl–aryl and aryl–aryl ether bonds (Jørgensen *et al.*, 2007; Wei, 2016).

Lignin encases cellulose to provide structural support and protection against microbial attack and is the main barrier to the degradation of lignocellulosic biomass due to its recalcitrant properties which are enhanced by ester and ether cross-linkages made with cellulose and hemicellulose (Fig. 2.1) (Jørgensen *et al.*, 2007; Lee *et al.*, 2014).

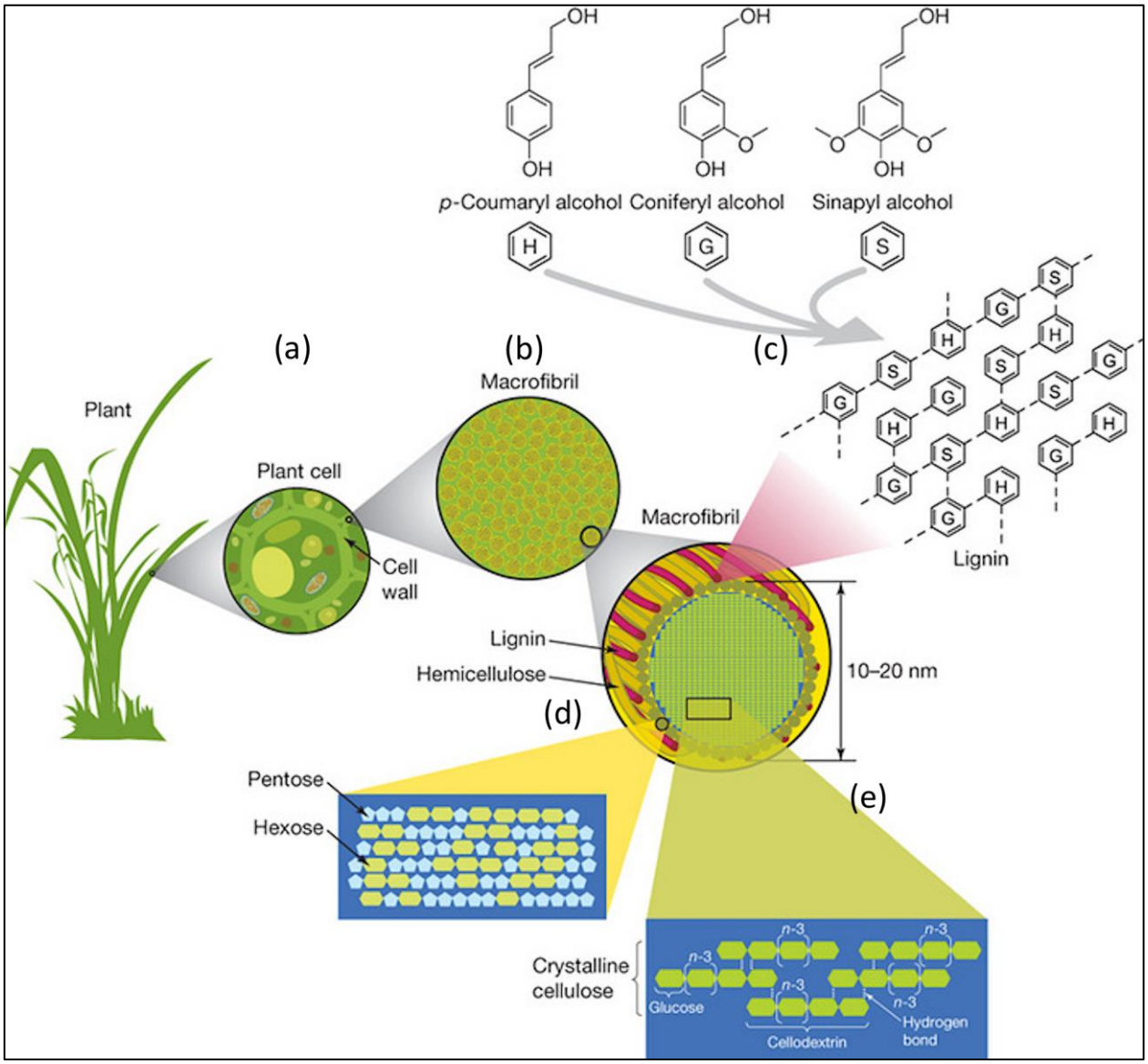

Fig. 2.1 Cross-section of (a) a plant cell wall highlighting (b) the microfibril containing a matrix of (c) lignin, hemicellulose and (e) cellulose.
Source: as cited by (Mohapatra *et al.*, 2017)

The aim of the pre-treatment of lignocellulosic biomass such as GS for biogas production is to increase its digestibility by making crystalline cellulose more accessible to enzymatic attack by breaking open the lignin (Nasir and Mohd Ghazi, 2015; Chen *et al.*, 2017). An optimal approach to pre-treatment is a combination of different methods that is cost-effective and avoids excessive parasitic energy demands and do not introduce by-products from lignin degradation such as furfural, hydroxymethyl furfural and levulinic acid that inhibit anaerobic microorganisms (Chen *et al.*, 2008; Nizami *et al.*, 2009; Carrere *et al.*, 2016; Mohapatra *et al.*, 2017).

Mechanical pre-treatment reduces the biomass particle size in order to increase contact surface area for chemical or enzymatic treatment and involves dry or wet crushing, ball mill grinding or compression (Carrere *et al.*, 2016; Chen *et al.*, 2017). Nizami *et al.* (2009) note that parasitic energy demand limits particle size reduction of GS to >1mm in commercial AD systems.

Under microwave pre-treatment, substrates are preheated within a short time period under normal pressure and temperature (NTP). Carbohydrates are hydrolysed to low weight molecular polysaccharides, proteins to saturated and unsaturated acids, ammonia and carbon dioxide and lipids to palmitic, stearic and oleic acids. Although pre-treatment time is short, the high capital cost of the equipment may extend payback periods for on-farm AD systems (Chen *et al.*, 2017).

Ultrasound pre-treatment uses sonic waves to create cavitation within plant cells and regions of liquid vapour, causing shearing forces and reactive H^+ and OH^- radicals that decompose lignin and hemicellulose molecules (Rodriguez *et al.*, 2017). The effect ultrasonic pre-treatment on cellulose is limited, however a combination of ultrasound and alkali pre-treatment enhances lignin degradation (Chen *et al.*, 2017).

In thermal pre-treatment, heat ranging from 50-250°C is applied to the feedstock to degrade lignin and hemicellulose as well as break the hydrogen bonds in crystalline cellulose and lignocellulose. At temperatures above 150-180°C hemicellulose and lignin begin to solubilize (Hendriks and Zeeman, 2009).

The high temperatures used in thermal pre-treatment of lignocellulosic biomass (LB) also provide the added benefit of pathogen removal and benefits facilities where the waste is stored prior to being processed. Thermal pre-treatment has high parasitic energy demand therefore it is usually implemented alongside acid or alkali chemical pre-treatment methods to improve its effectiveness. Steam explosion is an alternative thermal pre-treatment where pressurised saturated steam is applied to milled LB for a few minutes after which the pressure is quickly removed resulting in an explosive decompression that fractures and degrades hemicellulose and lignin and alters the crystalline cellulose matrix increasing its biodegradability (Nizami *et al.*, 2009; Carrere *et al.*, 2016; Chen *et al.*, 2017). Steam explosion is a widely used physico-chemical pre-treatment method due to its low environmental impact, short duration and low capital investment cost (Estevez *et al.*, 2012; Chen *et al.*, 2017). However, longer reaction times may lead to the production of compounds such as furfural and phenolic compounds that inhibit the AD process downstream (Neshat *et al.*, 2017).

Chemical pre-treatment of LB involves the application of either acids, alkalis, ionic liquids, organic acids, acetyl-salicylic acid and salicylic acid to degrade hemicellulose and lignin and hydrolyse the crystalline structure of cellulose (Nizami *et al.*, 2009; Chen *et al.*, 2017; Mohapatra *et al.*, 2017). In alkaline pre-treatment methods, alkalis such as NaOH, $Ca(OH)_2$, KOH or NH_4OH saponify the ester linkages within the LB, enhancing its porosity and therefore susceptibility to microbial attack. Alkaline pre-treatments are beneficial to the AD process since residual alkali can increase and maintain process alkalinity during the acidogenesis step of the AD process (Carrere *et al.*, 2016; Rodriguez *et al.*, 2017). Nizami *et al.* (2009) note that dilute acid pre-treatment is more preferential for GS due to its enhanced xylan-degrading properties. Both acid and alkali pre-treatment methods produce compounds that inhibit the AD process however, alkali pre-treatments are more often applied on an industrial scale as they may be less expensive than acids (Rouches *et al.*, 2016).

Biological pre-treatment methods involve the application of fungal, bacterial and enzymes to degrade LB (Rodriguez *et al.*, 2017). White rot fungi are the most effective for LB degradation because of their ability to degrade lignin (Sánchez, 2009; Rouches *et al.*, 2016). Biological pre-treatment methods also include the application of cellulose, hemicellulose or starch-degrading enzymes or a consortia of bacteria and/or fungi with lignolytic properties (Rouches *et al.*, 2016). Commercial enzyme cocktails which are produced by fungi, typically contain cellulases and xylanases and are expensive since extra steps are required to produce and extract the enzymes. An alternative is to pretreat with a known consortia of micro-organisms.

The ensiling process is also a form of biological pre-treatment in which lignocellulosic biomass (LB) such as grass, maize or sorghum undergo anaerobic lactic fermentation. Ensiling enhances the biomethanation process by converting resistant polysaccharides into simple sugars and VFAs, mainly lactic, acetic, propionic and butyric acids (Nizami *et al.*, 2009; Weiland, 2010; Rouches *et al.*, 2016). It facilitates wet storage which reduces dry matter loss and is considered a low cost, low energy pre-treatment method that can increase biogas yield and is therefore easily mixed with manure slurries (Allen *et al.*, 2016; Carrere *et al.*, 2016; Rouches *et al.*, 2016). The use of ensiled grass can increase biogas yield by 17% (Prochnow *et al.*, 2009; Esposito *et al.*, 2012; Wall *et al.*, 2014; Tsapekos *et al.*, 2017). Organic acids produced by the ensiling process reduce the process pH to between 3 and 4 further enhancing the degradation of hemicellulose and inhibiting the growth of unwanted microorganisms (Weiland, 2010). Rodriguez *et al.* (2017) note that biomass dry matter and composition along with microbial community influence the ensiling process. Biological pre-treatments are slow and require long reaction times. However since farmers already utilise ensiling to preserve forage for the winter, this biological pre-treatment of LB such as grasses provides a readily available feedstock to co-digest with their manure waste streams to improve biogas production.

2.2 The Anaerobic Digestion Process

Methane production from the AD of agricultural waste such as cattle slurry (CS) and grass silage (GS) consists of three phases namely pre-treatment, anaerobic hydrolysis and methane production (Hendriks and Zeeman, 2009). Agricultural waste such as CS and GS contain complex polymers of carbohydrates, lipids and proteins (Esposito *et al.*, 2012; Triolo *et al.*, 2013; Allen *et al.*, 2016). Theoretical gas yields for carbohydrates and proteins are lower than that obtained from lipids however lipids take longer to degrade under anaerobic conditions (VDI, 2006; Esposito *et al.*, 2012).

Triolo *et al.* (2013) note that lignocellulose content in plant biomass and animal slurry range from 49 – 82.8% and 30 – 80% respectively. They also observed that the lignin content in animal slurry volatile solids (VS), in particular dairy cows, is higher (18 ± 2.1%) than the lignin content in the VS contained in grass (3.6 – 10.5%) and reported that lignin content inversely correlates to the biochemical methane potential (BMP) of LB. Barakat *et al.* (2014) also conducted BMP tests on xylan-lignin composite substrates and observed that BMP decreased with increased levels of lignin content and molecular mass. Pre-treatment of agricultural waste such as CS and GS should increase the solubilisation of the complex polymeric matrices in LB in order to accelerate the hydrolysis step within the AD process which is considered rate-limiting (Weiland, 2010; Esposito *et al.*, 2012; Wei, 2016). This suggests the importance of employing an optimised pre-treatment method with co-substrates during anaerobic co-digestion since hydrolysis impacts on the AD process rate (Hendriks and Zeeman, 2009; Angelidaki *et al.*, 2011).

2.2.1 Hydrolysis

The AD process involves the biologically mediated degradation of biomass in the absence of oxygen which can be divided into four steps namely hydrolysis, acidogenesis, acetogenisis and methanogenesis (Batstone *et al.*, 2002; Campanaro *et al.*, 2016). The microbial community can be categorised into three distinct groups, which are primary fermenting bacteria, anaerobic oxidising bacteria and methanogenic archaea (De Francisci *et al.*, 2015).

During the AD process, biochemical reactions catalysed by intra- and extra-cellular enzymes excreted by hydrolysing and fermenting micro-organisms initially disintegrate composite organic material into particulate polymers i.e. carbohydrates, proteins and lipids inert material (Fig. 2.2) (Batstone *et al.*, 2002; Weiland, 2010; Campanaro *et al.*, 2016).

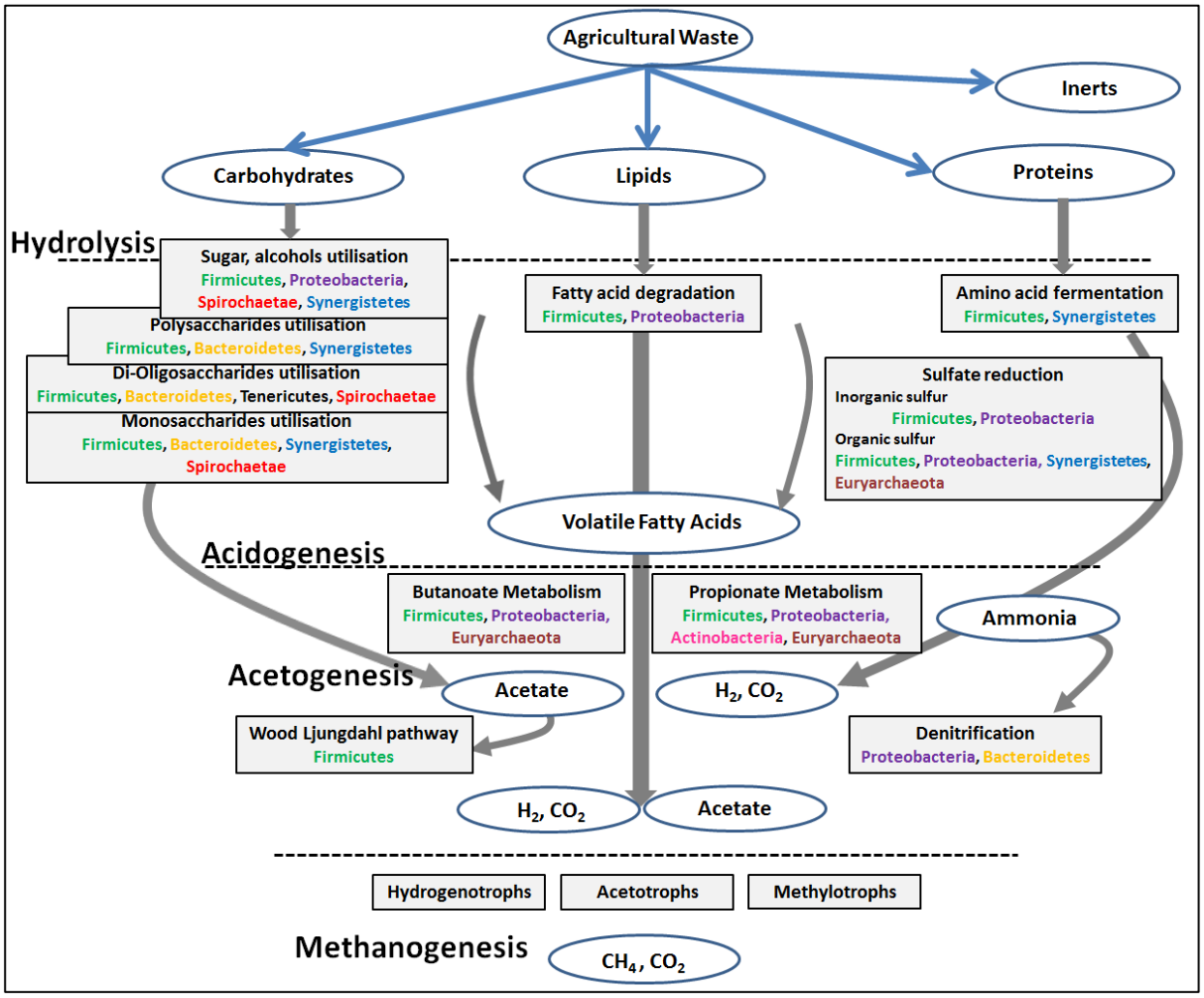

Fig. 2.2 The Anaerobic Digestion Process modified from Campanaro *et al.* (2016).

The disintegration step is followed by the hydrolysis stage where soluble monomeric compounds such as sugars, long-chain fatty acids (LCFA) and amino acids are respectively produced from the hydrolysis and fermentation of complex carbohydrates, lipids and proteins primary fermenting bacteria (Angelidaki *et al.*, 2011).

Cellulose, lipids and proteins are slowly hydrolysed to monomeric compounds within days by enzymes such as xylanases, cellobiases, cellulases, amylases, lipases, and proteases which are excreted by primary fermenting bacteria, while soluble carbohydrates are hydrolysed within hours (Weiland, 2010). Cattle slurry is an excellent co-substrate because it contains high levels of microbes that are capable of hydrolysing lignocellulose (Ward *et al.*, 2008).

The lignin component of LB is relatively non-degradable or inert under anaerobic conditions, however the degradation of lignin-derived compounds such as phenolic compounds, furfural and 5-hydroxymethyl-2-furaldehyde have an inhibitory effect on the AD process (Chen *et al.*, 2008; Ward *et al.*, 2008; Wei, 2016). Angelidaki *et al.* (2011) note that the depolymerisation and solubilisation processes that occur at the initial stages of the AD process are all hydrolytic since bio-transformations involving the cleavage of disulphide bonds can be oxidative or reductive. The disintegration/hydrolysis step is considered the rate-limiting step in the AD process (Mata-Alvarez *et al.*, 2014; Mason and Stuckey, 2016).

2.2.2 Acidogenesis

During the acidogenic or fermentative stage primary fermenting bacteria further convert a wide range of organic compounds such as unsaturated fatty acids, glycerol, monosaccharides and amino acids to acetate, higher organic acid-base pairs, hydrogen and carbon dioxide (Angelidaki *et al.*, 2011). Monosaccharides are fermented either by the Emben-Mayerhof-Parnas (EMP) or the Entner-Doudoroff (ED) pathways to yield lactate or propionate or acetate/butyrate/caproate via acetyl-CoA enzyme while amino acids are fermented via the Strickland reaction where mixed amino acids degrade in pairs to produce acetate and ammonia. Where there is a shortfall in amino acids, uncoupled oxidation may also occur under low levels of hydrogen (Angelidaki *et al.*, 2011). Bacteria belonging to the phyla *Firmicutes*, *Bacteroidetes* and *Proteobacteria* are part of a complex consortium of microbes that participate in the hydrolysis and fermentation stages of the AD process (Weiland, 2010; Wirth *et al.*, 2012; Campanaro *et al.*, 2016). Angelidaki *et al.* (2011) note that *Clostridia spp*., a member of the bacterial phylum *Firmicutes* and other low GC, gram positive bacteria are capable of fermenting monosaccharides and amino acids.

Wirth *et al.* (2012) used high throughput next generation sequencing (NGS) to characterise the microbial community in a commercial AD plant treating pig manure slurry and plant biomass and identified primary fermenting bacteria belonging to the bacterial phyla *Firmicutes*, *Tenericutes*, *Bacteroidetes* and *Spirochaetae* involved in the hydrolysis and acidogenesis stages of the AD process.

Campanaro *et al.* (2016) used NGS, online databases and a novel binning technique to characterise the microbial community of eight AD plants and also provided putative functional roles to "genome bins" (GB) that were assigned species names. They reported bacterial phyla *Proteobacteria*, *Synergistes* additional to those reported by Wirth *et al.* (2012) involved in the hydrolysis and acidogenesis stage of the AD process (Fig. 2.2) and noted that functions within the earlier stages of the AD process were shared by bacteria belonging to different taxonomic ranks which then competed for substrate, however the function of the microbes became gradually more specialised towards the latter stages of the AD process.

2.2.3 Acetogenesis

During acetogenisis obligate anaerobic bacteria utilise the reductive acetyl CoA or Wood-Ljungdahl pathway (WLP) for energy conservation, the synthesis of acetyl CoA and cell carbon from CO_2 (Ragsdale and Pierce, 2008; Angelidaki *et al.*, 2011). Anaerobic oxidising bacteria belong to the phyla *Spriochaetae*, *Proteobacteria*, *Actinobacteria*, however most of the acetogens identified belong to the phylum *Firmicutes* (Ragsdale and Pierce, 2008; Stams and Plugge, 2009; Campanaro *et al.*, 2016). Hydrogen-utilising acetogens or homoacetogens synthesize acetate via the reduction of CO_2 while hydrogen-producing acetogens produce acetate by oxidising organic acids such as butyrate and propionate and alcohols such as ethanol (Angelidaki *et al.*, 2011). Electrons produced from organic acid oxidation are transferred to protons (H^+) and bicarbonate (HCO_3^-) to produce hydrogen and formate respectively (Stams and Plugge, 2009).

Acetogens can also perform the reverse WLP in that they oxidise acetate syntrophically with a partner hydrogenotrophic methanogen (Campanaro *et al.*, 2016). Acetogens that obligately oxidise organic acids use protons H^+ and CO_2 as electron acceptors in partnership with hydrogenotrophic methanogens because the oxidation reaction by acetogenic bacteria of organic acids such as butyrate and propionate on their own is limited by unfavourable energetics (Stams and Plugge, 2009; Angelidaki *et al.*, 2011; Ahlert *et al.*, 2016). Consequently, there is an obligate dependence of acetogens that oxidise propionate and butyrate on hydrogenotrophic methanogens and hydrogen scavengers such as sulphate reducing bacteria (SRB) to maintain low levels of hydrogen making the oxidation reaction more thermodynamically favourable. The mechanism, referred to as interspecies electron transfer utilises hydrogen and/or formate as electron acceptors to oxidise organic acids in syntrophy with partner hydrogenotrophic methanogens (Stams and Plugge, 2009).

A third mechanism of inter-species electron transfer (IET) called direct inter-species electron transfer (DIET) was purported by Gorby *et al.* (2006) who observed electrically conductive pili or bacterial nanowires on *Shewanella oneidensis MR-1* and *Geobacter spp.* in a direct response to electron acceptor limitation. Gorby *et al.* (2006) also note that bacterial nanowires were also produced by *Pelotomaculum thermopropionicum*, a thermophilic syntrophic propionate oxidising bacterium (Imachi *et al.*, 2002), and suggest that DIET represents a common bacterial strategy for electron transfer. Propionate, butyrate and ethanol are ubiquitous fermentation products in stable-operating AD plants, however their accumulation can lead to inhibition of bacteria and archaea within the AD microbial community (Barredo and Evison, 1991; Chen *et al.*, 2008; Ahlert *et al.*, 2016). Propionate degrading acetogenic bacteria are more sensitive to increased levels of free ammonia (FA) than methanogens (Boe *et al.*, 2010; Xie *et al.*, 2014). Propionate degradation is therefore considered a limiting factor to the anaerobic biomethanation process and is an important focal point for the improvement of biogas production and quality from agricultural resources since increasing levels may be an indicator of process stress (Boe *et al.*, 2010; Ahlert *et al.*, 2016).

2.2.4 Methanogenesis

The final step of the AD process involves the conversion of C_1 methylated compounds, acetate and H_2/CO_2 to methane by strictly anaerobic methanogenic archaea in environments where O_2, NO_3^-, Fe^{3+} and SO_4^{2-} are limited (Angelidaki *et al.*, 2011). Methanogenic archaea belong to the phylum *Euryarchaeota* and seven phylogenetic orders have been identified from previous research namely *Methanosarcinales*, *Methanobacteriales*, *Methanomicrobiales*, *Methanococcales*, *Methanopyrales*, *Methanocellales* and *Methanomassiliicoccales* (Angelidaki *et al.*, 2011; Vanwonterghem *et al.*, 2016). The three main methanogenic pathways for methane production all incorporate the WLP (Angelidaki *et al.*, 2011; Borrel *et al.*, 2016; Campanaro *et al.*, 2016; Vanwonterghem *et al.*, 2016) and are:

- Acetoclastic methanogenesis- which involves the cleavage of acetate resulting in the production of CH_4 and CO_2.
- Hydrogenotrophic methanogenesis- where H_2 and CO_2 are reduced to form CH_4
- Methylotrophic methanogenesis- where the methyl group, originating from derivatives of pectin degradation, forms C_1 methylated compounds such as methanol, methylamines, methyl-mercaptopropionate, di-methylsulphides etc. (Sprenger et al., 2007; Angelidaki et al., 2011; Tracy et al., 2012; Henderson et al., 2015).

Acetoclastic and hydrogenotrophic methanogenesis are the two main pathways for methane production with 70% of the methane produced from acetate (Angenent *et al.*, 2004; Angelidaki *et al.*, 2011). Recent advances in molecular techniques have however challenged traditional views regarding the diversity of the archaeal community in ecological niches such as AD plants treating LB and the functions of archaea previously well documented in the literature providing opportunities for the improvement of biogas production and quality. Rotaru *et al.* (2014) used NGS, metatranscriptomic analysis coupled with fluorescence *in-situ* hybridization with specific 16S rRNA probes to analyse the methanogenic community sampled from an AD plant treating brewery waste and found that *Methanosaeta spp*. was the most abundant archaeal phylotypes present. Genes related to the complete reduction of CO_2 were also highly expressed in the samples.

They subsequently isolated *Methanosaeta harundinacea* which grew in co-culture with *Geobacter metallireducens* where they observed growth via DIET to degrade ethanol supported by highly expressed genes from *M. harundinacea* for the putative CO_2 reduction pathway and highly expressed genes for PilA from *G. metallireducens* which is a putative indicator of DIET. Rotaru *et al.* (2014) therefore note that *Methanosaeta spp.*, previously purported as an obligate acetoclastic archaea (Garcia *et al.*, 2000), is more versatile since it can produce methane from CO_2 via DIET during anaerobic fermentation. They note that the importance of DIET in methanogenic communities remains unknown but the ability of *Methanosaeta spp.* to produce methane via DIET may add to their competitive advantage. Recently, Vanwonterghem *et al.* (2016) reported the discovery of a methylotrophic archaeal cluster belonging to a new archaeal phylum *Verstraetearchaeota* after conducting metagenomic analyses on environmental samples two of which were obtained from a mesophilic anaerobic digester degrading cellulose and a mesophilic digester treating effluent from a palm oil mill. In addition to methane production, Vanwonterghem *et al.* (2016) report that *Verstraetearchaeota spp*. appear to be capable of utilising sugars as a carbon source to produce acetyl-CoA via the EMP pathway and pyruvate ferredoxin oxidoreductase and note that the putative fermentative capability of *Verstraetearchaeota spp*. suggest substrate diversification for the newly discovered archaea. The stability of the AD process is dependent on maintaining a delicate balance between producers and consumers of volatile fatty acids (VFA) (Rajagopal *et al.*, 2013). Increased levels of short chain VFA, long chain volatile fatty acids (LCFA) and/or ammonia along with other toxins within the digester can result in reduced process efficiencies or process failure by inhibiting methanogens (Batstone *et al.*, 2006; Chen *et al.*, 2008; Mata-Alvarez *et al.*, 2014).

2.3 Anaerobic Digestion Process Inhibition

Along with possible artefacts of the pre-treatment process such as furfural, 5-hydroxy methyl furfural, levulinic and aromatic carboxylic acids, agricultural feedstocks may contain a wide variety of substances that may result in shifts in the microbial community or inhibit microbial growth resulting in process disturbances or failures during AD (Chen *et al.*, 2008; Rajagopal *et al.*, 2013). Process instability can result from issues related to digester feeding, insufficient mixing, process temperature variation, micronutrient limitation and the presence of inhibitory and toxic substances (Angelidaki *et al.*, 2005; Drosg, 2013).

2.3.1 Ammonia

Ammonia is an important waste stream resource that promotes microbial growth. However agricultural wastes such as CS contain levels of ammonia greater than that required for microbial growth as well as urea and proteins which release ammonia as they degrade (Chen *et al.*, 2008; Chen *et al.*, 2014). Levels of total ammonia nitrogen (TAN) greater than 1.5g NH_4^+-N/L with a pH >7.4 can inhibit the micro-organisms within the AD process (Chen *et al.*, 2008; Lin *et al.*, 2013b; Rajagopal *et al.*, 2013). The ammonium ion (NH_4^+) and free ammonia (NH_3) are the two forms of inorganic ammonia nitrogen present in aqueous solution within the digester. Ammonia is more toxic in its non-ionised form FA, the concentration of which increases with rising pH and temperature (Ward *et al.*, 2008; Lin *et al.*, 2013b; Chen *et al.*, 2014). Free ammonia inhibits methanogens by diffusing into the cell membrane, causing a proton and/or K^+ deficiency, while ionized ammonium is reported to directly inhibit enzymes involved in methane synthesis (Sprott *et al.*, 1984; Sprott and Patel, 1986; Kayhanian, 1999; Drosg, 2013). The morphology of methanogens determine their susceptibility to FA inhibition on the basis of a diffusion rate of kilograms of NH_3 absorbed by a kilogram of cell mass per hour.

Therefore *Methanosarcina spp.*, having a higher volume ratio with a larger spherical shape are less susceptible to FA inhibition than smaller rod-shaped archaeal phylotypes belonging to *Methanotrix* (renamed *Methanosaeta*) *spp.* (Wiegant and Zeeman, 1986). Chen *et al.* (2008) note that the literature is inconclusive as to whether hydrogenotrophic or acetocleastic methanogens are the most sensitive to ammonia toxicity. Increased levels of ammonia concentration may also select the dominant methanogenic pathway within the AD process. Fotidis *et al.* (2014a) used fluorescent *in-situ* hybridisation (FISH) coupled with ^{14}C – labelled acetate in the methyl group to analyse the methanogenic community of samples taken from eight commercial biogas plants mainly treating agricultural waste, three operating at mesophilic and five at thermophilic temperatures (i.e. between 45 -65°C). They defined three categories of TAN levels (high, medium and low) that influenced the selection of methanogenic archaea within the AD plants and noted that:

- Syntrophic acetate oxidation followed by hydrogenotrophic methanogenesis (SAO-HM) was the dominant methanogenesis pathway for biogas plants operating with high levels of TAN (>2.8g NH_4^+-N/L) and FA levels (> 0.44g NH_3-N/L). These results were in agreement with Ahring et al. (1995) and Demirel and Scherer (2008).
- Plants operating at medium levels of TAN (1.5 – 2.8g NH_4^+-N/L) and low levels of TAN (<1.5g NH_4^+-N/L) were dominated by the acetoclastic methanogenesis pathway (ACM). They also observed that biogas plants operating at low levels of TAN had more stable dominant methanogenic populations.

The shift in archaeal composition to increasing relative abundance of hydrogenotrophic methanogens was also supported by more recent research and may help to improve biogas production and quality by decreasing total VFA levels (Ahlert *et al.*, 2016; Mosbaek *et al.*, 2016; Poirier *et al.*, 2016; Westerholm *et al.*, 2016; Hagos *et al.*, 2017). Microbial communities can adapt or acclimatise over time to a wide range of TAN levels ranging from (1.7 – 14g NH_4^+-N/L) which may result in a 50% reduction in methane production at mesophilic process temperature within the AD process (Angelidaki and Ahring, 1994; Chen *et al.*, 2008; Drosg, 2013).

Fotidis *et al.* (2014a) studied the effect of ammonia on the methanogenic pathway in eight Danish mesophilic AD plants operating under different concentration levels of ammonia and concluded that all the AD plants treating manure in the experiment were operating under an "inhibitive steady-state" condition where the process was affected by ammonia and/or free ammonia inhibition, with their profitability reduced up to 30%. This was supported by previous research that indicated AD plants treating manures are typically more stressed and operate at elevated VFA levels, thereby losing potential biogas production in digester effluent (Angelidaki and Ahring, 1994; Hansen *et al.*, 1998; Angelidaki *et al.*, 2005; Drosg, 2013).

2.3.2 Process Temperature

The AD process operating temperature of the bioreactor may also affect process stability. There is no significant difference in specific methane yields from mesophilic temperatures to thermophilic temperatures. Operating at mesophilic temperatures is reported to be more stable than thermophilic temperatures since the microbial community is more diverse and active (Ward *et al.*, 2008; Appels *et al.*, 2011). However thermophilic temperatures facilitate increased organic loading rates and shorter hydraulic retention times which are desired features of an optimised AD process (Angelidaki and Ahring, 1994; Bocher *et al.*, 2008). Angelidaki and Ahring (1994) operated laboratory scale reactors treating cattle manure at temperatures ranging from 40 to 64°C at two levels of TAN (2.5 and 6g NH_4^+-N/L) and observed that inhibition due to free ammonia increased with operating temperature. These findings were supported by Bayr *et al.* (2012) who co-digested rendering and slaughterhouse waste in laboratory-scale CSTR digesters. Distinct differences in process stability may occur when process temperature was varied within both the mesophilic and thermophilic ranges (El-Mashad *et al.*, 2004; Garcia and Angenent, 2009). Garcia and Angenent (2009) reported a 45% reduction in methane production while operating anaerobic sequence batch reactors (ASBR) treating swine waste at 25°C at TAN and FA levels of >4g NH_4^+-N/L and 0.08g NH_3-N/L as opposed to a 13% reduction in methane when operating at 35°C at higher levels of FA ca. 250g NH_3-N /L. The digesters were able to efficiently process waste at a TAN concentration of 5.24g N/L.

During the thermophilic AD treatment of cattle manure, El-Mashad *et al.* (2004) noted that FA adversely affected the hydrolysis and acidogenesis steps as well as affected acetate-utilising bacteria and they observed a lower methane production rate at an operating temperature of 60°C as opposed to 50°C in laboratory-scale CSTR digesters.

2.3.3 Process pH

pH inhibits all groups of micro-organisms and further exacerbates ammonia toxicity because increasing pH raises the concentration of FA (Batstone *et al.*, 2002; Chen *et al.*, 2008). Methane formation takes place in a narrow pH band of 6.5 to 8.5 and the AD process becomes inhibited if the pH falls below 6.0 or increases beyond 8.0 (Ward *et al.*, 2008; Lee *et al.*, 2009; Weiland, 2010). Since the hydrolysis/acidogenesis stages have a lower optimal pH range from the acetogenisis and methanogenesis stages of the AD process, a two-stage bioreactor design is reported to provide increased process stability (Khanal *et al.*, 2004; Nasir *et al.*, 2012; Peña Muñoz and Steinmetz, 2012).

The quantity of ammonia that is available from the anaerobic biodegradation of biomass can be estimated using the following stoichiometric relationship (Tchobanoglous *et al.*, 1993):

$$C_aH_bO_cN_d + \frac{4a-b-2c+3d}{4} H_2O \rightarrow \frac{4a+b-2c-3d}{8} CH_4 + \frac{4a-b+2c+3d}{8} CO_2 + dNH_3 \quad \text{Eq. 2.1}$$

Grass silage is characterised by high C:N ratios and poor buffer capacity due to the lactic acid and VFA content and pH (3.8 – 5.0). The AD of GS can produce large amounts of VFA during the acidification step of the AD process, if the rate of acidification is higher than the methanogenesis step rate, which may lead to inhibition (Weinberg and Muck, 1996; Angelidaki *et al.*, 2005; Siegert and Banks, 2005; Mata-Alvarez *et al.*, 2014). The mono-digestion of low carbon to nitrogen ratio (C:N) feedstocks such as cattle and pig slurry are more sensitive to small changes in pH and can become unstable due to FAN inhibition and provide a low methane yield (Drosg, 2013; Rajagopal *et al.*, 2013; Mata-Alvarez *et al.*, 2014).

Animal slurries, due to their high TAN levels, provide surplus alkalinity during the AD process when NH_4OH reacts with carbon dioxide to produce bicarbonate (Kayhanian, 1999; Chen *et al.*, 2014):

$$CO_2 + H_2O + OH^- \leftrightarrow HCO_3^- + H_2O \quad \text{Eq. 2.2}$$

The high buffering capacity of animal slurries along with the inherent buffering capacity of the AD process make CS an ideal co-substrate with GS (Drosg, 2013). This enables intermediate metabolites such as propionic and butyric acids to be metabolised into acetate H_2 and CO_2 by acetogens and ultimately converted to methane by methanogenic archaea (Angelidaki and Ellegaard, 2003; Weiland, 2010; Chen *et al.*, 2014; Mata-Alvarez *et al.*, 2014). Most digesters treating manures are stable at total VFA concentrations between 1-2g/L (up to 33mM) and most operate up to 4g/L, likely due in part to ammonia inhibition (Angelidaki *et al.*, 2005). However, total VFA of levels above 4g/L (66mM) can inhibit the fermentation of glucose which is an intermediate metabolite in the anaerobic fermentation of feedstocks rich in lignocellulose such as CS and GS (Ragsdale and Pierce, 2008; Ward *et al.*, 2008; Wei, 2016).

2.3.4 Trace elements

Farmyard manures have a mean pH of 7.3 and also contain micronutrients that micro-organisms utilise for growth in important biochemical processes, such as the WLP in the latter stages of the AD process where metals are used as cofactors to degrade VFA (Weiland, 2010; Karlsson *et al.*, 2012; Westerholm *et al.*, 2012a; Triolo *et al.*, 2013). Cobalt, nickel, copper, selenium, iron, molybdenum, tungsten and boron are essential for methanogenic growth and metabolism (Ward *et al.*, 2008; Demirel and Scherer, 2011; Vintiloiu *et al.*, 2013). Selenium, molybdenum and tungsten are used to synthesise the formate dehydrogenase, an enzyme used to oxidise formate (Rajagopal *et al.*, 2013). Shifts in pH or temperature can also affect the bioavailability of micronutrients from trace metals in bioreactors because they form poorly insoluble precipitates with carbonate and sulphide, preventing microbial uptake and causing process instability due to inhibition (Pobeheim *et al.*, 2010; Drosg, 2013). Trace element supplementation assisted in the degradation of long chain fatty acids (LCFAs) and the reduction of VFA at higher lipid loading rates (Jiang *et al.*, 2012).

However, trace element deficiency does not usually limit farm-based AD systems since animal slurries normally contain sufficient concentrations (Drosg, 2013).

2.3.5 Hydrogen Sulphide

Cattle slurry however contains low levels of sulphur since their diet is mainly grass which is high in carbohydrates and low in protein (Koch *et al.*, 2010; Triolo *et al.*, 2013; Westerholm *et al.*, 2016). Sulphur reducing bacteria (SRBs) reduce sulphates to hydrogen sulphide which are toxic to SRBs acetotrophic and hydrogenotrophic methanogens (Stams *et al.*, 2003; Chen *et al.*, 2014). The undissociated sulphide is the only form that is toxic due to its ability to migrate across the cell membrane (O'Flaherty *et al.*, 1998; Drosg, 2013). Hydrogen sulphide inhibited acetoclastic methanogens more than hydrogenotrophic methanogens with a half maximal inhibitory concentration (IC_{50}) of hydrogen sulphide for acetoctrophic and hydrogenotrophic methanogens reported to be 160 and 220 mg $H_2S_{(aq)}$/L (Yamaguchi *et al.*, 1999). Sulphate-reducing bacteria compete with acetogens and methanoganeic archaea for common substrates such as propionate, butyrate, acetate methanol and hydrogen under anaerobic conditions (Stams *et al.*, 2003; Paulo *et al.*, 2004; Chen *et al.*, 2008). The outcome of the competition between dissimilatory sulphate reduction and methanogenesis dictates the quality of biogas produced (Colleran *et al.*, 1995; Paulo *et al.*, 2004). Sulphides can also form metal precipitates with trace elements resulting in a reduction in micronutrient availability essential for AD biochemical processes and microbial growth thereby causing inhibition (Demirel and Scherer, 2011; Shakeri Yekta *et al.*, 2014).

2.3.6 Organic and Hydraulic Overload

Process inhibition can occur when solids accumulate within the digester, increase VFA concentration levels and effectively reduce reactor volume and microbial access to biomass (Angelidaki *et al.*, 2005; Ward *et al.*, 2008; Neshat *et al.*, 2017). This inhibition is caused by organic overload when the amount of organic matter fed into the digester exceeds the degradation capacity of the microbial community (Drosg, 2013). Inhibition due to hydraulic overload is caused by operating at retention times that are so low that it does not facilitate the individual growth rates of the microbes.

Propionate degraders and methanogens have very slow growth rates of 0.49 and 0.60 d^{-1} respectively (Table 2.1) (Angelidaki *et al.*, 1999). The wash out of slow-growing microbial consortia degrading the biomass can limit biogas production (Demirer and Chen, 2004; Angelidaki *et al.*, 2005; Drosg, 2013). Therefore AD plants must operate at retention times that exceed the doubling time of the microbial consortia which is typically 15 – 30 days at mesophilic conditions and 10 – 20 days at thermophilic conditions (Angelidaki *et al.*, 2011; Westerholm *et al.*, 2016).

Table 2.1 Maximum specific growth rates of bacterial and archaeal groups within the anaerobic digestion process modified from (Angelidaki *et al.*, 1999)

Bacterial/Archaeal Group	**Maximum Specific growth rate μ_{MAX} (d^{-1})**
Carbohydrate Enzymatic	1
Protein Enzymatic	1
Glucose Acidogens	5.10
Lipolytic	0.53
LCFA Degraders	0.55
Amino Acid Degraders	6.38
Propionate Degraders	0.49
Butyrate Degraders	0.67
Valerate Degraders	0.69
Methanogens	0.60

However, microbial doubling times obtained from cultures under optimal laboratory conditions are typically less than microbial doubling times within on-farm AD plants which may become longer due to the presence of inhibitory substances or changes in operating conditions. Hydraulic microbial wash-out becomes more likely as plant operators seek to improve process efficiency, resulting in increased levels of VFA due to hydraulic overload inhibition (Griffin *et al.*, 1998; Angelidaki *et al.*, 2011; Drosg, 2013). Process improvements such as the recirculation of a portion of the effluent to the main digester minimises the likelihood of microbial wash-out by increasing the effective retention time (Angelidaki *et al.*, 2011). Attachment of cellulolytic microorganisms to particulates within the feed prevents them from being washed out of the digester and is an essential step for hydrolytic bacteria in the AD of LB (Bayané and Guiot, 2010). The co-digestion of cattle manure with GS silages and the mono-digestion of GS were successfully achieved at high OLR levels ranging for 2-4g VS/m^3 per day in laboratory-scaled CSTR by recirculating a portion of the effluent, obtaining methane yields of 198-366 L CH_4/kg VS per day (Wall *et al.*, 2014).

Estevez *et al.* (2014) experienced organic overload when co-digesting cow manure with steam exploded salix using 6L laboratory-scale CSTRs, two of which were operated with digestate recirculated to the main tank to dilute the feedstock and increase the HRT. A 16% higher methane yield was achieved with the CSTR operating with recirculated digestate as opposed to recirculated water and FA, furfural and 5-hydroxy methyl furfural were below inhibitory levels. However, methane production began to reduce in the latter HRT due to solids accumulation, limiting microbial access to the biomass. The literature illustrates the current challenge faced by AD plant operators, which is how to retain slow-growing microbial biomass while simultaneously increasing process efficiency by decoupling the HRT and sludge retention times (Madsen *et al.*, 2011; Horváth *et al.*, 2016).

2.4 Types of Bioreactors used in the Co-digestion of Agricultural Waste

Reactor design configuration along with feedstock pre-treatment methods are important considerations for biogas improvement from the AD of agricultural resources as these influence retention times (Ward *et al.*, 2008; Nizami and Murphy, 2010). Capital investment cost as well as AD operating parameters such as degree of mixing, retention time, number of tanks, process temperature and feedstock characteristics are all critical factors to consider in bioreactor design (Igoni *et al.*, 2008; Mir *et al.*, 2016). Cattle slurry (CS) is low in total solids (TS) concentration with pig slurry ranging between (5 – 7%) and cattle and dairy cows between (7 – 9%) and is therefore treated using wet AD which are designed to treat solid waste with TS <16% (Angelidaki and Ellegaard, 2003; Ward *et al.*, 2008). Although low TS concentrations in animal slurries result in low methane yields (ca.10 – 20m^3/ ton), it provides an excellent carrier for more dry feedstocks such as GS which have a TS concentration of 20% by minimising line blockages when both feedstock are combined for anaerobic co-digestion (Angelidaki and Ellegaard, 2003; Ward *et al.*, 2008; Nizami and Murphy, 2010; Nasir *et al.*, 2012). About 90% of newly erected bioreactors facilitate wet fermentation (Weiland, 2006). Digester configurations used in research to enhance biogas production from biomass such as agricultural waste are classified into three broad categories, namely low rate, high rate and plug flow (Fig. 2.3) (Ward *et al.*, 2008; Nizami and Murphy, 2010; Shah *et al.*, 2015).

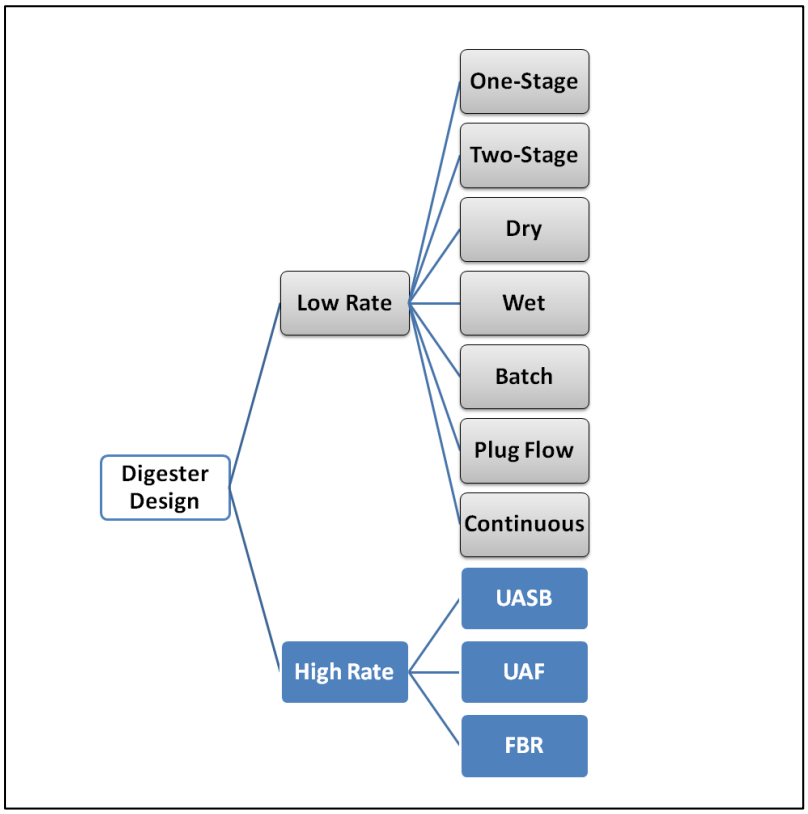

Fig. 2.3 Reactor types used in Anaerobic Digestion modified from (Shah *et al.*, 2015). UASB-upflow anaerobic sludge blanket, UAF-uplow anaerobic filter, FBR- fluidised bed reactor

2.4.1 Batch versus Continuous Digesters

A recently conducted meta-analysis of 115 research papers by Miranda *et al.* (2016) reviewed on-farm AD bioreactor types used to treat dairy cattle manure globally and indicated that the most common types were batch, continuously stirred tank reactor (CSTR), fixed film, plug-flow, and upflow anaerobic sludge blanket (UASB) reactors. Agricultural feeds such as a mixture of CS and GS, with varying feed type and a high fraction of particulate organic biomass are not suitable for digestion by high rate digesters e.g. UASB, fixed film digesters and fluidised bed reactors which are designed to treat industrial wastewater (Chan *et al.*, 2009; Shah *et al.*, 2015; Horváth *et al.*, 2016).

Weiland (2006) noted that most AD plants treating animal slurry co-digested with energy crops in Germany operate at low loading rates between 1 – 3 kg VS/m^3 per day. About 90% of modern wet AD plants are of the vertical tank CSTR configuration with retention times <30 days and can be configured to operate in either a batch or continuous mode. During batch digestion, the digester is loaded once with feedstock which is then left for a specific period of time to sufficiently degrade. A portion of the reactor contents is then removed leaving the balance as inoculum for subsequent feedstock loading (Ward *et al.*, 2008; Nizami and Murphy, 2010; Singh *et al.*, 2011; Nasir *et al.*, 2012).

Continuous digesters are categorised into plug flow or continuously stirred tank reactors (CSTR) while batch digesters operate in one-stage reactor (Ward *et al.*, 2008; Nizami and Murphy, 2010; Wirth *et al.*, 2012; Shah *et al.*, 2015). In continuous digesters, feedstock is continuously added to the digester while the digester contents is slowly mixed to avoid scum and bottom layers, resulting in improved degassing and microbial access to biomass (Weiland, 2006; Ward *et al.*, 2008; Mir *et al.*, 2016). Continuous systems facilitate rapid acidification and produce higher levels of VFA. However excessive mixing may disrupt granules containing syntrophic microbial communities oxidising intermediates such as propionate and butyrate, reduce biogas production and consequently lead to increased levels of total VFA concentration and digester instability (Angelidaki *et al.*, 2005; Ward *et al.*, 2008; Wirth *et al.*, 2012). Plug flow digesters are low cost, semi-continuous, wet digestion systems with relatively small design volumes ranging from 2.4 to 7.5m^3 where unmixed feedstock (TS concentration <11 – 13%) is fed through a horizontal reactor as a plug (Nasir *et al.*, 2012; Shah *et al.*, 2015; Mir *et al.*, 2016). Plug flow reactors do not require mixing and have low capital investment cost because of minimal auxiliary equipment requirement, but they have a lower biogas yield and are prone to clogging and feedstock channelling.

2.4.2 One versus Two-Stage Reactors

Commercial one-stage bioreactors are more popular than two-stage reactor systems because of their simplicity of design resulting in reduced occurrences of technical problems (Nizami and Murphy, 2010; Shah *et al.*, 2015). One-stage bioreactors however have longer retention times (10 – 60 days) than two-stage bioreactors (10 – 15 for both stages) hence two-stage designs facilitate increased throughput of waste. Two-stage systems improve biogas production and quality by separating AD processes that require different optimal environmental conditions and are able to treat waste streams with a range TS concentration (2 – 40%) than one-stage bioreactors (10 – 40%). Two-stage systems are also considered more stable than one-stage designs especially when treating easily hydrolysable feedstocks and they offer more protection against AD process imbalances caused by variations in organic loading rate, feedstock heterogeneity, and inhibitor concentration levels (Ward *et al.*, 2008; Khalid *et al.*, 2011). AD process stability is increased by controlling the acidification phase within the first stage, thereby preventing the inhibition of the methanogenic population within the second stage digester (Ward *et al.*, 2008). Two-stage designs are more versatile than one-stage reactors since they facilitate the recovery of both H_2 and CH_4 by optimising environmental conditions for dark fermentation within the first stage digester and methane production in the second stage digester (Ghimire *et al.*, 2015; Hagos *et al.*, 2017). Higher levels of energy recovery from two-stage systems result in higher volatile solid (VS) destruction than one-stage systems where there is a higher likelihood of feedstock short-circuiting i.e. feedstock passing through the digester at shorter retention times than the designed HRT (Ward *et al.*, 2008; Shah *et al.*, 2015; Carrere *et al.*, 2016).

However, Schievano *et al.* (2012) investigated the microbial community of a thermophilic, two-stage CSTR process and compared it to a one-stage CSTR reactor both of which treated swine manure co-digested with fruit and vegetable market waste and found no significant difference between the methane produced from both reactor types. They also observed a higher VS destruction rate and energy recovery from the single-stage reactor as well as higher bacterial and archaeal diversity.

A similar finding was made by Monlau *et al.* (2015) who also observed similar methane yields from pre-treated sunflower in the one-stage (150 ± 3.5mL CH_4/g VS) and two-stage (152 ± 3.5mL CH_4/g VS) bioreactor configurations but a molecular analysis of the microbial community was not undertaken. Moreover, hydrogen accumulation within the first stage can inhibit acidogenic bacteria within a two-stage design (Hagos *et al.*, 2017). One-stage on farm digesters remain more popular as a result of their simplicity of design and lower start-up capital cost (Ward *et al.*, 2008; Nizami and Murphy, 2010; Shah *et al.*, 2015; Hagos *et al.*, 2017). However, Wall *et al.* (2013) indicate that there is less extensive reporting of methane yields from the co-digestion of manures and GS. Jagadabhi *et al.* (2008) co-digested cattle manure with GS (30% of feed VS) at a loading rate of 2g VS/m^3 per day in laboratory-scale CSTRs, configured with a recirculation line to feed a portion of digester effluent back into the reactor and obtained methane yields of 180-185 L CH_4/kg VS but also reported that a scum layer had formed on the surface of the liquid phase and feed accumulated within the reactor. Lehtomäki *et al.* (2007) co-digested cow manure with four levels of GS in one-stage laboratory-scale CSTRs with loading rates ranging from 2 – 4g VS/m^3 per day and obtained methane yields ranging from 133 – 220 L CH_4/kg VS. Wall *et al.* (2014) reported an optimised version of the previous study conducted by Lehtomäki *et al.* (2007) by recirculating the separated liquor of the digestate to the reactors operating at higher loading rates and achieved methane yields of 198 – 366 L CH_4/kg VS.

2.5 Process Indicators and the impact of Molecular Ecological Tools

Imbalances within the AD process can be detected from changes in the concentration of intermediates produced during hydrolysis, acidogenesis, acetogenisis and methanogenesis (Bruni *et al.*, 2013). These changes depend upon the cause of the imbalance and the parameters that influence the composition of the microbial community within the digester (Boe *et al.*, 2010; Bruni *et al.*, 2013). Previously, optimisation of biogas production and quality was determined empirically and the AD process was monitored by changes in individual VFA concentrations within the digester (Ahring *et al.*, 1995).

Individual VFA concentration, particularly butyrate, isobutyrate, isovalerate were suggested as good indicators of ammonia inhibition in a thermophilic digester treating cattle and pig manure (Nakakubo *et al.*, 2008). The suggestion of isobutyrate and isovalerate as good indicators of ammonia inhibition was also supported by research investigating the response of a thermophilic digester treating chicken litter to shock ammonia loads (Bruni *et al.*, 2013). However Pind *et al.* (2003) noted that all individual VFA were important for control purposes but concentrations of isoforms of VFA are affected by interactions with other VFA such as propionate and acetate and that the concentration of VFA isoforms must therefore be evaluated along with their conversion during the AD process which complicates the analysis. While the literature agrees individual VFA measurement provides useful information on specific information for process diagnosis, the online monitoring of VFA is considered complex (Ahring *et al.*, 1995; Boe *et al.*, 2010).

Boe *et al.* (2010) took a more comprehensive approach and assessed the suitability of a range of AD process parameters namely biogas production, pH, individual VFA, dissolved H_2, and gas phase CH_4 and H_2 content as potential indicators of process inhibition. A thermophilic digester treating manure was subjected to hydraulic overloading by varying the HRT and to organic overload by simulating carbohydrate, protein and lipids overload with the addition of glucose, gelatine and rapeseed oil respectively. They suggested that a combination of acetate, propionate and biogas production were good indicators of AD process balance. Nielsen *et al.* (2007) reported successfully optimising the biogas production of a laboratory scaled CSTR treating manure using propionate as an indicator of process imbalance however, Boe and Angelidaki (2012) reported quite different results when using propionate concentration to optimise biogas in a pilot-scale digester treating manure, noting that the controller could not distinguish between decreased biogas production due to decreased feed levels or process inhibition. Drosg (2013) also recommends the use of a combination of AD process parameters such as total and individual VFA, alkalinity ratio and H_2 for AD process monitoring and control. They also suggested stability limits for mesophilic one-stage CSTR digesters or the second stage of a two-stage CSTR plant (Fig. 2.4).

	Range of the parameter		Interpretation
Total VFA [mg L^{-1}]	<1,000 mg L^{-1}		Stable process
	1,000–4,000 mg L^{-1}		Range in which stable as well as unstable processes are possible. In biogas processes using feedstocks relatively hard to digest (e.g. energy crops with high TS content) where the rate limiting step is the hydrolysis step, the concentration of total VFA is normally lower than in waste digesters where the feedstock is readily degradable. Increased VFA concentrations can also be an indication of a lack of trace elements.
	>4,000 mg L^{-1}		High VFA concentrations are normally an indication of process problems, especially if VFA concentrations are increasing rapidly. Yet, also stable degradation processes are possible at higher VFA concentrations, e.g. at higher ammonia concentrations. The concentration of VFA which will lead to a decrease in pH and consequently to process problems depends on the buffer capacity and is plant specific.
Individual VFA	Acetic acid	<1,000 mg L^{-1}	Stable process
		1,000–4,000 mg L^{-1}	Stable as well as unstable processes are possible
		>4,000 mg L^{-1}	High probability of unstable process
	Propionic acid	<250 mg L^{-1}	Stable process
		250–1,000 mg L^{-1}	Stable as well as unstable processes are possible
		>1,000 mg L^{-1}	High probability of unstable process
	Longer chained VFA (butyric, valeric)	<50 mg L^{-1}	Stable process
		>50 mg L^{-1}	If longer chained VFA (and especially branched isomers) accumulate, severe process problems occur
	Ratio acetic/ propionic acid	>2	Stable process
		1-2	Stable as well as unstable processes are possible
		<1	High probability of unstable process
Alkalinity ratio (FOS/TAC)	<0.3		Alkalinity ratios below 0.3 are in general considered to indicate stable processes
	0.3–0.8		As alkalinity ratios are not comparable between different biogas plants it is very difficult to generalise. Stability limits have to be defined for every specific biogas plant. The maximum limits reported in literature for stable processes range from 0.3 to 0.8.
	>0.8		Unstable process
H_2	<100 ppm		Stable process
	100-500 ppm		In practice, it is quite difficult to guarantee accurate H_2 measurements. For this reason the range where stable as well as unstable processes are possible is assumed to be quite big. If at a biogas plant accurate H_2 measurements can be guaranteed, a smaller range of stability limits can be defined.
	>500 ppm		Unstable process

Fig. 2.4 Stability limits of parameters that provide early indication of process instability (Drosg, 2013). Parameter ranges where the AD process is considered stable are shown in green, parameter ranges where stable and unstable processes are possible are shown in yellow and parameter ranges the AD process id considered unstable are shown in red.

Changes in pH take place only after a process imbalance due to the buffering capacity of the AD process hence pH is not a good early indicator of process imbalance for manure digesters. Gas phase measurement responses such as H_2 are slow because of digester headspace effects (Boe *et al.*, 2010; Drosg, 2013). Additionally, hydrogen measurements can provide valuable information regarding increased levels of individual VFA, however H_2 measurement is difficult in practice and currently not recommended. The relevance of specific individual VFA still remains unclear and, in the case of most AD plant installations VFA determination will require the use of external laboratories (Madsen *et al.*, 2011). Delays caused by offline analyses of process parameters may lead to worsening conditions within the digester. Total VFA may increase in AD plants operating at high levels of ammonia and remain elevated although the AD process is proceeding in a stable manner. Proper monitoring and control strategies are required in order to achieve process stability at higher conversion efficiencies within AD plants treating agricultural wastes with high levels of TAN such as CS since inhibition due to ammonia, organic overload and hydraulic overload are typical causes for digester instability and AD plant process failure (Ward *et al.*, 2008; Boe *et al.*, 2010; Drosg, 2013). However AD process parameters presented in the literature give poor direct information about the cause of the process imbalance or respond too slowly to process perturbation. They do not reflect the complexity of the microbial mediated AD process steps nor the disruptions to the delicate balance between the microbes caused by substrate inhibition (Ward *et al.*, 2008; Boe *et al.*, 2010; Madsen *et al.*, 2011; Lauwers *et al.*, 2013).

Advances in high throughput next generation sequencing (NGS) and molecular fingerprinting tools, within the last 20 years have facilitated the bio-monitoring of the complex interactions between consortia of diverse micro-organisms involved in the AD process and have provided an alternative process monitoring tool (Talbot *et al.*, 2008; Horváth *et al.*, 2016). It has been observed that during anaerobic co-digestion, methanogens are more affected by VFA and ammonia concentration levels than by the addition of co-substrate while bacteria are more diverse and seem to be more affected by co-substrate (Mata-Alvarez *et al.*, 2014).

This is supported by Wang *et al.* (2009) who analysed high throughput 16S rRNA amplicon sequences from samples taken from mesophilic laboratory digesters co-digesting three different feedstocks i.e. GS, wheat straw and sugar beet tops with cow manure. They observed Clostridia and unidentified bacteria from the phylum Bacteroidetes in all three digesters however the dominant phyla in each digester were clearly different. They also reported that the digester treating GS had the highest methane yield and was dominated by *Clostridia spp*. However, no statistical certainty was provided with their assessment of the beta diversity neither was there any correlation with digester operational parameters to establish any putative causes for the GS feedstock producing more methane. The phylogenetic characterisation of the microbial community via Polymerase Chain Reaction (PCR) amplification of the 16S rRNA conserved marker gene followed by Sanger sequencing was used to conduct a meta-analysis of the microbial diversity with anaerobic digesters and found that *Firmicutes*, *Bacteroidetes* and *Chloroflexi* were the dominant phyla however, nearly 60% of all sequences could not be classified to any known genus (Nelson *et al.*, 2011). Subsequent research used 454 pyrosequencing technology to assess bacterial and archaeal diversity within full scale AD plants and correlated changes in diversity to function, AD operating conditions, differences in substrate and the dominant methanogenic pathway (Werner *et al.*, 2011; Sundberg *et al.*, 2013).

The development of molecular ecology techniques can provide more detailed information regarding the characterisation of the microbial community, community changes over time, microbial quantification and an assessment of microbial function (Werner *et al.*, 2014; Cabezas *et al.*, 2015; Campanaro *et al.*, 2016; Westerholm *et al.*, 2016). Molecular fingerprinting tools such as Denaturing Gradient Gel Electrophoresis (DGGE), and Terminal Restriction Fragment Length Polymorphism (T-RFLP) as well as techniques that quantify different groups within the AD microbial community such as Quantitative Polymerase Chain Reaction (qPCR) and Fluorescent *in-situ* Hybridisation (FISH) have led to increased research in AD process improvement.

Sun *et al.* (2013) used T-RFLP and qPCR to investigate microbial shifts that occurred when co-digesting cattle manure with two samples of wheat straw, each undergoing a different pre-treatment at mesophilic and thermophilic temperatures. They noted that cellulose degrading bacteria dominated all digesters and that the community degrading cattle manure and steam exploded wheat straw was slightly different to that of the manure only digester and community diversity increased with operating temperature but no difference was observed in digester performance and gas yield. No correlation was made with additional AD parameters such as TAN, FA and pH to explore any causal relationships.

Results obtained from high throughput sequencing data and molecular fingerprinting techniques can be correlated to AD plant performance using multivariate statistical tools to identify patterns and putative causal factors relating to AD process improvement and stability (Ramette, 2007; Cabezas *et al.*, 2015). Werner *et al.* (2011) used a times series analysis of the microbial community of AD plants and used multivariate statistical techniques on the 16S rRNA sequence data, and found that bacterial composition were correlated with operating temperature and organic loading rate as well as substrate removal efficiency and methanogenic activity. They recommended correlating community structure and function to optimise the AD process based on their findings. A similar approach was taken by subsequent research (De Francisci *et al.*, 2015; Theuerl *et al.*, 2015; Sun *et al.*, 2016; Morrison *et al.*, 2017). Wirth *et al.* (2012) characterised the microbial community of an AD Plant treating pig manure slurry and plant material using NGS technology along with protein analysis for functional profiling and suggested that microbial diversity along with H_2 partial pressure regulation appear to be the driving forces optimising biogas production in microbial communities. They reported that bacteria were much more diverse than archaea and most of the genes expressed were related to carbohydrate metabolism which was expected from a digester treating LB. No correlation of the microbial dynamics with AD parameters was undertaken, but only 11% of the sequences produced could not be phylogenetically assigned which was a significant improvement from previous work (Nelson *et al.*, 2011) that affirms the superiority of NGS sequencing over T-FRLP where 58% of the bacteria was unassigned (Theuerl *et al.*, 2015).

More recently high throughput sequencing and molecular tools have facilitated recent discoveries of the previously unknown methanogenic archaea *Verstraetearchaeota spp*. with putative fermentation capabilities (Vanwonterghem *et al.*, 2016) as well as the increased versatility of *Methanosaeta spp.* to reduce CO_2 and produce methane via DIET (Rotaru *et al.*, 2014). These works indicated how little is known regarding the archaeal diversity and dynamics within AD and the role of advanced molecular techniques in its elucidation.

Chapter 3 Assessing the effect of the anaerobic fermentation of grass silage on AD process parameters in batch mode

3.1 Introduction

The worsening global energy crisis along with regulatory drivers such as the EU Landfill and Renewable Energy Directives (EC, 2008; EC, 2009) have facilitated the installation of biogas production plants that process waste streams (Evangelisti *et al.*, 2013). Anaerobic digestion (AD) is a sustainable approach to biogas production from organic matter primarily originating from waste. The agricultural sector within the EU currently benefits from AD technology as a means of managing waste and reducing energy demand from the national grid (Van Stappen *et al.*, 2016). The efficiency of the AD process is reliant upon a well-balanced equilibrium between the four process stages (Tchobanoglous *et al.*, 1993).

Cattle slurry (CS) contains adequate amounts of micronutrients and buffering capacity to sustain microbial growth during AD process stages such as methanogenesis (Takashima and Speece, 1989; Weiland, 2010; Frear *et al.*, 2011). However, AD plants processing low C:N ratio feedstocks such as cattle slurry are prone to experience a reduction in biogas production or process failure due to ammonia inhibition (Chen *et al.*, 2008; Ward *et al.*, 2008; Rajagopal *et al.*, 2013) since free ammonia is toxic to methanogens (Chen *et al.*, 2008). The inefficiency of farm-based AD plant performance, due to a steady-state tolerance to ammonia inhibition, may result in sub-optimal profit margins (Fotidis *et al.*, 2014a). The mono-digestion of high C:N ratio, readily available lignocellulosic biomass (LB) such as grass silage and energy crops also run the risk of reduction in biogas due to volatile fatty acid (VFA) accumulation and trace element washout (Chen *et al.*, 2008; Demirel and Scherer, 2011; Wall *et al.*, 2013). Anaerobic co-digestion of farm-based manures with grass silage is preferred since it improves biogas quality and production as the combined feedstock supplies the microbial consortia with trace elements, a high carbon source and buffering capacity to stabilize pH within an optimal range for methanogens (Seppälä *et al.*, 2013; Osborne Homeky *et al.*, 2016).

Batch fermentation tests (BFTs) are used to evaluate the biogas and methane potential yield along with the kinetics of the AD process (VDI, 2006). Popular protocols for performing BFTs include the Møller *et al.* (2004), Hansen *et al.* (2004) and VDI (2006) methods compared in Pham *et al.* (2013) and the BFT method outlined by Angelidaki *et al.* (2009). Previous research uses BFTs to highlight the advantages of co-digesting of agricultural waste with biomass having higher C:N ratios and its impact on the physico-chemical parameters of the AD process (Nasir *et al.*, 2012; Rajagopal *et al.*, 2013; Risberg *et al.*, 2013). Some BFT studies utilise a three parameter, modified Gompertz model (MGM) described by Zwietering *et al.* (1990) to quantitatively evaluate BMP and degradation process kinetics (Khanal *et al.*, 2004; Wall *et al.*, 2013; Allen *et al.*, 2016). Angelidaki *et al.* (2009) indicate that anaerobic degradation of biomass can be described using a two parameter first order model which can be used to describe the hydrolysis constant (k_h) and BMP of the biomass assuming that hydrolysis is the rate-limiting factor.

This study seeks to highlight the physico-chemical and biological changes that occur during the co-digestion of CS and GS operating in batch mode in an effort to improve the process efficiency of AD plants digesting agricultural waste. Process parameters such as VFAs, pH and free ammonia are correlated with biogas and methane production in order to establish relationships that govern the AD process. The objectives of this research are to:

- Assess the performance of the mesophilic anaerobic co-digestion in batch mode of CS with two distinct levels of GS and compare it with CS only and GS only treatments;
- Correlate the physico-chemical process parameters with AD process parameters in order to evaluate reactor performance;
- Identify key parameters that can be used to improve biogas production and quality.

3.2 Experimental Design

Two batch experiments were conducted to compare the BMP of co-digesting cattle slurry (CS) with two different levels of GS. Treatments were mixed on a volatile solids (VS) basis comprising of CS:GS ratios of CS mixed with GS (80:20) and (60:40) as recommended by Wall *et al.* (2013).

The experiments (Batch 1 and Batch 2) were repeated in order to assess the reproducibility and consistency of the results. The first run of Batch Experiment 1 (B1-01) commenced on 13/3/15 and ran for 20 days while the second run (B1-02) began on 22/4/15 and ran for 18 days. Experiments were conducted under mesophilic conditions at a temperature of 37°C. The design operating temperature falls within the optimal growth range of 30°C – 45°C for most methanogenic archaea previously identified in the AD process (Ward *et al.*, 2008). It was important to achieve a stable AD process in order to assess the impact of co-digesting cattle slurry (CS) and grass silage (GS). The diverse methanogenic population creates multiple pathways for metabolite degradation which results in the likelihood of a more stable AD process. Additionally, the average long-term temperature of Antigua and Barbuda is 30°C (CLISEC, 2011). A potential mesophilic AD plant installation would likely require considerably less parasitic energy demand and will therefore more financially viable project.

Batch experiments were terminated when the mean daily biogas production volume for each treatment was less than 1% of the cumulative biogas produced at that point in time (VDI, 2006). The working volume for Batch Experiment 1 was 350ml which comprised of 250ml of inoculum and 100ml of substrate. The concentration of the feed substrate and inoculum within the batch mixture were determined in accordance with VDI (2006) which advocates:

- The ratio of feedstock VS to inoculum VS should not exceed 0.5.
- The standardisation of the course of the fermentation process whereby each batch reactor should contain 1.5% - 2% by mass of VS from the inoculum. This criterion ensures that there is sufficient microbial activity within the inoculum.
- The use of microcrystalline cellulose (MC) (Sigma-Aldrich 435236-250G) as a substrate for the reference batch to assess effectiveness of the microbial community within the inoculum to degrade biomass.
- The preparation and use of a suitable inoculum as a control. Fresh inoculum was used for each run.

Triplicate bottles were used for each of the five treatment mixtures (CS only, 80:20, 60:40, reference and zero batches); giving a total of 15 bottles used (Fig. 3.1).

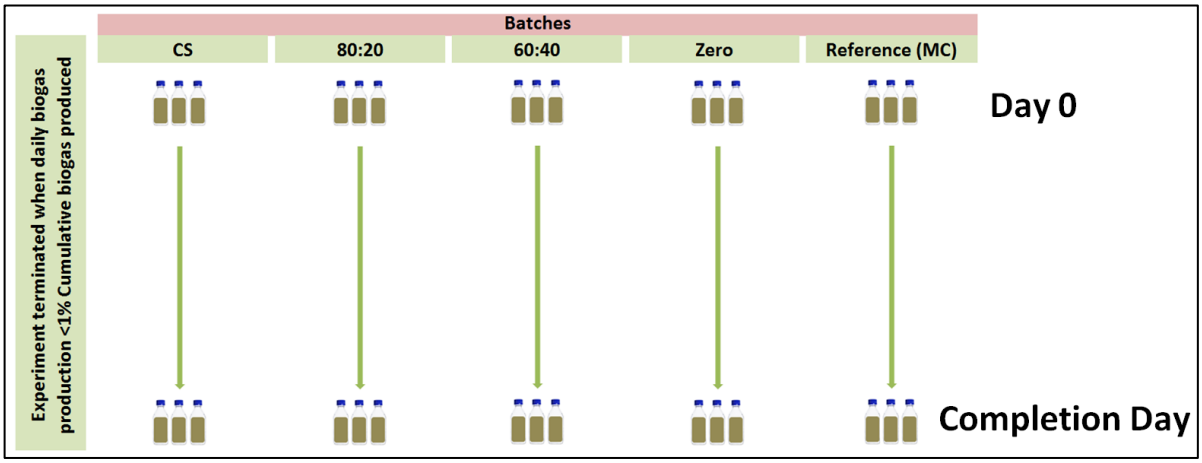

Fig. 3.1 Experimental design for Batch Experiment runs B1-01 and B1-02. MC = microcrystalline cellulose which was used as a reference substrate to assess the inoculum activity. Zero = the inoculum-only batch reactors

The first (B2-01) and second (B2-02) runs of Batch Experiment 2 commenced on the 13/9/15 and 3/11/15 respectively, and ran for 24 days under the same operating conditions as Batch Experiment 1. The design of the second batch experiment was modified to determine the BMP of five treatments (CS only, 80:20, 60:40, GS and inoculum only as a control) (Fig. 3.2).

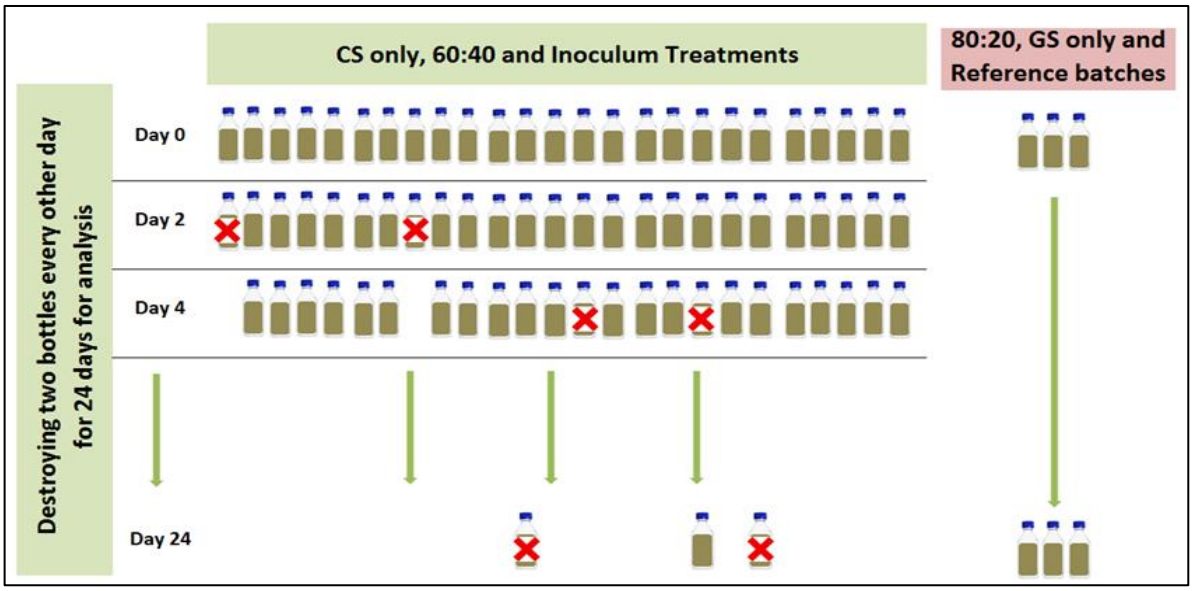

Fig. 3.2 Design for Batch Experiment runs B2-01 and B2-02. Microcrystalline cellulose was used as a reference substrate to assess the inoculum activity.

The experimental design also featured sacrificing a pair of batch bottles chosen at random containing the 60:40, cattle slurry (CS) and inoculum treatments every other day for further analysis. Effluent from each batch reactor was taken to determine Volatile Fatty Acids (VFA), Total Kjeldahl Nitrogen (TKN), Total Ammonia Nitrogen (TAN), Free Ammonia Nitrogen (FAN), Total Solids (TS), Volatile Solids (VS) and pH.

The remaining effluent from the CS only, 60:40 and inoculum only treatments was then dried in separate foil containers at 55°C in an incubator for 72 hours. Samples were then sent to an external laboratory (Sciantec Analytical Services, UK) for the determination of Acid Detergent Fibre (ADF), Acid Detergent Lignin (ADL) and Neutral Detergent Fibre (NDF) according to existing wet chemistry methods (Mertens, 2002). Triplicate bottles for the grass silage (GS) only, 80:20 and reference batch bottles were therefore maintained throughout the course of the experiment for cumulative biogas production. A total of 84 batch bottles were used for each experimental run for Batch Experiment 2. This figure comprised 25 bottles each for the CS only, 60:40 and inoculum treatments and three bottles each for the 80:20, GS only and reference batches. 500ml Duran bottles (Sigma Aldrich Z305197) were used for Batch Experiment 2 because there were an insufficient number of bottles of the type used in Batch Experiment 1. The working volume for Batch Experiment 2 was 450ml; comprising of 350ml of inoculum and 100ml of substrate of the respective treatments. The 80:20 mixture was not sampled for physico-chemical nor molecular analysis because preliminary BMP results from Batch Experiment 1 indicated that there was no significant difference between the performance of the 80:20 and the CS only treatments.

3.2.1 Primary Feedstock Collection and Storage

The primary feedstocks of CS and GS used in the batch experiments were obtained from a university owned, 75kW mesophilic on-farm AD facility at Cockle Park Farm, Newcastle upon Tyne, UK (latitude 55:15:51N; longitude 1:41:08W) which processes cattle and pig slurry. Cattle slurry, GS and inoculum samples were collected on the 8/3/15 for B1-01 and on the 11/9/15 for B2-01. Sampling from this facility were taken for the batch experiment since previous research indicated that the AD process was stable due to the abundance and diversity of bacterial and archaeal genera identified (Wade, 2012).

Grass silage was sampled 0.5m from the exposed surface of the silage clamp at Cockle Park Farm and placed in vacuum sealed bags. The GS comprised primarily of perennial rye grass, white clover and timothy, cut to 50-100 mm lengths. It was preserved with an ensiling agent (Ecosyl, Ecosyl 100) which contains a unique high performance strain of *Lactobacillus plantarum* to enhance fermentation (Ecosyl, 2018). Most of the air was removed from the bag on-site to prevent loss of volatile compounds from the sample. On returning to the laboratory, the remaining air was evacuated from the bag with a vacuum pump in preparation for storage at 4°C.

In Batch Experiment 1, the CS was collected from a manhole that provided access to the slurry coming from the dairy cattle shed at Cockle Park Farm. The CS was placed in a sealed 30L drum to limit exposure to oxygen. However, it was subsequently determined that the manhole where the CS was sampled may also contain a small amount of pig slurry (PS) since both waste streams flowed into the system the manhole accessed. Consequently, CS for both runs of Batch Experiment 2 was taken directly from the floor of the dairy stalls when the herd was brought in for milking during the afternoon. The inoculum for both batch experiments was also collected in a 32L container from the continuously stirred tank reactor (CSTR) primary digester of the AD system at Cockle Park Farm. The GS, CS primary feedstock sources and inoculum were then all stored at 4°C.

3.2.2 Apparatus Design and Assembly

The temperature of various regions of the water bath was monitored for uniformity using 10K25 thermistors (RS 250-0158) and a portable temperature controller (WILLHI model WH7016E). Batch reactors for B1-01 and B1-02 were assembled using borosilicate glass bottles and solid BS 2775 23mm bottom diameter rubber bungs (Fischer Scientific 11582922). Each bung was drilled and a 60mm long stainless steel tube with a diameter of 6mm (RS Components 437-3699) was inserted to facilitate the biogas take-off line (Fig. 3.3a). The biogas take-off system consisted of a 1L gas bag (Supelco 30221-U) attached to the stainless steel tube via a 7.5mm (outer diameter) plastic tubing with tube clip (Fischer Scientific 12376347).

Plastic sheath netting was placed around each reactor to minimise projectile travel in the event of over pressurisation within the reactor which may cause the vessel to explode.

Fig. 3.3 Assembled reactors used in Batch Experiments 1 (a) and 2 (b)

Several design changes were implemented for Batch Experiment 2. There was an insufficient quantity of reactor bottles of the type used in Batch Experiment 1 for Batch Experiment 2. Therefore to maintain uniformity of headspace eighty-four, 500ml grade 3.3 borosilicate glass Duran bottles (VWR 215-1594) were used for Experiment 2. Larger, 29mm bungs (Fischer Scientific 11512932) were also used to create an airtight seal at the lid. The most significant change however was with the biogas take-off system (Fig. 3.3b). During Experiment 1, it was observed that the flexibility and length of the 7.5 mm plastic tube caused it to droop or bend. This led to effluent from the reactor travelling up the tube into the gas bag. Therefore a small loss of VS occurred and a design change was required to eliminate or minimise this occurrence. The 250mm length of the biogas take-off tubing was also a concern since about 14-28ml of biogas between tube clip and the gas bag's inlet valve would have not been accounted for when taking daily readings. A much shorter gas take-off line was designed to eliminate any reading errors. The steel tube was replaced with the outer body of a 1ml syringe with luer lock tip (Scientific laboratory Supplies SYR6001) which provided a low cost and quick assembly solution. This facilitated the use of a three-way valve with luer lock capability to isolate the batch reactor from its gas bag. The gas bag was also changed from a 1L to a 600ml capacity (Supelco 30284-U).

3.2.3 Inoculum Preparation

The inoculum was poured into a smaller 10L container and degassed for 7-10 days prior to the commencement of each batch experiment by incubating the 10L container in a 800 X 600 X 220mm water bath (Plastor 3-221-0) regulated at 37°C using a circulating water heater (Grant model T100). The total solids (TS) and volatile solids (VS) of the GS and CS primary feedstocks along with the degassed inoculum were determined in accordance with APHA Standards (APHA, 2005) 24 hours prior to the commencement of each experimental run.

The fermentation process was standardised such that each batch reactor contained between 1.2 and 1.3% by weight of VS derived from the inoculum and the ratio of feedstock VS to seeding sludge VS was 2:1 (VDI, 2006). The working level for each batch test was chosen in order to determine the amount of treatment substrate to be added to the inoculum. The inoculum volume chosen in both B1-01 and B1-02 was 250ml. The inoculum prepared for run B1-01 was standardized to achieve 4.25g VS in 250ml (17g VS/L) or approximately 1.2% by weight of VS in each reactor bottle by adding distilled water. Based on a 2:1 VS ratio, 1.42g VS of each treatment was required to be added to triplicate batch reactors containing 250ml of inoculum. CS was therefore added to the inoculum and degassed for 12 days by incubating at 37◦C. After 12 days the TS and VS were re-evaluated and values of 32.0 ± 0.5g TS/L and of 21.4 ± 0.5g VS/L were obtained.

The inoculum was then standardized to 4.25 gVS in 250ml by adding the requisite amount of distilled water. Fig. 3.4 illustrates the variation of inoculum VS before and after standardisation for Experiments 1 and 2.

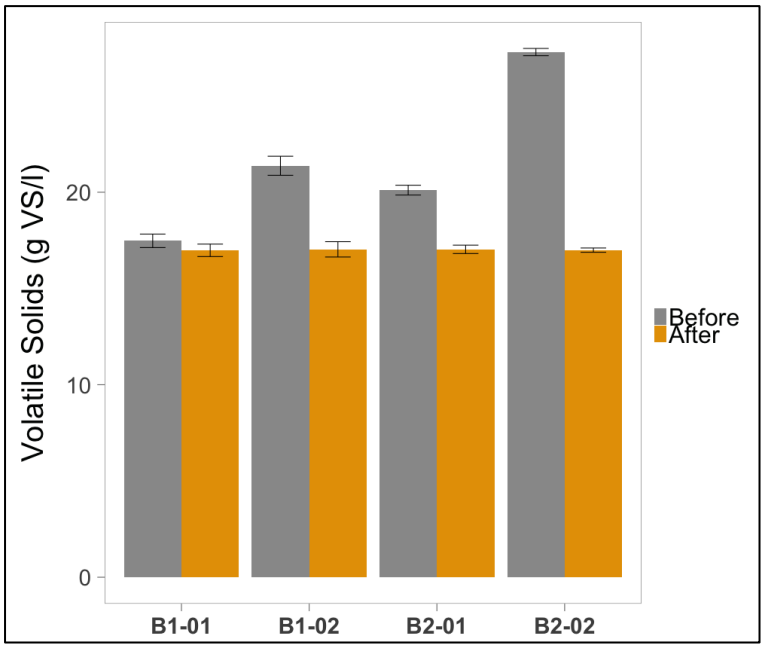

Fig. 3.4 Inoculum VS before and after standardisation. Values are expressed as means (n=3) with standard error bars.

The working level was changed to 450ml for both runs in Experiment 2 to facilitate the new reactor bottles and minimise headspace effects. The inoculum was standardised to 5.95g VS in 350ml (17g VS/L) or approximately 1.3% by weight of VS in each batch reactor. Mean inoculum VS values before standardisation for B2-01 and B2-02 were 20.1 ± 0.3g VS/L, while mean values after standardisation were 27.3 ± 0.2g VS/L and 17.0 ± 0.2g VS/L and 17.0 ± 0.1g VS/L.

3.2.4 Treatment Preparation

The CS, 80:20, and 60:40 treatments in B1-01 and B1-02 were initially prepared from the primary feedstock obtained from Cockle Park Farm to yield a total of 2.0g VS in 100ml. The CS, 80:20 and 60:40 co-digested feeds were prepared on a VS basis by combining the requisite portions of CS and GS and homogenising for three minutes using a blender (Kenwood SB054).

The VS and TS were then determined for the 60:40, 80:20, and CS feed mixture samples. Each feed mixture was then stored in a Nalgene HDPE 2L amber bottle (2009-0064) at 4°C. The CS, 80:20, and 60:40 treatments in B2-01 and B2-02 were also prepared from the primary feedstock obtained from Cockle Park Farm to provide a total of 2.5g VS in 100ml using the method previously described. The particle size for the GS treatment was reduced to less than 5mm with a pair of scissors before weighing. The mass of GS added to triplicate batch reactors was calculated by determining the wet weight of GS required to yield 2.5g VS. 100ml of distilled water was then added to bring the reactors up to the working level. Batch reactors were flushed with nitrogen gas for 10 seconds prior to incubation. The batch reactors were then incubated at 37 °C in a water bath (Fig. 3.5). Polystyrene packaging material was placed in the water bath to reduce the loss of water due to evaporation.

Fig. 3.5 Completed set up for (a) Experiment 1 and (b) Experiment 2, showing different gas take-off configurations and the use of polystyrene packing to prevent water evaporation.

Ambient conditions remained fairly constant throughout the batch experiments with the mean atmospheric pressure of 1011.1 ± 2.6 millibars and a mean temperature of 22.8 ± 0.5 °C. The contents of each reactor in both experiments were mixed on a daily basis to prevent crust formation and to facilitate the release of methane from the liquid phase into the gas bags. In runs B1-02 and B2-02 methane content was measured daily up to day 16 and then every other day until the batch test was completed.

3.2.5 Data Handling

In order to facilitate speed and reproducibility, the experimental design included a methodology of capturing and storing the raw data, recording design steps taken, formularising a method to ensure all calculation steps are visible and documented with the use of the statistical package R version 3.3.2 (R Core Team, 2013). A data handling process (Fig. 3.6) was used to create an R Markdown file whose pseudo code along with a list of the R-packages used are provided (Appendix 1). The data handling and manipulation methods were chosen to establish a fully accessible repository for the raw data, data processing and data analysis of the physico-chemical parameters and operational conditions.

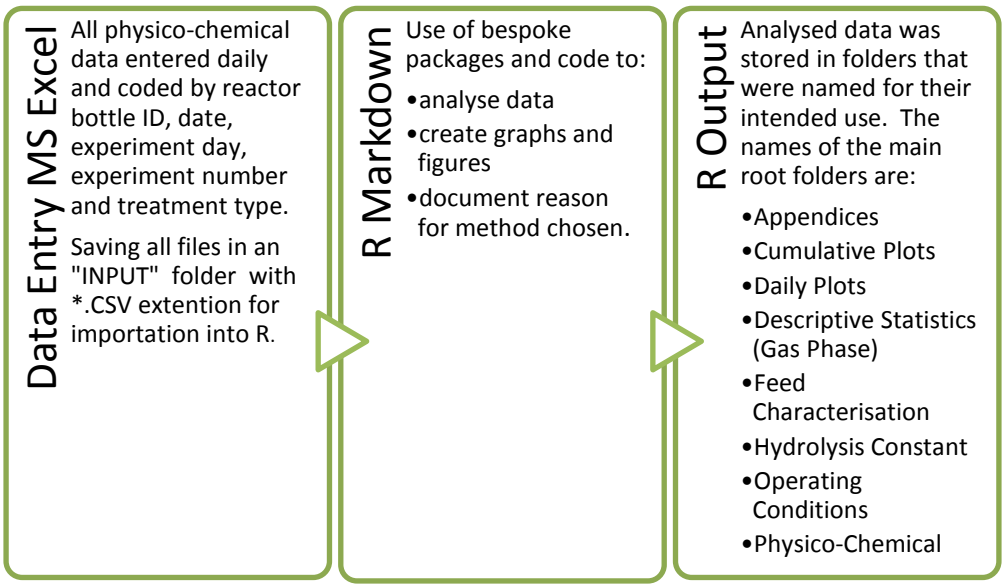

Fig. 3.6 Data handling process summary

3.3 Methods

Measurement of pH was accomplished using a Jenway model 3310 benchtop pH meter in accordance with the (APHA, 2005) standard method 4500-H^+. The meter was calibrated against standard pH 4.0 and 7.0 buffer solutions prior to use. During Experiment 2, the temperature of the liquid phase within each sacrificed reactor was measured using a temperature controller (WILLHI model WH7016E) along with a 10K25 thermistor probe (RS 250-0158).

3.3.1 Total and Volatile Solids

TS and VS of feed and effluent samples were conducted in accordance with APHA standards (APHA, 2005). For samples such as GS and CS obtained from Cockle Park Farm the TS and VS were calculated by mass.

3.3.2 Biogas Production

Biogas production (BP) was measured after methane content was measured. For B1-01 and B1-02, BP measurement involved repeat operation of the gas bag valve (Fig. 3.7). Biogas was measured daily for B1-01 and B2-01. In B1-02 and B2-02 biogas was measured daily up to day 14 and then every other day until the end of the experiment.

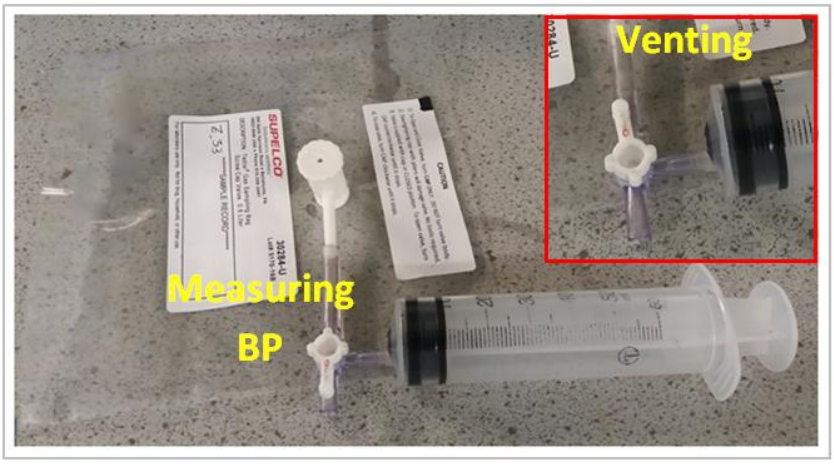

Fig. 3.7 Biogas measurement apparatus with valve positions for measuring and venting biogas.

Prior to taking measurements, the plunger was removed from the syringe body and dipped in mineral oil (Sigma Aldrich M8410-1L) to reduce errors in recording low BP volumes by minimising internal friction. Once the measurement was taken, the three-way valve was repositioned to vent the gas to the atmosphere and the process was then repeated. This reduced the frequency of operation of the gas bag inlet valve; resulting in increased longevity. The normal volume of biogas was calculated in accordance with VDI (2006) using atmospheric pressure data from Wennström (2015). Biogas volumes were reported as dry gas by correcting for water vapour content using the Goff-Gratch equation (VDI, 2006; Vömel, 2013):

$$V_{Dry} = V * \frac{(p - p_w) * T_0}{p_0 * T} \qquad \text{Eq. 3.1}$$

where:

V_{Dry} = Volume of dry gas in the normal state, (ml_N)

V = Biogas volume as measured from gas bag, (ml_N)

p= Pressure in the gas bags at the time of reading obtained from Wennström (2015), (hPa)

T_0= Normal temperature, (273 K)

T= Temperature of the biogas, K

p_w = Vapour pressure of the water as a function of the ambient temperature calculated by the Goff-Gratch equation (Vömel, 2013):

$$6.112 * e^{\left(17.62*T/(243.12+T)\right)}, \text{(hPa)} \qquad \text{Eq. 3.2}$$

p_0= Normal pressure, (1013 hPa)

e = Euler's number defined by exp(1)

Each gas bag's inlet valve was closed and removed from its respective batch reactor bottle. Biogas samples were analysed using a gas chromatograph (GC) (Carlo Erba, HRG 5160) with thermal conductivity detection in accordance with APHA standards (APHA, 2005) standard method index 2720 C. The GC column used hydrogen carrier gas and had a HP-Plot Q column (30m long, 0.32mm internal diameter, 20 micron film) installed. GC data was collected and displayed using Atlas Data Collection Software. The instrument was calibrated using a standard gas mixture having a ratio of methane: carbon dioxide of 10:90 for days 1 and 2 and either 80:20 or 70:30 for the remainder of the experiment. Equipment calibration involved setting the GC split valve to 5. Triplicate samples of 50µl biogas obtained from each bag were tested for methane content. Methane composition was also corrected for headspace effects and water vapour (VDI, 2006) which was 200 ml for bottles in Batch Experiment 1 and 150 ml in Batch Experiment 2.

The BMP for each feed mixture was determined by fitting the mean net specific cumulative methane yield to a modified Gompertz curve proposed by Zwietering *et al.* (1990):

$$f(t) = A * \exp\left(-exp\left[\frac{\mu_m * e}{A}\right] * (\lambda - t) + 1\right) \qquad \text{Eq. 3.3}$$

where:

f(t) Cumulative net specific methane production from the organic substrate, (ml_N/g VS) or (L_N /kg VS)

t = time that has elapsed since the batch began, (days)

μ_m= tangent at the point of inflection; defined as the maximum daily rate of specific methane production, (ml_N/g VS per day) or (L_N /kg VS per day)

A = BMP is described by the asymptote reached when the growth rate reaches zero (ml_N dry gas/g VS) or (L_N dry gas/kg VS)

λ = x-axis intercept of the tangent formed by μ_m and defines the time that elapses prior to microbial growth, (days)

e = Euler's number defined by exp(1)

The three parameters μ_m, A and λ were first estimated using the MATLAB® curve fitting toolbox (MathWorks, 2002). Parameter estimates were then used as starting values for the Non-Linear Regression NLS Toolbox in R to obtain a nonlinear regression fit of the modified Gompertz model with the daily net cumulative methane production yields from each reactor bottle (Eq. 3.4) (Baty *et al.*, 2015).

$$y = f(\theta, x) + \epsilon, \epsilon \sim N(\delta^2, 0) \quad \text{Eq. 3.4}$$

where:

y = response variable i.e. mean net cumulative methane yield, in (L_N /kg VS of treatment)

x = elapsed time since experiment began, (days)

θ = A vector of model parameters- μ_m, A and λ) that characterises the relationship between x and y.

ϵ = residual error term that is assumed to be normally distributed with a standard deviation of δ and centred about 0.

3.3.3 Volatile Fatty Acids

Duplicate samples were taken from each bottle and poured into 2ml micro-centrifuge tubes (VWR 211-0034). Tubes were then centrifuged at 13000 rpm for 15 minutes (MSE Micro Centaur, MSB010. CX1.1). The supernatant of each sample was extracted with a 1ml syringe and filtered with a 0.2µm filter (VWR 514-0072); placing the filtrate into a clean 2ml Eppendorf tube. 0.4ml of filtrate was placed in another clean 2ml tube along with 0.4ml of octanesulphonic acid (ThermoFisher Scientific,035362) in accordance with Manning and Bewsher (1997). The samples were then sonicated for 40 minutes. The samples were centrifuged at 13000 rpm for 15 minutes to pellet any suspended precipitate.

Duplicate samples of filtrate were then prepared for analysis by the Dionex ICS 1000 ion chromatograph having the following features:

- Column: Ionpac HPICE AS1 (250 X 4mm)
- Guard column: OnGuard-P
- Conductivity Detector
- Injector: Automatic loop filling with flow rate of 0.16ml/min
- Suppressor: Dionex AMMS ICE II with 5mM tetrabutylammonium hydroxide cation regenerant
- Mobile phase: 1.0 mM heptafluorobutyric acid.

The Ion Chromatograph was calibrated with commercially sourced standards of Formic, Acetic, Propionic, Isobutyric, Butyric, Isovaleric, and Valeric acids; each diluted with deionised water (18MΩ-cm) to concentration levels of 5, 50, 100 and 500ppm. Deionised water (18MΩ-cm) was used as a blank.

3.3.4 Elemental Analysis

At the end of Batch Experiment 2, run 2 (B2-02), CS and GS were prepared for elemental analysis (EA) by drying the wet samples at 55°C for 48 hours (Combs *et al.*, 2003). Dried samples were then passed through a 1mm sieve. Duplicate replicates of 2g were then weighed and sent for analysis to an external laboratory (Elemental Microanalysis Ltd, UK). The theoretical methane production of the CS and GS samples was determined using the stoichiometric equation containing each element (C, H, O, N and S) originally proposed by Buswell and Mueller (1952) but subsequently adapted to include N and S (Boyle, 1977).

Theoretical methane production (BMP_{Th}) was calculated using Buswell's equation that was subsequently modified (Boyle, 1977):

$$C_a H_b O_c N_d S_e + \left(a - \frac{b}{4} - \frac{c}{2} + \frac{3d}{4} + \frac{e}{2}\right) H_2O \rightarrow \left(\frac{a}{2} + \frac{b}{8} - \frac{c}{4} - \frac{3d}{8} - \frac{e}{4}\right) CH_4$$

$$+ \left(\frac{a}{2} - \frac{b}{8} + \frac{c}{4} + \frac{3d}{8} + \frac{e}{4}\right) CO_2 + dNH_3 + eH_2S \qquad \text{Eq. 3.5}$$

Elemental analysis of microcrystalline cellulose and mean values for CHON and S as percentage TS are listed in Table 3.1

Table 3.1 Elemental analysis (EA) of microcrystalline cellulose (MC).

% TS Composition					Source
C	**H**	**N**	**O**	**S**	
43.37	6.11	0.48	49.81	nd[b]	Li *et al.* (2015)
44.4	5.8	0.3	49.3	0.1	Couhert *et al.* (2009)
44.7	6.31	0.19	48.76	nd	Venkatakrishnan *et al.* (2015)
43.52 (0.21)[a]	6.30 (0.11)[a]	<0.05 (0.001)[a]	49.3 (0.73)[a]	nd	Current study

[a]Values are expressed as means (± standard error), n=2.
[b]nd = Not Detected.

Mean values of each element were then used to calculate the theoretical value of MC. The C, H, O, N, S composition of the 80:20 and 60:40 treatments in both runs of Experiment 2 were estimated based on elemental analysis of the CS only and GS only samples and their proportions within the co-digested treatments.

Elemental analysis results were measured on a TS basis, however the co-digested mixtures were blended using a VS basis. Consequently, Eq. 3.6 was formulated to estimate the elemental composition (E) for each blend on a TS basis:

$$E_{a:b} = \frac{\left[\left(\frac{a}{a+b}\right) * E_{cs} * TS_{cs} * VS_{cs}\right] + \left[\left(\frac{b}{a+b}\right) * E_{gs} * TS_{gs} * VS_{gs}\right]}{VS_{a:b}} \qquad \text{Eq. 3.6}$$

where:

a = composition of CS in treatment blend, (%)

b = composition of GS in treatment blend, (%)

$E_{cs} =$ element's percentage composition in CS, (%TS)

$TS_{cs} =$ TS of CS treatment, (g/L)

$VS_{cs} =$ VS of CS treatment, (%TS)

$E_{gs} =$ element's percentage composition in GS, (%TS)

$TS_{gs} =$ TS of GS treatment, (g/L)

$VS_{gs} =$ VS of GS treatment, (%TS)

$VS_{a:b} =$ VS of co-digested mixture, (g/L)

Moles of methane produced were then converted to volume at standard temperature and pressure (STP) using the Ideal Gas Law.

The biodegradability (BD) of each treatment was reported by calculating the ratio of BMP determined experimentally to its respective theoretical value (Triolo *et al.*, 2011) (Eq. 3.7):

$$BD = \frac{BMP_{EX}}{BMP_{\mathrm{TH}}} * 100 \qquad \text{Eq. 3.7}$$

where:

BD = biodegradability of each substrate, (%)

BMP_{EX} = Experimentally derived BMP

BMP_{TH}= Theoretical BMP

The percentage relative error between BMP values obtained via experiments and their respective adjusted theoretical BMP values was calculated using Eq. 3.8:

$$\Delta \text{ error} = \frac{(BMP_{\mathrm{EX}} - BMP_{THBD}) * 100}{BMP_{\mathrm{EX}}} \qquad \text{Eq. 3.8}$$

3.3.5 First Order Hydrolysis Constant

The first order hydrolysis constant (k_h) was calculated using the following formula (Angelidaki *et al.*, 2009):

$$ln\left(\frac{B_\infty - B}{B_\infty}\right) = -k_h * t \qquad \text{Eq. 3.9}$$

where:

t = time that has elapsed since the batch began, (days).

B_∞ = ultimate methane production which is equivalent to the asymptote reached when the growth rate is zero (BMP), (ml_N dry gas/g VS) or (L_N dry gas/kg VS)

B = cumulative methane production at time =t; (ml_N dry gas/g VS) or (L_N dry gas/kg VS)

k_h = first order hydrolysis constant, (day^{-1})

3.3.6 Total Kjeldahl Nitrogen and Total Ammonia Nitrogen

Samples for total Kjeldahl nitrogen (TKN) and total ammonia nitrogen (TAN) were acidified to pH <2 with concentrated H_2SO_4 and stored at a temperature of 4°C prior to analysis. Analysis was conducted in accordance with APHA standards (APHA, 2005). A standard of 20mg/L TKN was prepared by adding 0.0429g of anhydrous urea (CH_4N_2O) to 1L of deionised water and a standard of 20mg/L NH_3-N was prepared by adding 0.0628g of anhydrous Ammonium Chloride (NH_4Cl) to 1L of deionised water.

Free ammonia nitrogen (FAN) was calculated from TAN data along with the pH and temperature data using the formula stated in Koch *et al.* (2010):

$$FAN = \frac{TAN}{1 + 10^{(pK_a - pH)}} \quad \text{Eq. 3.10}$$

where:

TAN = total ammonia nitrogen (NH_3-N), (mg/L)

pK_a = dissociation constant of the Ammonium ion, and is given by the formula:

$$pK_a = 0.09018 + \frac{2729.92}{T + 273.15} \text{ (Eq. 3.11) where}$$

T = temperature of the sample at the time the sample was taken, (°C).

pH = pH of the sample at the time the sample was taken

3.3.7 Chemical Oxygen Demand

Samples were acidified to a pH <2 with concentrated H_2SO_4 and stored at a temperature of 4°C prior to analysis. The closed reflux method (APHA, 2005) was used to measure chemical oxygen demand (COD). A standard of 500mg O_2/L was prepared by adding 0.0425g of anhydrous potassium hydrogen phthalate ($C_8H_5KO_4$) to 1L of deionised water.

3.3.8 Cellulose, hemicellulose and lignin determination

At the end of Batch Experiment 2 run 2 (B2-02) cattle slurry (CS) and grass silage (GS) samples taken from the primary feedstock, along with samples taken from the batch reactors that were sacrificed every 2 days throughout Batch Experiment 2 were dried separately in foil containers at 55°C for 72 hours. Dried samples were then passed through a 1mm^2 sieve and placed in sealed containers.

Aliquots of samples from days 2, 6, 14 and 24 for the CS, 60:40 and zero batches along with samples of CS and GS were sent to an external laboratory (Sciantec Analytical, UK) for the determination of dry matter percentage composition of acid detergent fibre (ADF) acid detergent lignin (ADL) and neutral detergent fibre (NDF).

The NDF, ADF, and ADL results were reported on a percent TS basis, however the co-digested treatments were blended using a VS basis. Eq. 3.6 was modified and used to estimate the NDF, ADF and ADL content of the 80:20 and 60:40 co-digested blend on a TS basis. It was assumed that the CS and GS sampled for NDF, ADF and ADL analysis came from a feedstock of uniform concentration and that changes in the ratio of NDF:ADF:ADL content of the primary feedstock due to storage was negligible. Cellulose, hemicellulose and lignin concentration were determined using the following formulae (Koch *et al.*, 2010):

$$Cellulose = ADF - ADL \qquad \text{Eq. 3.12}$$

$$Hemicellulose = NDF - ADF \qquad \text{Eq. 3.13}$$

$$Lignin = ADL \qquad \text{Eq. 3.14}$$

3.3.9 Raw Protein and Lipids

Raw protein (RP) was calculated from the nitrogen composition (N) in the dry matter from samples sent for elemental analysis. RP measured in percent TS was calculated by multiplying N% by 6.25 (Van Os *et al.*, 1995). Raw lipids (RL) were determined with Soxhlet apparatus in accordance with the ether extract method (EE, AOAC official Method 920.39). Samples were dried at 55°C for 96 hours and then crushed and passed through a 1mm sieve. All thimbles (Fisher Scientific 11734043) and round bottom flasks used were dried at 105°C for 24 hours prior to the experiment and later stored in a desiccator. Their weights were then recorded using a 4 figure balance after they had cooled to room temperature. Aliquots of each treatment weighing approximately 1.5g were then measured out in triplicate and placed into each thimble and the weight was taken.

Each thimble was then capped with cotton wool and placed in the Soxhlet apparatus where lipids were extracted with petroleum ether (40-60◦C) (Fisher Scientific, 10568060) for approximately 6 hours. Afterwards, round bottom flasks containing the extracts were placed in a drying oven at 105°C for 24 hours. They were subsequently placed in a desiccator for cooling and then weighed. RL measured in percent TS was determined using the following equation:

$$RL = \frac{(W_{FE} - W_F) * 100}{(W_{ThS} - W_{Th})} \quad \text{Eq. 3.15}$$

where:

W_{FE} = weight of flask with extract, (g)

W_F = weight of flask, (g)

$W_{ThS} =$ weight of thimble and sample, (g)

$W_{Th} =$ weight of thimble, (g)

3.3.10 Structural and Non-Structural Carbohydrates

Volatile solids (VS) for each feedstock can be disaggregated into raw lipids (RL), raw proteins (RP) and carbohydrates (Koch *et al.*, 2010). Therefore carbohydrate content within each feedstock was determined by subtracting the sum of RP and RL from the total VS content (Eq. 3.16) and was reported on a %TS basis:

$$Carbohydrates\ (\%TS) = 100\% - (RP + RL) \quad \text{Eq. 3.16}$$

where:

RP = Raw protein content, (%TS)

RL = Raw lipids, (%TS)

3.3.11 Statistical Analyses

All statistical analysis was done in R version 3.3.2 (R Core Team, 2013) with the packages described in Appendix 1. Data were evaluated using Analysis of Variance (ANOVA) along with appropriate posthoc analyses, t-tests were reported with bonferroni corrected p values using a significance level (α) of 0.05. Model assumptions were validated by visually assessing graphical depictions of normality and autocorrelation of the residuals along with the standard normality tests (Field, 2012; Baty *et al.*, 2015) (Appendix 2). Repeated Measures ANOVA with type III error sums were conducted using a linear mixed model (LME) fit by maximum likelihood developed by Bates *et al.* (2015). Linear mixed models were used to investigate the effect of time and type of treatment on the physico-chemical parameters and interaction effects were examined with the "Effects" R package (Fox, 2003).

3.4 Results

3.4.1 Characterisation of Feed and Inoculum

Total solids and VS of each treatment in Batch Experiment 1 shows that feeds with similar TS and VS values were used (Table 3.2).

Table 3.2 TS and VS of feedstocks used in Batch Experiment 1. Values are expressed as means (n=16) with standard error

Parameter	Run	Units	CS	60:40	80:20	GS	Inoculum
Total Solids (TS)	**B1-01**	**(%WW)**	2.7 ± 0	2.3 ± 0	2.5 ± 0	24.0 ± 0	2.4 ± 0.1
Volatile Solids (VS)	**B1-01**	**(%TS)**	81.2 ± 0.11	84.4 ± 0.41	82.4 ± 0.12	92.4 ± 0	71.5 ± 3.33
Total Solids (TS)	**B1-02**	**(%WW)**	1.4 ± 0.13	1.5 ± 0.09	1.4 ± 0.10	21.0 ± 0	3.2 ± 0.14
Volatile Solids (VS)	**B1-02**	**(%TS)**	79.1 ± 0.84	85.7 ± 0.19	81.6 ± 0.65	89.2 ± 0	66.8 ± 0.58

The characterisation of treatments and inoculum for Batch Experiments 1 and 2 into raw proteins, raw lipids, carbohydrates and lignocellulose fractions according to extended Weender analysis (Koch *et al.*, 2010) along with additional parameters is detailed in Table 3.3 and Table 3.4.

Table 3.3 Treatment characterisation for Experiment run B2-01. Values are expressed as means (n=16) with standard error.

Parameter	Units	CS	60:40	80:20	GS	Inoculum
Total Solids (TS)	**(%WW)**	3.2 ± 0	3.1 ± 0	3.1 ± 0	16.1 ± 0	2.9 ± 0
Volatile Solids (VS)	**(%TS)**	82.4 ± 0.1	84.7 ± 0	83.6 ± 0.1	91.7 ± 0.9	70.7 ± 0.3
Raw Protein (RP)	**(%TS)**	18.9 ± 0.1	19.7 ± 0.2	19.1 ± 0.2	19.3 ± 0.2	NR
Raw Lipids (RL)	**(%TS)**	3.3 ± 0.2	4.2 ± 0.4	3.8 ± 0.2	5 ± 0	NR
Carbohydrates	**(%TS)**	60.2 ± 0.2	60.8 ± 0.4	60.7 ± 0.3	67.4 ± 0.9	NR
Cellulose	**(%TS)**	18.6 ± 0.1	22.9 ± 0.5*	20.6 ± 0.6	27.4 ± 0.4	NR
Hemicellulose	**(%TS)**	15.6 ± 0.3	18.5 ± 1*	16.9 ± 1.1	21.5 ± 0.8	NR
Lignin	**(%TS)**	13.7 ± 0.1	10.3 ± 0.2*	11.9 ± 0.2	4.4 ± 0.1	NR
Carbon	**(%TS)**	41.2 ± 0.1	43.5 ± 0.5	42.0 ± 0.5	43.6 ± 0.1	NR
Hydrogen	**(%TS)**	5.7 ± 0	6.0 ± 0.1	5.8 ± 0.1	6.1 ± 0	NR
Nitrogen	**(%TS)**	3.02 ± 0.02	3.15 ± 0.04	3.06 ± 0.04	3.09 ± 0.03	NR
Oxygen	**(%TS)**	31.4 ± 0.1	33.8 ± 0.4	32.4 ± 0.4	34.8 ± 0.3	NR
Sulphur	**(%TS)**	0.49 ± 0.01	0.42 ± 0.01	0.45 ± 0.01	0.29 ± 0.01	NR
C/N ratio		18.2 ± 0.3	20.3 ± 0.3	19.7 ± 0.3	34.9 ± 1.6	NR
COD	**g/L O_2**	20.6 ± 0.50	21.7 ± 0.20	NR[a]	NR	NR
TKN	**g/L TKN**	2.5 ± 0.10	2.2 ± 0.10	NR	NR	2.2 ± 0.04
TAN	**g/L NH_3**	1.5 ± 0.11	1.5± 0.10	NR	NR	1.5± 0.02
Formic	**ppm**	2.0 ± 0.10	0.5 ± 0.02	NR	NR	0.7 ± 0.02
Acetic	**ppm**	292.7 ± 0.51	473.0 ± 0.02	NR	NR	17.8 ± 0.38
Propionic	**ppm**	nd[b]	nd	NR	NR	nd
Butyric	**ppm**	nd[b]	NR	NR	NR	nd
Isobutyric	**ppm**	1991.1 ± 1.77	1867.0 ± 0.16	NR	NR	2077.7 ± 0.09
Valeric	**ppm**	nd	nd	NR	NR	nd
Isovaleric	**ppm**	nd	nd	NR	NR	nd

[a] NR = Not Reported. Samples were not analysed due to equipment failure.

[b] nd = Not Detected

Table 3.4 Treatment characterisation for Experiment run B2-02. Values are expressed as means (n=16) with standard error.

Parameter	Units	CS	60:40	80:20	GS	Inoculum
Total Solids (TS)	**(%WW)**	3.0 ± 0	2.8 ± 0	3.1 ± 0	23.4 ± 0.1	3.6 ± 0
Volatile Solids (VS)	**(%TS)**	84.5 ± 0.2	87.0 ± 0.3	85.8 ± 0.2	83.6 ± 0.5	75.4 ± 0.3
Raw Protein (RP)	**(%TS)**	18.9 ± 0.1	18.9 ± 0.2	19 ± 0.2	19.3 ± 0.2	nd
Raw Lipids (RL)	**(%TS)**	3.3 ± 0.2	4.2 ± 0.4	3.8 ± 0.2	5 ± 0	nd
Carbohydrates	**(%TS)**	62.3 ± 0.3	63.9 ± 0.5	63.0 ± 0.3	59.3 ± 0.5	nd
Cellulose	**(%TS)**	18.6 ± 0.2	22 ± 0.5	20.4 ± 0.4	27.4 ± 0.3	nd
Hemicellulose	**(%TS)**	15.6 ± 0.3	17.8 ± 1	16.8 ± 0.9	21.5 ± 0.5	nd
Lignin	**(%TS)**	13.7 ± 0.1	9.9 ± 0.2	11.8 ± 0.2	4.4 ± 0	nd
Carbon	**(%TS)**	41.2 ± 0.1	41.9 ± 0.5	41.7 ± 0.4	43.6 ± 0.1	nd
Hydrogen	**(%TS)**	5.7 ± 0	5.8 ± 0.1	5.8 ± 0.1	6.1 ± 0	nd
Nitrogen	**(%TS)**	3.02 ± 0.02	3.03 ± 0.04	3.04 ± 0.03	3.09 ± 0.03	nd
Oxygen	**(%TS)**	31.4 ± 0.1	32.5 ± 0.4	32.1 ± 0.3	34.8 ± 0.3	nd
Sulphur	**(%TS)**	0.49 ± 0.01	0.41 ± 0.01	0.45 ± 0.01	0.29 ± 0.01	nd
C/N ratio		18.2 ± 0.3	20.3 ± 0.3	19.7 ± 0.3	34.9 ± 1.6	nd
COD	**g/L O_2**	27.4 ± 1.39	27.0± 1.78	NR	NR	nd
TKN	**g/L TKN**	1.4 ± 0.10	1.7 ± 0.31	NR	NR	1.3 ± 0.06
TAN	**g/L NH_3**	0.8 ± 0.01	0.8 ± 0.02	NR	NR	0.8 ± 0.04
Formic	**ppm**	3.6 ± 0	NR[a]	NR	NR	486.75 ± 0.71
Acetic	**ppm**	206.5 ± 0	NR	NR	NR	714.5 ± 0.11
Propionic	**ppm**	340.5 ± 20.00	NR	NR	NR	3586.3 ± 0.22
Butyric	**ppm**	nd[b]	NR	NR	NR	nd
Isobutyric	**ppm**	771.5± 17.88	NR	NR	NR	2168.81 ± 0.7
Valeric	**ppm**	nd	NR	NR	NR	nd
Isovaleric	**ppm**	nd	NR	NR	NR	nd

[a] NR = Not Reported. Samples were not analysed due to equipment failure.
[b] nd = Not Detected

3.4.2 Theoretical Determination of BMP

Buswell's equation (VDI, 2006; Nielfa *et al.*, 2015) was used to derive the molecular mass of each treatment and their theoretical methane yield adjusted for the presence of ammonia and sulphur. A theoretical specific biochemical methane potential (BMP_{TH}) of 408.1 L_N CH_4/kg VS was obtained for microcrystalline cellulose; using the elemental analysis (EA) data from the laboratory report (Table 3.5). The theoretical BMP value was marginally lower than the 415L_N CH_4/kg VS found in Wall *et al.* (2013), Sambusiti *et al.* (2014) and Wang *et al.* (2014).

However theoretical BMP does not account for elemental loss due to microbial community growth. The higher of the two theoretical BMP values calculated for each treatment was used to calculate the biodegradation (BD) index.

Table 3.5 Theoretical specific methane production as determined from Elemental Analysis

Run	Treatment	Initial Feed VS (g)	Buswell Parameters					Theoretical CH_4	
			a	b	c	d	e	CH_4 ml	BMP_{TH} CH_4 L_N/kg VS
B2-01	CS	1.4920	0.0622	0.1027	0.0355	0.0039	0.0003	0.7503	**502.9**
	60:40	1.4443	0.0618	0.1029	0.0361	0.0038	0.0002	0.7452	**515.9**
	80:20	1.4782	0.0619	0.1027	0.0358	0.0039	0.0003	0.7471	**505.4**
	GS	1.4876	0.0589	0.0990	0.0353	0.0036	0.0001	0.7082	**476.1**
B2-02	CS	1.4797	0.0601	0.0994	0.0344	0.0038	0.0003	0.7259	**490.6**
	60:40	1.4931	0.0614	0.1022	0.0358	0.0038	0.0002	0.7401	**495.7**
	80:20	1.4815	0.0607	0.1008	0.0351	0.0038	0.0002	0.7325	**494.4**
	GS	1.5790	0.0629	0.1058	0.0377	0.0038	0.0002	0.7565	**479.1**
	MC	n/a	0.0392	0.0639	0.0328	2E-04	3E-05	n/a	**408.1**

Organic fraction composition (OFC) analysis was another method used to calculate the theoretical specific methane production of each treatment. Table 3.6 below outlines the specific biogas production that can be expected from the organic fractions of carbohydrates, proteins and fats (lipids).

Table 3.6 Theoretical specific biogas yield and gas composition of OFC (VDI, 2006)

Organic Fraction	Theoretical Specific Biogas yield (L_N/kg VS)	Theoretical CH_4 and CO_2 composition (% by volume)	
Carbohydrate	750	50% CH_4	50% CO_2
Fat	1390	72% CH_4	28% CO_2
Protein	800	60% CH_4	40% CO_2

The theoretical calculation once again assumed that 100% of each organic fraction VS was converted to methane and does not take into account nutrient requirements to increase the microbial population nor the non-degradability of recalcitrant biomass within the treatments.

Table 3.7 Theoretical specific methane production as determined from OFC analysis

Run	Treatment	Initial Feed VS (g)	Raw Protein (g VS)	Raw Lipids (g VS)	Carbohydrates (g VS)	CH_4 (ml_N)	BMP_{TH} L_N/kg VS
B2-01	CS	1.4920	0.2819	0.0487	1.1613	619.6	415.3
	60:40	1.4443	0.2843	0.0599	1.1001	609.0	421.6
	80:20	1.4782	0.2830	0.0556	1.1396	618.8	418.6
	GS	1.4876	0.2873	0.0748	1.1256	634.8	426.7
B2-02	CS	1.4797	0.2796	0.0483	1.1518	614.5	415.3
	60:40	1.4931	0.2897	0.0620	1.1414	629.1	421.3
	80:20	1.4815	0.2847	0.0557	1.1410	620.3	418.7
	GS	1.5790	0.3050	0.0794	1.1947	673.8	426.7

3.4.3 Experimental Determination of BMP

BMP values for the treatments in Experiments 1 and 2 were calculated by fitting the modified Gompertz model (MGM) described by Eq. 3.3 to the experimental data depicting the mean specific net cumulative daily dry methane production (Table 3.8). BMP values obtained for microcrystalline cellulose were 412.1 ± 5.9 and 383.9 ± 8.8 L_N CH_4/kg VS for B1-01 and B1-02 respectively and 408.0 ± 1.9 and 370.6 ± 4.9 L_N CH_4/kg VS for runs B2-01 and B2-02 respectively. The biodegradability of microcrystalline cellulose for all 4 runs ranged from 89.3% to 99.3%; using the higher theoretical BMP value of 415 L_N CH_4/kg VS in Eq. 3.7 which infers the presence of an active microbial community.

Table 3.8 Summary of the modified Gompertz Model parameter estimates.

Run	Treatment	Gompertz Parameter			Goodness of Fit Parameters		
		A L_N CH_4/kg VS	μ_m L_N CH_4/kgVS*d	λ (days)	Residual Standard Error (RSE)	Degrees of Freedom (DF)	Residual Sum of Squares (RSS)
B1-01	60:40	289.9 ± 4.9 [a]	28.7 ± 1.2 [a]	1.2 ± 0.2 [a]	7.3	15	800
	80:20	249.5 ± 4.2 [b]	27.4 ± 1.3 [a]	1.2 ± 0.2 [a]	7.2	16	840
	CS	250.6 ± 5.2 [b]	25.5 ± 1.3 [a]	1.4 ± 0.2 [a]	8	16	1020
B1-02	60:40	381.7 ± 3.4 [a]	33.9 ± 0.4 [a]	3.0 ± 0.1 [a]	3	16	150
	80:20	327.0 ± 8.8 [b]	23.8 ± 0.6 [c]	3.0 ± 0.2 [a]	5	16	410
	CS	247.8 ± 6.8 [c]	21.7 ± 0.8 [c]	3.8 ± 0.2 [b]	5	16	400
B2-01	60:40	322.6 ± 4.7 [a]	34.9 ± 2.1 [a]	0.3 ± 0.3 [a]	13.2	22	3830
	80:20	307.2 ± 3.5 [b]	36.9 ± 1.9 [ab]	0.5 ± 0.2 [a]	10.4	22	2390
	CS	300 ± 4.5 [b]	29.1 ± 1.6 [a]	0.2 ± 0.3 [a]	11.2	22	2770
	GS	409.0 ± 4.5 [c]	47.0 ± 2.3 [c]	0.6 ± 0.2 [a]	13.1	22	3770
B2-02	60:40	371 ± 3.7 [a]	45.1 ± 1.6 [a]	1.8 ± 0.1 [a]	8	16	1010
	80:20	300.4 ± 4.1 [b]	41.6 ± 2.3 [ab]	1.3 ± 0.2 [a]	9.9	16	1560
	CS	222.9 ± 3.7 [c]	33.6 ± 2.4 [b]	1.2 ± 0.2 [a]	9.2	16	1360
	GS	410.7 ± 6.5 [d]	73.6 ± 5.8 [c]	2.3 ± 0.2 [a]	17.1	16	4700

[a,b,c] Means for each MGM parameter with different superscript letters are significantly different ($p<0.05$).

Validation of the MGM fits were based on an assessment of the goodness of fit parameters such as RSE and SSE values in Table 3.8 along with autocorrelation and normality plots of the residuals for each fit (Appendix 2). A comparison of the EA and OFC methods of determining theoretical BMP with the experimental results from Batch Experiment 2 indicate that the OFC method reported lower errors and was therefore more representative of the experimental values obtained (Table 3.9).

Table 3.9 Comparison of biodegradability indices using the EA and OFC methods for theoretical BMP estimates

Run	Treatment	Experiment BMP Estimate CH_{4TH} L_N/kg VS	EA Method			OFC Method		
			Theoretical BMP CH_{4TH} L_N/kg VS	BD_{EA} (%)	Δ $Error_{EA}$ (%)	Theoretical BMP CH_{4TH} L_N/kg VS	BD_{OFC} (%)	Δ $Error_{OFC}$ (%)
B2-01	**60:40**	322.6	515.9	63	37	421.6	77	23
	80:20	307.2	505.4	61	39	418.6	73	27
	CS	300	502.9	60	40	415.3	72	28
	GS	409	476.1	86	14	426.7	96	4
B2-02	**60:40**	371	495.7	75	25	421.3	88	12
	80:20	300.4	494.4	61	39	418.7	72	28
	CS	222.9	490.6	45	55	415.3	54	46
	GS	410.7	479.1	86	14	426.7	96	4

Energy requirements to grow and maintain the microbial community as well as the existence of non-degradable, recalcitrant fractions of each treatment are possible sources of relative error. GS was the most digestible treatment for runs B2-01 and B2-02 since the biodegradability index (BD) was 86% using the elemental analysis (EA) method and 96% using the OFC method; resulting in GS reporting the lowest relative error among all treatments. CS was the least digestible in both experimental runs with values of 60% and 45% with the EA method and 72% and 54% with the OFC method. The MGM fits representing the cumulative net specific CH_4 production for each treatment from Experiments 1 and 2 are shown in Fig. 3.8.

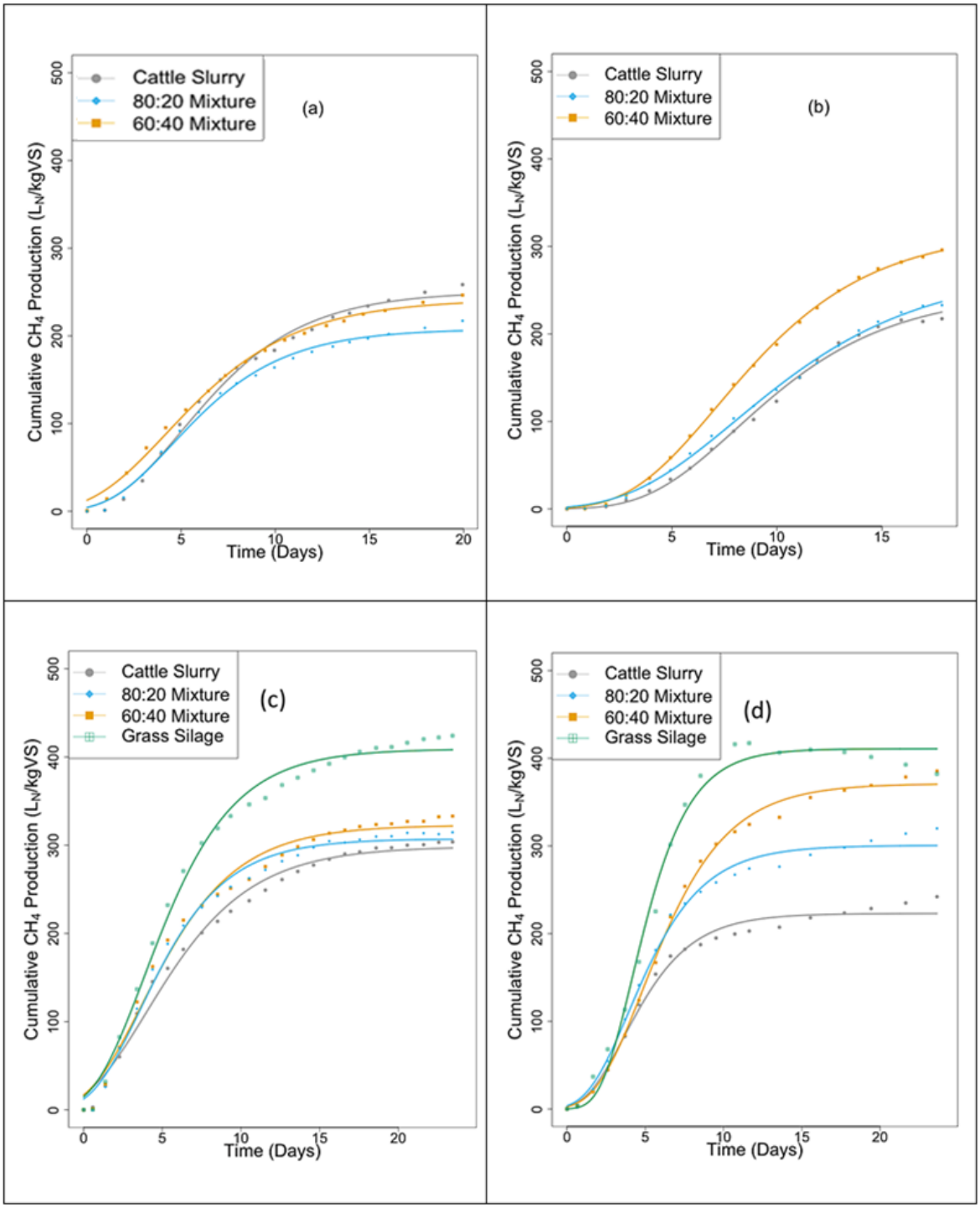

Fig. 3.8 Fitted modified Gompertz plots for Batch Experiment (a) B1-01, (b) B1-02, (c) B2-01(c) and (d) B2-02. Symbols represent mean of experimental data.

The CS treatment produced significantly less net specific cumulative methane ($\bar{x}$ = 119.4 ± 1.65) than the 60:40 ($\bar{x}$ = 132.8 ± 1.65, $p < 0.001$) and 80:20 ($\bar{x}$ = 129.0 ± 1.65, $p < 0.001$) treatments on day 6 in B1-01.

A similar trend was observed on day 10. At the end of the experimental run however, the specific cumulative methane production of the CS and 80:20 treatments were not significantly different. The 60:40 treatment had a significantly higher specific methane production compared to the CS treatment ($p < 0.001$). The trend on day 6 and in B1-02 was similar to that of B1-01 with the 60:40 ($\bar{x} = 95.8 \pm 1.8$, $p < 0.001$) and 80:20 ($\bar{x} = 72.3 \pm 1.8$, $p < 0.001$) co-digested treatments producing significantly higher net specific cumulative methane production than the CS only treatment ($\bar{x} = 44.1 \pm 1.8$). However, unlike run B1-01, 60:40 and 80:20 co-digested treatments continued to produce significantly higher specific methane yields than the CS treatment ($p < 0.001$) every day until the experimental run ended on day 18.

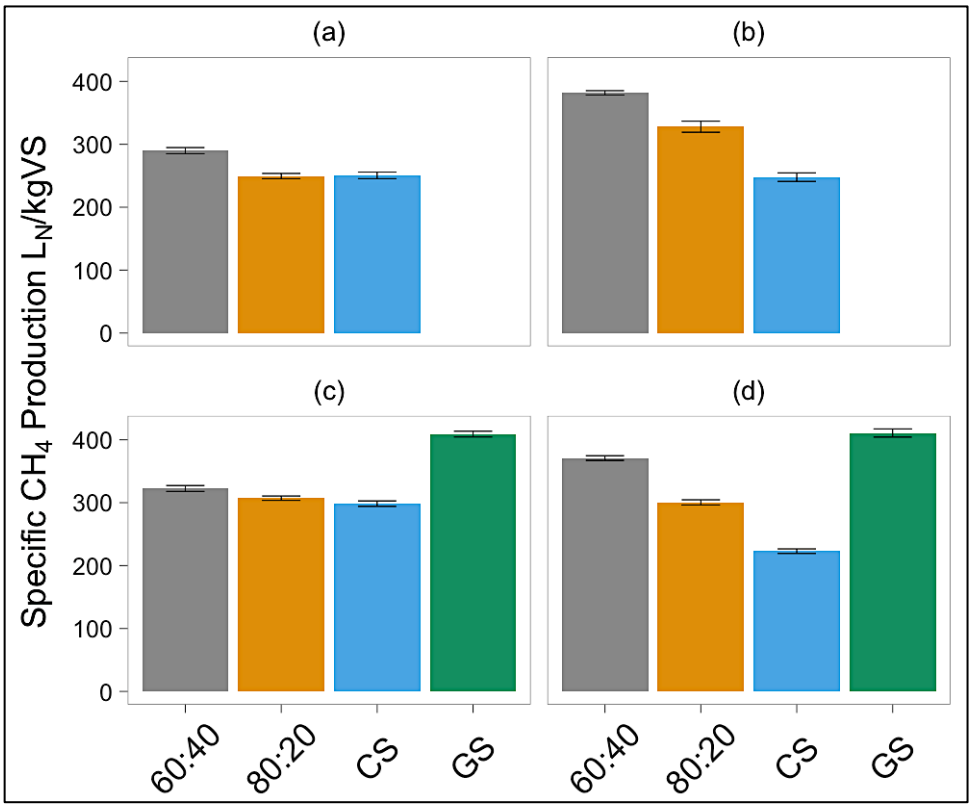

Fig. 3.9 BMP of treatments for Experiment 1 run B1-01 (a) and B1-02 (b) and Experiment 2 run B2-01 (c) run B2-02 (d). Values are expressed as means ± standard errors

The BMP estimates were verified as normal across treatments and experimental runs ($W = 0.91$, p = not significant (ns)) and having similar variances (Bartlett's $k^2 = 6.001$, $df = 4$). An analysis of variance (ANOVA) verified a main effect of treatment on BMP ($p < 0.001$), however there was no significant effect of experiment run on BMP.

Posthoc analyses using pairwise t-tests with bonferroni adjustment indicated that the CS BMP estimate ($\bar{x}$ = 254.9 ± 10.3) was significantly lower ($p < 0.05$) than the 60:40 co-digested treatment ($\bar{x}$ = 341.3 ± 8.5). However there was no significant difference between the CS and 80:20 BMP ($\bar{x}$ = 296.3 ± 11.1) treatments. The BMP for GS ($\bar{x}$ = 409.9 ± 8.0) was significantly higher than CS ($p < 0.001$) and the 80:20 ($p < 0.05$) treatments but not significantly different from the 60:40 treatment ($\bar{x}$ = 341.3 ± 8.5). The results summarised in Fig. 3.10 further support the trend that the most biodegradable treatment produced the highest BMP. The mean BMP for 60:40 co-digested treatments was 34% higher than the mean BMP for the CS ($p < 0.05$).

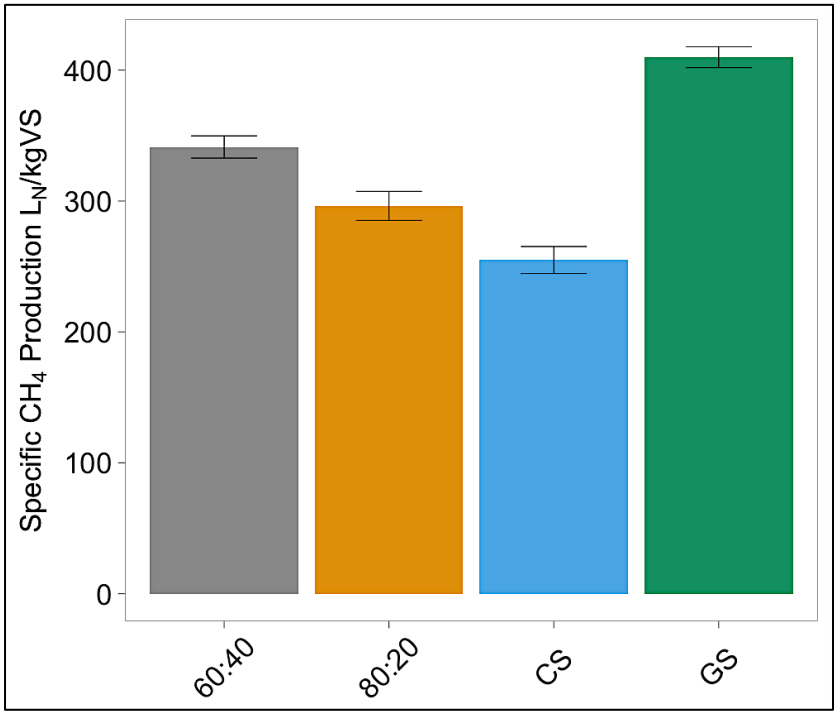

Fig. 3.10 Mean BMP estimates by treatment. Values are expressed as means ± standard errors

A linear regression of the experimental BMP of the CS, 80:20 , 60:40 and GS treatments in Experiment 2 versus the cellulose and hemicellulose content in each treatment indicate a significant positive relationship with both cellulose (R^2 = 0.84, $p < 0.001$) and hemicellulose (R^2 = 0.84, $p < 0.001$). However there was a significant negative relationship between BMP and lignin content (R^2 = -0.83, $p < 0.01$).

3.4.4 Process Kinetics: The First Order Hydrolysis Constant

Angelidaki *et al.* (2009) note that once the quality of the cumulative BMP plot is good then the hydrolysis rate of the substrate studied can be obtained provided that hydrolysis is limiting the anaerobic digestion process. A plot of $ln\left(\frac{B_\infty - B}{B_\infty}\right)$ versus time (t) (Eq. 3.9) produces a straight line with a gradient equivalent to the hydrolysis constant (k_h). Individual plots of the first order hydrolysis constant of each substrate shown in Table 3.10 by experimental run are provided (Appendix 3).

The relatively high adjusted R^2 values indicate that hydrolysis was the rate limiting step for the batch AD process. The k_h estimates were normal across treatments and experimental runs (W= 0.93) and had similar variances (Bartlett's k^2 = 2.33). An ANOVA highlighted significant effects of experiment run ($p < 0.001$) and treatment ($p < 0.05$) on k_h. Post-hoc analysis confirmed the trend shown in Table 3.10 i.e. the mean k_h in run B1-02 ($\overline{x}$ = 0.1 ± 0.01) was significantly less ($p < 0.001$) than that in run B1-01 ($\overline{x}$ = 0.157 ± 0.01). The mean k_h for run B2-01 was not significantly different to run B2-02. Table 3.10 summarises the k_h for each substrate and indicates the time taken to produce 50% and 90% of the ultimate methane yield.

With the exclusion of run B1-02 (due to inoculum effects), the CS and GS treatments both attained 50% of the methane potential in 4-5 days and attained 90% of the BMP within 12-16 days.

Table 3.10 Summary of k_h estimates by treatment and run.

Run	Treatment	Hydrolysis Constant K_h (day^{-1})	T50 (days)	T90 (days)
B1-01	60:40	0.161 ± 0.006 [a]	4	14
	80:20	0.169 ± 0.008 [a]	4	14
	CS	0.148 ± 0.008 [a]	5	16
B1-02	60:40	0.09 ± 0.009 [a]	8	25
	80:20	0.071 ± 0.006 [a]	10	32
	CS	0.068 ± 0.007 [a]	10	34
B2-01	60:40	0.177 ± 0.005 [a]	4	13
	80:20	0.200 ± 0.006 [ab]	4	12
	CS	0.157 ± 0.004 [a]	4	15
	GS	0.250 ± 0.007 [ab]	4	12
B2-02	60:40	0.181 ± 0.016 [a]	4	13
	80:20	0.224 ± 0.014 [a]	3	10
	CS	0.242 ± 0.014 [a]	4	12
	GS	0.356 ± 0.055 [a]	3	10

[a,b,c] Means followed by different superscript letter are significantly different ($p<0.05$)

3.4.5 Dry Biogas Production

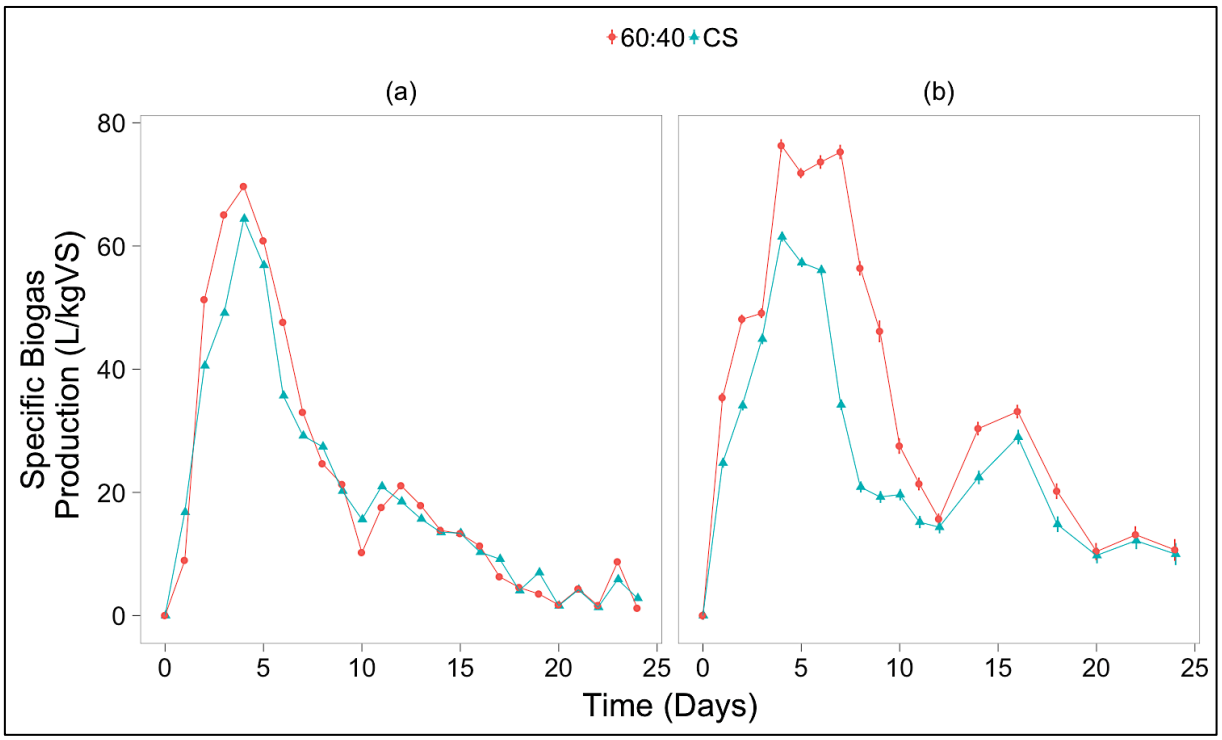

Fig. 3.11 Effect of treatment on daily specific biogas production for (a) B2-01 and (b) B2-02. Means shown with standard error bars. The 60:40 treatment is depicted in red and the Cattle Slurry only treatment in blue.

The results indicate that the specific dry biogas production of the 60:40 blend in B2-01 was significantly higher than that for CS between days 2-7 ($p < 0.01$) (Fig. 3.11). Specific net biogas production for both treatments increased between days 0-4 and then decreased from days 6-10. The 60:40 treatment's biogas production was higher than that of CS during days 1-2 while the CS treatment rate was higher during days 3-4. Specific dry gas production for CS was significantly higher than the 60:40 co-digested blend on days 10 and 11 only ($p < 0.01$).

Each treatment in both experimental runs feature two main peaks occurring around days 4 and 11 for run B2-01 and days 4 and 16 for run B2-02. For run B2-02 the specific dry biogas production for the 60:40 blend was significantly higher than CS for days 1-11 and days 14 and 18 ($p < 0.05$). The 60:40 treatment's specific biogas production rate was again higher than the CS treatment for the first two days. The rate reduced considerably between days 2-

3 compared to the CS rate over the same period but then increased once more to a higher level than the CS treatment between days 3-4.

Peak specific biogas production for both treatments was maintained for a longer time in run B2-02 than in run B2-01. However, specific production rates for the 60:40 treatment increased from days 5-7 while the CS treatment's biogas production rate decreased over the same period. In run B2-02, the CS treatment's specific biogas production rate decreased less rapidly than the 60:40 treatment between days 8-12.

3.4.6 Methane Production and Content

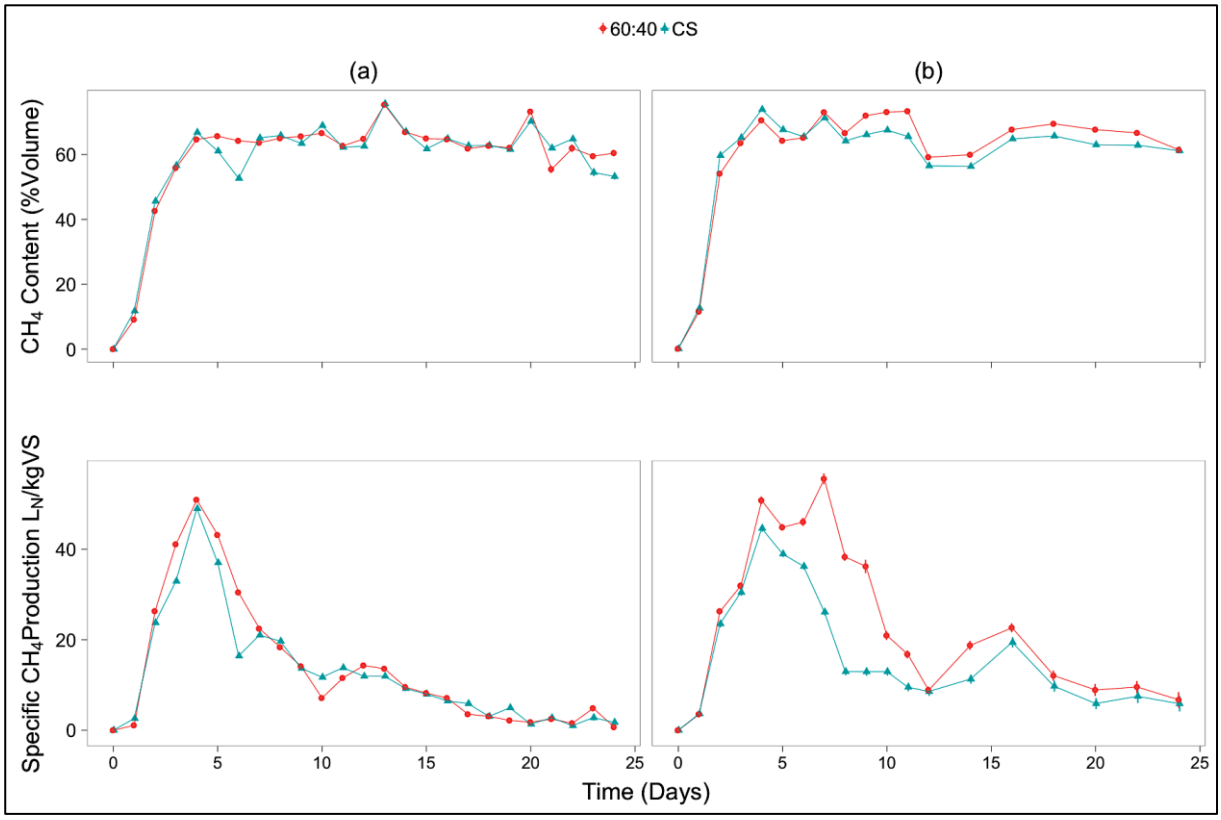

Fig. 3.12 Effect of treatment on specific methane production and methane content for (a) B2-01 and (b) B2-02. Means shown with standard error bars. The 60:40 treatment is depicted in red and the Cattle Slurry only treatment in blue.

The 60:40 blend produced significantly more methane in B2-01 on days 2-7 (Fig. 3.12) compared to CS ($p < 0.01$) while CS produced significantly higher methane on days 10,11 and 17 of the experiment ($p < 0.01$).

In run B2-02 however, methane production for the 60:40 treatment was significantly higher than the CS throughout most of the experiment as a result of a notably higher methane content from days 7-22 ($p < 0.001$). This resulted in a significantly higher specific daily methane production parameter (μ_m) for the 60:40 treatment ($\bar{x}$ = 45.1 ± 1.6) compared to the CS ($\bar{x}$ = 33.6 ± 2.4, $p < 0.05$) in the MGM for B2-02 (Table 3.8).

3.4.7 Total Nitrogen and pH

A mean total ammonia nitrogen (TAN) of 1945.2 ± 12.43 mg/L and 1938.0 ± 12.55 mg/L was observed during B2-01 for the CS and 60:40 treatments respectively. These mean TAN values were much higher ($p < 0.001$) than the TAN levels observed during run B2-02 i.e. CS ($\bar{x}$ = 1008.69 ± 13.1) and 60:40 treatments ($\bar{x}$ = 983.15 ± 12.2). This was associated with a significantly lower ($p < 0.001$) inoculum pH in run B2-02 ($\bar{x}$ = 7.19 ± 0.01) compared to run B2-01 ($\bar{x}$ = 7.48 ± 0.01) (Fig. 3.13). Significantly lower levels ($p < 0.001$) of free ammonia were also observed in the liquid phase in run B2-02 when compared to run B2-01 for both CS and 60:40 treatments. However, it is unlikely that the lower pH observed in run B2-02 would cause any process inhibition.

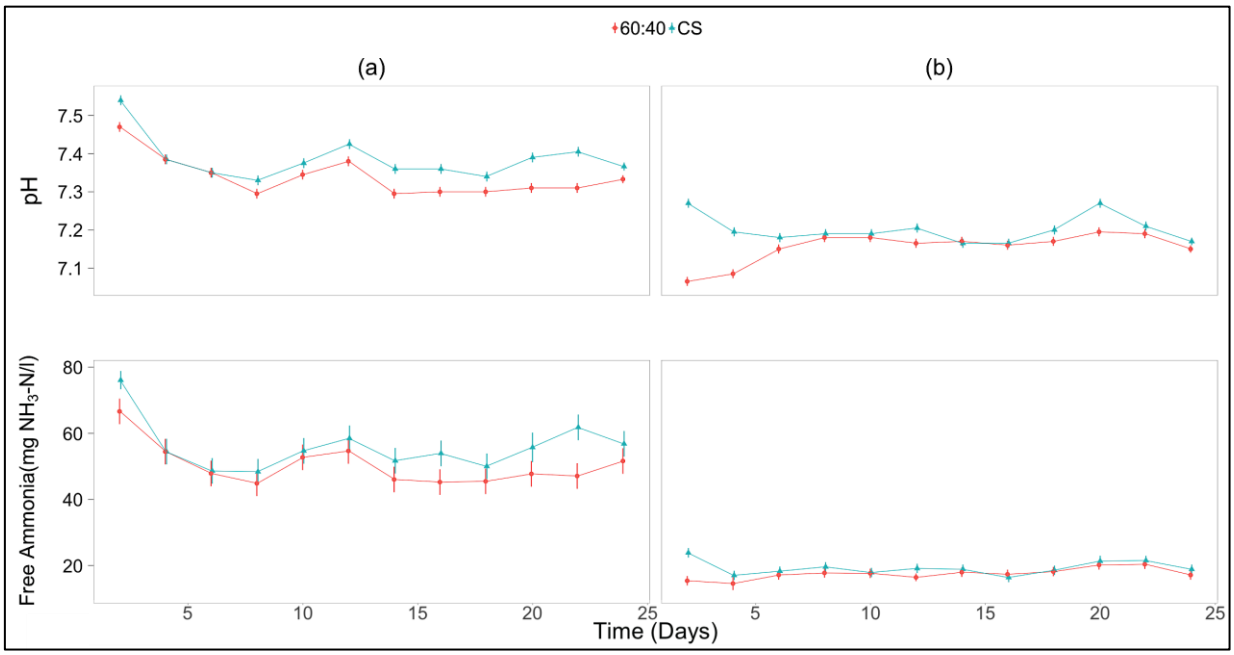

Fig. 3.13 Effect of treatment on pH and free ammonia (a) B2-01 and (b) B2-02. Means shown with standard error bars. The 60:40 treatment is depicted in red and the Cattle Slurry only treatment in blue.

The pH of CS was significantly higher ($p < 0.01$) than the 60:40 treatment on day 2 of run B2-01 and days 2 and 4 of run B2-02 ($p < 0.001$). An increase in the pH of the 60:40 treatment during days 2 to 6 of run B2-02 was associated with a general increase in methane content in the biogas over the same period (Fig. 3.12b).

There was no significant difference between mean total VFAs for the CS ($\bar{x}$ = 1173.71 ± 183) and 60:40 ($\bar{x}$ = 1389.57± 112.06) treatments in run B2-01. The total VFAs were mainly comprised of acetic and isobutyric acids during days 2 -10 (Fig. 3.14). On day 16 of run B2-01, total VFA comprised of acetic, propionic and isobutyric acids in the CS treatment. However on day 22, the concentration of isobutyric acid was reduced and formic, acetic, butyric, and propionic acids were the main constituents of total VFAs in the CS treatment. During the first four days of run B2-02, total VFAs in both treatments mainly comprised of acetic and propionic acids; with isobutyric acid also being observed on day 2 for the 60:40 treatment. Three distinct peaks in total VFAs were observed on days 6, 12, and 16 for the CS treatment where total VFAs were in excess of 50 mg/L. Propionic acid concentration was highest on two of those days i.e. 66.5mg/L on day 6 and 74.6mg/L on day 16 while 56.9mg/L of isobutyric acid was the main component of total VFAs on day 12. These spikes in total VFA dissipated after 2-4 days.

Fig. 3.14 Individual VFA composition for (a) B2-01 and (b) B2-02.

3.4.8 Total and Volatile Solids Reduction

Errors in TS and VS values for days 6-10 in B2-01 (Fig. 3.15) may have been due in part to the balance being disturbed or requiring calibration during that time. Therefore these time points have been excluded from the analysis. The TS and VS of all treatments declined with time. There was a 25% and a 22% reduction in VS from day 2 to 24 of run B2-01 for the 60:40 ($p < 0.001$) and CS treatments ($p < 0.001$) respectively. The same trend was observed for run B2-02 where 26% and a 25% of VS was removed from the CS ($p < 0.001$) and 60:40 treatments ($p < 0.001$) respectively. There was no difference between the CS and 60:40 treatments with regards to the level of TS and VS destruction.

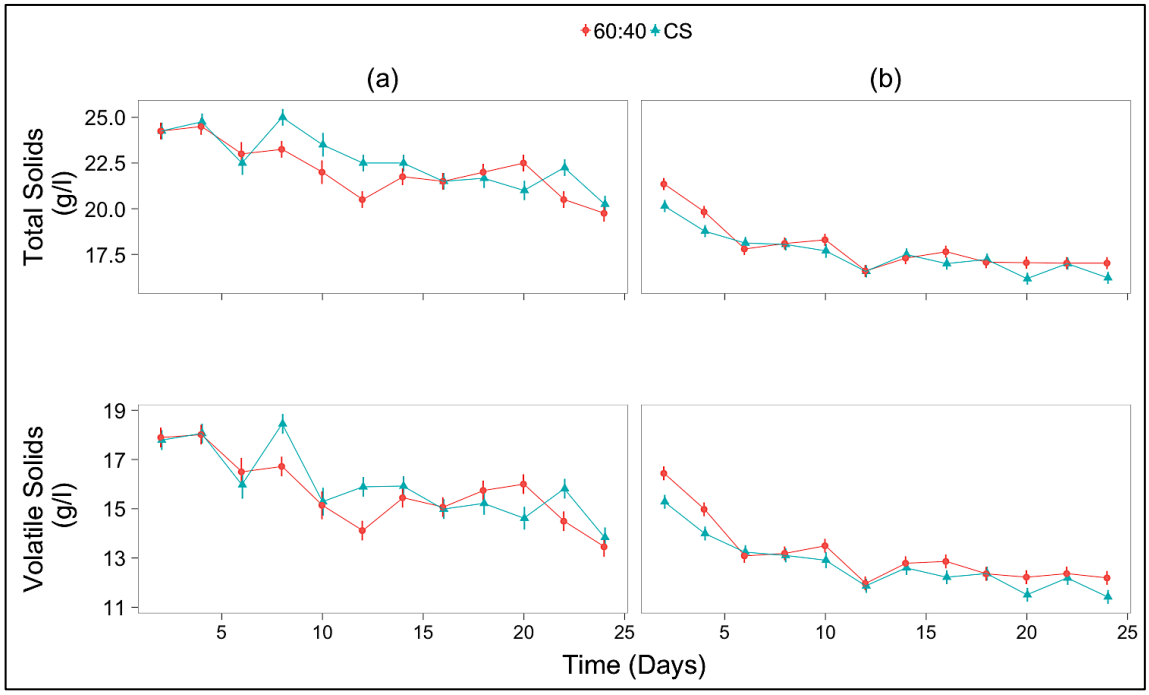

Fig. 3.15 The effect of treatment and time on TS and VS levels for (a) B2-01 and (b) B2-02. Means shown with standard error bars. The 60:40 treatment is depicted in red and the Cattle Slurry only treatment in blue.

In run B2-01, the cellulose content within the CS and 60:40 treatments degraded by 35% ($p < 0.001$) and 49% ($p < 0.001$) respectively from days 2 to 24. Approximately equal amounts of cellulose were removed in the CS and 60:40 treatments during run B2-02. In the 60:40 treatment however, most of the cellulose was removed within the first 4 days of the

experiment while cellulose degradation was slower in the CS treatment over the same time period (Fig. 3.16).

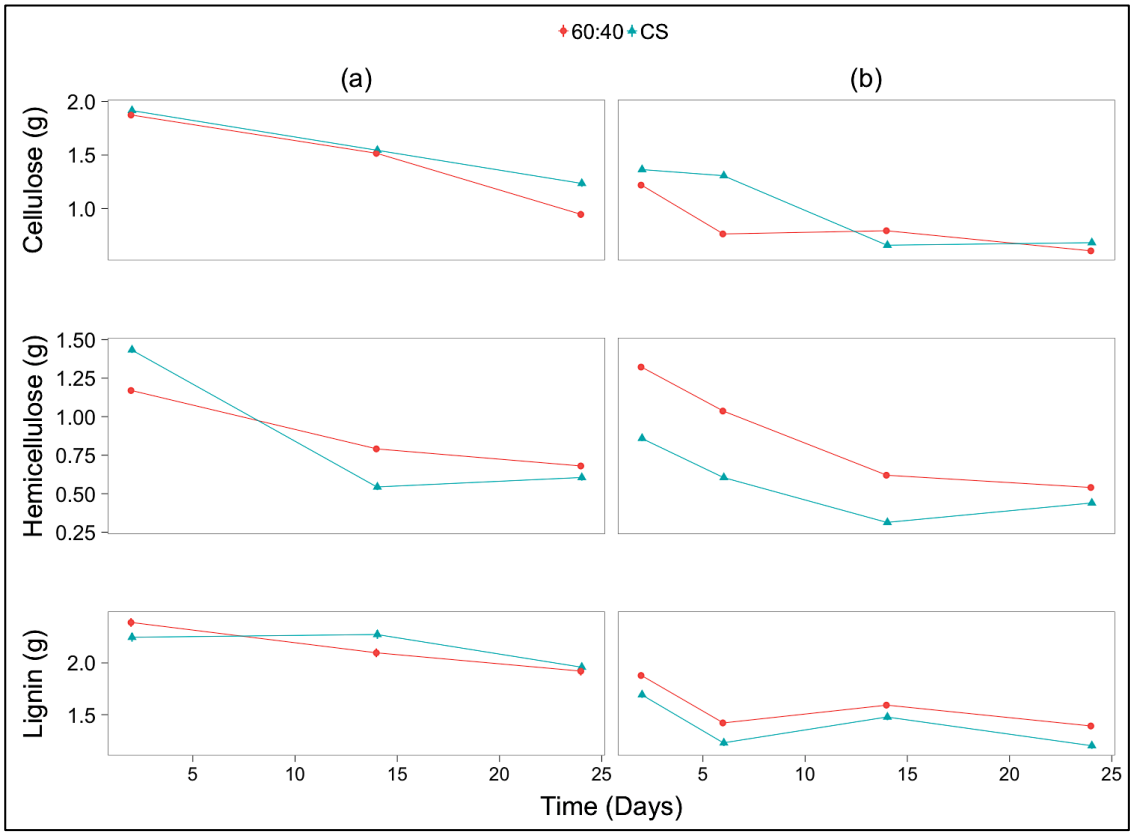

Fig. 3.16 Cellulose hemicellulose and lignin reduction for (a) B2-01 and (b) B2-02. The 60:40 treatment is depicted in red and the Cattle Slurry only treatment in blue.

The rate of degradation of hemicellulose was the greatest during the first 14 days of run B2-01. There was more hemicellulose in the 60:40 treatment than the CS treatment in run B2-02 and hemicellulose degraded at similar rates for both treatments. The overall lignin reduction in run B2-01 was 13% and 26% for the CS and 60:40 treatments respectively and 29% and 26% respectively in run B2-02.

3.4.9 Correlation of physico-chemical data

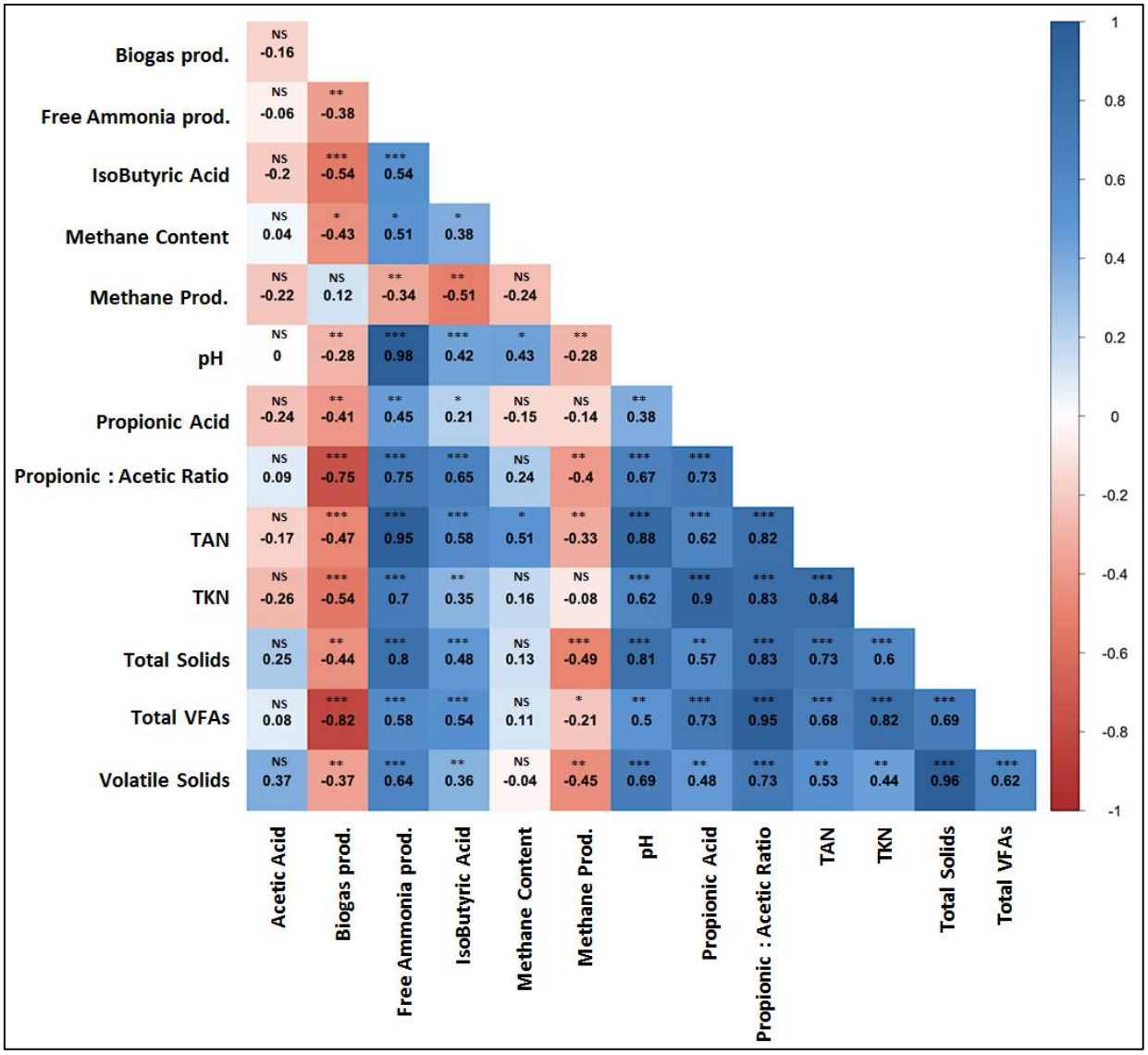

Fig. 3.17 Physico-chemical parameter correlation matrix. When statistical significance was observed, R^2 value is presented with the associated significance level. A negative sign (-) indicates a negative correlation (as shown in red) and no sign indicates a positive correlation (as shown in blue). *** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$, NS not significant

Free ammonia is negatively correlated with biogas (R^2 = -0.38,$p < 0.01$) and methane production (R^2 = -0.34,$p < 0.01$) and positively correlated with total VFAs (R^2 = 0.58,$p < 0.001$) and TS (R^2 = 0.8093,$p < 0.001$) (Fig. 3.17). Propionic to Acetic acid ratio negatively correlated to biogas production (R^2 = -0.75, $p < 0.001$) and methane production (R^2 = -0.4,$p < 0.01$).

Finally, the impact effect of pH on AD process efficiency is highlighted by its negative correlation with biogas (R^2 = -0.28, $p < 0.01$) and methane production (R^2 = -0.28, $p < 0.01$) as well as the positive correlation with biogas methane content (R^2 = 0.43, $p < 0.05$).

3.5 Discussion

Table 3.11 compares the BMP for the mono-digested and co-digested feedstocks obtained in this research with information from the literature.

Table 3.11 BMP of cattle slurry, grass silage and co-digested treatments. Mean values with standard deviation (SD) reported

BMP (L_N CH_4/kg VS)				**Reference**
CS	**80:20**	**60:40**	**GS**	
255(20.6)[a]	296(22.2)	341(17)	410(16)	current study
247(58)			320(22)	(Luna-del Risco *et al.*, 2011)
175(13.9)-239(9.5)			390(3.1)	(Allen *et al.*, 2016)
239(9)	250(8)	273(5)	400(4)	(Wall *et al.*, 2013)
243(60)				(Labatut *et al.*, 2011)
197(3.3),224(3.7)			374(35.7),421(22.2),439(21.8)	(Triolo *et al.*, 2011)
155(1.1),199(4.4),155(6.9)				(Pham *et al.*, 2013)

The mean CS BMP results obtained from the four runs in this research compared favourably with those found in the literature which ranged from 155 to 247 L_N CH_4/kg VS. Luna-del Risco *et al.* (2011), Labatut *et al.* (2011) and Allen *et al.* (2016) all reported similar values of 247, 243 and 239 L_N CH_4/kg VS respectively. Pham *et al.* (2013) reported BMP values for three BFT methods used (i.e. Møller method 155, Hansen method 199 and VDI method 155 L_N CH_4/kg VS). Pham *et al.* (2013) also reported a theoretical BMP for CS of 514.7 L_N CH_4/kg VS which is comparable to the findings of this research (497 L_N CH_4/kg VS). Therefore, the differences observed may be due to differences in inoculum since the mean BMP of cellulose also reported as 329 L_N CH_4/kg VS (Pham *et al.*, 2013) was less than that obtained in this study 394 L_N CH_4/kg VS.

Triolo *et al.* (2011) obtained CS and GS samples from 10 different farms with considerable variations in the CS BMP reported which may be due to geographical, physico-chemical and biological factors e.g. age, diet etc.

Allen *et al.* (2016) reported variations in CS BMP values from dairy slurries due to seasonal variations i.e. BMPs ranging from 175 L_N CH_4/kg VS for samples taken in autumn to 239 L_N CH_4/kg VS for those taken in summer. Therefore seasonal variation due to dietary changes i.e. grass in summer versus concentrate in winter, might have also influenced the BMP results for Batch Experiments 1 and 2 in this research which ranged from 222 to 300 L_N CH_4/kg VS. This is further supported by results obtained in Batch Experiment 2 where there was variation in the BMP reported for CS and co-digested treatments as opposed to minimal variation for the BMP reported for GS (Table 3.8). Theoretical CS BMP values of 491 and 502 L_N CH_4/kg VS obtained from this research, using the EA method (Section 3.4.2), were similar to values ranging from 389 to 606 L_N CH_4/kg VS reported by Allen *et al.* (2016).

The BMP for GS found in the literature ranged from 320 - 439 L_N CH_4/kg VS is consistent with those reported by Allen *et al.* (2016) who noted GS BMP ranging from 374 – 400 L_N CH_4/kg VS based on geographical location in Ireland (i.e. 389.61 in the North, 374.48 in the South and 399.56 L_N CH_4/kg VS in the Midlands). Wall *et al.* (2013) reported a BMP value of 400 L_N CH_4/kg VS from GS that comprised of perennial rye grass; similar to the grass used in this study. Grass silage theoretical values (EA method) from runs B2-01 (471 L_N CH_4/kg VS) and B2-02 (479 L_N CH_4/kg VS) were also similar to those reported by Allen *et al.* (2016) and Wall *et al.* (2013); which were 472-503 and 443 L_N CH_4/kg VS respectively.

3.5.1 The effect of biodegradability on BMP

The mean biodegradability index (BD) results obtained in this research i.e. (CS 53%, 80:20 treatment 61%, 60:40 treatment 69% and GS 86%) also compares favourably with those reported by Allen *et al.* (2016) i.e. CS (26-62%) and GS (78-90%) and Wall *et al.* (2013) i.e. CS 61%, 80:20 treatment 63%, 60:40 66% and GS 90%. There is a clear discrepancy in BD value for the 80:20 treatment reported by Wall *et al.* (2013) i.e. BD of 63% when experimental BMP was 250 and predicted BMP 271. In this research, the theoretical BMP values were obtained by first calculating the equivalent percentage composition of each element in the elemental analysis (EA) data on a VS basis before using the values to predict the theoretical BMP of the co-digested mixtures (Eq. 3.6).

The determination of acid detergent fibre (ADF), neutral detergent fibre (NDF) and acid detergent lignin (ADL) indicated that CS contained higher levels of lignin than GS (Table 3.12). Consequently, based on Eq. 3.6, proportionate VS contributions of CS and GS in the treatment blends resulted in the treatment with the higher proportion of GS having significantly lower levels of lignin.

Table 3.12 Proximate analysis of CS and GS feedstocks

Parameter	Grass Silage	Cattle Slurry
ADF (% TS)	31.80	32.31
NDF (%TS)	53.30	47.90
ADL (%TS)	4.39	13.68

The feedstock characteristics in Table 3.3, indicate that the CS treatment contained 19% cellulose, 16% hemicellulose and 14% lignin on a TS basis. The GS contained 27% cellulose, 22% hemicellulose and 4% lignin on a TS basis.

The composition of CS and GS related to lignocellulose content was consistent with that found in the literature (Table 3.13).

Table 3.13 Lignocellulose content of grass silage and cattle slurry. Mean values expressed as a % TS basis.

Feedstocks	Cellulose	Hemicellulose	Lignin	References
Grass Silage	27.4[a]	21.5[a]	4.4[a]	current study
	27	21.7	5.3	(Koch *et al.*, 2010)
	13-20	17-20	13-18	(Osborne Homeky *et al.*, 2016)
	41.4	12.6	4.9	(Mönch-Tegeder *et al.*, 2014)
Cattle Slurry	18.6[a]	15.6[a]	13.7[a]	current study
	21.0	19.0	11.0	(Raju *et al.*, 2013)
	24.3	8.0	14.8	(Budde *et al.*, 2014)
	24.1	34.1	10.1	(Kalamaras and Kotsopoulos, 2014)

[a]Values were calculated from the Neutral Detergent Fibre (NDF), Acid Detergent Fibre (ADF) and Acid Detergent Lignin (ADL) content in the cattle slurry and grass silage samples in Table 3.12.

Cattle slurry is less degradable since most of the degradable VS have already been consumed within the ruminant's intestinal tract as opposed to the higher degradable VS of GS (Lehtomäki *et al.*, 2007; Triolo *et al.*, 2013). The co-digested treatments contained levels of lignocellulose in proportion to percentage VS of CS and GS from which the treatment mixtures were made. Lignin forms a matrix with cellulose and hemicellulose fibres that supresses the extracellular enzymatic hydrolysis of the complex structural carbohydrate polymers by holocellulolytic microorganisms (Jørgensen *et al.*, 2007; Triolo *et al.*, 2013; Tsapekos *et al.*, 2017). Lignin content negatively correlated with BMP (R^2 = -0.83, $p < 0.01$). This also results in the trend observed where the treatment with the highest BD i.e. GS also had the highest BMP. The highest BMP was observed with the 60:40 co-digested treatment because it contained more degradable TS than the 80:20 treatments.

The general trend is that there was no difference between the hydrolysis constants for the mono-digested and co-digested feedstocks. High R^2 values obtained in determining k_h (Appendix 3) infers that hydrolysis was the rate limiting factor for the AD process; which is typical of lignocellulose AD (Cirne *et al.*, 2007; Bayané and Guiot, 2010; Sun *et al.*, 2015; Tsapekos *et al.*, 2017).

The time taken to attain 50 percent of BMP (T50) for all treatments was around 3-10 days. Allen *et al.* (2016) reported T50 values of 4 days for CS collected in spring and summer and 5 days for GS collected in the north of Ireland. Wall *et al.* (2013) reported slightly higher T50 values i.e. 8 days for GS, 80:20 and 60:40 feedstock and 10 days for CS but concluded that the hydraulic retention time (HRT) in an AD plant for mono-digested GS and co-digested CS:GS would be similar. However this study found that the k_h for CS did not differ from GS nor the co-digested treatments. This may be due to the fact that the microbial degradation in the rumen compromises the structure of lignocellulose thus making the cellulose and hemicellulose fibres in CS more susceptible to microbial attack during AD (Triolo *et al.*, 2013). The rate of degradation of cellulose and hemicellulose was greatest during the first 15 days of the batch run. The rapid 33% degradation of cellulose contained in the 60:40 treatment in run B2-02 was most likely due to the lower inoculum pH in B2-02 facilitating increased hydrolysis. This effect of pH can be observed in the degradation of hemicellulose and lignin as well.

3.5.2 The effect of co-digestion physico-chemical process parameters

Carbohydrates (75-78%TS) were the main component fraction for both mono-and co-digested treatments. Raw protein was the second highest component (19-20%TS) followed by raw lipids (3-5%TS). Differences in specific biogas production between the CS and 60:40 treatments during the first 10 days were primarily due to the 60:40 treatment having more digestible VS than the CS. Holocellulytic microorganisms in the 60:40 treatment may have had greater access to convert polysaccharides such as mannose, arabinose, xylose into simple sugars, i.e. glucose and fructose and alcohols during the hydrolysis stage of AD (Jørgensen *et al.*, 2007) because of the treatment's higher biodegradability.

Additionally, ensiling reduces the resistance of holocellulose to holocellulytic bacteria and produces VFAs ready to be used by methanogens for biomethanation (Nizami *et al.*, 2009). Secondary peaks shown in both experimental runs are likely to have been due to the methanogens processing metabolites originating from more recalcitrant lignocellulose, and microorganisms that would have died due to substrate availability becoming more limited (Batstone *et al.*, 2002).

There was a clear effect of pH on biogas methane content observed in run B2-02. Inoculum pH differed significantly between runs because of a decrease in pH of the distilled water i.e. (pH = 4.5) used to prepare the inoculum and the treatments. The initial pH for the 60:40 blend was lower than that of CS possibly due to the lactic acid and VFAs it contained from the GS and the lower buffering capacity of GS compared to CS (Mata-Alvarez *et al.*, 2014). However the 60:40 effluent's pH increased as the VFAs were metabolised by the methanogens. The formation of ionised ammonia from the metabolised amino acids would also assist in increasing pH in the liquid phase of the 60:40 treatment. This resulted in a corresponding increase in biogas methane content since the pH approached the upper range reported as ideal for AD, i.e. a pH of 6.8 – 7.2 (Nizami *et al.*, 2009; Khalid *et al.*, 2011; Neshat *et al.*, 2017). A lower inoculum pH enhanced the hydrolysis of lignocellulose since the optimum pH for hydrolytic and acidogenic bacteria ranges from 5.5 to 6.5 (Ward *et al.*, 2008). An enhanced hydrolysis phase is likely to have led to higher concentrations of intermediate metabolites such as propionate within the CS only treatment in experimental run B2-02.

The reduction in pH in run B2-02 also caused a reduction in free ammonia concentration even though concentration levels were below levels that may cause inhibition i.e. 0.22g NH_3-N/L (Chen *et al.*, 2008). During acetogenisis, long chain fatty acids (LCFA), i.e. propionic acid and butyric acids are oxidised by acetogenic bacteria to produce acetate, carbon dioxide, and hydrogen (Angenent *et al.*, 2004). Methane is then produced via three main pathways, two of which involve acetoclastic methanogens converting acetic acid to methane and carbon dioxide via acetate decarboxylation or a reduction of hydrogen and carbon dioxide by hydrogenotrophic methanogens (Angenent et al., 2004; Glass and Orphan, 2012). The initial accumulation and subsequent removal of acetic and propionic acids during the AD of both treatments suggests the presence of hydrogenotrophic and acetoclastic methanogenesis pathways. The syntrophic relationship between hydrogenotrophic methanogens and acetogens facilitates the oxidation of propionate and butyrate via interspecies hydrogen transfer (IHT) or interspecies formate transfer (IFT) where hydrogen and/or formate are used as terminal electron acceptors.

The thermodynamic conditions required for propionate and butyrate oxidation are low hydrogen partial pressures and/ or low levels of formate concentration (Stams and Plugge, 2009; Aymerich *et al.*, 2013).

IHT was the most likely propionate oxidation mechanism since there was no accumulation of formate detected within the CS treatment on days 6 nor 16. This suggests that the accumulation of propionate within the CS treatment which resulted in clear spikes in total VFAs, particularly during days 6 and 16 of run B2-02, was likely due to a decoupling of the biochemical syntrophic interaction between the hydrogenotrophic methanogens and propionate oxidising bacteria which may have been caused by a temporal increase in hydrogen partial pressure beyond the narrow window of 10^{-4} to 10^{-6} atm. This confirms the decline of the methanogenic population as substrate availability became limiting.

3.5.3 Conclusions

The salient points of this chapter are:

- The low BMP of CS justifies its co-digestion with GS. The GS treatment has the highest BMP followed by the 60:40, 80:20 and CS treatments in descending order. Therefore co-digesting with a CS:GS mixture of 60:40 in batch mode will be of commercial benefit to farmers for increasing the efficiency of AD;
- Degradation of holocellulose occurred within the first 15 days of the BFT. Therefore 15 days can be considered as a minimum retention time for a commercial AD co-digesting a CS:GS mixture that contains a maximum 60% GS;
- Process parameters such as pH, free ammonia and the Propionic : Acetic acid ratio were negatively correlated with biogas and methane production. Low pH levels may decouple the syntrophic relationship between propionate oxidising bacteria and their partner hydrogenotrophic methanogens and lead to an accumulation of propionate which may reduce biogas production or quality;
- The high spikes in VFA observed in run B2-02 were caused by a temporary accumulation of propionate due to a decoupling of the syntrophic oxidisation of propionate via the IHT mechanism.

Chapter 4 Molecular characterization of an AD microbial community operating in batch mode and its correlation with process parameters

4.1 Introduction

Advances in technology have focused on unravelling complexities related to the dynamics that control the AD process and their relationship to physico-chemical process parameters. The optimisation and stability of the process is still limited by insufficient knowledge regarding the phylogeny of the microbial consortia and their function with respect to the metabolic pathways involved in biogas production (Werner *et al.*, 2011; Wirth *et al.*, 2012; Tsapekos *et al.*, 2017). This is because many of the microorganisms have not yet been identified due to limited sequencing depth and restricted resolution of molecular fingerprinting methods or they simply cannot be cultured (Werner *et al.*, 2011). The development of polymer chain reaction (PCR) (Saiki *et al.*, 1985) together with recent advances in molecular tools have narrowed the knowledge gap by facilitating the characterisation of taxa directly associated with various stages of the AD process (Pycke *et al*., 2011; Wirth *et al*., 2012).

Research is required to further enhance the characterisation of the microbial consortia, their function within each AD process stage and correlate community shifts with environmental factors that influence the AD process (Wang *et al.*, 2010b; Cadavid-Rodríguez and Horan, 2013; Mata-Alvarez *et al.*, 2014). Molecular tools such as denaturing gradient gel electrophoresis (DGGE) (Muyzer *et al.*, 1993), terminal restriction fragment length polymorphism (T-RFLP) (Liu *et al.*, 1997), fluorescence *in-situ* hybridization (FISH) (Wagner *et al.*, 2003) and real time quantitative PCR (RT qPCR) (Harms *et al.*, 2003) have been developed in recent years to further characterise microbial communities in tandem with the use of online reference databases (Fig. 4.1).

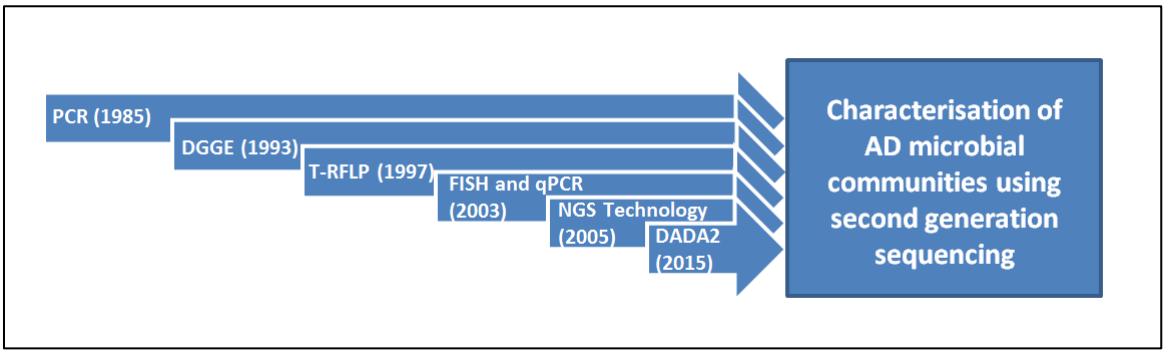

Fig. 4.1 Molecular techniques used in the microbial ecology of Anaerobic Digesters

Significant cost reductions in high-throughput next generation sequencing (NGS) of hypervariable regions of the 16S rRNA gene amplicon clone libraries and the accessibility of curated repositories of both cultured and uncultured microorganisms in recent years have facilitated its usage in microbial ecology (Li *et al.*, 2011; Kopylova *et al.*, 2016; Kulski, 2016). However the molecular techniques such as PCR, used in library preparation, are prone to errors (Farrelly *et al.*, 1995; Li *et al.*, 2011; Rosselli *et al.*, 2016). The effectiveness of probes used to bind uncultured organisms and the unknown and possibly variable amplification efficiencies may lead to a distortion of the relative abundances of the microorganisms within a sample (Rosselli *et al.*, 2016). Therefore the interpretation of results using these molecular techniques is quantitatively uncertain because of variability in genome sizes and gene copy numbers. Bioinformatics workflows seek to reduce uncertainty by denoising or removing erroneous artefacts introduced into high-throughput NGS data from upstream processes.

One such step is to cluster 16S rRNA next generation amplicon sequences together, within a threshold radius of similarity i.e. typically 97% (Callahan *et al.*, 2016a; Kopylova *et al.*, 2016). Kopylova *et al.* (2016) note 3 OTU methods currently used by bioinformatics software namely:

- Closed reference - input sequences are assigned to operational taxonomic units (OTUs) using reference sequence databases such as Greengenes (DeSantis et al., 2006), RDP (Cole et al., 2014) and Sylva123 (Yilmaz et al., 2014) in a process referred to as phylotyping (Caporaso *et al.*, 2010; Schloss and Westcott, 2011).
- De novo- OTUs are created by pairwise similarity of input sequence with all the sequences in the dataset.
- Open reference- OTUs are initially created with a closed reference step. Sequences that failed to be characterised by the reference database are then clustered using the de novo method.

Molecular characterisation of AD microbial communities using NGS is therefore dependent on the OTU clustering and phylotyping methods used. While Callahan *et al.* (2016a) and Rosen *et al.* (2012) concede that threshold similarity OTU clustering minimises misinterpretation of sequences having both amplicon and sequencing errors as biological variation, they also note that such methods prohibit the ability to discriminate between legitimate fine scale biological variation i.e. where sequences may differ by one nucleotide. OTU clustering methods over-estimate diversity in the presence of noise larger than the threshold cut-off and are unable to assess biological diversity on a scale higher than the chosen threshold. Such fine scale variation can provide information regarding microorganism ecological niches (Koeppel and Wu, 2013), population structure and temporal dynamics (Callahan *et al.*, 2016a). Molecular characterisation of AD microbial communities can benefit from such fine scale discrimination to adequately assess diversity, population structure and temporal shifts that occur during the AD process.

This research aims to increase knowledge regarding the anaerobic co-digestion of cattle slurry (CS) and grass silage (GS) in batch mode by characterising the microbial community using a bioinformatics algorithm called Divisive Amplicon Denoising Algorithm (DADA2) (Callahan, 2016). High-throughput NGS 16S rRNA cloned libraries were used to characterise the microorganisms into unique ribosomal sequence variants (RSV) as opposed to traditional OTUs (Callahan *et al.*, 2016a). This research compares the microbial community shifts within an AD batch operated system digesting CS- only feedstock and CS co-digested with GS at a CS:GS ratio of 60:40. The objectives of this research are to:

- Characterise the microbial communities digesting agricultural substrates at a higher nucleotide resolution; therefore providing more insight into the microorganisms that are present and the roles they may play in anaerobic co-digestion.
- Evaluate the phylogenetic differences within and between treatments to determine whether phylogenetic differences in the microbial community correlated with biogas production or quality.
- Correlate microbial community shifts to physico-chemical data collected during the batch experiment to determine which environmental variables had the largest impact on microbial community composition and diversity.

4.2 Materials and methods

The samples for analysis were collected and prepared in accordance with the experiment design and methods outlined in Chapter 3. Samples for microbial analysis were taken from the CS and 60:40 treatments on days 0, 6, 10 and 24 for each experimental run of the BMP test. Sampling points were chosen for runs B2-01 and B2-02 after visual inspection of the cumulative BMP curve (Fig. 4.2).

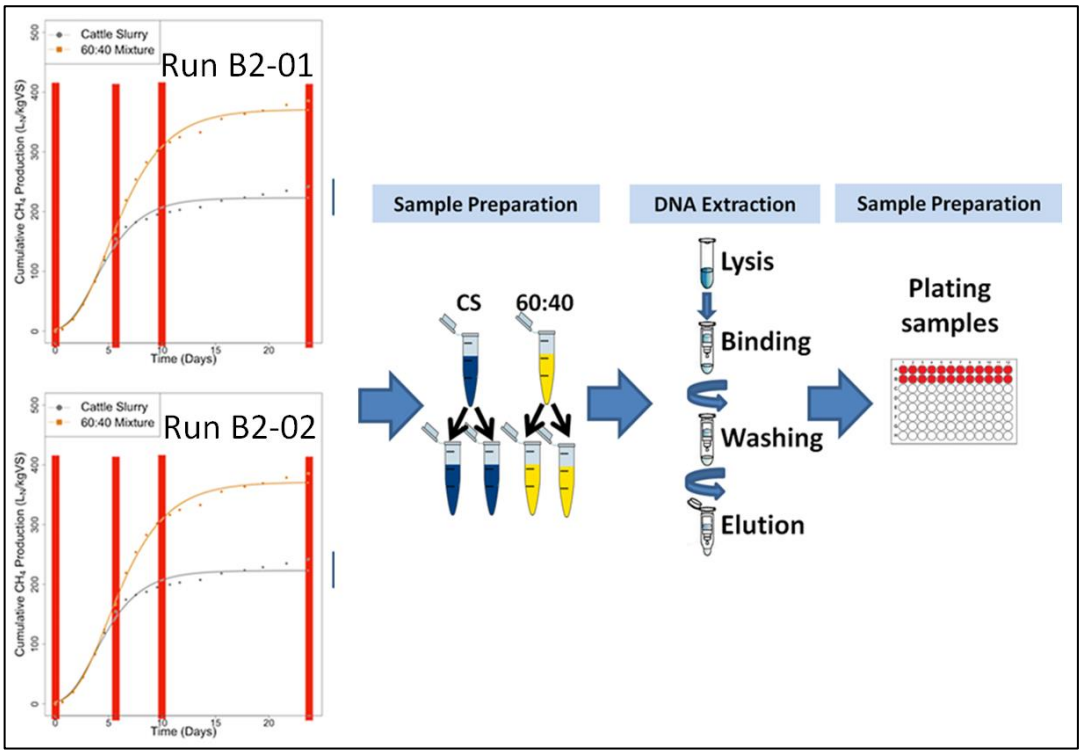

Fig. 4.2 Sample selection and preparation. Sampling times are indicated by red lines.

Day 0 was chosen to provide insight into the initial diversity and population of the microbial community. Day 6 represents the linear portion of the BMP curve where hydrolysis was rate limiting and the maximum specific methane production rate was observed. The other 2 sampling points represent stages in the AD process when microbial access to substrate either began to be limited (day 10) or became very limited (day 24). Two technical replicates of one biological sample for each treatment at each time were taken for analysis.

Samples were stored at -20°C in sterile 50ml centrifuge tubes (VWR, 525-0402) prior to genomic DNA extraction. DNA extraction was conducted using the FastDNA™ SPIN Kit for Soil (MP Biomedicals LLC., 116560200). Modifications were made to the original protocol (MP Biomedicals LLC., 2007) since the samples contained faecal and plant material. Only 250 µl of sample was used in the process to minimise the adverse effect of humic acids on downstream PCR amplification (Trochimchuk *et al.*, 2003). A procedural blank of 250 µl microbiological grade sterilised water (Microzone, UK) was processed to determine the presence or absence of kit contaminants.

The homogenizing step during the lysis stage was extended so that the samples were homogenized 4 times for 40 seconds at 6m/s using the FastPrep®-24 instrument (MP Biomedicals LLC., 116004500) in order to facilitate lysis of plant material. Samples were cooled at 4°C in a refrigerator for 4 min between runs instead of being placed on ice for 2 minutes as per the instruction manual. Samples were then stored at -20°C prior to further analysis. 100µl of extracted DNA extracts from each sample was plated on to a sterile 96 well plate and sent to an external laboratory (NU-OMICS, Northumbria University, UK) for polymerase chain reaction (PCR) amplification, library preparation and high-throughput 2 X 250 amplicon sequencing of the V4 region of the 16S rRNA gene using the Illumina MiSeq Personal Sequencer in accordance with the 16S Sequencing with the Illumina MiSeq Personal Sequencer protocol (Kozich *et al.*, 2003). Library clean up and normalisation was performed using the SequalPrep Plate Normalisation kit (Invitrogen A10510-01). Extracted DNA were amplified with 515f and 806r primers which targeted variable region 4 (V4) of the 16S rRNA gene and were capable of detecting both bacteria and archaea (Caporaso *et al.*, 2011; Walters *et al.*, 2011). The de-multiplexed FastQ files were subsequently processed using the DADA2 bioinformatics workflow.

4.3 Bioinformatics and Analysis Workflow

DADA2 uses a de-novo, reference free method to infer unique RSVs and is more sensitive than traditional OTU clustering since it is able to discriminate between sequence variants differing by a single nucleotide (Callahan *et al.*, 2016a). The algorithm uses an error model that quantifies an error rate (λ_{ji}) defined as the rate at which a particular sequence 'i' is produced from sample sequence 'j' as a function of sequence composition and its quality score. The error rate is then used to parameterise a Poisson model in order to calculate p-values for the null hypothesis that the abundance of sequence 'i' is consistent with the error model. These p-values are then used as the division criteria for an iterative partitioning algorithm where sequencing reads are binned into distinct groups consistent with having originated from their central sequence (Callahan *et al.*, 2016a).

DADA2 is also implemented in the R statistical software (R Core Team, 2013) and therefore facilitates an open source bioinformatics workflow with access to bespoke molecular and statistical tools for downstream analysis of NGS 16S rRNA sequence data (Callahan *et al.*, 2016b). De-multiplexed FastQ files relating to runs B2-01 and B2-02 were processed separately using the DADA2 R package in a workflow described by Callahan *et al.* (2016b).

The workflow mainly depends on the use of 4 main packages in R i.e. DADA2 (Callahan *et al.*, 2016a), DECIPHER (Wright, 2016), Phangorn (Schliep, 2011) and Phyloseq (McMurdie and Holmes, 2013). Six pairs of forward and reverse reads, 250 bases in length were randomly sampled from the dataset of each run in order to visually assess their respective quality score plots (Appendix 4).

The forward reads consistently had Phred scores above 30 throughout the read length however quality dropped towards latter base sequences of each read. Quality scores for bases greater than the 220^{th} nucleotide for the reverse reads had a Phred score of around 26 or lower. Therefore nucleotide sequences beyond the 220^{th} base in each reverse read were truncated. The first 10 bases for both forward and reverse reads were trimmed given that the first 10 bases for Illumina datasets often contain pathological errors (Callahan *et al.*, 2016b). All paired reads were then trimmed and truncated based on the previous observations using the criteria developed i.e. trim the first 10 nucleotides on both forward and reverse reads and truncate the reverse reads beyond the 220^{th} nucleotide. The reads were processed by FastQC (Andrews, 2016), before and after filtering, in order to validate the reports obtained from DADA2. The “derep” function in DADA2 was used to dereplicate the reads in each sample in order to identify unique ribosomal sequence variants (RSVs) from redundant sequences contained in the dataset. Respective forward and reverse reads were then paired using the mergePairs function and separate sequence tables were created for both experimental runs (B2-01 and B2-02) comprising of inferred RSVs as columns and their respective abundances in each sample as rows. Chimeric RSVs were then removed from each sequence table using DADA2’s "removeBimeraDenovo” algorithm.

In the dataset for run B2-01, 7,793 unique RSVs were found, 3,035 of which were removed due to chimeras. For run B2-02, 8,338 unique inferred RSVs were found in the dataset where 3,206 were removed due to chimeras. In both datasets chimeric sequence variants represented about 7 % of total reads. Silva123 reference database (Quast *et al.*, 2013) was used for taxonomic assignment of the non-chimeric sequences from runs B2-01 and B2-02. The *DECIPHER* R package (Wright, 2016) was used for multiple alignment of the reads and *Phanghorn* R package (Schliep, 2011) to create a phylogenetic tree. Phyloseq (McMurdie and Holmes, 2013) was then used to create a phyloseq object that combined the taxonomy table, sequence table, metadata table and phylogenetic tree that was used in downstream analysis. A total of 3.6M reads were processed using the Silva 123 reference database; resulting in the classification below.

Table 4.1 Classification of RSVs at the kingdom taxonomic rank

Run	Bacteria	Archaea	Eukaryota	Unassigned
B2-01	4,647	97	7	7
B2-02	5,025	94	6	7

The unassigned sequences were BLASTed against the National Center for Biotechnology (NCBI) database (NCBI Resource Coordinators, 2016) but did not return any hits and were therefore removed along with sequences classified as Eukaryota as these were most likely due to contamination (Callahan *et al.*, 2016b) and represent 0.07% of combined reads. KRONA software (Ondov *et al.*, 2011) was used to describe the relative composition of bacteria and archaea within each treatment's microbial community. The reference database of all of the RSV contained within the cleaned sequence table and assigned to their respective taxonomic ranks is provided in a Microsoft Excel file in Appendix 5. RSV not previously characterised to the species rank by the Sylva123 database were identified by BLASTing against the NCBI database.

Chao1 and Shannon diversity indices were calculated using the taxonomically filtered dataset. Only the top 15 bacterial phyla present in 2.5% of both treatment samples were used to indicate changes in relative abundances throughout the experiment. However no prevalence filtering was conducted on archaeal relative abundances. Process variables used in the analysis were produced from mean daily values of biogas and methane production, TS, VS, pH, TAN, TKN, FAN and total VFAs were measured on days 0, 6, 10 and 24 for each treatment during experiment run B2-01 and B2-02.

4.4 Statistical analysis

Indirect gradient analysis techniques i.e. principal coordinate analysis (PCoA) and non-metric multidimensional (NMDS) scaling with Bray-Curtis (Bray and Curtis, 1957) dissimilarity were used to visualise the differences between sample clusters resulting from phylogenetic variation. Sample counts were transformed to relative abundances as a variance stabilising measure and z-scores of the process variables (Table 4.2) were used for ordination analysis (Ramette, 2007; Borcard *et al.*, 2011). Chao1 and Shannon richness indices along with species evenness were used to describe the alpha diversity of both treatments.

Table 4.2 Table of mean values of selected process parameters.

Experiment Run	Treatment	Day	Methane Produced (mL_N CH_4/d)	Volatile Solids (g/L)	pH	TAN (g/L)	Total Volatile Fatty Acids (g/L)
B2-01	60:40	0	0	21	7.81	1.48	21.5
		6	30	16	7.35	1.93	17.69
		10	7	15	7.35	2.00	8.15
		24	1	13	7.34	1.97	14.43
	CS	0	0	19	7.83	1.49	21.38
		6	16	16	7.35	1.96	14.61
		10	11	15	7.38	1.91	5.99
		24	2	14	7.39	1.96	12.84
B2-02	60:40	0	0	19	7.61	0.78	56.04
		6	26	13	7.18	0.97	12.49
		10	19	13	7.2	0.94	10.46
		24	8	12	7.17	0.99	0.874
	CS	0	0	19	7.65	0.78	57.02
		6	33	13	7.14	0.97	36.58
		10	10	13	7.18	0.96	28.12
		24	8	11	7.15	1.06	0.146

The top 30 genera seen in 10% of the samples were selected for ordination. The most influential process variables were determined using the vegan function “bioenv” where the 'best' group of process variables was the grouping whose Euclidean distance matrix had the highest correlation with the Bray-Curtis dissimilarity matrix resulting from their response data. Using the null hypothesis that no difference existed between the relative abundance and composition of taxa with each sample, PERMANOVA (Anderson, 2001) tests were used to assess differences between treatment type, experimental run and day that each sample was taken. Using the “adonis” function in R, this non-parametric test uses permutation to compare within group phylogenetic differences to between group phylogenetic differences using a pseudo F-test. Phylogenetic differences between groups were considered significant if more than 5% of the permuted F-statistic was less than the observed statistic i.e. ($p < 0.05$). Homogeneity of group dispersion was assessed using the “betadisper” function in R which is a multivariate equivalent to the Levene’s test for equal variance implemented (Anderson, 2001) in order to ensure the robustness of the dissimilarity test results.

Canonical correspondence analysis (CCA) was used to investigate the influence of the process variables on the diversity of the microbial population. It is based on the assumption that microbial response to these factors is unimodal and uses chi-squared (χ^2) distances of association to relate the likelihood of species abundances (Ramette, 2007). The main process variables and individual genera that correlated with community composition were fitted to the plots using the “envfit” function in the *vegan* R package. Significance of process parameters was determined by Monte Carlo testing with 1000 permutations. Perpendicular projections of samples or species points along a process variable’s arrow provide an approximation of their ranking along that specific variable. Constrained CCA plots therefore provide a meaningful summary of species-process variable relationships (Ramette, 2007; Borcard *et al.*, 2011).

4.5 Results

Mean reads per sample of 27,651 and 27,995 were obtained for run B2-01 and B2-02 samples respectively. Sequence depth ranged from 176,536 to 395,476 in run B2-01 and 197,716 to 250,439 in run B2-02. The 60:40 treatment for run B2-01 on day 24 had a much higher read count than other samples. This was due to possible errors made in the normalization step in the Illumina MiSeq Personal Sequencer protocol. Diversity indices were assessed for both treatments; with and without rarefying the data. The rarefied dataset was created by rarefying to 176,000 using the phyloseq function “rarefy_even_depth”. However, the analysis was conducted on the original dataset since no significant difference was observed between the diversity indices for each treatment of the rarefied and unrarefied datasets. Rarefying would also have resulted in the loss of valuable species data (McMurdie and Holmes, 2014).

4.5.1 Characterization of the microbial community by treatment

The CS and 60:40 treatments exhibited RSV classification profiles similar to the overall profile (Fig. 4.3). Appendix 6 provides a summary of the overall microbial community profiles of the CS-only and 60:40 treatments.

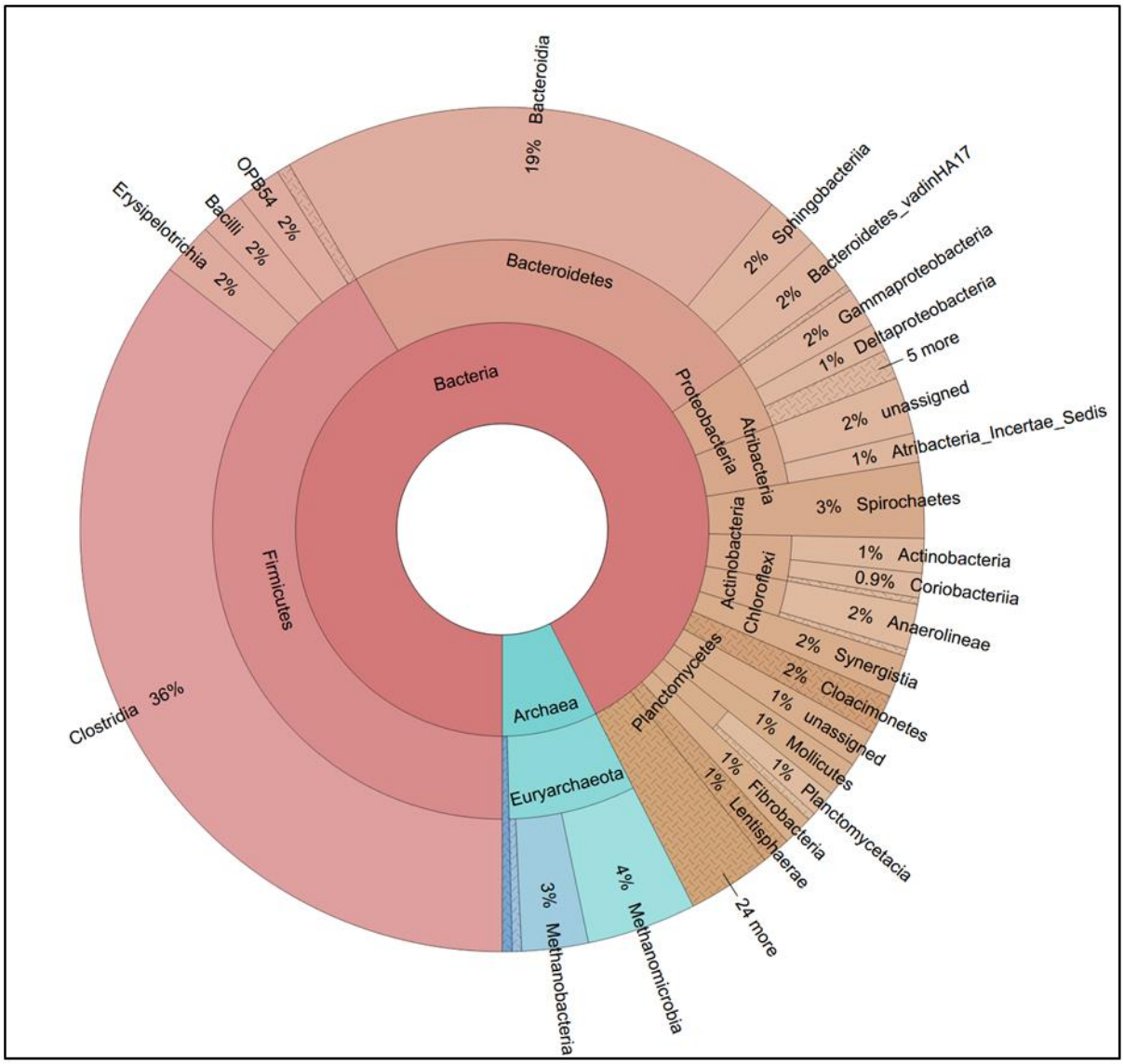

Fig. 4.3 Relative composition of combined taxa found

Ninety-three percent of both CS and 60:40 treatment samples were dominated by bacteria with only 7% classified as archaea. *Firmicutes* was the most predominant bacterial phyla i.e. 46% of which 86% belonged to the class *Clostridia* along with fairly similar abundances (ca.

4%) of classes *Bacilli*, *OPB54* and *Erysipelotrichia*. Ninety-eight percent of the members belonging to class *Clostridia* were of the order *Clostridiales*. The phylum *Bacteriodetes* had the second largest abundance in both the CS and 60:40 treatment microbial communities; making up 26% of total bacterial counts. Over 80% percent of the phylum *Bacteroidetes* was dominated by class *Bacteroidia*; 96% of which were of the order *Bacteroidales* and 4% were of the order *Bacteroidia Incertae Sedis*. Much lower abundances of bacteria were observed for the phyla *Proteobacteria* (4%), *Atribacteria* (3%) and *Spirocaetes* (3%), *Actinobacteria* (2%), *Chloroflexi* (2%), *Synergistia* (2%), *Mollicutes* (2%) and *Cloacimonetes* (2%).

Archaea in both treatments were dominated by phylum *Euryarchaeota* i.e. 95% in the CS treatment and 93% in the 60:40 treatment. Approximately 6% of the archaea were unassigned at the phylum rank. Phylum *Euryarchaeota* mainly consisted of archaea of classes *Methanomicrobia* (59%), *Methanobacteria* (35%) and *Thermoplasmata* (5%). Members of class *Methanomicrobia* were of the order *Methanosarcinales* (85% and 80%) and *Methanomicrobiales* (15% and 20%) for the CS and 60:40 treatments respectively. Members of phylum *Thermoplasmata* were of the order *WCHA1-57* (61%) and *Thermoplasmatales* (39%) in the CS treatment's microbial community but more evenly divided in the 60:40 treatment's microbial community with *WCHA1-57* (51%) and *Thermoplasmatales* (49%).

4.5.2 Assessing the alpha diversity of the treatment microbial communities

The number of reads ranged from 167,536 to 395,476 in run B2-01 and 197,716 to 250,439 in run B2-02. The range in sequence depth between days 0 and 24 samples for run B2-01 was higher than that for run B2-02. This was most likely caused by normalisation errors prior to sequencing. Such variation in sequencing depth marginally biased the trend in richness estimates for run B2-01 as more rare RSVs were detected due to the higher number of reads (Sims *et al.*, 2014). Species richness estimates for both treatments generally decreased with time presumably as available substrate became limited (Fig. 4.4).

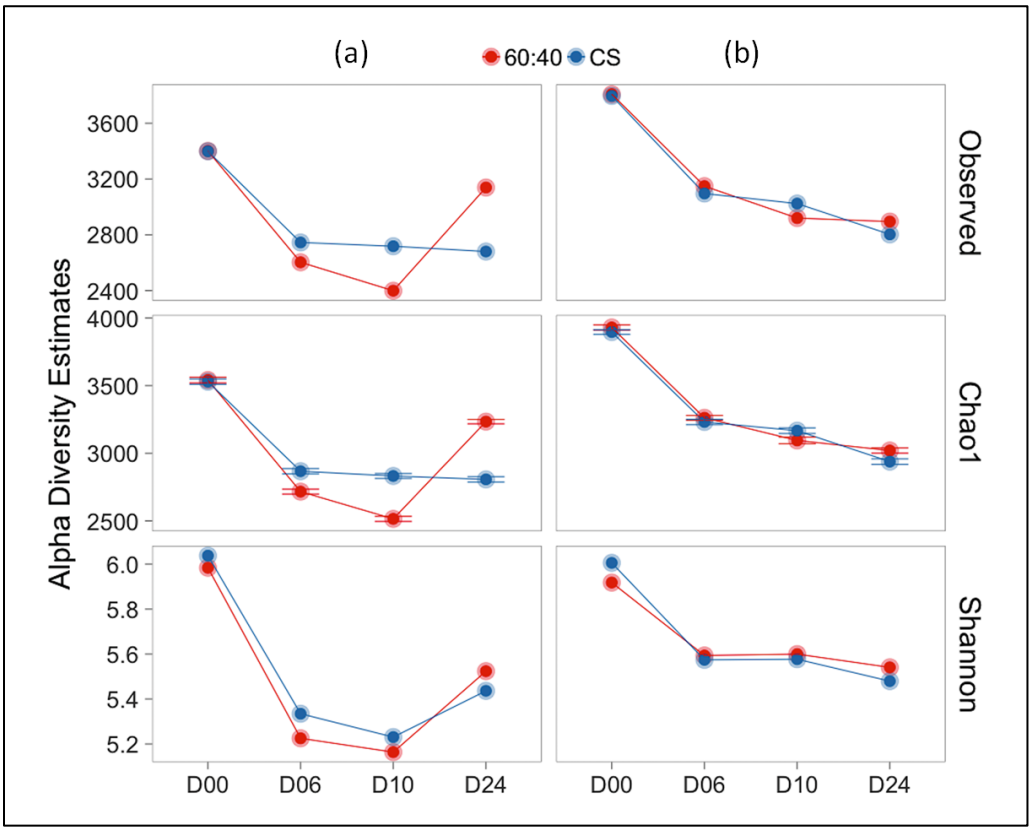

Fig. 4.4 Observed richness, Chao1 richness and Shannon diversity estimates versus time for the 60:40 and CS treatments for experimental runs (a) B2-01 and (b) B2-02. The 60:40 treatment is depicted in red and the Cattle Slurry only treatment in blue. The higher richness estimates observed on Day 24 for the 60:40 treatment in run (a) B2-01 were due to a normalisation error which occurred prior to sequencing.

Chao1 and Shannon richness estimates on day 0 were significantly higher than on days 6, 10 and 24 (all $p < 0.05$) for the CS treatment. However there was only a significant difference between day 0 and day 10 for the 60:40 treatment ($p < 0.05$). There was no difference between the species richness estimates of days 6 to 24; which coincided with species evenness becoming somewhat constant (Fig. 4.5). The Chao1 richness index is calculated using the following:

$$S_{Chao1} = S_{obs} * \frac{F_1^2}{2F_2} \quad \text{Eq. 4.1}$$

where S_{obs} is the observed number of RSV within a sample, F_1 is the number of observed RSV represented by a single sample (singletons) and F_2 the number of observed RSV represented by two samples (doubletons). Chao1 is therefore dependent upon abundance data (Magurran, 2013).

The error made during the pooled normalisation step caused more RSV to be detected in run B2-01 day 24, 60:40; resulting in a higher index which is counterintuitive to the observed reducing trend from days 6 to 24 for the remaining samples.

Shannon's richness index (H') is given by:

$$H' = -\sum p_i * \ln(p_i) \quad \text{Eq. 4.2}$$

where the quantity p_i is the proportion of individuals observed in the i^{th} species (Magurran, 2013). The Shannon index has a dual purpose in that it can be used to convey the evenness of the samples as well as sample richness due to its proportion component. This duality has also been reported as a disadvantage as it relates to the 60:40 treatment in run B2-01, day 24 where increases in the index can either be due to an increase in evenness or richness or both. It is most likely that, given the trends for the other samples that the increase in the 60:40 treatment day 24 sample in run B2-01 observed in the Shannon index is due solely to the increase in abundance due to the normalisation error.

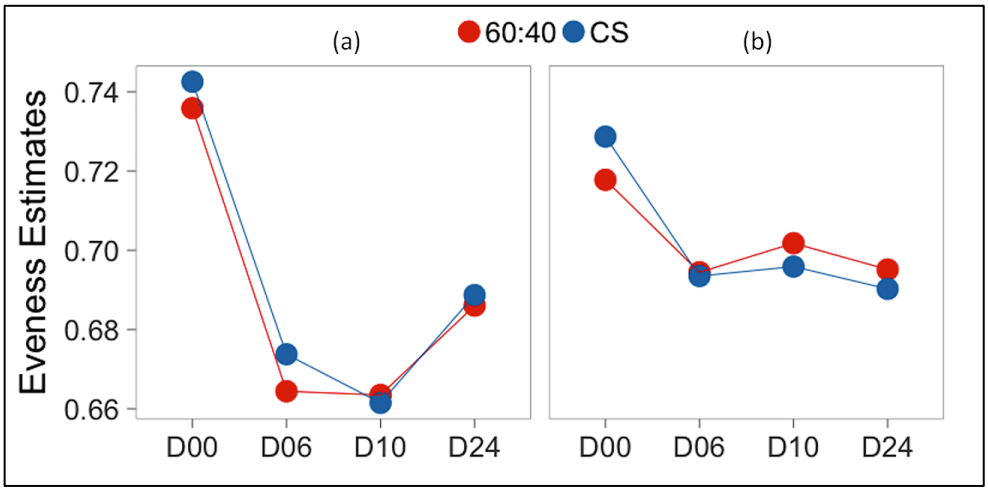

Fig. 4.5 Evenness estimates of the CS and 60:40 microbial communities versus time for experimental runs (a) B2-01 and (b) B2-02. The 60:40 treatment is depicted in red and the Cattle Slurry only treatment in blue. The higher richness estimates observed on Day 24 for the 60:40 treatment in run (a) B2-01 were due to a normalisation error which occurred prior to sequencing.

The apparent increase in diversity estimates from days 10 to 24 for the 60:40 and CS treatments in run B2-01 was due to an increased number of reads for samples taken on day 24. Mean reads per sample of 27,651 and 27,995 were obtained for runs B2-01 and B2-02 respectively.

4.5.3 The archaeal microbial community

The relative abundance of archaeal phyla consisted of *Miscellaneous_Crenarchaeotic_Group* (MCG) (5-7%) and *Euryarchaeota* (93-95%) for the CS-only and CS:GS treatments. Archaeal phylotypes for MCG were however not defined at the genus level and therefore not incorporated into the alpha or beta diversity analyses. The relative abundance of archaeal genera belonging to phylum *Euryarchaeota* for both treatments exhibited a similar trend in that evenness reduced with time (Fig. 4.6) as dominant archaeal genera increased in relative abundance from day 0 to day 24 of the batch experiment. The archaeal community's diversity was established by day 6 of both experimental runs, which coincides with the period of greatest methane production for both treatments reported in Chapter 3. The two most dominant archaeal phylotypes during days 0 to 6 in both treatments were *Methanosaeta spp.* (obligate acetotrophic methanogens), *Methanobrevibacter spp.* (hydrogenotrophic methanogens) (Garcia, 1990) with relative abundances of 38% and 27% respectively of the archaeal community within both treatments. The dominance of *Methanosaeta* and *Methanosarcina* indicates the presence of both acetotrophic and hydrogenotrophic methanogenesis pathways within the CS-only and CS:GS treatments. Other archaea of lesser relative abundances that were present in the CS-only and CS:GS treatment during days 0 to 24 were of the genera *Methanosarcina*, *Methanospirillum*, *Methanoculleus*, and *Methanobacterium*. The majority of the archaeal phylotypes belonged to the taxonomic class of *Methanomicrobia* and *Methanobacteria* (Fig. 4.3).

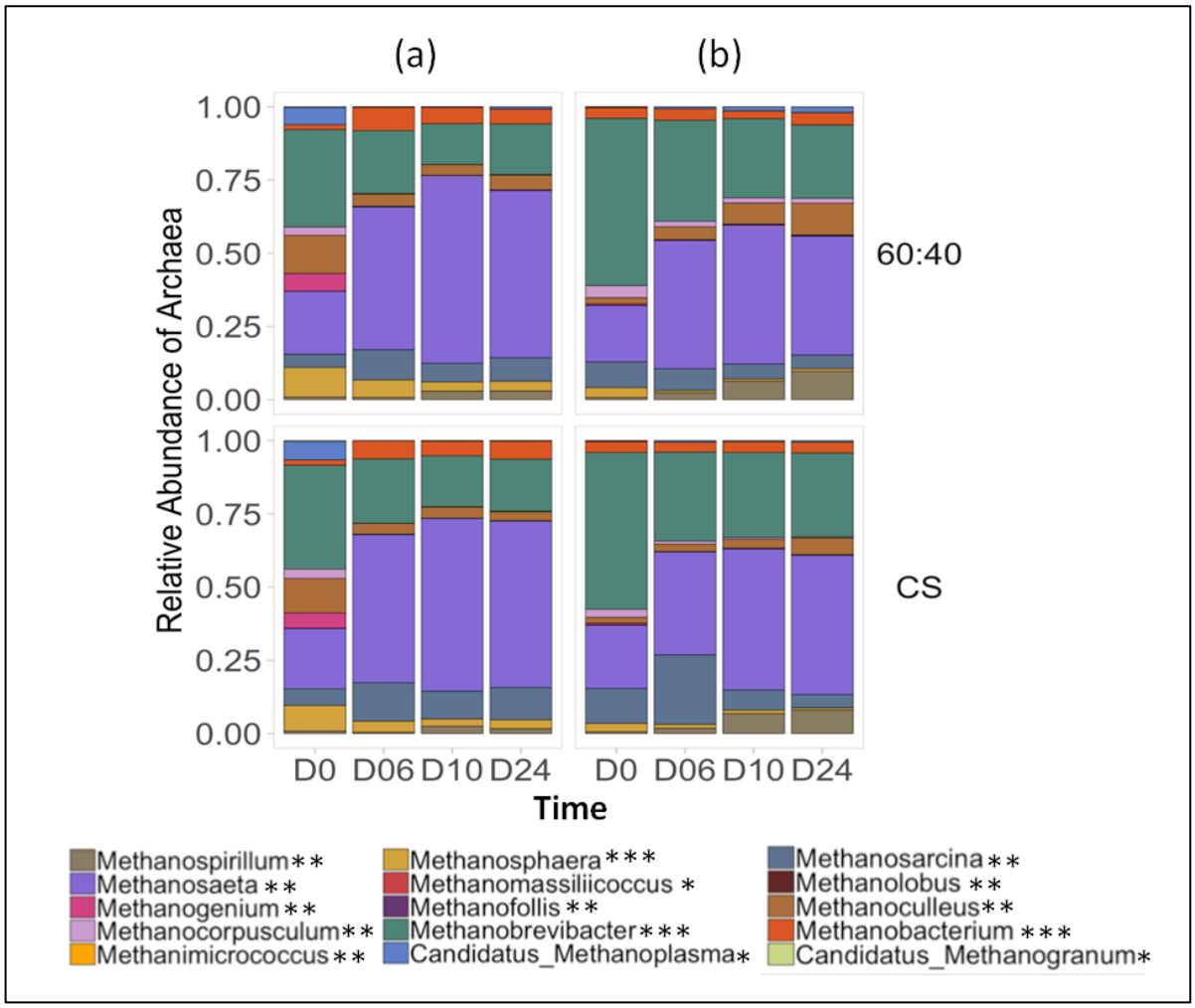

Fig. 4.6 Relative abundance of the archaeal microbial community belonging to phylum *Euryarchaeota* for the CS and 60:40 treatments in batch experiment (a) B2-01 and (b) B2-02. *Archaeal genera belonging to class *Thermoplasmata*, **Archaeal genera belonging to class *Methanomicrobia*, ***Archaeal genera belonging to class *Methanobacteria*

Methanoculleus spp. had an overall relative abundance of 5% of the archaeal phylotypes within both the CS-only and CS:GS samples. The relative abundance of *Methanospirillum spp.* increased 6-fold within the archaeal community for both treatments throughout the course of the batch experiment- from 0.7- 4.0% of the CS-only treatment and 0.8-5.0% of the CS:GS treatment for day 0 to 24 samples respectively. Differences between the relative abundance of *Candidatus_Methanoplasma spp.* and *Methanogenium spp.* within the CS-only treatment day 0 samples for Batch Experimental runs B2-01 and B2-02 were also observed within day 0 samples for the CS:GS treatment and indicates that the archaeal community within the inoculums used for each experimental run was phylogenetically different. However there was no discernable phylogenetic difference between the archaeal community of the CS-only and CS:GS treatments.

The methane production plots in Chapter 3 indicate that the largest methane production was recorded during days 0 to 6 of the batch experiment. During this time, the relative abundance of *Methanosaeta spp.* and *Methanosarcina spp.* increased from 20-41% and from 19-43% of archaeal phylotypes within the CS-only and CS:GS treatments respectively. However *Methanobrevibacter spp.* decreased in relative abundance from 42-25% and from 42-27% within the CS-only and CS:GS treatments respectively. During days 6 to 24, as substrate availability became limiting and daily methane production decreased, the relative abundance of *Methanosaeta spp.* increased from 41-43% within the archaeal community for the CS-only treatment but remained constant at 43% within the CS:GS treatment.

4.5.4 The bacterial microbial community

Samples taken at day 0 for each treatment differed in phylogenetic composition between experimental runs (Fig. 4.7). This trend occurred in both archaeal and bacterial communities. The phylogenetic differences such as the relative abundance of *Psychrobacter spp.* are likely due to variations in the composition of the microbial community in the inoculum.

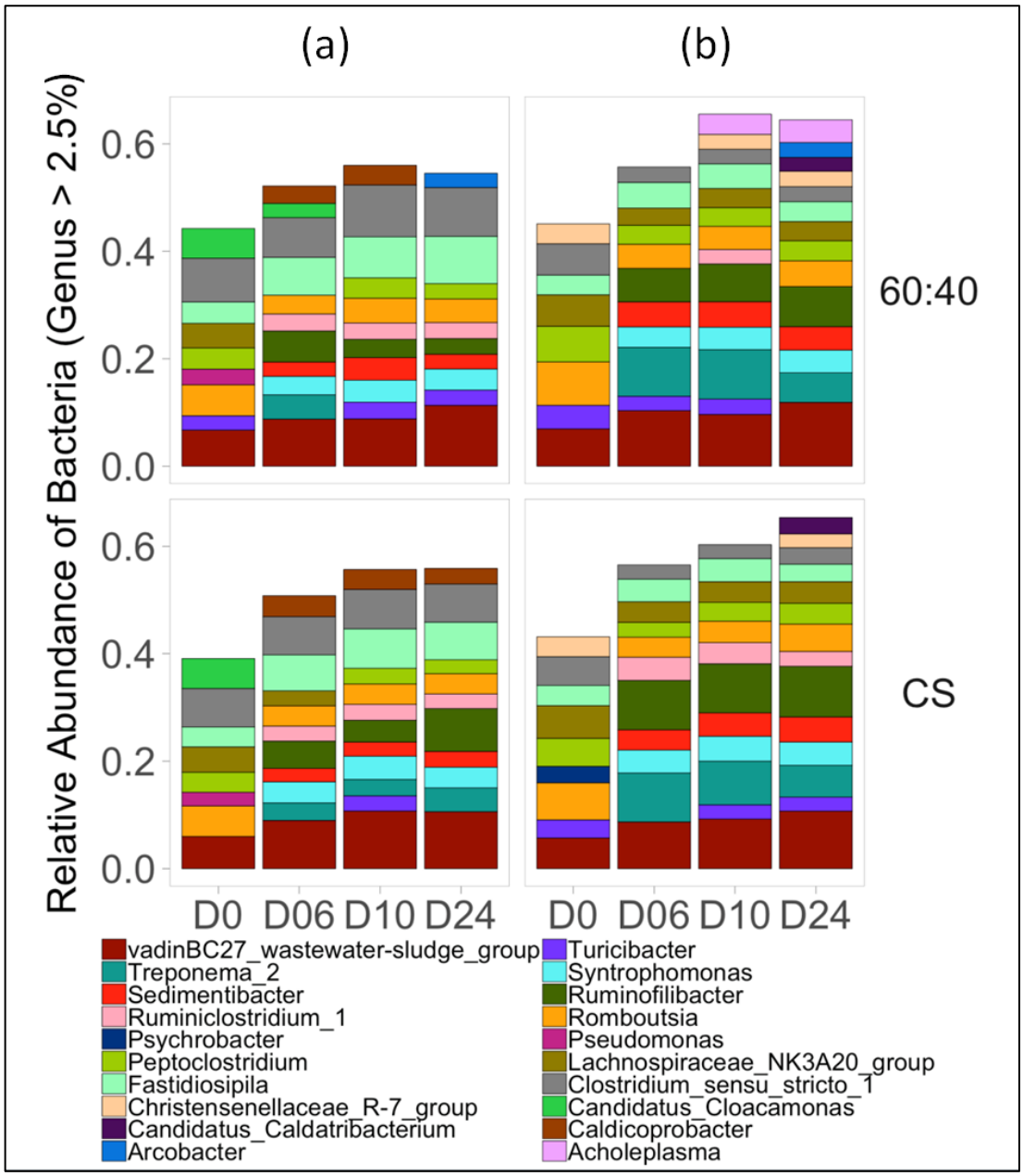

Fig. 4.7 Relative abundance of bacterial genera for the CS and 60:40 treatments in Batch Experiment (a) B2-01 and (b) B2-02

Sub-populations of *Sedimentibacter spp.*, *Sytrophomonas spp*. and *Ruminofilibacter spp*. were only evident from 6 to 24 within the CS-only and CS:GS treatments. Phylotypes belonging to *Romboutsia spp.*, *Fastidiosipila spp.* and *VadinBC27 spp.* were present in all samples for both treatments. Dominant bacterial phylotypes present in the CS-only and CS:GS treatments from days 6 to 24 suggest that they played a role in the degradation of recalcitrant biomass as substrate became limiting. *VadinBC27 spp.* had the largest relative abundance within the CS-only (5%) and CS:GS (6%) treatments.

The relative abundance of *Acholeplasma spp*. in day 10 and 24 samples of the CS:GS treatment in experimental run B2-02 as well as *Arcobacter spp*. in day 24 samples indicate the existence of some phylogenetic differences between treatments.

4.5.5 Assessing the beta diversity between treatments

Principal co-ordinate analysis (PCoA) with Bray-Curtis distance matrix explained 90% of the variation between samples with the first 3 principal components (PC) (Fig. 4.8a). The first two principle components, PC1 and PC2, explained 83% of the variation observed. The first PC discriminates between samples based on time (Fig. 4.8b) while PC2 separates group centroids based on experimental run (Fig. 4.8c).

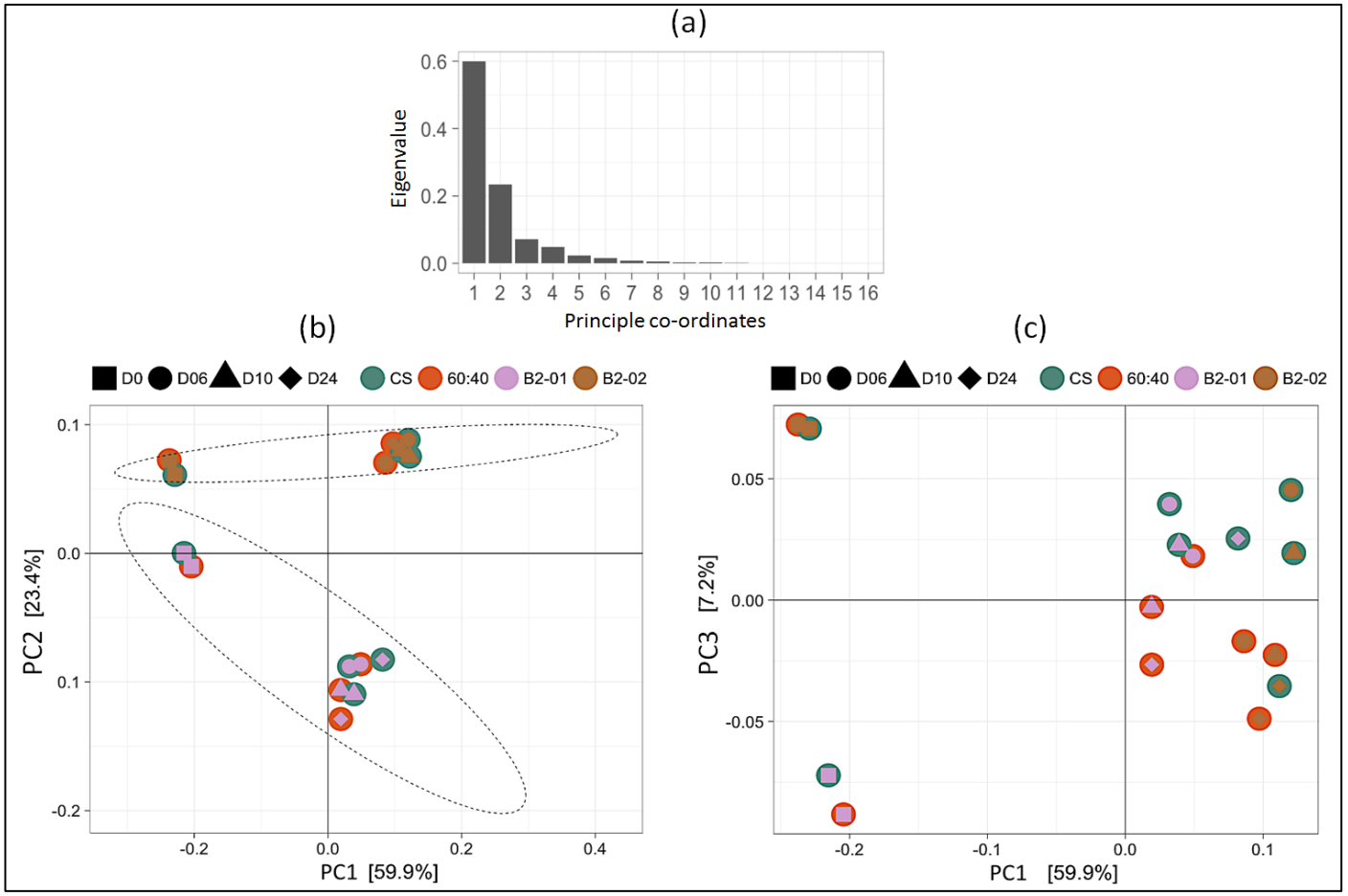

Fig. 4.8 PCoA with Bray-Curtis dissimilarity matrix of CS and 60:40 treatments showing axes (a) scree plot of ordination (b) PC1 vs PC2 with subsamples grouped by experimental run along with 95% normal confidence areas and (c) PC1 vs PC3

Adonis test results indicate that samples taken on day 0 were significantly different to those taken subsequently ($p < 0.01$) while run B1-01 samples were also significantly different to those taken in B2-02 ($p < 0.05$). No significant difference was observed between treatments from the samples taken. A test for homogeneity of group dispersion did not yield any significant results. Therefore the null hypothesis of variance homogeneity across groups could not be rejected. Borcard *et al.* (2011) warn against interpreting results from the Adonis test; noting that false positives may occur if group dispersion is not homogenous. More certainty can therefore be placed on the fact that the treatments are not phylogenetically different.

Volatile solid (VS) destruction, pH, TAN and total VFA were initially selected as the best combinations of process variables to use in CCA ordination since they produced the highest rank correlation with community differences. Methane production was used instead of VS destruction in order correlate species abundances with this parameter and to identify RSV from each treatment whose relative abundances responded to changes in methane production during the course of the experiment (Ramette, 2007). There was a significant correlation of the canonical axes with the chosen explanatory variables ($p < 0.001$). The unconstrained CA plot is shown in Appendix 7.

4.5.6 Assessing microbial community correlation with process variables

The top 30 genera observed at least once in 10% of both treatment samples were dominated by bacteria of the order *Clostridiales*, *Bacteroidales*, *Spirochaetales*, *Bacteroidia_Incertae_Sedis* and *Erysipelotrichales*. Archaea of the order *Methanosarcinales* and *Methanobacteriales* were the most prevalent. Ninety-five percent of the observed phylogenetic variation between the treatment samples in the unconstrained CCA plot was explained by variations in pH, TAN, total VFA and methane production (Fig. 4.9).

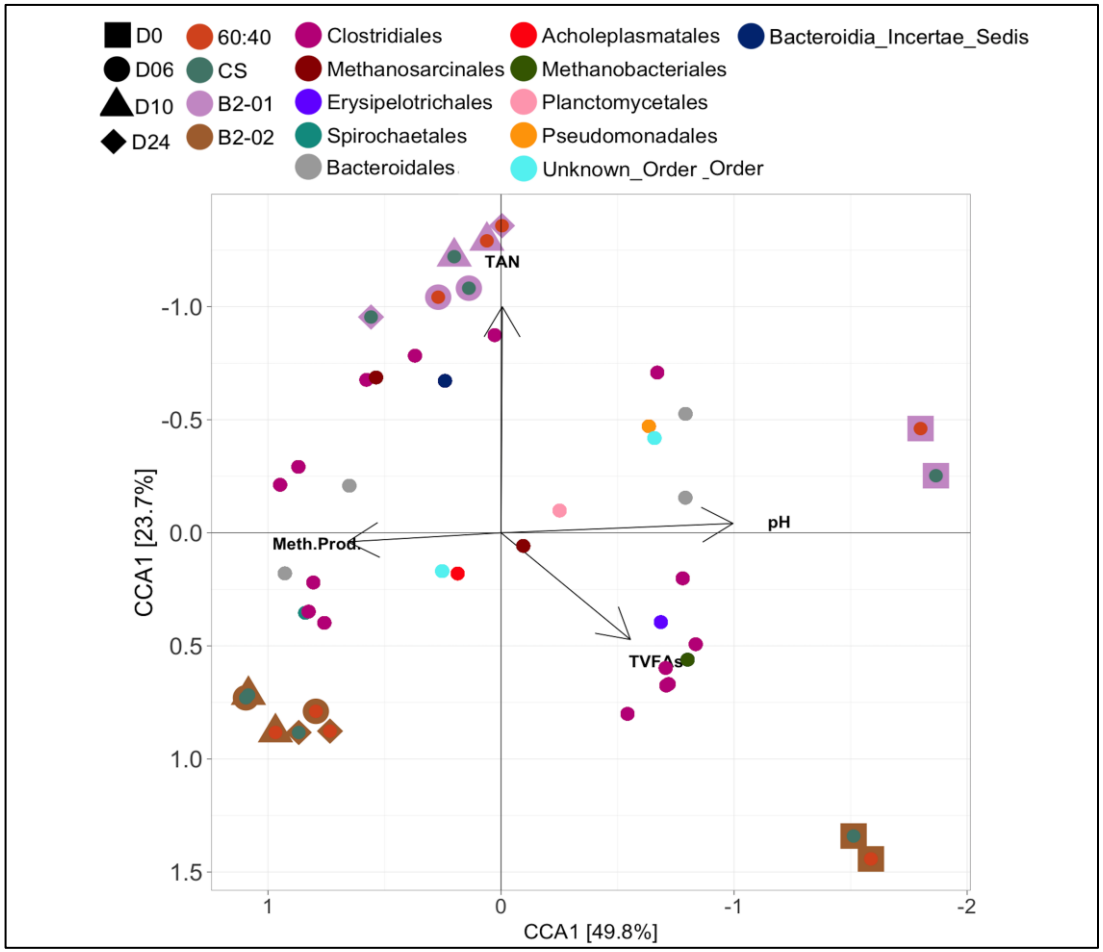

Fig. 4.9 CCA tri-plot of samples and top 30 genera coloured by order with vectors showing the correlation of process variables. Meth. Prod. =Methane production, TAN = Total Ammonia Nitrogen, TVFAs = Total Volatile Fatty Acids

CCA analysis further confirmed no phylogenetic differences between treatment groups from the samples taken. Instead, phylogenetic differences were clearly observed between experiment run and time. The plot of methane production versus time in Chapter 3 indicated that methane production for both treatments was highest between days 0 and 6. This suggests that the highest concentration of metabolites from degradable substrates were converted to methane during the first 6 days of the batch experiment. Bacterial phylotypes that peaked in relative abundance in the day 0 CS-only and CS:GS treatment samples and were associated with mid to high levels of total VFA and pH as well as decreasing levels of TAN belonged to *Christensenellaceae_R-7_group spp.* (2%), *Mogibacterium spp.* (1%), *Romboutsia spp.* (4%), *Lachnospiraceae_NK3A20_group spp.* (3%), *Peptoclostridium spp.* (2%), *Turicibacter spp.* (2%) and *Eubacterium_coprostanoligenes_group spp*. (1%). *Petrimonas spp*. (1%), *Candidatus_Cloacamonas spp.(*2%) and *Pseudomonas spp.* (1%) peaked in relative abundance within both the CS-only and CS:GS treatments and were associated with mid to high levels of total VFA and pH as well as mid to low levels of TAN. Peaks in relative abundances within the CS-only and CS:GS treatments were also observed for the bacterial phylotypes *Proteiniphilum spp.* (0.9%) and *Clostridium_sensu_stricto_1 spp.* (4%) at similar levels of total VFA and pH but mid to high levels of TAN. Peaks in relative abundances for bacterial phylotypes in the day 0 samples were also associated with a peak in relative abundance in *Methanobrevibacter spp.* (42%) and *Methanosarcina spp.* (7%) within the archaeal community of both the CS-only and CS:GS treatments when TAN levels were decreasing; suggesting a preference of these archaeal phylotypes for lower TAN concentrations (Appendix 7).

Bacterial phylotypes associated with mid to high levels of methane production and decreasing levels of TAN, pH and total VFA, that peaked in relative abundance on days 6 within both the CS-only and CS:GS treatment samples, belonged to *Ruminococcaceae_UCG-010 spp.* (0.8%), *Sedimentibacter spp.* (2%), *Treponema_2 spp.* (4%), *Mobilitalea spp.* (0.9%) and *Ruminofilibacter spp.* (4%). High methane production at mid to low TAN levels, decreasing pH and total VFA was associated with bacterial phylotypes belonging to *VadinBC27 spp.* (5%,6%), *Ruminiclostridium_1 spp.* (2%) and *Syntrophomonas spp.* (2%)

peaking in relative abundances within both the CS-only and CS:GS treatments. *Caldicoprobacter spp.* (2%) peaked in relative abundance within the CS-only and CS:GS treatments at mid to high levels of methane production and TAN as well as decreasing levels of pH and total VFA.

Mid to low levels of methane production and decreasing levels of TAN, pH and total VFA were associated with respective peaks in relative abundance within the days 10 to 24 CS-only and CS:GS treatment samples for phylotypes belonging to *Acholeplasma spp* and *Candidatus_Caldatribacterium spp.* Peaks in relative abundance in bacterial phylotypes belonging to *Mariniphaga spp*. and *Fastidiosipila spp*. were associated with mid to low levels of methane production and mid to high levels of TAN as well as decreasing levels of pH and total VFA. Archaeal phylotypes belonging to *Methanosaeta spp.* (44%, 42%) peaked in relative abundance in the CS-only and CS:GS day 24 treatment samples respectively during mid to high levels of TAN and methane production and decreasing pH and total VFA. Apparent couplings between bacterial phylotypes (Fig. 4.9 and Fig. 4.10) as evidenced by the proximity of *Syntrophomonas spp.* to *Ruminiclostridium_1 spp* and *VadinBC27 spp.* on the plots; signifying that they peaked in relative abundance in unison.

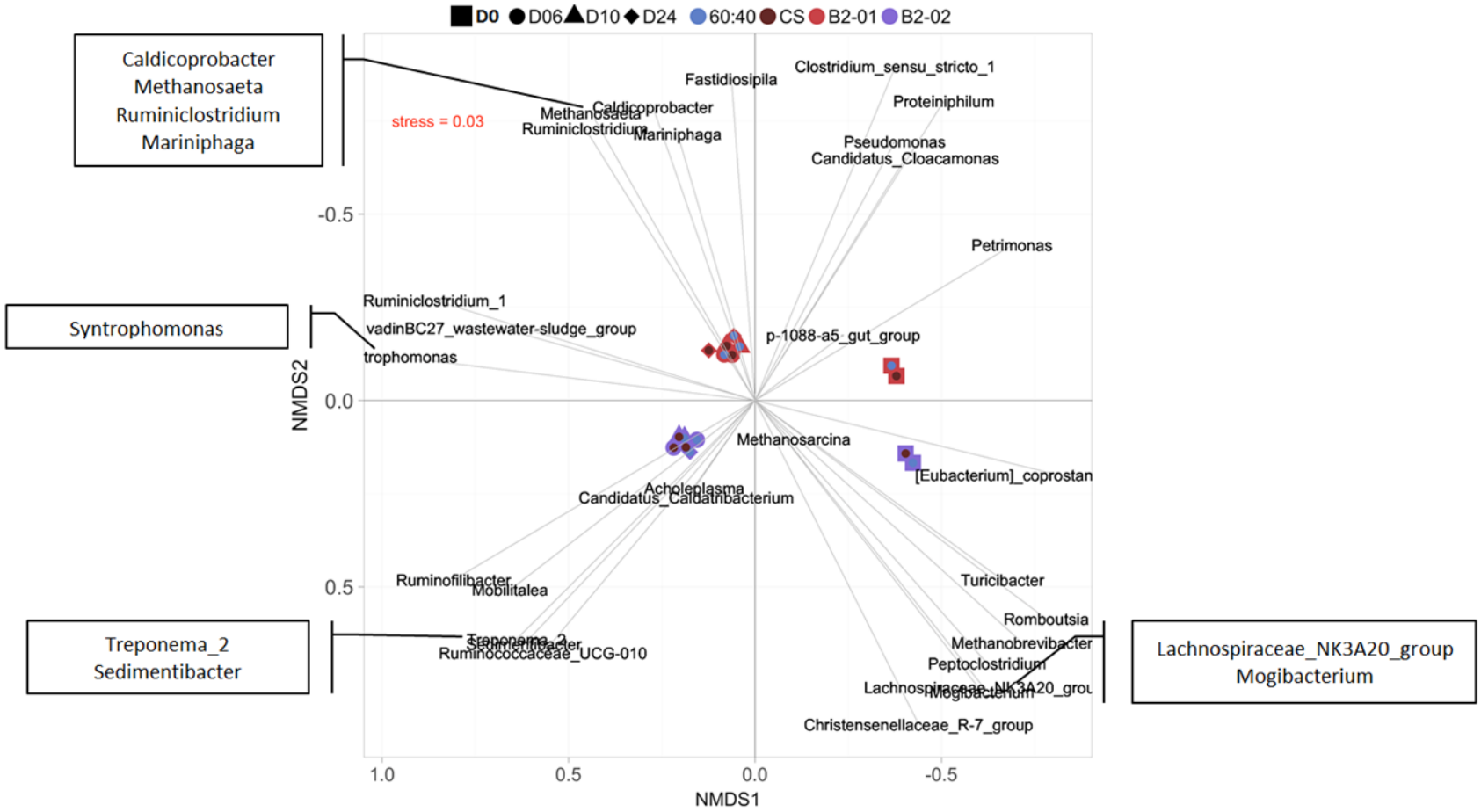

Fig. 4.10 Non-metric dimensional scaling (NMDS) of CS and 60:40 treatments and the top 30 genera observed at least once in 10% of all samples

Bacterial-archaeal apparent coupling was evidenced by the proximity of *Ruminiclostridium spp*., *Mariniphaga spp., Caldicoprobacter spp.* and *Methanosaeta spp* as well as *Romboutsia spp*., *Peptoclostridium spp.* and *Mogibacterium spp.* and *Methanobrevibacter spp*. in the NMDS plot (Fig. 4.10). This proximity suggests a close relationship between producers and consumers of metabolites generated during the AD process.

4.6 Discussion

In both experiments, the microbial community degraded soluble fractions from the VS obtained from the feed and inoculum. A 2:1 ratio of inoculum VS: feed VS was used in the batch experiments to avoid inhibition of the batch process (VDI, 2006). The CS:GS treatment produced 34% higher methane than the CS treatment ($p<0.05$). However there were no significant phylogenetic differences between treatments samples taken. According to the plot of methane production versus time for each treatment in Chapter 3, the majority of the difference in methane production between treatments was observed within the first 5 days of the batch experiment. The sampling method was developed using the cumulative BMP curve to determine the days (0, 6, 10 and 24) that samples were taken for analysis. However subsequent analysis of the data suggests that a fifth sampling time, taken between days 0 and 6 might have captured phylogenetic differences between the treatments that may have been associated with significantly different levels of methane produced. Further research is required to determine whether decreasing the sampling interval between days 0 to 6 will produce results different to what was observed during this current study.

4.6.1 General composition of the microbial community

The relative composition of the CS and CS:GS treatments compared favourably with findings by Wirth *et al.* (2012) who characterised the microbial community of an AD plant processing pig manure and plant biomass and reported the five- most dominant phyla as *Firmicutes*, *Proteobacteria*, *Bacteroidetes*, *Mollicutes* and *Actinobacteria*. Nelson *et al.* (2011) conducted a meta-analysis of microbial communities found in AD and reported *Firmicutes*, *Bacteroidetes*, *Chloroflexi* and *Proteobacteria* were among the dominant phyla. This was also

supported in findings by Theuerl *et al.* (2015) and Ziganshin *et al.* (2013), who also characterised the microbial communities digesting farm waste at mesophilic temperatures. In the current study, *Firmicutes* (46%), *Bacteroidetes* (26%) were the major phyla and *Proteobacteria* (4%), *Atribacteria* (3%), *Actinobacteria* (3%) were the minor phyla identified in both treatments. The dominance of *Firmicutes* and *Bacteroidetes* within the microbial community compared well with the literature, with some phylotypes belonging to these phyla being known for their efficient cellulolytic and saccharolytic capabilities (Nelson *et al.*, 2011; Regueiro *et al.*, 2012; Wirth *et al.*, 2012; Theuerl *et al.*, 2015). The composition of phyla identified within this study is therefore consistent with findings within the literature.

The relative abundance of lactic acid bacteria (LAB) belonging to *Lactobacillus spp.* was 6 times higher in the co-digested treatment than the cattle slurry only treatment (i.e. 0.06% for the CS:GS versus 0.01% for the CS-only treatment). *Lactobacillus spp.* were likely to be artefacts of the grass ensiling process since *Lactobacillus plantarum* is an active bacterial strain used in the Ecosyl 100 ensiling agent that was applied (Ecosyl, 2018). Some RSVs belonging to *Lactobacillus spp.* were identified as *L. ruminis* strain NBRC 102161 (Sequence ID NR_041611.1, Identity 99%) and *L. pontis* strain LTH 2587 (Sequence ID NR_036788.2, Identity 98%). However *L. acetotolerans* strain JCM 3825 (Sequence ID NR_117073.1, Identity 100%), a homofermentative, facultative anaerobe was more prevalent (Li and Nishino, 2011). Li and Nishino (2011) also identified *L. acetotolerans* in maize silage that was treated with a cocktail of lactic acid bacteria which included *L. plantarum*. They noted that *L. acetotolerans* were found in ensiling bunkers where the samples were taken at a depth of approximately 0.5m but were not present in samples taken at greater depths. This is consistent with the findings of this research where GS samples were taken at a similar depth as indicated in Chapter 3. *Lactobacillus acetotolerans* strains grow optimally at a pH ranging between 3.3 - 6.6 and temperatures between 23° - 40°C (Entani *et al.*, 1986). The relatively low relative abundance of *Lactobacillus spp* was likely resulted from inhibited growth due to the AD process pH which ranged between 7.1 and 7.5 as indicated from the results in Chapter 3.

4.6.2 The role of dominant bacteria

Hydrolysis was associated with fermenting bacteria of the order *Clostridiales*, *Bacteroidales*, *Pseudomonadales*, *Planctomycetales*, *Spirochaetales*, *Erysipelotrichales* and phylotypes of genus *Candidatus_Cloacamonas* spp. Limam *et al.* (2014) conducted a mesophilic batch experiment using ^{13}C-cellulose as substrate and reported the members of *Candidatus_Cloacamonas spp.* were involved in anaerobic cellulose degradation. A species belonging to *Candidatus_Cloacamonas*, *Candidatus_Cloacamonas acidaminovorans* has expressed genes similar to those involved in syntrophic propionate oxidation (Ahlert *et al.*, 2016). More research is therefore required to determine the role *Candidatus_Cloacamonas spp.* play within the AD batch degradation of lignocellulosic biomass. The temporal phylogenetic difference between day 0 and 6 to day 24 of the CS-only and CS:GS treatments was partly due to an increase in relative abundance in day 0 samples of *Planctomycetales spp., Pseudomonadales spp.* and *Erysipelotrichales spp.* while bacteria of the order *Bacteroidia Incertae Sedis spp., Spirochaetales spp.* and *Acholeplasmatales spp.* increased in relative abundance within the day 6-24 CS-only and CS:GS samples.

Bacteria of the order *Clostridiales* and *Bacteroidales* have been associated with the AD of agricultural waste and the degradation of lignocellulosic biomass (Nelson *et al.*, 2011; Wirth *et al.*, 2012; Mata-Alvarez *et al.*, 2014; Theuerl *et al.*, 2015). BLASTing RSVs from day 0 samples related to *Romboutsia spp.* of the order *Clostridiales* within the CS-only and CS:GS treatments against the NCBI reference database (Zhang *et al.*, 2000) identified *R. sedimentorum* strain LAM201 (Sequence ID NR_134800.1, Identity 100%). *R. sedimentorum* strain LAM201 utilises monosaccharides, disaccharides as a carbon source and can produce acetate, ethanol, isobutyric and isovaleric acids as products of glucose fermentation (Wang et al., 2015). Another species, *R. timonensis* strain DR1 (Sequence ID NR_144740.1, Identity 100%) was recently isolated from the human gut (Ricaboni *et al.*, 2016) but has not yet been defined. A bacterium belonging to *Clostridium_sensu_stricto_1 spp*., *Clostridium disporicum* strain DS1 (Sequence ID NR_026491.1, Identity 100%) that was identified within this current study, was reported to possess saccarolytic capabilities and can also degrade xylose with acetic and lactic acid as its major products of fermentation (Horn, 1987).

Also identified was *C. butyricum* strain VPI3266 (Sequence ID NR_042144.1, Identity 100%) which is a degrader of complex carbohydrates (Biebl and Spröer, 2002). Species belonging to phylum *Bacteroidetes* were identified by BLASTing RSVs from day 0 CS-only and CS:GS samples which were taxonomically assigned to the genera *Proteiniphilum spp.* and *Petrimonas spp.* using the Silva123 reference database.

Proteiniphilum acetatigenes strain TB107 (Sequence ID NR_043154.1, Identity 94-95%), a proteolytic bacterium that was initially isolated from a mesophilic reactor which metabolises L-arginine, pyruvate, glycine and peptone to produce acetic acid and NH_3 (Chen and Dong, 2005) and *Petrimonas sulfuriphila* strain BN3 (Sequence ID NR_042987.1, Identity 95%), a bacterium that ferments intermediate metabolites of complex carbohydrates to produce acetate, H_2 and CO_2 (Grabowski et al., 2005) were identified by the NCBI online database. However RSV for both *Proteiniphilum spp.* and *Petrimonas spp.* showed a higher similarity to a novel bacterium, *Fermentimonas caenicola* strain ING2-E5B (Sequence ID NR_148809.1, Identity 98-100%), which was isolated from a laboratory-scale mesophilic digester co-digesting cow and pig manure with maize silage (Hahnke *et al.*, 2016). *F. caenicola* strain ING2-E5B ferments carbohydrates and complex proteinaceous substrates. Also identified were the bacteria *Pseudomonas caeni* strain HY-14 (Sequence ID NR_116388 .1, Identity 99%) a denitrifying bacterium isolated from the sludge of an ammonium-oxidising AD system and *P. caeni* strain HY-14, a bacterium that utilises caprate and malate as its sole carbon source (Xiao *et al.*, 2009).

Sun *et al.* (2015) identified bacterial phylotypes of the order *Erysipelotrichales* at an AD plant operating at mesophilic temperatures, processing cattle manure (CM) and CM co-digested with straw. However no description as to its function was provided. This study was able to identify two species belonging to this order by BLASTing RSVs using the NCBI database. *Kandleria vitulina* strain JCM 1143 (Sequence ID NR_041305.1, Identity 100%) metabolises complex carbohydrates to produce acetic and lactic acids (Salvetti *et al*., 2011). *Turicibacter sanguinis* strain MOL 361 (Sequence ID NR_0288161, Identity 99%) *is* a gram positive bacteria that metabolises Maltose and 5-ketogluconate to produce lactic and acetic acid (Bosshard *et al.*, 2002).

Bacterial phylotypes associated with days 6-24 samples belonged to the orders *Clostridiales*, *Bacteroidales*, *Bacteroidia_Incertae_Sedis*, *Spirochaetales* and *Acholeplasmatales*. Two strains of *Syntrophomonas spp.,* bacterial phylotypes that belong to the order *Clostridiales*, were identified in both the CS-only and CS:GS day 6-24 samples. *Syntrophomonas bryantii* strain CuCal (Sequence ID NR_104881.1, Identity 97%) and *S. zehnderi* strain OL-4 (Sequence ID NR_044008.1, Identity 97%), syntrophically degrade butyrate, 2-methyl butyrate and long chain VFA to acetic acid and propionate via syntrophic pathways involving hydrogenotrophic methanogens (Wu *et al.*, 2006; Sousa *et al.*, 2007; Stams and Plugge, 2009; Mathai *et al.*, 2015). Another bacterium that was identified which belonged to the order *Clostridiales* was *Sedimentibacter saalensis* strain ZF2 (Sequence ID NR_025498.1, Identity 95%) a proteolytic bacterium that is associated with the hydrolysis of polypeptides and amino acids and was also found in AD systems processing agricultural waste (Sun *et al.*, 2015; Theuerl *et al.*, 2015). *S. saalensis* strain ZF2 is also purported to metabolise pyruvate and produces acetate and butyrate as fermentation products (Breitenstein *et al.*, 2002).

Cardinali-Rezende *et al.* (2016) identified bacteria of the genus *Fastidiosipila* from samples taken from the AD of municipal solid waste (MSW) and described them as proteolytic, non-saccarolytic fermenters which produce small amounts of acetic and butyric acids (Falsen *et al.*, 2005) which appeared to prefer high ammonium ion concentration levels (greater than 4g/L). The relative abundance of *Fastidiosipila spp.* in this current study attained a maxima within the CS-only (3%) and CS:GS (4%) treatments during day 24 of the batch experiment possibly due to degrading proteins and amino acids from dead bacteria albeit at a lower concentration levels of TAN. Another bacteria identified that were associated with days 6-24 CS-only and CS:GS treatment samples were *Mobilitalea sibirica* strain P3M-3 (Sequence ID NR_134091.1, Identity 95%) which is a halotolerant polysaccharide degrader that produces acetate, ethanol, H_2 and CO_2 (Podosokorskaya *et al.*, 2014). *Ruminofilibacter xylanolyticum* and *VadinBC27 spp.* are purported to be efficient degraders of xylan, cellulose and hemicellulose in anaerobic environments (Nissilä *et al.*, 2012; Xie *et al.*, 2014; Morrison *et al.*, 2017) and are both members of the order *Bacteroidales* which peaked in relative abundance in the day 10 CS-only (24%) and CS:GS samples (23%). Chapter 3 indicates that the highest methane production occurred within the first 10 days of the batch experiment

when the relative abundances of *Sedimentibacter spp*., *Syntrophomonas spp.*, *Treponema_2 spp.* and *VadinBC27 spp.* likely attained their respective maxima.

Relative abundances of bacterial phylotypes belonging to orders *Acholeplasmatales*, *Spirochaetales* and *Bacteroidia_Incertae_Sedis* attained their respective peaks during the latter stages of the batch experiment when substrate availability was limiting and methane production was at mid to low levels. The biomass consisted of more recalcitrant particles according to the cellulose, hemicellulose and lignin composition for days 10 and 24 (results presented in Chapter 3). All of the species identified within this study that belonged to the orders *Acholeplasmatales*, *Spirochaetales* and *Bacteroidia_Incertae_Sedis* were reported as carbohydrate degraders in the literature. *Acholeplasma morum* strain 72-043 (Sequence ID NR_042959.1, Identity 92%) degrades glucose and hydrolyses esculin (Rose *et al.*, 1980). *Mariniphaga anaerophila strain* Fu11-5T metabolises simple and complex carbohydrates to produce succinic and acetic acids (Iino *et al.*, 2014) and *Treponema zuelzerae* strain DSM 1903 and *T. brennaborense* strain DD5/3 utilise carbohydrates to produce lactic, acetic and succinic acids (Veldkamp, 1960; Schrank *et al.*, 1999). Most of the RSVs that were identified in this study were purported to play a role in the degradation of complex carbohydrates contained within the cellulose, hemicellulose content of the CS-only and CS:GS feedstocks processed by the AD batch operated system. This is consistent with the findings in Chapter 3 where carbohydrates were the highest organic fraction (75-78 %TS) of the CS-only and CS:GS treatments.

Bacterial phylotypes belonging to the minor phyla *Proteobacteria* and *Actinobacteria* may have also played important roles during days 6-24 of the batch experiment. Humic acids can inhibit the AD process and are abundant in AD systems treating manure and agricultural waste (Fernandes *et al.*, 2015; Azman *et al.*, 2017). Azman *et al.* (2017) reported a 40% reduction in the hydrolytic efficiency and a reduction in the relative abundance of hydrogenotrophic methanogens within an AD system treating cellulose and xylan while increasing the level of humic acid over time. The addition of enzymes was reported to have counteracted the negative effect of humic acid addition to the AD system.

In this current research RSVs from the phylum *Actinobacteria* were identified as *Corynebacterium humireducens* strain MFC-5 (Sequence ID NR_117357.1, Identity 100%), an alkaliphile that metabolises carbohydrates and uses antraquinone-2,6-disulfonate (humic acid) at pH 7-11 as the sole terminal electron acceptor under anaerobic conditions (Wu *et al.*, 2011). Lin *et al.* (2013a) increased VFA from the hydrolysis of pig manure at a pH 10 and enriched with *Corynebacterium humireducens.* Therefore improved biogas production and quality is likely if CS:GS feedstocks are enriched with this bacterium.

The RSVs BLASTed against the NCBI database identified three syntrophs responsible the degradation of intermediate metabolites produced by the anaerobic degradation of the CS-only and CS:GS treatments. *Syntrophus aciditrophicus* strain SB (Sequence ID NR_102776.1, Identity 100%) metabolises VFA, amino acids, sugars, hydrocarbons and benzoate in syntrophy with partner hydrogen and/or formate consuming methanogens or sulfate reducers (McInerney *et al.*, 2007; Kato *et al.*, 2015).

Kato *et al.* (2015) identified *S. aciditrophicus* which appeared to degrade benzoate to acetate H_2/CO_2 in a culture enriched with lignin-derived mono-aromatic compounds. Acetate and H_2/CO_2 are then converted to CH_4 via the purported pathway:

$$C_7H_5O_2^-(benzoate) + 7.75H_2O \rightarrow 3.75CH_4 + 3.25HCO_3^- + 2.25H^+ \quad \text{Eq. 4.3}$$

Pelotomaculum isophthalicicum strain JI (Sequence ID NR_041320.1, Identity 96%) was another benzoate degrading syntroph identified which can also degrade 3-hydroxybenzoate and phthalate isomers in co-culture with *Methanospirillum hungatei*. Chen *et al.* (2008) note that aromatic carboxylic acids were mildly toxic to methanogens but phenolic acids in high concentration levels can inhibit methane production. Further research is required to determine whether *Syntrophus aciditrophicus* strain SB and or *Pelotomaculum isophthalicicum* strain JI and *Cryptanaerobacter phenolicus* strain LR7.2 (Sequence ID NR_025757.1, Identity 95%), a bacterium identified in this current study that transforms phenol into benzoate (Juteau *et al.*, 2005), played a role in the marginal degradation of lignin content within the CS and CS:GS treatments reported in Chapter 3.

The relative abundance of *Pelotomaculum schinkii strain HH* (Sequence ID NR_044877.1, Identity 94%) increased 3-fold and 10-fold in the CS-only and CS:GS treatments respectively from days 0 to 24. *P. schinkii strain HH* oxidises propionate via a randomising methylmalonyl-CoA pathway to convert propionate to acetate in syntrophy with *Methanospirillum hungatei* (De Bok *et al.*, 2005). The general increase in relative abundance during days 0 to 24 of *Methanospirillum spp.* (0.7-4%) and (0.8-5%) of the archaeal community within the CS-only and CS:GS treatments was concomitant with a three and tenfold change in relative abundance for *Pelotomaculum schinkii strain HH* and suggests the occurrence of the syntrophic oxidation of propionate during the duration of the batch experiment. *Smithella propionica* strain LYP (Sequence ID NR_024989.1, Identity 97%) metabolises propionate in syntrophy with a partner hydrogen and/or formate utilising methanogen via a non-randomising pathway that dismutates propionate to butyrate and acetate (De Bok *et al.*, 2005; Stams and Plugge, 2009; Ahlert *et al.*, 2016). Butyrate is then converted to acetate via β-oxidation (De Bok *et al.*, 2001). The presence of *Smithella propionica* strain LYP and *Pelotomaculum schinkii strain HH* therefore indicates that redundant syntrophic pathways for propionate oxidation were present in both the CS-only and CS:GS treatments.

4.6.3 The role of dominant archaea

Molecular hydrogen is produced as bacteria, such as the ones previously described, degrade the cellulose and hemicellulose contained within the CS-only and CS:GS treatments during the acidogenesis and acetogenesis phases. Hydrogen inhibits the hydrogenase of H_2-producing bacteria and favours the formation of more reduced metabolites such as butyrate over acetate (Bułkowska *et al.*, 2012). Therefore low hydrogen partial pressures and/or levels of formate are important parameters within the AD process because they provide favourable thermodynamic conditions whereby key metabolic intermediates such as propionate and butyrate can be oxidised to acetate (Stams *et al.*, 2003; Angenent *et al.*, 2004; Bułkowska *et al.*, 2012). Ward *et al.* (2008) note that propionic and butyric acid accumulation can inhibit methanogens and propionate concentration levels of 3g/L can lead to digester failure.

Hydrogenotrophic methanogens play a crucial role as hydrogen and or formate consumers in AD systems thus maintaining low levels of H_2 and or formate (Demirel and Scherer, 2008). The relative abundance of hydrogenotrophic methanogens was greatest during days 0-6 for both treatment samples. This finding is consistent with findings of Moset *et al.* (2015) and Walter *et al.* (2016) who observed hydrogenotrophic methanogenesis was dominant in reactors co-digesting manure and cellulosic biomass. *Methanosarcina spp.* however reduced in relative abundance from day 6 to 24 in both the CS-only and CS:GS treatments as substrate became limited. *Methanosarcina spp.* consisted of *Methanosarcina soligelidi* strain SMA-21 (Sequence ID NR_109423.1, Identity 98%), which grows on H_2/CO_2, acetate and methanol to methane (Wagner *et al.*, 2013) and *Methanosarcina subterranea* strain HC-2 (Sequence ID NR_134763.1, Identity 100%) grows on methanol, mono- , di- and tri-methylamine and dimethylsulphide but not on H_2/CO_2 or acetate. Methanol is a product of the degradation of pectin in plant material within the CS and CS:GS treatments (Jørgensen *et al.*, 2007; Sprenger *et al.*, 2007; Nizami *et al.*, 2009). *Methanosarcina spp.* is therefore mixotrophic since its versatility facilitates the conversion of H_2/CO_2, acetate and methyl compounds to methane via hydrogenotrophic, acetotrophic and methylotrophic methanogenesis pathways (Garcia, 1990).

Methanosphaera cuniculi strain 1R7 (Sequence ID NR_104874.1, Identity 99%) was another archaeon identified in the current study which requires both H_2 and methanol for growth (Biavati *et al.*, 1988; Glass and Orphan, 2012). Relative abundances of *M. cuniculi* strain 1R7 however reduced from day 0 to day 24 likely due to being outcompeted for substrate by *Methanosarcina spp. Methanoculleus palmolei* strain DSM 4273 (Sequence ID NR_028253.1, Identity 100%) was isolated from a biogas plant treating wastewater from a palm oil mill and grows on H_2/CO_2, formate, 2-propanol/CO_2, 2-butanol/CO_2 and cyclopentanol/CO_2 (Zellner *et al.*, 1998). Reductions in relative abundance from day 0 to day 6 for the CS-only and CS:GS treatments is also likely due to being outcompeted for substrate by *Methanosarcina spp.* and obligate acetotrophs such as *Methanosaeta spp. Methanospirillum hungatei* strain JF-1 (Sequence ID NR_074177.1, Identity 96%) was isolated from sewage sludge and grows on H_2/CO_2 and formate (Gunsalus *et al.*, 2016) and partners with syntrophs to oxidise butyrate and propionate (De Bok *et al.*, 2001).

The relative abundance of *M. hungatei* strain JF-1 increased from day 0 to day 24 within the CS-only and CS:GS treatments. This increase was concomitant with increases in relative abundance in *Smithella spp.* during days 0 to 24 for both treatments and suggests that syntrophy benefitted both microbial organisms even as substrate availability was limiting. *Methanobrevibacter spp.* were the most dominant hydrogenotrophic methanogen within the archaeal communities for the CS-only and CS:GS treatments. Both treatments contained *Methanobrevibacter olleyae* strain KM1H5-1P (Sequence ID NR_042785.1, Identity 99%) and *Methanobrevibacter millerae* strain ZA-10 (Sequence ID NR_042785.1, Identity 100%), both of which convert H_2/CO_2 and formate to methane (Rea *et al.*, 2007). *M. millerae* was one of four formate utilising archaea isolated from the ovine and bovine rumen (Rea *et al.*, 2007). The relative abundance for *Methanobrevibacter spp.* reduced from days 6 to 24 in the CS-only and CS:GS treatments as substrate availability became limiting. *Methanobacterium ferruginis* strain Mic6c05 was also identified within the CS-only and CS:GS treatments and only grows on H_2 (Mori and Harayama, 2011). *Methanosaeta spp.,* obligate acetotrophic methanogens (Patel, 1984; Patel and Sprott, 1990), were also identified within the archaeal community of the CS-only and CS:GS treatments with all RSV being associated with the strain *Methanosaeta concilii* strain GP6 (Sequence ID NR_102903.1, Identity 99%). *Methanosaeta spp.* increased in relative abundance from days 6 to 24 in both treatments at the expense of dominant hydrogenotrophic methanogens such as *Methanosarcina spp*.

Evenness decreased with time as the dominant bacterial and archaeal subpopulations outcompeted other strains for available substrate. Stams *et al.* (2003) note that competitive interactions between microorganisms can be categorised into either kinetic competition or thermodynamic competition. They state that fermentative competitive interactions are typically due to differences in microbial growth rates and are therefore based on kinetic competition. *Methanosaeta spp*. and obligate hydrogenotrophic methanogens such as *Methanobrevibacter spp., Methanoculleus spp.*, and *Methanobacterium ferruginis* strain Mic6c05 as well as mixotrophic methanogens namely *Methanosarcina spp.* were present in both treatments.

Having a lower maximum specific growth rate (μ_{max}) and half saturation (K_s) concentration for acetate gives *Methanosaeta spp.* a kinetic advantage when competing for substrate at low levels of acetate concentration (Conklin *et al.*, 2006; Demirel and Scherer, 2008; Chen and He, 2015). This resulted in an increase in relative abundance of obligate acetoclastic methanogens *Methanosaeta spp.* from days 0 – 24 for each treatment as acetate availability became limiting. Therefore the comparatively low relative abundance of *Methanosarcina spp.* infers it was outcompeted by *Methanosaeta spp.* via thermodynamic competition.

The identification of *Methanosarcina subterranea* strain HC-2 (Sequence ID NR_134763.1, Identity 100%), and obligate methylotrophic methanogen, in both the CS-only and CS:GS treatments (Shimizu *et al.*, 2015) confirms that the three main methanogenesis pathways were present (Glass and Orphan, 2012; Borrel *et al.*, 2016) namely:

- Methylotrophic methanogenesis which involves the production of methane from methyl sulfides, methyl amines and alcohols.
- Hydrogenotrophic methanogenesis where methane is produced from the reduction of H_2/CO_2.
- Acetotrophic methanogenesis, considered the main pathway and involves methane production from acetate.

Very little is known about archaeal phylum *Miscellaneous_Crenarchaeotic_Group* (MCG) also known as *Candidatus Bathyarchaeota*. Their relative abundance increased 4- 5-fold between day 6 to day 24 within the CS-only and CS:GS treatments and was concomitant with an increase in the relative abundance of *Methanosaeta spp.* over the same time period. Weber *et al*. (2017) used ^{13}C assimilation into RNA via stable isotope probing to examine the association of archaea assigned to MCG with the anaerobic oxidation of methane (AOM) in low sulfate iron rich environments and reported that while AOM was active, some ^{13}C from the ^{13}C – labelled methane was incorporated into the 16S rRNA of all of the clones belonging to MCG.

He *et al.* (2016) state that phylotypes belonging to MCG are capable of AOM and also stated that some lineages may be capable of homoacetogenesis and the degradation of proteins, cellulose, chitin and aromatic compounds since genes were identified that encoded metabolic pathways for:

- Acetyltransferase and acetate kinase for acetate production/assimilation;
- Glycolysis/gluconeogenesis and beta-oxidation;
- Benzoyl degradation.

Further research is required to verify whether AOM, homoacetogenesis as well as the metabolism carbohydrates and aromatic compounds were indeed present during the later stages of the AD process in batch mode. The 4-5 fold increase in relative abundance of *Candidatus Bathyarchaeota spp.* during days 6 to 24 for both treatments may therefore lead to a clearer understanding of the degradation of highly recalcitrant organic matter and improved biogas production and quality.

4.6.4 The effect of microbial community dynamics on pH, TAN and VFA

During the AD process, effluent pH reduced from depolymerisation of complex carbohydrates such as xylose, arabinose, mannose and cellulose from the lignocellulosic material (Jørgensen *et al.*, 2007), by fermenting bacteria primarily of the order *Clostridiales*, *Bacteroidales*, *Spirochaetales*, *Bacteroidia_Incertae_Sedis* and *Erysipelotrichales*. These complex macromolecules were depolymerised by enzymes (e.g. cellulase, lipase, protease and amylase) excreted by fermenting bacteria (Bajpai, 2017). The increasing concentration of total VFA that resulted from the hydrolysis and acidogenesis steps reduced effluent pH. With the highest relative abundance within the archaeal community of both treatments, *Methanobrevibacter spp.* peaked in relative abundance during high levels of total VFA. This suggests that *Methanobrevibacter spp.* facilitated low levels of H_2 partial pressures and formate concentration during the period of maximum methane production (days 0 to 6) to facilitate propionate and/or butyrate oxidation (Stams and Plugge, 2009). The reduction in pH was however counter-balanced in part by the increase in ammonium hydroxide which reacts with CO_2 to produce bicarbonate as a result of the biomethanation process (Möller and Müller, 2012).

Batstone *et al.* (2002) stated the main acid/base pairs of equilibrium reactions involved in the AD process such as CO_2/HCO_3^- (pKa = 6.35) and VFA/VFA^- (pKa≈4.8) and notes that CO_2, a gas which constitutes approximately 50% of biogas, has medium solubility. The pH range for the AD batch process for both treatments was 7.14 - 7.39 during days 6 to 24. This was within the range of 6.6 to 7.3 for methanogenesis to occur efficiently (Garcia, 1990; Demirel and Scherer, 2008). *Methanosaeta spp.* dominated in both treatment groups where levels of TAN concentration ranged from 1.0 -2.0g NH_4^+-N/L between days 6 to 24 of the batch experiment. This finding is consistent with Fotidis *et al.* (2014a) who reported hydrogenotrophic methanogenesis was the main pathway for concentrations of ammonia ranging from (2.8-4.57g NH_4^+-N/L) and acetotrophic methanogenesis was dominant at TAN levels of (<1.21g NH_4^+-N/L) for mesophilic full scale AD plants. A higher upper threshold of 2.0g NH_4^+-N/L observed in this current study may be due to the acclimatisation of *Methanosaeta spp.* to increased TAN levels.

The temporal propionate accumulation observed in the CS-only treatment on day 6 and day 16 that was described in Chapter 3 was likely due to the enhanced hydrolysis and acidogenesis stages caused by the use of distilled water of pH 4.5 to make the CS-only and 60:40 feeds. TAN levels ranging from 1.7 to 14g NH_4^+-N/L can inhibit methanogens with *Methanosaeta spp.* being more sensitive to Increased TAN levels than *Methanosarcina spp.* (Chen *et al.*, 2008; Ward *et al.*, 2008). A temporal inhibition of hydrogenotrophic methanogens would have therefore led to a disruption in H_2 partial pressures which would likely affect the propionate oxidising bacteria. The diauxic nature of the degradation of cellulosic biomass as seen by the daily methane production plots in Chapter 3 and supported by the literature (VDI, 2006; Walter *et al.*, 2016) resulted in a subsequent temporal accumulation of propionate on day 16 of experimental run B2-02. Therefore temporal process imbalances within an AD system operating at the upper threshold of TAN level concentrations are likely if the hydrogenotrophic methanogens are unable to maintain low H_2 and/or formate levels.

4.6.5 Conclusions

The salient points from this chapter are:

- The phylogenetic similarity observed between treatments was likely as a result of insufficient sampling points between days 0 and 6 of the batch experiment when methane production was the highest. Most of the bacteria identified from the two main phyla *Firmicutes* and *Bacteroidetes* were purported to play a role in the degradation of carbohydrates;
- Temporal phylogenetic variations were observed between days 0 and days 6-24; highlighting that a significant change within the microbial community occurred within the first 6 days of the batch experiment. Phylogenetic differences between days 0 and 6-24 were partly due to the increase in relative abundance of *Bacteroidia Incertae Sedis spp.*, *Spirochaetales spp.* and *Acholeplasmatales spp.* increased in relative abundance within both the CS-only and CS:GS samples;
- 90% of the variation observed between phylogenetically distinct groups of samples was due to microbial community changes associated with variations in the AD process parameters i.e. total VFA, TAN and pH. Relative mixing of the biomass and inoculum with an AD batch operation must be done in order to keep TAN levels within a range that is not inhibitory to methanogens i.e. <2g NH_4^+-N/L;
- Further research is required to investigate whether *Corynebacterium humireducens* strain MFC-5 from phylum *Actinobacteria* can be exploited to metabolise carbohydrates and reduce humic acid levels in AD batch operated systems thereby increasing biogas production and quality;
- Further research is also required to investigate whether *Syntrophus aciditrophicus* strain SB, *Pelotomaculum isophthalicicum* strain JI, *Cryptanaerobacter phenolicus* strain LR7.2 along with archaea belonging to *Candidatus Bathyarchaeota* can be exploited to improve lignocellulosic degradation and biogas production and quality within AD batch operated systems.

- The dominant archaea were *Methanosaeta spp., Methanobrevibacter spp., Methanosarcina spp., Methanobacterium spp.* and *Methanoculleus spp.* which indicated the existence of the 3 main methanogenesis pathways i.e. acetotrophic, hydrogenotrophic and methylotrophic methanogenesis. Evenness decreased from days 0-24 as the relative abundance of acetoclastic methanogens became the most dominant of the three pathways during days 6-24;
- The presence of *Smithella propionica* and *Pelotomaculum schinkii* indicated that redundant syntrophic pathways for propionate oxidation were present in both treatments. The butyrate- and LCFA-degrading syntroph, *Syntrophomonas spp.* was also present within the CS-only and CS:GS treatments. A balance between hydrogenotrophic methanogens and propionate degraders is required to avoid process imbalances within a batch operated system.
- Further research is required to investigate the role the bacteria *Fermentimonas caenicola* strain ING2-E5B which ferments carbohydrates and proteinaceous compounds along with *Pseudomonas caen*i strain HY-14 which utilises caprate and malate as sole carbon sources in the hydrolysis of the CS and CS:GS substrates. Improving the efficiency of the hydrolysis step will lead to an overall enhancement of the AD process since hydrolysis is considered to be rate limiting in the AD of feedstock rich in complex carbohydrates.

Chapter 5 Assessing the effect of the anaerobic fermentation of grass silage and cattle slurry on AD process parameters in continuous operation

5.1 Introduction

The number of biogas plants co-digesting farmyard manure (FYM) with other waste streams has increased over the last 20 years as a consequence of the EU Renewable Energy and Waste Directives (EC, 2008; EC, 2009). Approximately 14.9 million tonnes of oil equivalent energy (Mtoe) was produced from AD plants within the EU in 2014 (Bacenetti et al., 2016); representing a 6.6% increase on the previous year. In 2013, there were 53 on-farm AD digesters in the UK; representing 21% of AD installations for bioenergy generation (Edwards *et al.*, 2015). The number of on-farm AD plants within the UK increased by 177% to 147 as of 2016 (WRAP, 2017). However, UK AD installations still trail behind those in Germany (8726) and Italy (1700) (Bacenetti *et al.*, 2016).

The increase in the deployment of AD plants to treat agricultural waste such as FYM and slurries is because the process reduces GHG emissions by decreasing the organic matter content of the waste while simultaneously producing useable heat and power requirements together with useable fertilizer (Fruergaard and Astrup, 2011; Möller and Müller, 2012; Bacenetti *et al.*, 2016). Since the calorific value of biogas is derived from its methane content, there is increasing interest in improving biogas quality from agricultural waste streams. AD systems using FYM only substrates such as CS produce low methane yields (Lehtomäki *et al.*, 2007; Frear *et al.*, 2011; Seppälä *et al.*, 2013). Biogas production and quality are limited by its low solids content, increased levels of total ammonia nitrogen (TAN) and recalcitrant biomass such as lignin and low carbon to nitrogen (C:N) ratios (Triolo *et al.*, 2013; Tsapekos *et al.*, 2017).

Low C:N ratio feedstocks such as CS however provide the requisite microbial consortia, buffering capacity and macro and micro nutrients, while higher C:N ratio feedstocks provide the carbon source required for methanation (Mata-Alvarez *et al.*, 2014; Neshat *et al.*, 2017). Co-digesting manures with crops may not lead to improved biogas production since crops with poorly lignified cell walls are readily degraded in an AD process while crops having highly lignified cell walls are not (Wang *et al.*, 2009). The use of ensiled grass as a second-generation feedstock in biogas production is an attractive alternative due to its high availability and sustainability and the fact that the ensiling process itself improves the digestibility of the grass, which can increase biogas yield by 17% (Prochnow *et al.*, 2009; Esposito *et al.*, 2012; Wall *et al.*, 2014; Tsapekos *et al.*, 2017).

Apart from co-digestion studies, research relating to the microbiology behind biogas production and quality has gained interest in recent years (Ward *et al.*, 2008; Wirth *et al.*, 2012; Theuerl *et al.*, 2015; Xie *et al.*, 2016; Tsapekos *et al.*, 2017). Although the co-digestion of lignocellulosic biomass (LB) such as cattle slurry (CS) and grass silage (GS) has already been addressed the literature correlating the physico-chemical and microbiological factors within CS:GS remains limited. Previous studies also focused on the efficiency of AD systems co-digesting CS and GS as it relates to the organic loading rate (OLR), or CS:GS ratio at which maximum biogas is produced (Lehtomäki *et al.*, 2007; Wall *et al.*, 2014; Osborne Homeky *et al.*, 2016), yet loading rates for AD systems remain between 5-6kg VS m^3/per day (Mason and Stuckey, 2016). Hydrolysis, which involves cellulolysis, is considered the rate limiting step for the anaerobic degradation of LB (Xie *et al.*, 2011; Mata-Alvarez *et al.*, 2014; Sun *et al.*, 2015; Mason and Stuckey, 2016). Volatile fatty acid (VFA) intermediates such as propionate and pH can inhibit both hydrolytic and methanogenic microbes and reduce biogas production and quality (Chen *et al.*, 2008; Ward *et al.*, 2008). Therefore the hydrolysis step must be enhanced in order to improve biogas production and quality (Ziganshin *et al.*, 2011).

Aspects of the AD process such as functions of the degrading bacteria, inhibition mechanisms and the oxidation of short chain VFA such as propionate and butyrate being not fully understood, and research into effective process modelling, control and optimisation are ongoing challenges in AD (Weiland, 2010; Curry and Pillay, 2012; Rajagopal *et al.*, 2013; Stromberg *et al.*, 2013). The biochemical methane potential (BMP) of CS-only and CS co-digested with two different levels of GS was determined by batch fermentation tests (BFTs) in Chapter 3. However, BFTs do not provide information relating to the process dynamics between VFA concentration, pH and TAN that may lead to process imbalance within a continuously fed AD system (VDI, 2006).

This study aims to highlight the physico-chemical and biological processes taking place within the co-digestion of CS and GS using semi-continuously fed, laboratory scale continuously stirred tank reactors (CSTRs) in order to improve biogas production and quality. The objectives of this research are to:

- Assess the performance of the mesophilic anaerobic co-digestion of CS with two distinct levels of GS using CSTRs at low OLR levels;
- Correlate the physico-chemical process parameters with AD process parameters in order to evaluate reactor performance;
- Identify key AD process parameters that can be used to improve biogas production and quality.

5.2 Experimental Design

An operating temperature of 37°C was chosen since most commercial AD systems operate within the mesophilic range and operating temperature can affect AD process stability (Ziganshin *et al.*, 2013; Mata-Alvarez *et al.*, 2014; Xie *et al.*, 2016). Process instability and operating temperature can affect the diversity of the microbial community (Chen *et al.*, 2008; Theuerl *et al.*, 2015). Operating an AD system at mesophilic temperature minimises the risk of instability as the rate of microbial degradation is slower (Moset *et al.*, 2015).

The three feed mixtures studied in Chapter 3, i.e. CS and CS mixed with GS (80:20) and (60:40) on a VS basis, were prepared as feed for CSTRs operating at 37°C with a hydraulic retention time (HRT) of 25 days. From the Batch BMP Experiment B2-01 and B2-02 conducted in Chapter 3, 90% of the BMP for the CS-only and CS:GS treatments was achieved within 12-15 days. The HRT of 25 days was chosen so that it was long enough to avoid VFA accumulation (Dareioti and Kornaros, 2014).

Each digester operated for 75 days or three HRTs to allow the system to attain steady-state. Each treatment used duplicate 5L CSTRs operating with a working volume of 4L. In a previous study, Wall *et al.* (2014) employed a feeding regime where reactors were only fed five days per week. For this study, reactors were fed every day of the week to more accurately mimic a commercial operating system. Two CSTR experiments each with repeated runs were conducted in order to assess the repeatability of the results. The primary focus of this study was to observe the physico-chemical and biological changes that occur during the co-digestion process. Since the changes in the inoculum can affect the microbial community within an AD system (Karakashev *et al.*, 2005; Shah *et al.*, 2014), it was decided that each experimental run would have the same inoculum source. Fresh inoculum obtained from the AD unit at Cockle Park Farm was used for each repeat run. Low Organic Loading Rates (OLRs) were also chosen in order to minimise occurrences of process imbalance. Laboratory scale CSTRs were fed with 0.5g VS per m^3/d for 75 days during experimental runs C1-01 and C1-02. Upon termination of CSTR Experiment 1, it was decided to increase the OLR because a cursory review of the reactor performances indicated that VFA levels in the effluent were very low. The OLR was therefore increased to 1g VS per m^3/d to obtain reliable VFA measurements while operating the reactors at an OLR low enough to avoid AD process instability. Therefore each reactor was fed with 1g VS per m^3/d for 75 days during experimental runs C2-01 and C2-02. The VS contribution of CS was initially calculated by volume from the primary feedstock collected from Cockle Park Farm.

The VS contribution of the GS in the CS:GS mixtures was calculated by mass. However, this method was changed for the second CSTR experiment where the CS and GS contributions were both calculated by mass since it was no longer feasible to measure the primary feedstock CS volumetrically due to its viscosity. All feeds were diluted with distilled water to deliver the required OLR in 160 ml of feed.

The process parameters biogas production, methane %, pH, Total Ammonia Nitrogen (TAN), Free Ammonia Nitrogen (FAN), Total Kjeldhal Nitrogen (TKN), chemical oxygen demand (COD), individual volatile fatty acids (VFA), volatile solids (VS) total solids (TS) and temperature were measured throughout each experimental run (Table 5.1) at time intervals in accordance with VDI (2006) and (APHA, 2005). Samples of both reactor feedstocks and effluent were taken for analysis.

Table 5.1 AD process parameters monitored throughout each experimental run.

Process Parameter	**Units**	**Measurement Frequency**
Biogas Production	ml	at least every 2 days
Methane Concentration	% volume	at least every 2 days
pH		daily
TAN	mg NH_4-N	weekly
FAN	mg NH_3-N	calculated
TKN	mg-N	weekly
COD	mg O_2/L	weekly
Temperature	$^{\circ}$C	daily
VFA	mg/L	weekly
VS	g/L	weekly
TS	g/L	weekly

Unless stated otherwise, all process parameters were reported by determining their mean values during the third HRT i.e. during days 50 to 75, i.e. when the reactors attained steady-state conditions.

5.3 Materials and Methods

The primary feedstocks of CS and GS together with the inoculum used in the CSTR experiments were obtained from a 75kWe on-farm AD facility at Cockle Park Farm, Newcastle-upon-Tyne, UK (latitude 55:15:51N; longitude 1:41:08W) that processes cattle and pig slurry. The handling and storage of both the GS and CS were conducted in accordance with the procedures previously mentioned in Chapter 3. The analyses of the physico-chemical parameters were also conducted in accordance with the methods outlined in section 3.3 of Chapter 3. Performance measures, biomethane efficiency (BE) and biodegradability (BD), previously defined in Chapter 3, were also assessed. Biomethane efficiency is defined as the quotient of specific methane production (SMP) of the substrate treated in a CSTR and the SMP of the substrate treated under AD batch conditions (Wall *et al.*, 2014).

The reactor assembly was constructed from Quickfit® compatible components and consisted of three main sections, namely the gas take-off system, feeding mechanism and stirrers.

An airtight seal was obtained on all reactors using a 5-port head plate (Thermofisher, MAF2/52) that facilitated the feeding and gas take-off assemblies (Fig. 5.1).

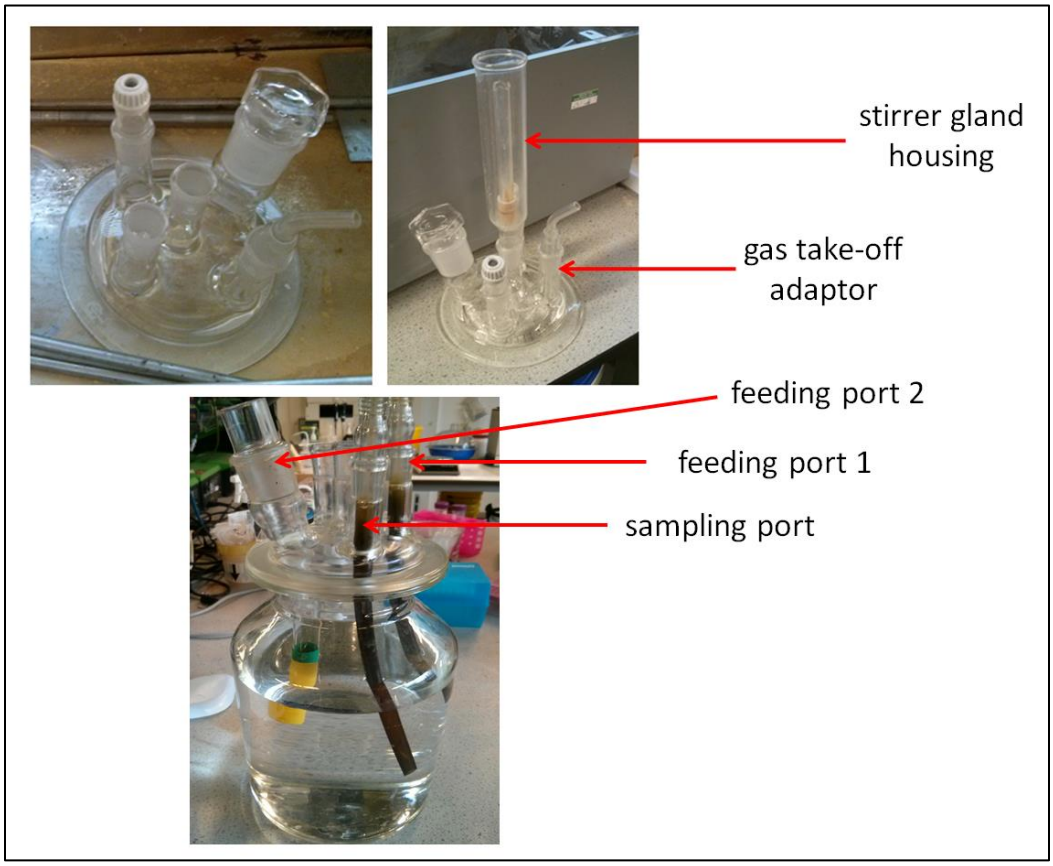

Fig. 5.1 Main components of the CSTR assembly

Two of the five ports were earmarked for feeding/sampling (Fig. 5.1). Plastic tubing of sufficient length to penetrate the designed working level were attached to both the feeding/sampling ports. This novel retrofit ensured that feeding, sampling and liquid phase temperature measurements were conducted without allowing much ambient air into the reactor headspace. The retrofit also improved upon the observed common practice of feeding and sampling where ambient air was allowed to enter the headspace through the largest port on the head plate when using a ladle. The ingress of cool air into the headspace would then expand when the port was closed causing a temporary and erroneously high reading in biogas production. Transient errors such as this would result in a higher than actual SMP reported.

A larger tube e.g. feeding port 2, fabricated from glass was used during the second CSTR experiment in order to facilitate larger feed particulates and eliminate blockages. Feeding was accomplished by modifying a 200ml plastic syringe along with the use of two ports on the CSTRs design to accept a 14 mm diameter tube. Feeding was done once per day and the time recorded. 160ml of digestate was first removed which was then followed by the addition of 160 ml of prepared feed to retain the same working volume each day. Another important feature of the port is the threaded male end, which enables air to enter the tubing column to allow the trapped fluid to freely enter the digester (Fig. 5.2).

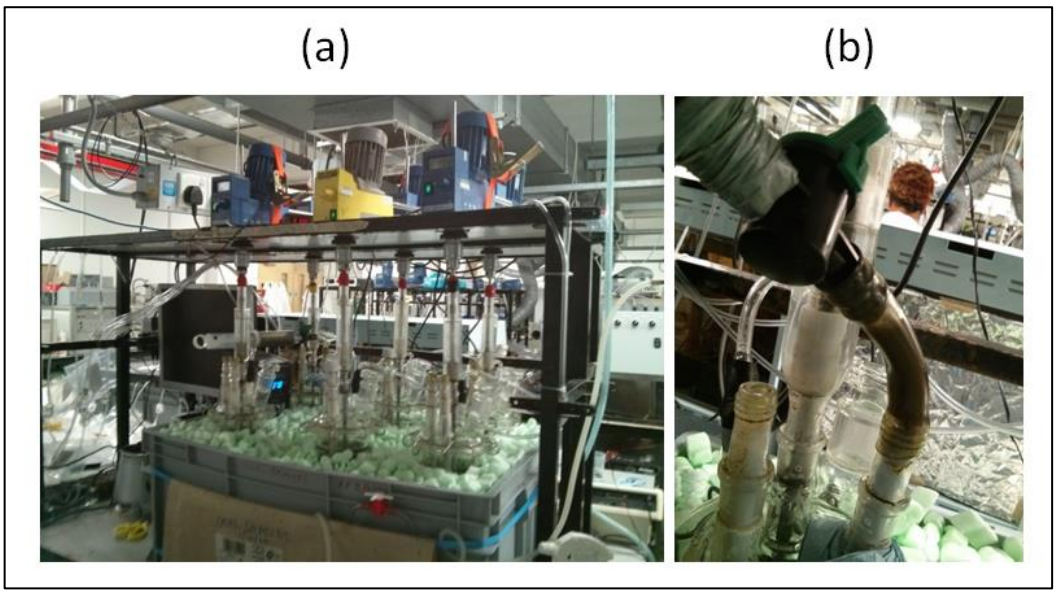

Fig. 5.2 The CSTR experiment setup showing (a) full assembly and (b) feeding mechanism

The centre port was fitted with a gland seal through which the metal shaft for the stirrer was inserted. The seal was then filled with water to prevent air from entering the reactor.

The gas take-off system was assembled by attaching a 1 mm^2 diameter plastic tube with a tube clip to a 2L gas bag (Supelco, 30273-U). Five litre gas bags (Supelco, 30274-U) were used during CSTR Experimental runs C2-01 and C2-02 due to increased levels of biogas production. The reactors were then labelled and incubated in a water bath at 37°C using an automatic temperature controlled heater (Grant, T100). The headspace for each reactor was then flushed with nitrogen for two minutes and overhead stirrers operating between 100-130 rpm were used to provide constant mixing.

The inoculum was allowed to degas at 37^{o}C for seven days within each reactor and the biogas produced was collected in gas bags in order to assess whether the assembly was gas tight. Packing material was used to reduce water losses in the water bath due to evaporation and minimise the movement of reactors within the water bath.

There was a reduction in biogas output between days 30 to 35 and 60 to 65 of CSTR run C2-01 and days 30 to 35 and 52 to 67 of run C2-02. All reactors remained gas tight however it was discovered that the distilled water used to make the feed mixtures had a pH of 4.7. A decision was made to dose each reactor with 0.5g of sodium carbonate (Sigma Aldrich, S7795). Each reactor was dosed every other day starting from day 31 until day 50 when biogas daily production had increased and become steady. A daily split-feed regime was instituted for run C2-02 from day 52 to day 75, as a substitute for dosing, when it was observed that biogas production began to fall once more. During the split feed, each reactor had half of its daily feed in the morning with feeding of the additional 80ml no less than 7 hours later. Days 60 to 65 of CSTR run C2-01 and days 52 to 67 of run C2-02 were treated as outliers and were, therefore, excluded from calculations to determine the specific biogas and methane production.

The biomethane efficiency (BE) was calculated for each treatment at both organic loading rates used in the CSTR Experiment. The BE is calculated by dividing the specific methane production (SMP) from the CSTR Experiment by the SMP for the Batch Experiment (Wall *et al.*, 2014).

5.3.1 Statistical Analyses

Statistical analysis was performed in R version 3.3.2 (R Core Team, 2013). Data were evaluated using analysis of variance (ANOVA) along with appropriate posthoc-analyses, t-tests were reported with bonferroni corrected p-values at a significance level (α) of 0.05 to determine whether differences between groups were significant. Mean parameter estimates for each treatment during the third HRT were reported for CSTR Experiments 1 and 2.

Correlation of the physico-chemical process parameters with AD process parameters was made using Spearman correlation with the *cor.test* function within the *corrplot* R package (Wei and Simko, 2016).

5.4 Results

The C:N ratios of GS and CS used to make the feed were 34.9 ± 1.58 and 18.2 ± 0.32 respectively. The C:N ratio of the CS:GS treatments i.e. 80:20 ($\bar{x}$ = 19.7 ± 0.31) and 60:40 ($\bar{x}$ = 20.3 ± 0.28) were significantly higher than the CS by 9% ($p < 0.01$) and 11% ($p < 0.001$), respectively. There was no significant difference between the C:N ratios for the 80:20 and 60:40 treatments. The actual OLR by treatment for each reactor was kept essentially constant through all experimental runs. The mean daily OLRs during the third HRT of both runs in CSTR experiment 1 for the CS, 80:20 and 60:40 treatments were 1.97 ± 0.04, 2.01 ± 0.02 and 1.98 ± 0.03g VS respectively. The mean daily OLRs during the third HRT of both runs of CSTR experiment were 4.03 ± 0.06g VS, 4.00 ± 0.04g VS and 4.23 ± 0.12g VS for the CS, 80:20 and 60:40 treatments respectively. Variations in OLR from the designed feed rate were due to the particulate nature of mixtures; resulting in difficulties in obtaining samples of uniform TS and VS for solids analysis.

5.4.1 Effect of OLR on AD process efficiency

The mean steady-state TS in the effluent for each treatment in CSTR Experiment 2 was 40% higher than the respective effluent TS at steady state in CSTR Experiment 1 ($p < 0.001$) (Table 5.2 and Table 5.3).

Table 5.2 Treatment feed and effluent characterisation at an OLR of 0.5g VS per m^3/d (Experimental run C1). Values are expressed as means (n=42) ± standard error

		CS		80:20		60:40	
Parameters	**Units**	**Feed**	**Effluent**	**Feed**	**Effluent**	**Feed**	**Effluent**
TS	**g/L**	15.3 ± 0.11	12.5 ± 1.99	15.1 ± 0.11	11.5 ± 0.65	14.6 ± 0.18	10.1 ± 0.31
VS	**g/L**	12.3 ± 0.27	9.0 ± 0.33	12.6 ± 0.13	8.2 ± 0.53	12.4 ± 0.19	7.3 ± 0.26
COD	**g O_2/l**	24.8 ± 1.58	12.0 ± 0.64	23.3 ± 0.86	11.4± 0.79	23.3 ± 0.86	11.4 ± 0.79
pH		7.3 ± 0.01	7.5 ± 0.02	7.0 ± 0.02	7.4 ± 0.02	6.6 ± 0.03	7.4 ± 0.02
C:N Ratio		18.2 ± 0.32	NR	19.7 ± 0.30	NR	20.3 ± 0.30	NR
TKN	**g/L**	0.89 ± 0.117	1.11 ± 0.068	0.81 ± 0.119	1.22 ± 0.066	0.63 ± 0.130	1.24 ± 0.080
TAN	**g/L**	0.20 ± 0.170	0.62 ± 0.250	0.14 ± 0.110	0.56 ± 0.025	0.10 ± 0.012	0.49 ± 0.24
TKN/TAN		0.22	0.56	0.17	0.46	0.16	0.39
Formic	**mg/L**	2.9 ± 1.08	9.9 ± 5.48	34.6 ± 6.23	1.6 ± 0.59	111.0 ± 26.29	2.0 ± 0.78
Acetic	**mg/L**	646.5 ± 87.29	2.9 ± 0.42	1001.5 ± 361.24	4.7 ± 1.03	456.2 ± 97.6	4.2 ± 0.66
Propionic	**mg/L**	105.6 ± 18.57	2.8 ± 1.11	87.6 ± 11.98	2.6 ± 1.04	45.7 ± 10.75	5.7 ± 1.68
Butyric	**mg/L**	10.9 ± 0.76	19.3 ± 0.02	9.0 ± 0.04	19.3 ± 0.03	10.4 ± 0.02	nd
Isobutyric	**mg/L**	40.8 ± 12.65	50.0 ± 14.18	17.7 ± 4.96	27.1 ± 5.88	18.4 ± 5.48	20.1 ± 4.35
Isovaleric	**mg/L**	nd	nd	nd	7.22 ± 0	nd	18.1 ± 0.04
Valeric	**mg/L**	nd	nd	nd	nd	nd	nd

NR = Not Reported

nd = Not detected

Table 5.3 Treatment feed and effluent characterisation at an OLR of 1g VS per m^3/d (Experimental run C2). Values are expressed as means (n=42) ± standard error

		CS		80:20		60:40	
Parameters	**Units**	**Feed**	**Effluent**	**Feed**	**Effluent**	**Feed**	**Effluent**
TS	**g/L**	30.5 ± 0.40	20.7 ± 1.37	29.7 ± 0.32	18.7 ± 0.87	31.0 ± 0.77	18.0 ± 1.24
VS	**g/L**	25.2 ± 0.34	15.0 ± 0.22	25.0 ± 0.26	14.4 ± 0.13	26.5 ± 0.75	13.4 ± 0.20
COD	**g O_2/l**	40.0 ± 0.97	23.3 ± 0.90	40.0 ± 0.71	22.9± 1.23	39.0± 1.18	22.4 ± 0.85
pH		7.2 ± 0.02	7.4 ± 0.01	6.9 ± 0.20	7.4 ± 0.01	6.4 ± 0.03	7.4 ± 0.01
C:N Ratio		18.2 ± 0.32	NR	19.8 ± 0.30	NR	20.3 ± 0.30	NR
TKN	**g/L**	0.99 ± 0.075	1.19 ± 0.042	0.90 ± 0.045	1.20 ± 0.038	1.03 ± 0.072	1.30 ± 0.091
TAN	**g/L**	0.18 ± 0.018	0.50 ± 0.015	0.18 ± 0.017	0.55 ± 0.020	0.17 ± 0.010	0.48 ± 0.015
TAN/TKN		0.18	0.42	0.20	0.46	0.16	0.39
Formic	**mg/L**	nd	1.7 ± 0.19	nd	1.4 ± 0.18	nd	1.2 ± 0.21
Acetic	**mg/L**	1579.0 ± 599.04	54.6 ± 9.46	2230.9 ± 678.28	42.1 ± 8.76	2087.7 ± 901.8	90.4 ± 25.82
Propionic	**mg/L**	14.7 ± 2.01	4.4 ± 0.88	19.2 ± 5.01	10.2 ± 0.21	13.5 ± 0.04	20.6 ± 7.63
Butyric	**mg/L**	24.7 ± 3.55	5.6 ± 04	31.8 ± 1.77	11.5 ± 10.63	27.2 ± 0.48	11.0 ± 8.01
Isobutyric	**mg/L**	14.6 ± 1.76	125.1 ± 27.03	14.6 ± 0.04	74.6 ± 16.82	17.3 ± 0.01	74.8 ± 16.65
Isovaleric	**mg/L**	3.6 ± 0. 39	nd	nd	7.4 ± 2.04	4.42 ± 0.39	10.6 ± 0.78
Valeric	**mg/L**	nd	nd	18.4 ± 0.6	nd	nd	nd

NR = Not Reported

nd = Not detected

Higher rates of TS and VS reduction were observed when feeding at the higher OLR of 1g VS per m^3/d as opposed to 0.5g VS per m^3/d ($p < 0.001$). Feeding reactors at an OLR of 0.5g VS per m^3/d resulted in 19%, 24% and 31% reductions in TS for the CS, 80:20 and 60:40 treatments respectively. A similar trend was observed with VS reduction in that the CS treatment had the lowest mean VS % reduction i.e. 27% compared to the 80:20 treatment (34%) and was significantly lower ($p < 0.001$) than the 60:40 treatment (41%). Total solids and VS reduction for the CS treatment was also significantly lower than that of the 60:40 treatment at an OLR of 1g VS per m^3/d ($p < 0.001$). Percentage TS reductions of 32%, 37%, and 42% were observed for the CS, 80:20 and 60:40 treatments respectively. Volatile solids for the CS treatment were also reduced by 40% and the 80:20 treatment by 43%. The percentage VS reduction in the 60:40 treatment (49%) was significantly higher than the VS reduction in the CS (40% $p < 0.001$). Higher operational efficiencies likely resulted from increased rates of microbial biomass immobilisation resulting from more particulates within the feedstock.

Significant reductions in COD were also observed in all treatments with the CS treatment having a 48% and 42%, the 80:20 treatment having a 51% and 43% and the 60:40 treatment having a 40% and 42% COD reduction at an OLR of 0.5g VS per m^3/d and 1g VS per m^3/d respectively. Differences in solid destruction efficiencies may be due to differences in inoculum and substrate used between CSTR Experiment 1 and CSTR Experiment 2.

5.4.2 Effect of treatment on gas phase parameters

The proportion of GS within the treatment determined the level of biodegradability (BD). The CS:GS treatment with the highest proportion of GS had the highest BD index while the lowest BD was observed in the CS-only treatment (Table 5.4).

Table 5.4 Performance parameters of treatments by OLR. Mean values with standard deviation.

OLR	Parameter	Units	CS	80:20	60:40
0.5g VS per m^3/d	SBP	L_N/kg VS	267 (21)	336 (37)	403 (46)
	SMP	L_N/kg VS	182 (15)	230 (38)	270 (47)
	CH_4 Concentration	% v/v	70.9 (4.0)	68.5 (4.0)	66.8 (4.0)
	BD		0.37	0.46	0.53
	BE		0.70	0.76	0.78
1g VS per m^3/d	SBP	L_N/kg VS	308 (23)	343 (10)	372 (21)
	SMP	L_N/kg VS	210 (16)	231 (6)	244 (14)
	CH_4 Concentration	% v/v	68.3 (1.0)	67.9 (2.0)	66.0 (4.0)
	BD		0.42	0.46	0.48
	BE		0.80	0.76	0.70

SBP = Specific Biogas Production
SMP = Specific Methane Production
BD = Biodegradability
BE = Biomethane Efficiency

At an OLR of 0.5g VS per m^3/d, the treatment with the highest BE was also the most biodegradable i.e. the 60:40 treatment. However, at the higher OLR of 4g per day, the CS treatment had the highest BE. This was most likely due to the reduction in biogas production of the CS:GS mixture during days 52 to 67 when the reactor feed was inadvertently made with distilled water with a low pH of 4.7. Mean methane content in dry biogas ranged from 66 – 71%; signifying the existence of a healthy microbial community, in particular the methanogen sub-population.

The 60:40 treatment recorded the highest specific biogas production (SBP) of all three treatments during HRT3 (Fig. 5.3). There were no significant differences between the SBP and SMP for the CS and CS:GS treatments at the two contrasting OLR levels.

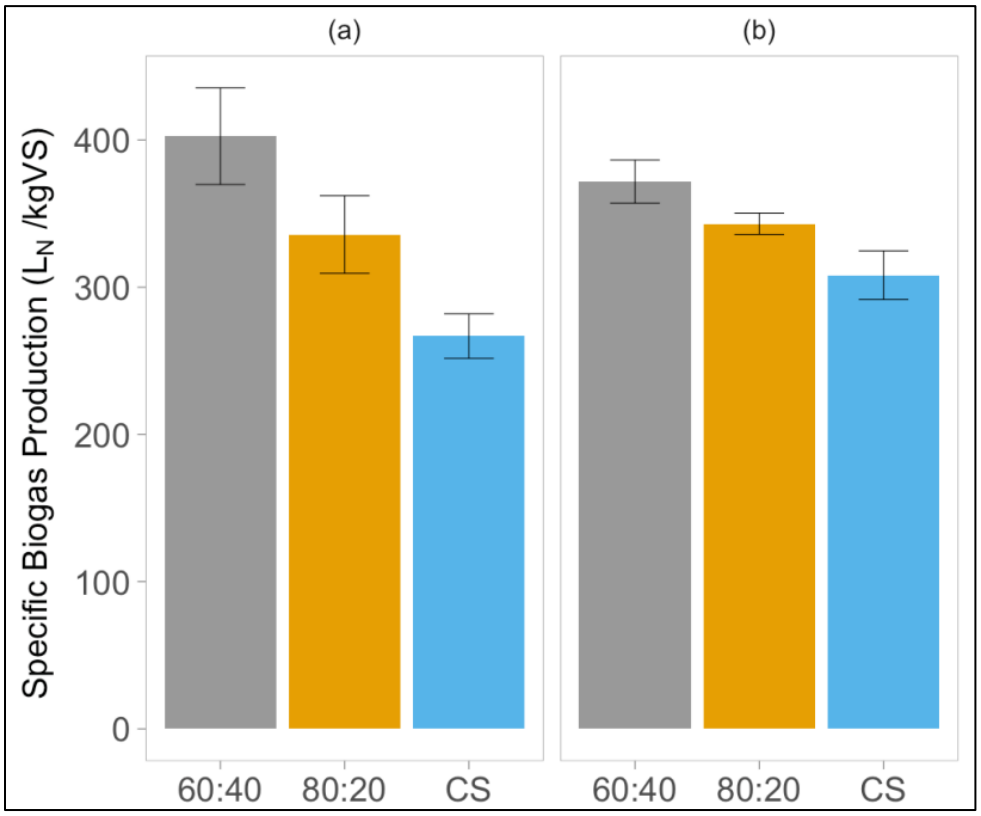

Fig. 5.3 Mean specific biogas production by treatment for (a) CSTR Experiment 1 and (b) CSTR Experiment 2

The overall mean SBP of the 60:40 treatment ($\bar{x}$ = 387.2 ± 17.2 L_N/kg VS) was 26% higher than the CS treatment ($\bar{x}$ = 287.5 ± 15.0 L_N/kg VS, $p < 0.001$). There was no significant difference between the specific biogas production of the 80:20 ($\bar{x}$ = 339.4 ± 11.4L_N/kg VS) and the CS and 60:40 treatments.

A similar trend was observed when comparing the overall mean SMP for each treatment in that the 60:40 treatment ($\bar{x}$ = 257.3 ± 16.0 L_N/kg VS) produced 31% more methane than the CS treatment ($\bar{x}$ = 195.9 ± 10.2 L_N/kg VS) (Fig. 5.4) and there was no significant difference between the 80:20 ($\bar{x}$ = 231.0 ± 11.0 L_N/kg VS) and the CS and 60:40 treatments.

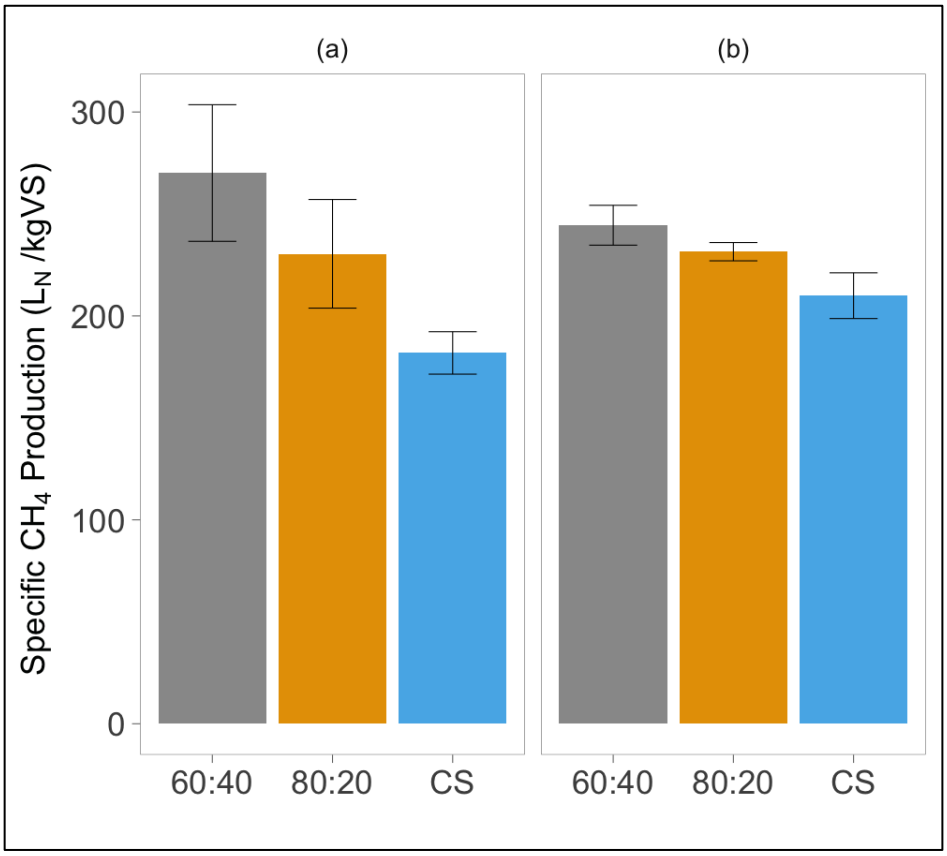

Fig. 5.4 Mean specific methane production by treatment for (a) CSTR Experiment 1 and (b) CSTR Experiment 2

Estimates for SBP and SMP for a GS only treatment were determined by assuming the contribution to biogas production from the CS fraction in the 80:20 and 60:40 treatments remain constant. The following equations were derived for biogas and methane production:

Specific biogas potential:

$$0.80 * CS + 0.20 * GS = 339.4 \quad \text{Eq. 5.1}$$
$$0.60 * CS + 0.40 * GS = 387.2 \quad \text{Eq. 5.2}$$

where CS = 287.5

Specific methane potential:

$$0.80 * CS + 0.20 * GS = 231.0 \quad \text{Eq. 5.3}$$
$$0.60 * CS + 0.40 * GS = 257.3 \quad \text{Eq. 5.4}$$

where CS = 195.9

Solving the equation for GS produces an SBP estimate of 753.9 L_N/kg VS and a SMP estimate of 356.7 L_N/kg VS for a GS only treatment.

No significant differences were observed in the mean methane concentration in dry biogas between the treatments at an OLR of 0.5g VS per m^3/d (Fig. 5.5).

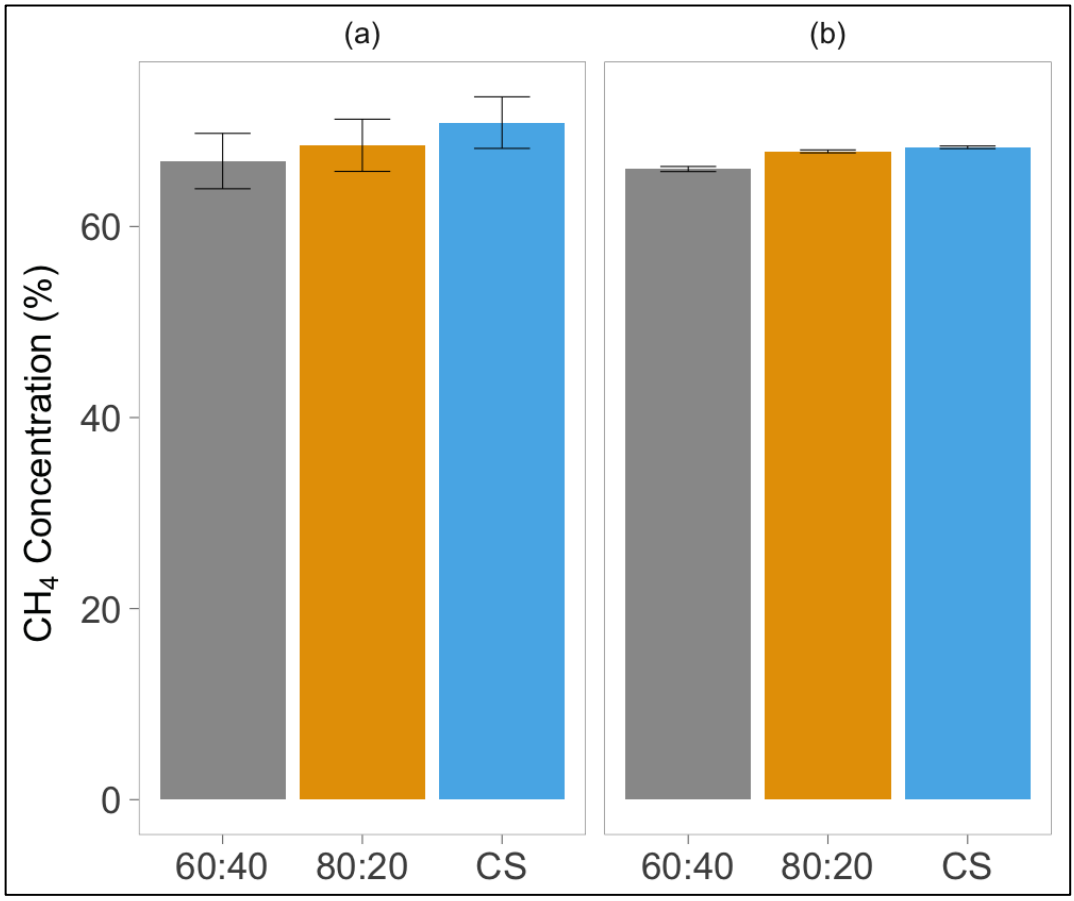

Fig. 5.5 Mean Methane concentration by treatment for (a) CSTR Experiment 1 and (b) CSTR Experiment 2

However, when the OLR was doubled to 1g VS per m^3/d the methane concentration in the 60:40 treatment ($\bar{x}$ = 66.0 ± 0.27% v/v) was less than that of the CS-only treatment ($\bar{x}$ = 68.3± 0.19% v/v) and the 80:20 treatment ($\bar{x}$ = 67.9 ± 0.15% v/v) during steady-state conditions ($p<0.05$). There was no significant difference between the methane concentration for the 80:20 treatment and the CS-only treatment.

This suggests that the observed trend in SMP between treatments is a result of the higher specific biogas production in the 60:40 treatment due to its higher biodegradability as evidenced by the lignin cellulose and hemicellulose content of the treatments (Table 5.5).

Table 5.5 Compositional analysis of feedstocks

Parameter	Units	CS	60:40	80:20	GS
Cellulose	**(%TS)**	18.6 ± 0.1	22.9 ± 0.5	20.6 ± 0.6	27.4 ± 0.4
Hemicellulose	**(%TS)**	15.6 ± 0.3	18.5 ± 1	16.9 ± 1.1	21.5 ± 0.8
Lignin	**(%TS)**	13.7 ± 0.1	10.3 ± 0.2	11.9 ± 0.2	4.4 ± 0.1
Carbon	**(%TS)**	41.2 ± 0.1	43.5 ± 0.5	42.0 ± 0.5	43.6 ± 0.1
Hydrogen	**(%TS)**	5.7 ± 0	6.0 ± 0.1	5.8 ± 0.1	6.1 ± 0
Nitrogen	**(%TS)**	3.02 ± 0.02	3.15 ± 0.04	3.06 ± 0.04	3.09 ± 0.03
Oxygen	**(%TS)**	31.4 ± 0.1	33.8 ± 0.4	32.4 ± 0.4	34.8 ± 0.3
Sulphur	**(%TS)**	0.49 ± 0.01	0.42 ± 0.01	0.45 ± 0.01	0.29 ± 0.01

The CS-only treatment contains more lignin than the GS and the co-digested treatments. Elemental analysis (EA) of the feedstocks also indicate that, although sulphur content is very low within all treatments (< 0.50%), the CS-only treatment contains more sulphur than the co-digested treatments. Being more biodegradable, the 60:40 treatment produced on average more methane i.e. (19.2 ± 0.5L and 43.1 ± 0.8L) than the 80:20 (18.8 ± 0.7L and 39.6 ± 0.9L) and CS (15.7 ± 0.7L and 31.6 ± 0.8L) treatments at daily OLR of 2g VS and 4g VS respectively (Fig. 5.6 and Fig. 5.7). Individual plots for each reactor by experimental run are provided in Appendix 8.

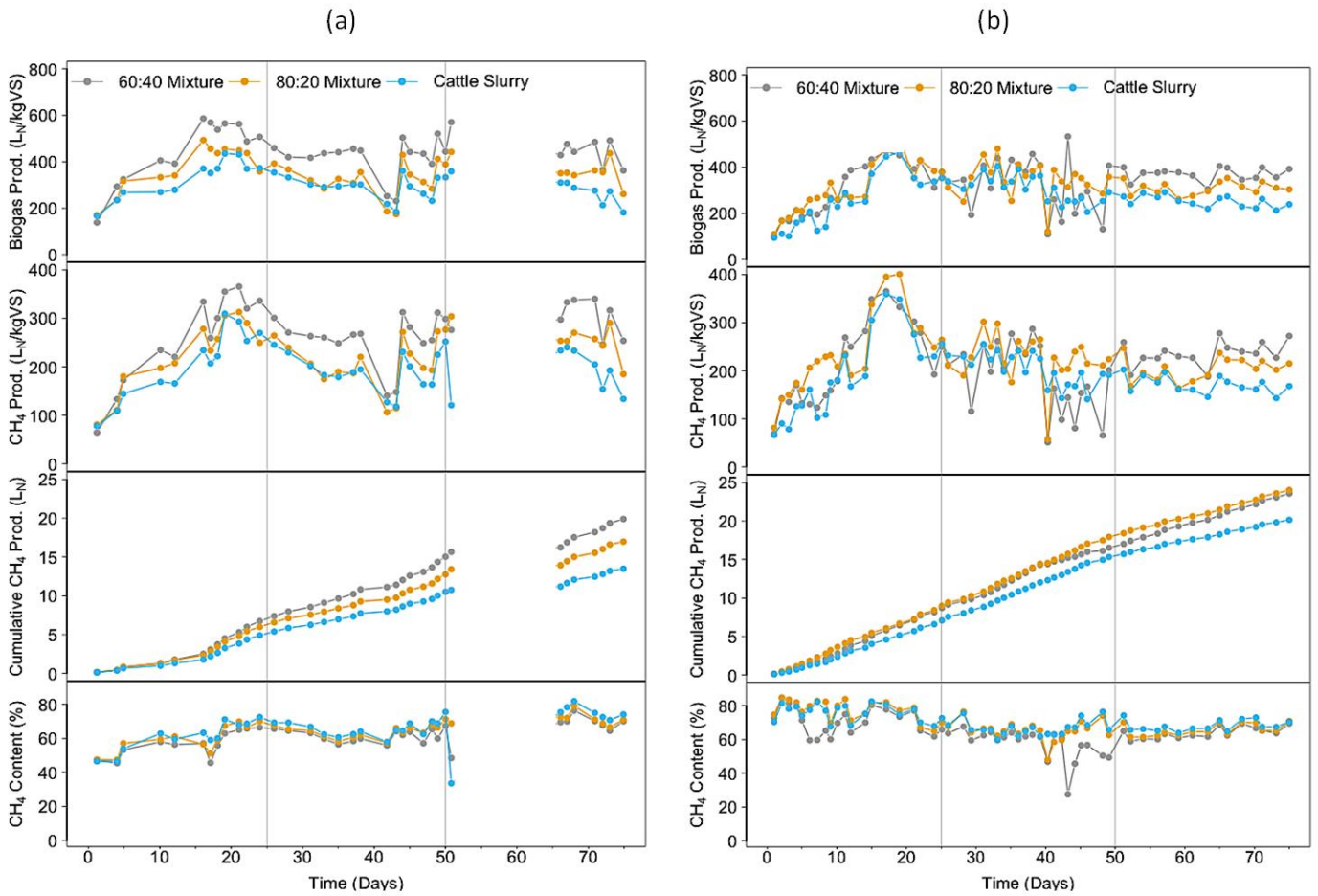

Fig. 5.6 Biogas, methane production (L_N/kg VS), cumulative methane production and methane concentration in biogas for CSTR Experiment 1 (a) run C1-01 and (b) run C1-02. Missing values during days 51 to 65 of run C1-01 were due to the closure of the laboratory facilities.

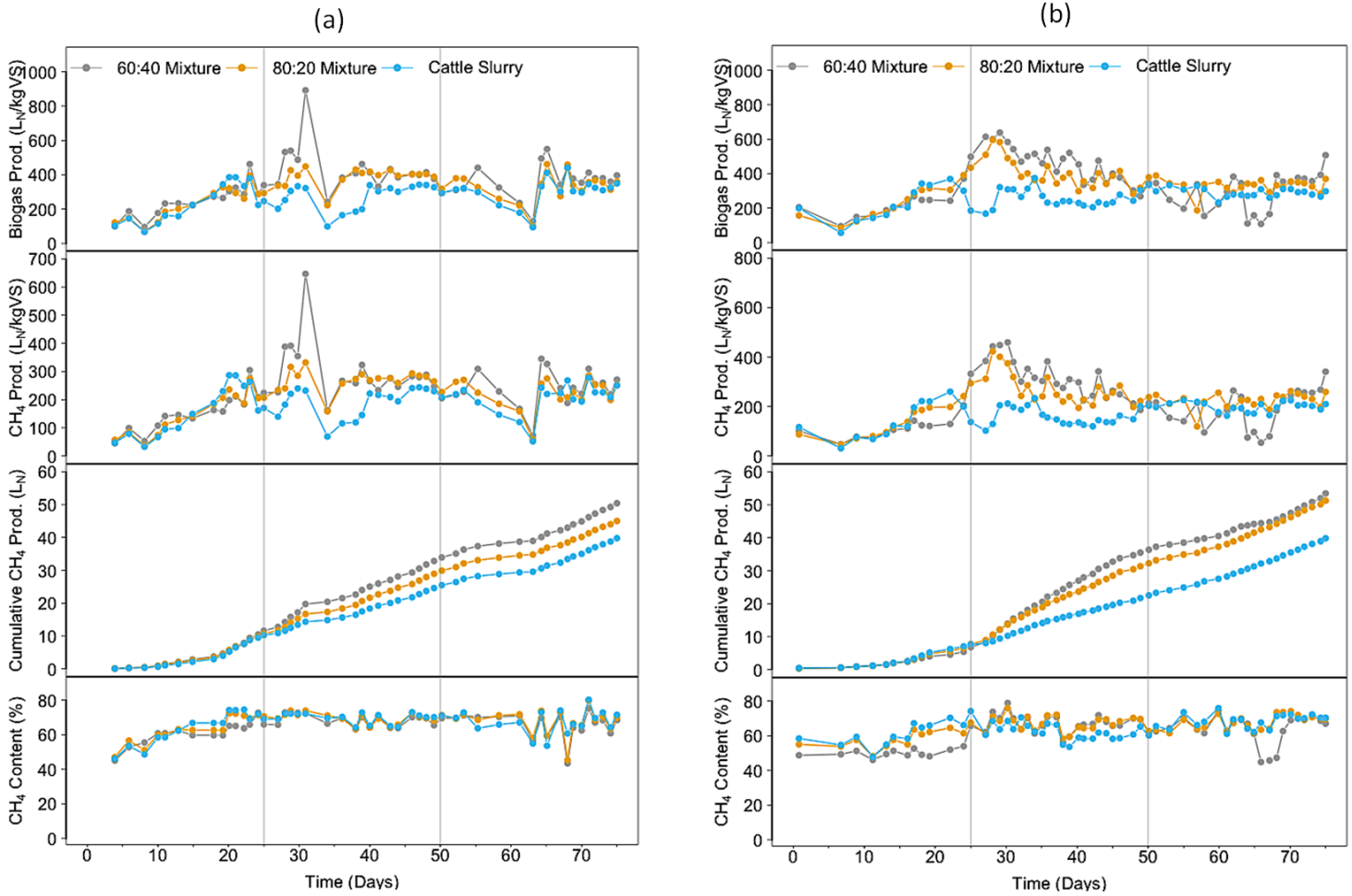

Fig. 5.7 Biogas, methane production (L_N/kg VS), cumulative methane production and methane concentration in biogas for CSTR Experiment 2 (a) run C2-01 and (b) run C2-02.

5.4.3 Effect of treatment on pH

There was a clear influence of treatment on feed pH due to the proportion of GS added to the feed. The mean pH of the CS feed ($\bar{x}$ = 7.31 ± 0.02) was significantly higher than the 60:40 ($\bar{x}$ = 6.59 ± 0.02, $p < 0.001$) and the 80:20 ($\bar{x}$ = 6.94 ± 0.02, $p < 0.02$) feeds at both OLR levels (Fig. 5.8). The mean pH for the 80:20 was also significantly different to both the CS and 60:40 treatments ($p < 0.05$).

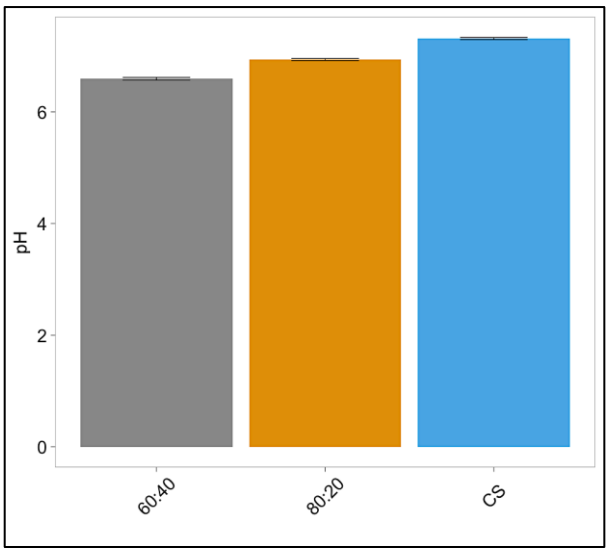

Fig. 5.8 Levels of feed pH for the different treatment regimes

However, there was no significant difference between treatments in effluent pH (Fig. 5.9) at HRT3. Nevertheless, there was a clear trend where the effluent pH decreased from HRT1 to HRT3 ($p < 0.01$).

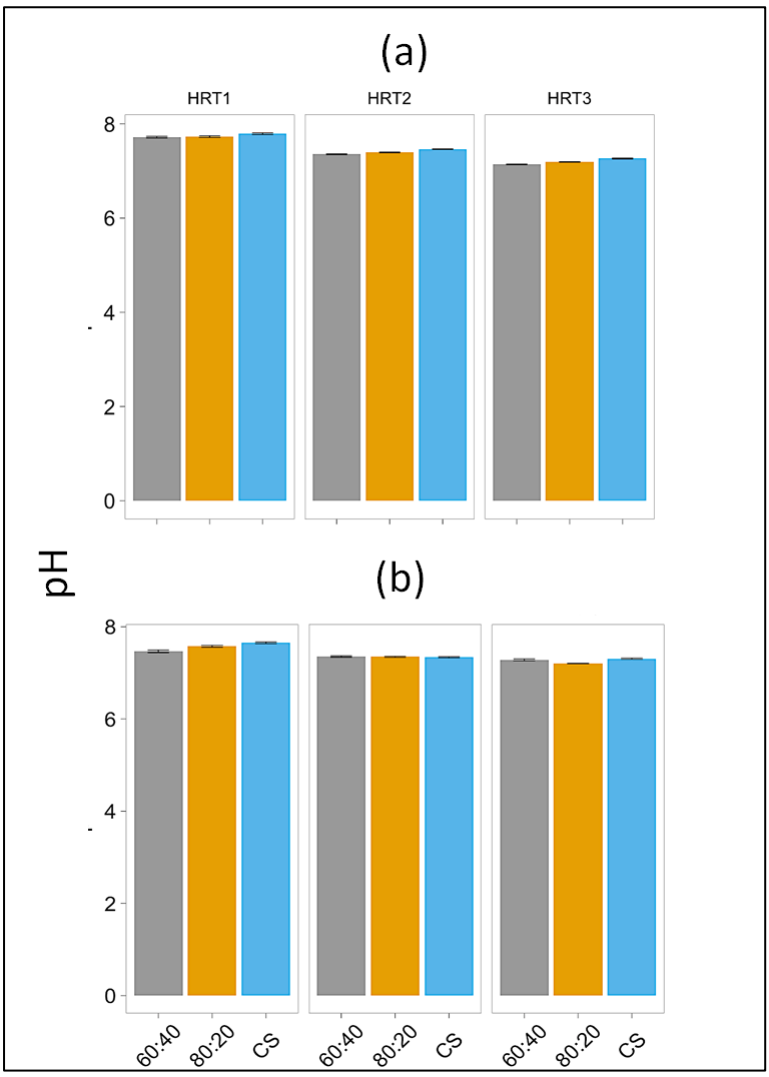

Fig. 5.9 Effluent pH by treatment and HRT for CSTR (a) Experiment 1 and (b) Experiment 2

Average effluent pH for each HRT was significantly different from each other when operating at an OLR of 0.5g VS per m^3/d. The effluent pH during HRT1 ($\bar{x}$ = 7.74 ± 0.01) was significantly higher than HRT2 ($\bar{x}$ = 7.40 ± 0.09) and HRT3 ($\bar{x}$ = 7.20 ± 0.01) ($p < 0.001$). A similar trend was also observed while operating at an OLR of 1g VS per m^3/d, in that the highest effluent pH for all treatments was observed during HRT1 ($\bar{x}$ = 7.56 ± 0.18, $p < 0.05$). However, there was no significant difference between treatments in effluent pH during HRT2 ($\bar{x}$ = 7.34 ± 0.12) and HRT3 ($\bar{x}$ = 7.26 ± 0.01) when the reactors were dosed with Na_2CO_3 between days 31 and 50.

5.4.4 Effect of treatment on TAN, TKN and FAN

There were no significant differences between the mean effluent TKN or TAN levels across all treatments during steady-state conditions operating at both OLR levels (Table 5.3). TAN levels were above 1.5 NH_4^+-N/L for the first 15 days of both CSTR experiments. TAN levels were maintained at levels below 1.5g NH_4^+-N/L across all treatments from day 20 until the experiment was terminated on day 75 (Fig. 5.10). This resulted in higher Free Ammonia Nitrogen (FAN) levels within the effluent for each treatment at both OLR levels. FAN levels fell below 50mg NH_3-N/L from the beginning of HRT2 until the end of the experiment.

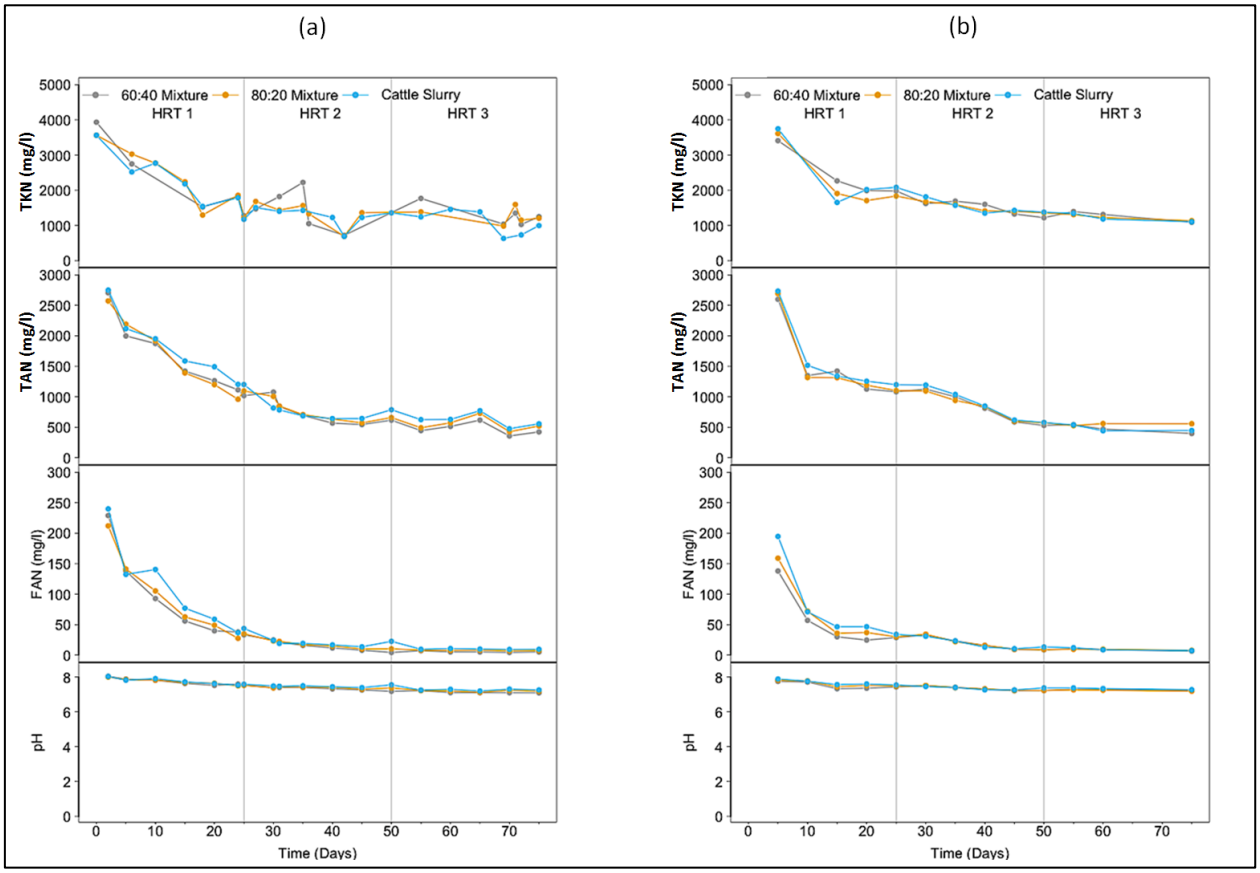

Fig. 5.10 Mean effluent TKN, TAN, FAN and pH levels at OLR of (a) 0.5g and (b) 1g VS per m^3/d

The CS:GS feed with the highest proportion of GS had the lowest TAN percentage share of TKN i.e. 16% at both OLR levels while the CS feed had the highest i.e. 22% during CSTR Experiment 1 and 18% during CSTR Experiment 2.

There was also a clear trend, at both OLR levels, where the percentage share of TAN in TKN for each treatment was higher in the effluent than the feed.

5.4.5 Effect of treatment on VFA

Isobutyric acid was the most dominant VFA present in the reactors for both OLR levels of operation. Mean levels of concentration of 9, 8 and 7g/L within the 60:40, 80:20 and CS treatments were initially high but were subsequently reduced to <0.1g/L by the third HRT. A similar trend in the consumption of Isobutyric acid was observed during operation at the higher OLR level. Total VFA in the effluent of all treatments was reduced to <1g/L when operating at an OLR of 0.5g VS per m^3/d; signifying a stable AD system. Isobutyric was the most abundant VFA since short chain individual VFA were consumed as soon as they were produced. However significant reductions in specific biogas and methane production that were observed while operating at an OLR of 1g VS per m^3/d coincided with increased formic, acetic and propionic acids in the effluent; resulting in increases in total VFA (TVFA) (Fig. 5.11).

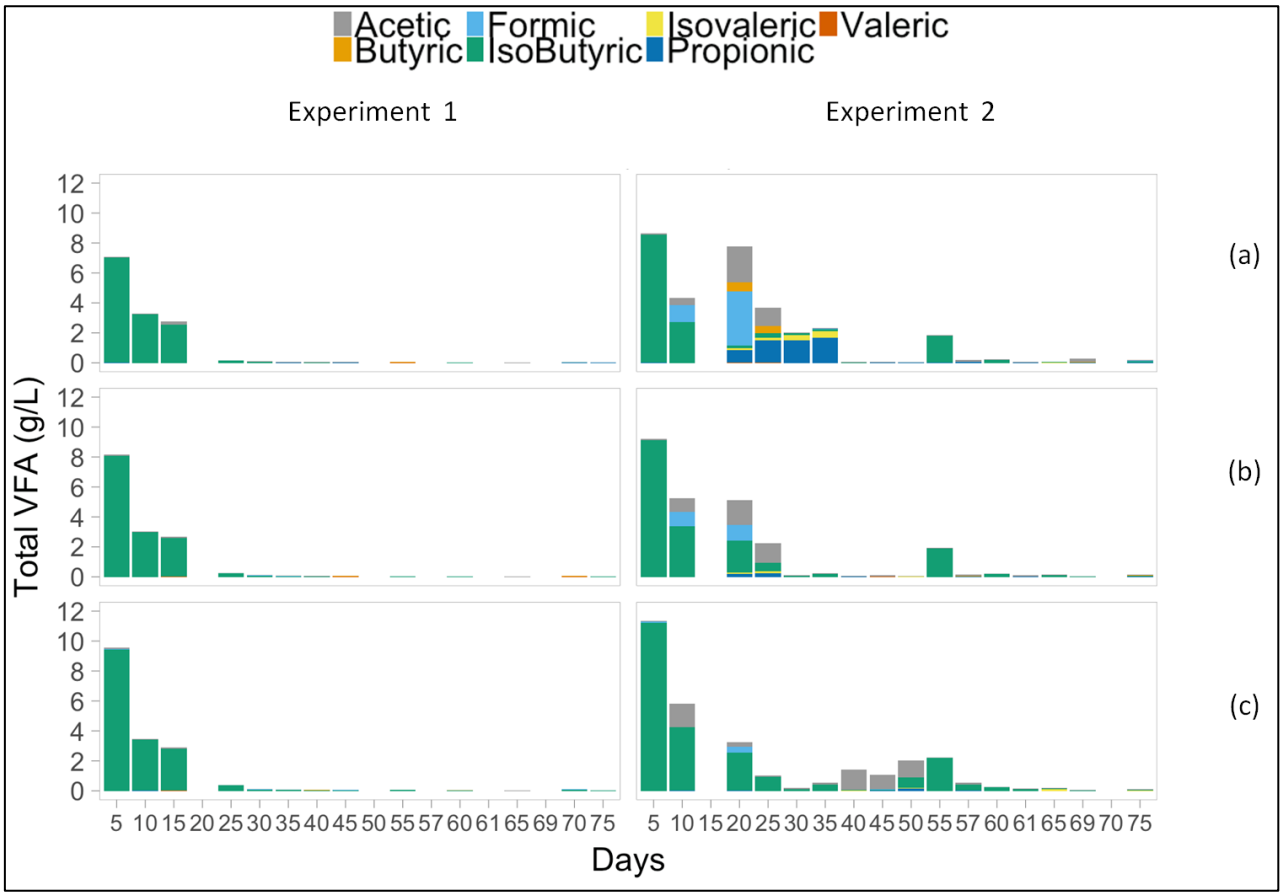

Fig. 5.11 Mean total VFA in effluent for CSTR Experiment 1 and 2 for the (a) 60:40 (b) 80:20 and (c) CS treatments

The 60:40 treatment produced the largest amount of propionate during HRT2; operating at an OLR of 1g VS per m^3/d. During this period, the total VFA in the 60:40 effluent ranged from 2-8g/L, while the total VFA in the 80:20 and CS treatments ranged from 2-4g/L. Formic and acetic acids initially began to accumulate by day 10 for the co-digestion treatments. However only acetic acid accumulation was observed by day 10 within the CS-only treatment. The propionic to acetic ratio exceeded 1.4 on days 30 and 35 for the 60:40 treatment.

Levels of Isobutyric were high, ranging from 6-10 g/L at the beginning of the CSTR Experiments but were reduced to <0.1g/l by as it is metabolized along with butyrate and propionate by acetogenic bacteria to produce acetate, H_2 and CO_2 (Nielsen *et al.*, 2007).

The accumulation of acetic and formic acids means for each treatment during CSTR Experiment 2 indicates that acetotrophic and/or hydrogenotrophic methanogens might have been inhibited within the first 10 days of both runs for CSTR Experiment 2. The first 10 days of CSTR Experiment 2 were also associated with TAN levels above 1.5g/L, which could have precipitated methanogenic inhibition. Formic and acetic acid accumulation on day 10 was then followed by an accumulation of propionate and butyrate between days 20 to 35 for the 60:40 treatment. In the 60:40 treatment, propionate and butyrate gradually accumulated to a maximum of 2 and 1g/L respectively. The accumulation of propionate and butyrate during days 20 to 35 therefore suggests that changes in the environment also precipitated a disruption to the propionate and butyrate oxidation steps within the AD process. Acetate, formate and Isobutyrate accumulated within the CS-only and 80:20 treatments during days 20 to 35; suggesting that the accumulation of VFA within these treatments was most likely due to a disruption in methanogenic growth. Since hydrogenotrophic methanogens degrade butyrate and propionate in syntrophy with an oxidizing bacteria (Stams *et al.*, 2003), the evidence suggests that during days 10 to 55, environmental factors with the AD process inhibited the growth of acetoclastic and hydrogenotrophic methanogens with the 60:40 treatment ; resulting in a decoupling of syntrophic oxidation of propionate and butyrate. A similar perturbation occurred in the CS-only and 80:20 treatments; causing the decline in methane production due to methanogenic inhibition.

5.4.6 Correlation of physico-chemical data

The propionic acid to acetic acid ratio (P:A ratio) was negatively correlated with biogas production (R^2 = -0.52, p < 0.001) and methane production (R^2 = -0.49, p < 0.001) and positively correlated with FAN (R^2 = 0.54, p < 0.001) (Fig. 5.12). Propionic acid was also negatively correlated to acetic acid (R^2 = -0.51, p < 0.001). Effluent pH was positively correlated with biogas (R^2 = 0.4, p < 0.01). FAN negatively correlated with biogas (R^2 = -0.48, p < 0.001) and methane (R^2 = -0.55, p < 0.001) production.

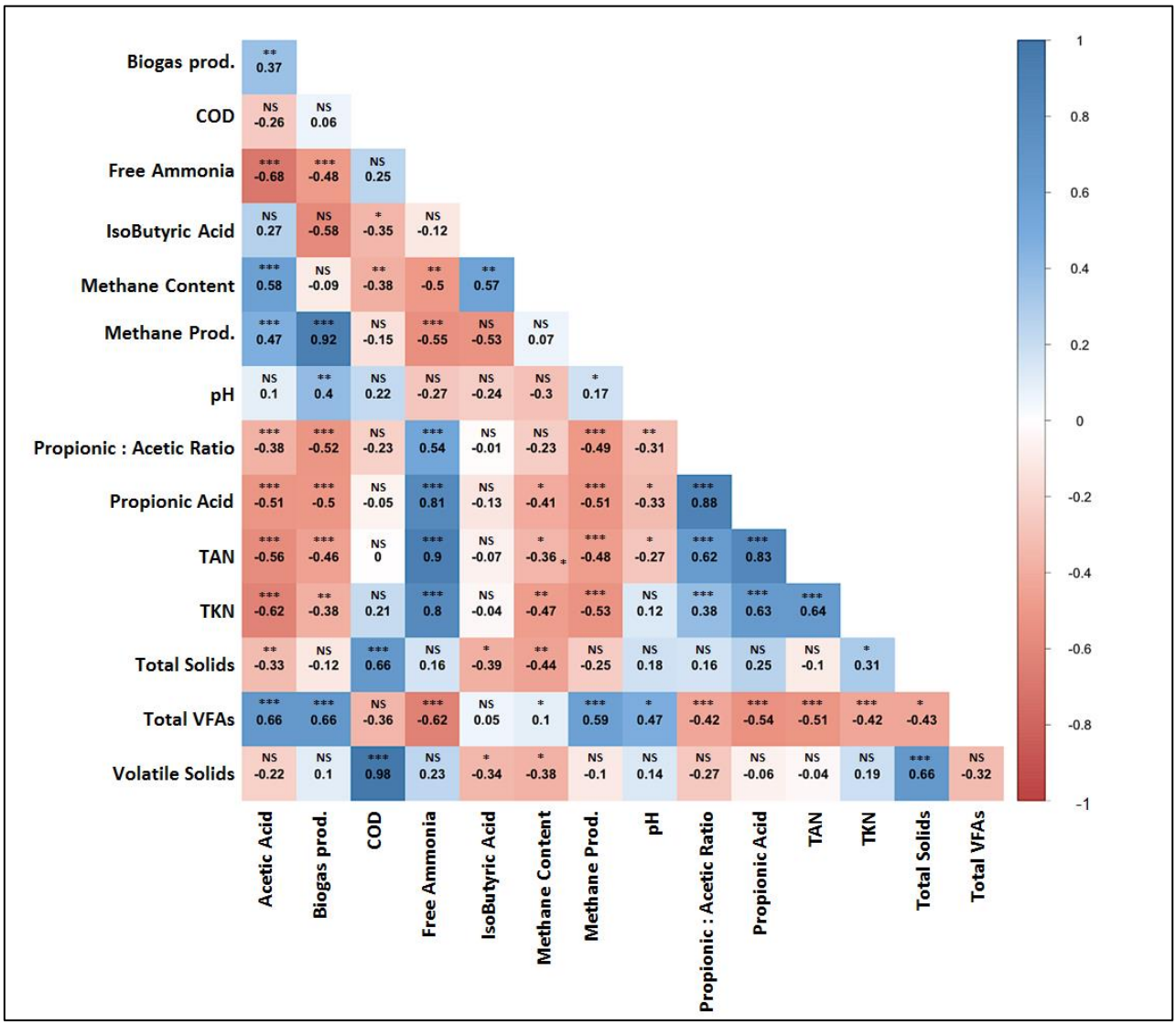

Fig. 5.12 Physico-chemical parameter correlation matrix. When statistical significance was observed, R^2 value is presented with the associated significance level. A negative sign (-) indicates a negative correlation (as shown in red) and no sign indicates a positive correlation (as shown in blue). *** p < 0.001, ** p < 0.01, * p < 0.05, NS not significant

Total ammonia nitrogen concentration was also negatively correlated with biogas (R^2 = -0.46, $p < 0.001$) and methane production (R^2 = -0.48, $p < 0.001$). Total VFA was negatively correlated with TAN (R^2 = -0.51, $p < 0.001$) and TKN (R^2 = -0.42, $p < 0.001$); signifying that TAN levels did not impede total VFA consumption at both OLR levels. However, TAN levels positively correlated to propionic acid production (R^2 = 0.83, $p < 0.001$) which infers TAN levels may have contributed to the process imbalance observed during CSTR experiment 2 HRT2 when propionic acid accumulated within the reactors treating the 60:40 blend. The negative correlation of propionate with methane content in dry biogas (R^2 = -0.41, $p < 0.05$), biogas production (R^2 = -0.50, $p < 0.001$) and methane production (R^2 = -0.51, $p < 0.001$) suggests that the accumulation of propionate within the AD process, adversely affected biogas production and quality. Methane production positively correlated with the concentration of acetic acid within the effluent (R^2 = 0.47, $p < 0.05$).

5.5 Discussion

The 60:40 treatment had the highest SBP and SMP because it contained the most degradable VS of the three treatments. The trend in % solids destruction among the CS and CS:GS treatments is consistent with findings obtained in the BMP experiments in Chapter 3 where it was determined that CS contains more lignin than the CS:GS treatments and is therefore less biodegradable. Cattle slurry contained approximately 13.7% lignin (% of TS) while the GS used to make the CS:GS feeds contained approximately 4.4% lignin. Lignin is recalcitrant biomass that impedes extracellular enzymatic hydrolysis of cellulose and hemicellulose fibres by cellulolytic microorganisms thereby significantly limiting VS destruction (Nizami *et al.*, 2009; Triolo *et al.*, 2011; Sambusiti *et al.*, 2014; Shah *et al.*, 2015; Tsapekos *et al.*, 2017). Cattle slurry contains more lignin that the co-digested treatments because it consists of biomass that has been up-concentrated in the rumen by the digestive process (Triolo *et al.*, 2011). The resulting low BD of CS was highlighted by Allen *et al.* (2016) who note that the BD of CS ranges from (0.26 – 0.62). Being slow to degrade, lignin is likely to have accumulated within the reactors since the CSTRs were fed semi-continuously for 75 days at an HRT of 25 days (Labatut *et al.*, 2011; Bułkowska *et al.*, 2012; Walter *et al.*, 2016).

Holocellulosic biomass was greater in the co-digested treatments, due to the contribution of the GS substrate, with the 60:40 treatment having more cellulose and hemicellulose content. The hydrophobic surface of cellulose along with its crystalline structure acts as a barrier to microbial enzymatic degradation while hemicellulose, with its amorphous structure is more readily hydrolysed by a dilute acid or base (Jørgensen *et al.*, 2007; Nizami *et al.*, 2009). A comparison of the mean biodegradability indices for the CS (40%), 80:20 (46%) and 60:40 (50%) treatments obtained are in agreement with the literature which have found animal manure to be higher in lignin than for mixtures co-digested with plant material (Triolo *et al.*, 2011; Triolo *et al.*, 2013; Wall *et al.*, 2013; Mata-Alvarez *et al.*, 2014; Wall *et al.*, 2014).

The literature supports the trend observed in the current study and the Batch Experiment (Chapter 3), where the treatment with the highest proportion of plant material also had the highest SMP. The percentage TS and VS destruction obtained for the CS and CS:GS treatments within this study are consistent with those found in the literature (Lehtomäki *et al.*, 2007; Seppälä *et al.*, 2013; Wall *et al.*, 2014); having similar substrates and AD process operating conditions. Lehtomäki *et al.* (2007) investigated the mesophilic co-digestion of cow manure (CM) with various ratios of agricultural feedstocks including GS at an OLR of 2g VS per m^3/d while Seppälä *et al.* (2013) varied the OLR from 2-3g VS per m^3/d in steps of 0.5g VS per m^3/d; co-digesting CM with several agricultural residues inclusive of GS. The co-digestion study conducted by Wall *et al.* (2014) studied the SMP of various CS:GS ratios; varying the OLR from 2-4g VS per m^3/d in steps of 0.5g VS per m^3/d. For ease of comparison, the OLR levels used in the current study were converted to g VS per litre reactor per day and AD performance measures obtained from the literature were limited to similar co-digestion ratios and OLR levels (Table 5.6).

Table 5.6 Comparison of selected AD parameters obtained in this study with the literature. Mean values with standard deviation.

Treatment	OLR (g VS per m^3/d)	HRT (days)	Temp. (°C)	TS Removal (%)	VS Removal (%)	SMP (L_N/kgVS*d)	CH_4 (%v/v)	TAN/TKN Feed	TAN/TKN Effluent	pH	BE	BD	References
60:40	2.0	20	35	41	46	250 (16)	52 (2)	0.33	0.39	7.3	0.95*	NR	(Lehtomäki *et al.*, 2007)
80:20	2.0	20	35	34	42	178 (9)	50 (2)	0.33	0.47	7.5	0.72*	NR	
CS	2.0	20	35	19	25	151 (48)	50 (4)	0.38	0.42	7.6	0.65*	NR	
60:40	2.0	30	35	41	48	259 (6)	52 (2)	0.28	0.35	7.4	0.96*	NR	(Seppälä *et al.*, 2013)
80:20	2.0	27	35	22	27	198 (7)	52 (1)	0.28	0.32	7.4	0.82*	NR	
CS	2.0	23	35	16	21	193 (2)	57 (2)	0.31	0.35	7.5	0.91*	NR	
60:40	2.0	38	37	NR	NR	233	51.6	NR	NR	7.6	0.85	0.77*	(Wall *et al.*, 2013; Wall *et al.*, 2014)
80:20	2.0	28	37	NR	NR	220	49.5	NR	NR	7.6	0.88	0.81*	
CS	2.0	28	37	NR	NR	112	50.1	NR	NR	7.6	0.47	0.29*	
60:40	0.5	25	37	31	41	270 (47)	66.8 (4.0)	0.16	0.39	7.4	0.78	0.53	Current study
60:40	1.0	25	37	42	49	244 (14)	66.0 (4.0)	0.13	0.38	7.4	0.7	0.48	
80:20	0.5	25	37	24	34	230 (38)	68.5 (4.0)	0.17	0.46	7.4	0.76	0.46	
80:20	1.0	25	37	37	43	231 (6)	67.9 (2.0	0.20	0.46	7.4	0.76	0.46	
CS	0.5	25	37	19	27	182 (15)	70.9 (4.0)	0.22	0.56	7.5	0.7	0.37	
CS	1.0	25	37	32	40	210 (16)	68.3 (1.0)	0.18	0.42	7.4	0.8	0.42	

*Calculated from values reported.

NR = Not Reported

The substrate C:N ratio is an important factor to consider in the operation of AD systems. The AD process may fail due to VFA inhibition if the C:N ratio is too high while a low C:N ratio increases system buffer capacity but may cause system failure due to ammonia inhibition (Esposito *et al.*, 2012; Rajagopal *et al.*, 2013). The literature provides conflicting reports regarding the optimal C:N ratio required to maximise biogas production and quality. Wu *et al.* (2010) and Alvarez *et al.* (2010) found that a C:N ratio of 20:1 was the optimal ratio when co-digesting agricultural waste while other literature report that an optimal C:N ratio for AD is between 25:1 and 30:1 (Ward *et al.*, 2008; Allen *et al.*, 2016). A wider range of 20:1 to 30:1 was also reported (Esposito *et al.*, 2012). Co-digesting the CS-only treatment with GS increased the C:N from 18.2 ± 0.32 for the CS-only treatment to 19.7 ± 0.31 and 20.3 ± 0.28 for the 80:20 and 60:40 treatments respectively. Both the 80:20 and 60:40 treatments have C:N ratios that are comparable to the optimum reported by Wu *et al.* (2010), Esposito *et al.* (2012) and Alvarez *et al.* (2010). The 60:40 treatment produced more methane than the CS-only treatment, with a mean methane concentration ranging from 66-67%, because of the additional carbon source provided by the 40% VS obtained from the GS. The methane concentration reported by Lehtomäki *et al.* (2007), Seppälä *et al.* (2013) and Wall *et al.* (2014) ranged from (50 – 57%) for all treatments however the respective SMP reported for each treatment was similar to that obtained in this study.

5.5.1 The effect of co-digestion on physico-chemical process parameters

The estimate of SMP for 357L_N/kg VS for a GS only treatment calculated from the mean values obtained from the CS-only, 80:20 and 60:40 treatments are consistent with that reported by Wall *et al.* (2014) who reported a SMP for GS of 414L_N/kg VS and 455L_N/kg VS Thamsiriroj *et al.* (2012) using 5L and 472L CSTRs respectively and operating at an OLR of 2g VS per m^3/d. The difference between the two SMP values is likely due to differences in the OLR. Wall *et al.* (2014) used an OLR of 39 days and Thamsiriroj *et al.* (2012) used an HRT of 60 days as opposed to 25 days used in the current study. Therefore a higher methane production would be produced since the microbes have a longer time to degrade the biomass.

Lehtomäki *et al.* (2007), Seppälä *et al.* (2013) and Wall *et al.* (2014) reported that the SMP of their 60:40, CM:GS co-digested treatments were 65%, 34% and 108% respectively higher than the manure only treatment used in their studies at an OLR of 2g VS per m^3/d.

In the current study, the 60:40 treatment was 48% and 16% higher than the CS-only treatment at OLR levels of 0.5g VS per m^3/d and 1g VS per m^3/d respectively, which is in consistent with findings of Lehtomäki *et al.* (2007) and Seppälä *et al.* (2013). Hagelqvist and Granström (2016) observed a higher methane yield from GS and manure substrates sampled during the spring as opposed to autumn months. Nizami *et al.* (2009) note that the maturity of the crop and leaf-stem ratio while Osborne Homeky *et al.* (2016) cite silage duration as factors that affect the BMP of GS. Therefore, differences between SMP values reported in the literature and the current study are likely due to variations in substrate and inoculum due to changes in feed, the maturity at which the GS was harvested and the duration of the ensiling process. Lower values obtained at the OLR of 1g VS per m^3/d were likely because the reactors treating the 60:40 treatment were operating sub-optimally as they were recovering from the process imbalance experienced in HRT2.

Lehtomäki *et al.* (2007) increased the OLR of the CM:GS, 60:40 treatment to 3 and 4g VS per m^3/d and reduced the HRT to 18 and 16 days respectively without experiencing any process imbalance. However SMP at an OLR of 4g VS/ m^3 (186 ± 23 L_N/kg VS) was significantly lower than that at an OLR of 3g VS/ m^3. Seppälä *et al.* (2013) increased the composition of maize silage in a co-digested treatment containing CS to 67%. However, as the composition of maize silage in the treatment increased beyond 40%, a proportion of digestate was recirculated back to the reactor to prevent overloading. A 35% reduction in SMP was reported as the daily OLR was increased from 2.5 to 3g VS/ m^3 and maize silage composition increased from 60-67%, while keeping the same HRT of 25 days. Wall *et al.* (2014) were able to utilise the recirculation of or portion the digestate to increase the % composition of GS within a CS:GS co-digested mixture to 60%. Unlike Lehtomäki *et al.* (2007) and Seppälä *et al.* (2013), Wall *et al.* (2014) reported increasing SMP as the % composition of GS in the co-digested treatment increased from 40-60%. At a maximum OLR of 4g VS per m^3/d.

Wall *et al.* (2014) also noted TAN levels of 2.5g/L and that the system was bordering on the upper limits cut-off of AD process stability.

The mean SMP obtained for treatments in this study i.e. (CS = 197 L_N/kg VS, 80:20 = 228 L_N/kg VS, 60:40 = 249 L_N/kg VS) were lower than those reported for the Batch Experiment in Chapter 3 i.e. (CS = 255 L_N/kg VS, 80:20 = 296 L_N/kg VS, 60:40 = 341 L_N/kg VS). This resulted in bio-methane efficiency (BE) values < 1, ranging from 0.70 to 0.80, which were also congruent with existing literature. Likely reasons for differences are the humic acid concentration and biomass such as lignin content within the reactors in the CSTR experiments that would have been in higher concentration in the reactors in the CSTR experiment (fed daily) as opposed to the Batch Experiment. Humic acid has been reported to adversely affect fermentative hydrolytic bacteria and hydrogenotrophic methanogens degrading cellulosic biomass (Azman *et al.*, 2017). The daily feeding regime would have likely led to a higher concentration of humic acids within the reactors in the CSTR experiments as opposed to the BMP experiments where the VS of the feedstock is limited to 2% of overall VS contained within the batch reactor (VDI, 2006). By-products from the degradation of lignin i.e. phenol, compounds with aldehyde and carboxylic groups also inhibit the AD process; resulting in VFA accumulation (Chen *et al.*, 2008; Pokój *et al.*, 2015). Therefore a higher concentration of inhibitors within the reactors likely resulted in the lower SMP values reported in the CSTR experiment.

Lactic acid and VFA content in GS due to fermentation of sugars during the ensiling process (Nizami *et al.*, 2009; Prochnow *et al.*, 2009; Herrmann *et al.*, 2011) are likely to have altered the pH of each treatment resulting in the treatment having the highest proportion of GS having the lowest pH. Nousiainen *et al.* (2003) observed lactic acid content in GS ranging from 11-92g/kg TS while Wall *et al.* (2014) stated a mean lactic acid content in GS of 77g/kg TS. (Ward *et al.*, 2008) state that the ideal pH range for hydrolysis and acidogenesis was 5.5 – 6.5. The mean pH of 6.91 for the 60:40 treatment facilitates the growth of hydrolytic, fermentative bacteria as well as archaea.

The mean effluent pH at steady state within the CS-only, 80:20 and 60:40 reactors i.e. 7.4 was consistent with the range reported in the literature i.e. 7.3 to 7.6 (Lehtomäki *et al.*, 2007; Seppälä *et al.*, 2013; Wall *et al.*, 2014) and falls within the pH range of 6.5 to 7.5 that facilitates bacterial and archaeal growth (Ward *et al.*, 2008; Nizami *et al.*, 2009; Bayané and Guiot, 2010; Khalid *et al.*, 2011).

During the first 10 days of CSTR Experiment C2, TAN levels were within the 1.7 – 14g N/L range where methanogenic growth is inhibited (Chen *et al.*, 2008). Xie *et al.* (2011) state that a positive correlation of methane production and acetic acid indicates the presence of acetotrophic methanogens. Acetotrophic methanogens are more sensitive to inhibition due to elevated TAN levels than hydrogenotrophic methanogens (Chen *et al.*, 2008; Demirel and Scherer, 2008). The dilution of acetotrophic methanogens due hydraulic overload may have led to the initial accumulation of acetate. Similarly, the retardation of hydrogenotrophic methanogen growth precipitated the accumulation of a key substrate, formate. Free ammonia nitrogen (FAN) inhibition was an unlikely cause for the accumulation of VFA observed during HRT2 while operating at an OLR of 1g VS per m^3/d, since FAN levels were low i.e. 50mg NH_3-N/L across all treatments by day 10.

Batstone *et al.* (2002) state the main acid-base pairs that control the pH of the liquid phase within the CSTR reactors were due to equilibrium reactions involving NH_4^+/NH_3, CO_2/HCO_3^-, $H_2O/OH^-/H^+$ and VFA/VFA^-. There was no significant difference in methane concentration between the treatments at an OLR of 0.5g VS per m^3/d. At an OLR of 1g VS per m^3/d however, the methane concentration within the biogas of 60:40 treatment was significantly lower than the 80:20 and CS-only treatments. This was likely due to the low pH (4.7) distilled water being used to make the reactor feedstocks increasing the products of hydrolysis. The dominance of Isobutyric acid within the reactor effluent at both OLR levels is as a result of the degradation of proteins (Bruni *et al.*, 2013). TAN concentration is increased as amino acids are degraded; resulting in an increase in pH as ammonium hydroxide reacts with CO_2 to form bicarbonate (Möller and Müller, 2012). This increase in pH was countered by continuous production of VFA during the hydrolysis and acidogenesis phases of the AD process; providing a stable pH as steady state conditions was approached.

Möller and Müller (2012) also note that the digestate of biodegradable feedstock containing ammonia characteristically has higher levels of TAN as a proportion of total nitrogen (TKN). This was consistent with the findings of this study where the fraction of TAN in total nitrogen across all treatments was higher in the effluent samples taken during steady state. The highest TAN: total nitrogen ratio was observed in the feedstock with the lowest C:N ratio; highlighting characteristic accumulation of mineral N within the AD process and the high suitability for the digestate as a fertiliser to promote crop growth.

Dosing the reactors with Na_2CO_3 during days 31 to 50 and subsequent split-feeding assisted by increasing reactor pH to stimulate methanogenic activity/growth thereby reduce propionate and butyrate levels. The increase in the HPr:HAc to > 1.4 during days 30 and 35 for the 60:40 treatment was concomitant with reduced biogas and propionate levels between 1.5 -1.7g/L and is consistent with the literature (Marchaim and Krause, 1993; Aboudi *et al.*, 2016). Hill *et al.* (1987) conducted a review of the literature relating to the stability of AD systems treating cow or swine manures and determined that acetate levels > 0.8g/L and propionate: acetate (HPr:HAc) ratios greater than 1:1.4 are signs of imminent reactor failure. This was later validated by Marchaim and Krause (1993) by spiking reactors treating CS with glucose to induce instability. Marchaim and Krause (1993) noted that HPr:HAc ratios were effective at predicting instability and process failures in well buffered systems. However, other authors have indicated that, although HPr:HAc ratios can predict imminent reactor failure, the 1:1.4 cutoff varies with substrate type and reactor performance (Aboudi *et al.*, 2016). Pullammanappallil *et al.* (2001) observed HPr:HAc ratios as high as 56 and that reactors fed with propionate tolerated propionate levels of 2.8g/L without becoming unstable. Aboudi *et al.* (2016) observed stable reactor performance at HPr:HAc ratios between 0 and 2, and noted that propionate concentration ranging from 1.1 – 5.5g/L, in reactors co-digesting sugar beet by-product and cow manure, did not cause the reactors to become unstable. They also suggested that higher HPr:HAc ratios are associated with a decoupling of the metabolic rates between the acid-producing microbes and those responsible for VFA consumption.

The results of feedstock characterisation in Chapter 3 indicated that the CS-only and the CS:GS treatments mainly comprised of carbohydrates. The glycolysis of glucose, a primary monomeric subunit of hemicellulose and cellulose, produces pyruvate which is further metabolised into butyrate, lactate, acetate, 2,3 butan-1-ol, caproate, acetone, acetoine, ethanol and other metabolites (Tracy *et al.*, 2012). Lactate acid is further metabolised to propionate acid by fermentative bacteria. Propionate and butyrate oxidation take place in the presence of obligate syntrophic partner microorganisms via inter-species electron transfer (IET) using either hydrogen and/or formate as electron donors (Stams and Plugge, 2009; Zhao *et al.*, 2016).

The oxidation of propionate to acetate is thermodynamically possible when hydrogen partial pressure and or formate levels within the reactor are kept low by hydrogenotrophic methanogens. and is given by the following equations by Ahlert *et al.* (2016) and Stams *et al.* (2003):

$$Propionate^- + 3H_2O \rightarrow Acetate^- + HCO_3^- + H^+ + 3H_2 \quad \text{Eq. 5.5}$$

$$Butyrate^- + 2H_2O \rightarrow 2Acetate^- + H^+ + 2H_2 \quad \text{Eq. 5.6}$$

High hydrogen partial pressures and/or formate levels can inhibit propionate and butyrate oxidation of propionate and butyrate (Ozturk *et al.*, 1989; Stams and Plugge, 2009). Zhao *et al.* (2016) state that the reducing equivalents of IET, either hydrogen and/or formate, must be efficiently consumed by the partner methanogen in order for both syntrophic microorganisms to grow. In this study, the propionic to acetic acid (P:A) ratio within the effluent of the 60:40 treatment was greater than 1.4 between days 30-35 of CSTR experimental run C2-02. Therefore, the accumulation of propionic acid, formic and butyric acids for the all treatments during HRT2 while operating at 1g VS per m^3/d (Fig. 5.11) infers that an AD process imbalance was caused by an uncoupling of the syntrophic relationship characterised by inter-species formate transfer between propionate oxidising bacteria and their syntrophic methanogen. Theuerl *et al.* (2015) also observed VFA accumulation while monitoring CSTR digesting agricultural waste and energy crops and noted that the accumulation indicates a failing consumption of the produced VFA which led to a reduction in biogas production. Propionate accumulation was most severe with the treatment that has the highest level of digestible carbohydrates i.e. the 60:40 CS:GS mixture.

Split feeding the digesters during HRT3 limited the concentration of VFA within the liquid phase of the reactors and alleviated the process imbalance over time.

5.5.2 Conclusions

The salient points of this chapter are:

- The low SMP of the CS-only treatment justifies commercial co-digestion with GS. The C:N ratio of the 60:40 treatment was 11% higher than the CS-only treatment ($p < 0.001$); providing an additional carbon source for methane synthesis;
- The 60:40 treatment produced 31% more methane than the CS-only treatment thereby confirming the suitability of GS as substrate for co-digestion;
- The treatment with the highest proportion of GS had the lowest feed pH. Low pH facilitates early acidogenesis and increases the degradation rate. Higher OLRs may require recirculation of digestate, dosing with carbonates added buffering capacity and avoid process imbalances due to propionate accumulation;
- Process imbalances occurred at relatively low OLR i.e. 1g VS per m^3/d and were most severe in the 60:40 treatment. The cause of the disruption was most likely due to an uncoupling of the syntrophic relationship between propionate oxidising bacteria and archaea characterised by inter-species formate transfer;
- pH, TAN, total VFA, and propionic acid are key process parameters that should be monitored during the co-digestion of CS and GS. Regardless of the OLR level, TAN concentrations <1.5g/L, can decouple the syntrophic microbial metabolism of key intermediates such as propionate, resulting in sub-optimal AD performance.

Chapter 6 Microbial diversity changes in CSTR through the co-digestion of cattle slurry and grass silage

6.1 Introduction

Manure-only AD produces less biogas than co-digested mixtures because of its low total solids (TS) content (Seppälä *et al.*, 2013; Mata-Alvarez *et al.*, 2014) and low carbon to nitrogen ratio. High total ammonia nitrogen (TAN) levels have also caused inhibition or poor performance within manure fed digesters (Fotidis *et al.*, 2014b). Co-digestion with feedstocks having higher CN ratios have been shown to improve biogas quality and production in AD systems processing agricultural waste (Prochnow *et al.*, 2009; Seppälä *et al.*, 2013; Mata-Alvarez *et al.*, 2014; Allen *et al.*, 2016). The use of ensiled grass has been demonstrated to enhance AD process performance (Prochnow *et al.*, 2009; Tsapekos *et al.*, 2017). Low C:N ratio feedstocks such as cattle slurry (CS), provide the requisite microbial consortia, buffering capacity and macro and micro-nutrients, while grass silage (GS) provides the carbon source required for biomethanation (Mata-Alvarez *et al.*, 2014).

However, knowledge regarding the effect of how AD process parameter variations affect the syntrophic relationships between bacteria and archaea within AD systems and the impact on biogas quality remains limited (Stams *et al.*, 2003; Karakashev *et al.*, 2005; Williams *et al.*, 2013; Westerholm *et al.*, 2016). In Chapter 5, a process imbalance in the AD of CS and CS co-digested with GS led to the accumulation of VFA in the reactors. Volatile fatty acid degradation, particularly propionate, is considered rate-limiting in the AD process (Nielsen *et al.*, 2007; Ito *et al.*, 2012). Knowledge is limited regarding mechanisms such as inter-species electron transfer and the syntrophs involved in propionate and butyrate oxidation as well as the final stage of biomethanation that affect biogas production and quality (Stams *et al.*, 2003; Stams and Plugge, 2009; Zhao *et al.*, 2016).

Advances in molecular techniques such as polymer chain reaction PCR (Harms *et al.*, 2003), real-time polymer chain reaction (qPCR), Denaturing Gradient Gel Electrophoresis (DGGE) (Muyzer *et al.*, 1993), terminal restriction fragment length polymorphism (T-RFLP) (Liu *et al.*, 1997), fluorescence in-situ hybridization (FISH) (Wagner *et al.*, 2003) and high-throughput Next Generation Sequencing (NGS) of the variable regions of 16S rRNA amplicons (Reuter *et al.*, 2015) have provided a general knowledge of the nature and relationships between the trophic groups (Albertsen *et al.*, 2015; Callahan *et al.*, 2016b; Cardinali-Rezende *et al.*, 2016). However knowledge of co-digesting communities is still lacking. More research involving the characterisation of the microbial consortia, assessing their function within the AD process and correlating shifts in the microbial community with AD process parameters is also required (Wang *et al.*, 2010b; Cadavid-Rodríguez and Horan, 2013; Mata-Alvarez *et al.*, 2014; Sun *et al.*, 2015). Molecular techniques such as PCR used in library preparation are prone to errors (Farrelly *et al.*, 1995; Desai *et al.*, 2010). Bioinformatic tools used to cluster or bin denoised operational taxonomic units (OTUs) may over-estimate biological diversity in the presence of noise larger than the threshold cut-off (i.e. typically 97%) and are unable to assess biological diversity on a scale higher than the chosen threshold. Partitioning sequences using fine-scale binning algorithms can provide additional information regarding microbial ecological niches as well as community strucure and dynamics (Koeppel and Wu, 2013; Callahan *et al.*, 2016a).

This research aims to reduce the uncertainty associated with the characterisation of the microbial community degrading CS and CS:GS treatments through mesophilic AD operating in continuous mode using a novel algorithm called Divisive Amplicon Denoising Algorithm (DADA2) (Callahan, 2016). High-throughput NGS 16S rRNA cloned libraries were used to characterise the microorganisms into unique Ribosomal Sequence Variants (RSV) as opposed to the traditional OTUs.

Since there are no existing examples in the literature where DADA2 was used to characterise the microbial community of the AD process, this research will evaluate the power of this tool to provide more rigorous examination of the impact of GS addition and the AD process parameters on the microbial community dynamics and biogas quality.

The objectives of this research are to:

- Characterise the microbial communities digesting agricultural substrates at a higher nucleotide resolution than the standard OTU 97% clustering method, providing greater insight into the syntrophic relationships between the microorganisms within a continuous AD process.
- Evaluate phylogenetic differences within and between treatments as steady-state is approached within the reactors.
- Correlate the shifts in the microbial community to physico-chemical observations and determine the AD process parameters that have greatest influence on the dominant bacteria and archaea.

6.2 Materials and methods

All samples were collected and analysed in accordance with the experimental design outlined in Chapter 5 and the analytical methods described in Chapters 3 and 4. Samples for microbial community analysis were taken from reactors processing the 60:40 (CS:GS) and CS treatments only every five days. One biological sample, on days 20, 40, and 75, was taken from reactors R1, R2, R5 and R6 for each experimental run; i.e. runs C2-01 and C2-02 (Fig. 6.1). Hence, there were duplicate biological replicates for each treatment mixture per experimental run. Additional samples were taken on day 25 for reactors R1 and R2 and day 65 for reactors R5 and R6 for the CSTR Experimental run C2-02 in order to assess the microbial community dynamics during the time when biogas production was reduced due to propionate accumulation.

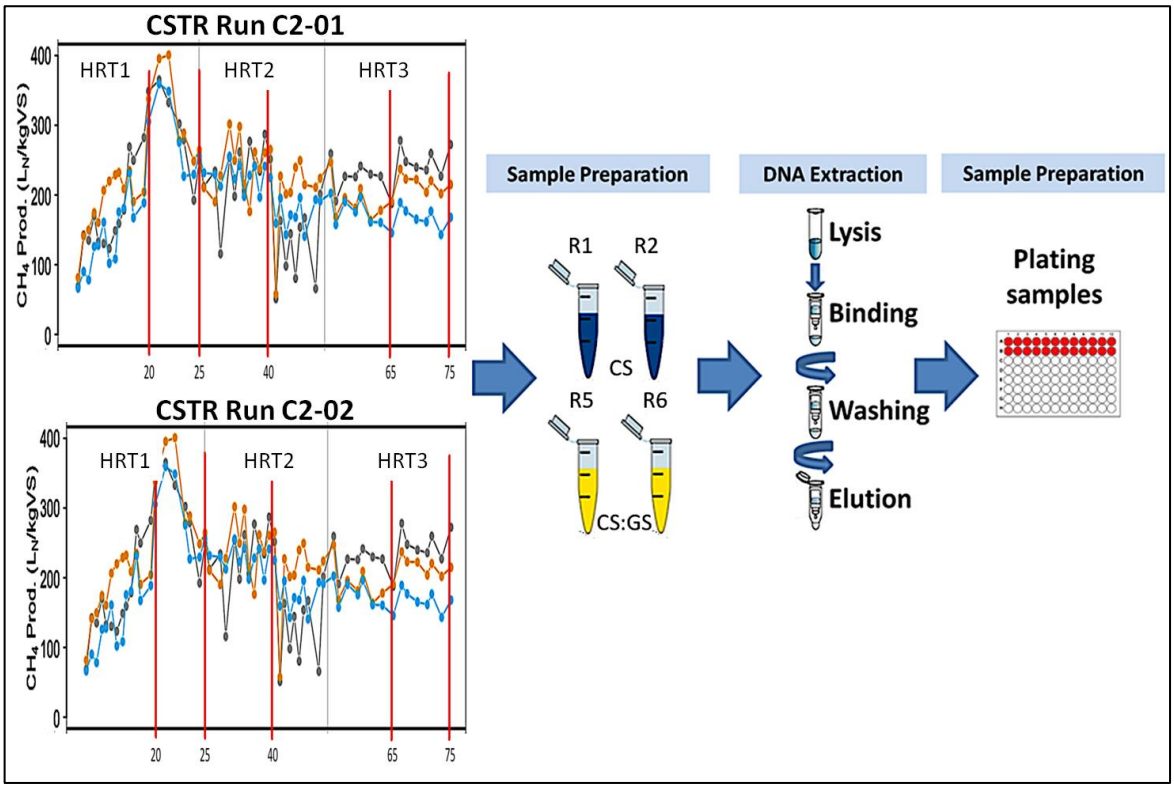

Fig. 6.1 Sample selection and preparation process. Sampling times are indicated by red lines.

For each treatment, days 20, 40 and 75 represented the state of the microbial community during the first, second and third hydraulic residence times (HRT1-3) of the AD process. Sample storage and DNA extraction were conducted in accordance with the protocol described in Chapter 4. DNA extracts were plated on a 96 well plate and sent to an external laboratory (NU Omics, Northumbria University, Newcastle-upon-Tyne, UK) for PCR amplification, library preparation and high-throughput amplicon sequencing of the V4 region of the 16SrRNA gene using an Illumina 2 X 250 MiSeq personal sequencer in accordance with the 16S Sequencing with the Illumina MiSeq Personal Sequencer protocol (Kozich *et al.*, 2003), as described in Chapter 4.

6.3 Bioinformatics and Analysis Workflow

Quality assurance and de-noising of the demultiplexed FastQ files received after Illumina MiSeq sequencing were once again performed using the DADA2 algorithm (Callahan *et al.*, 2016a) and quantification and qualification methods described in Chapter 4. The counts of biological replicates, i.e. samples taken on the same day from parallel reactors digesting the same treatment, were merged together. Samples from both experimental runs were combined to generate a phyloseq object for post-processing; using the *Phyloseq* package in R (McMurdie and Holmes, 2013) (Fig. 6.2).

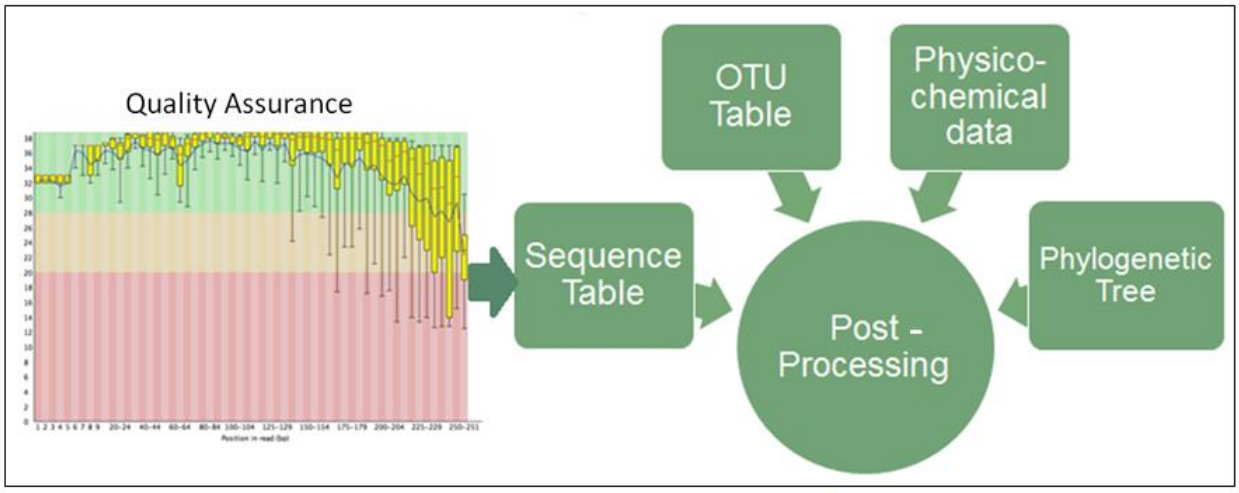

Fig. 6.2 Overview of the bioinformatics process for removing erroneous artefacts introduced into high-throughput NGS and inferring unique RSV

The sequence table created from de-noised, chimera free, merged reads that were classified to the species taxonomic rank using training set formatted for DADA2 developed from the recently updated Silva123 reference database (Quast *et al.*, 2013; Yilmaz *et al.*, 2014) Appendix 9. Sequences not yielding hits against the Silva123 database were subsequently identified by Blasting against the NCBI reference database (Zhang *et al.*, 2000).

Sequence alignment was performed using the *DECIPHER* package (Wright, 2016) while the phylogenic tree object was created using the *Phangorn* (Schliep, 2011) package in R. The Chao1 and Shannon richness indices were used to describe the alpha diversity of the CS and CS:GS samples. The Chao1 richness index is calculated as follows:

$$S_{Chao1} = S_{obs} * \frac{F_1^2}{2F_2} \qquad \text{Eq. 6.1}$$

where S_{obs} is observed number of RSV within a sample, F_1 is the number of observed RSV represented by a single individual (singletons) and F_2 the number of observed RSV represented by two individuals (doubletons). The Shannon richness index is given by:

$$H' = -\sum p_i * \ln(p_i) \qquad \text{Eq. 6.2}$$

where p_i is the proportion of individuals seen in the i^{th} species (Magurran, 2013). The metadata file containing the environmental variables was compiled by finding the means of the physico-chemical and AD process parameters grouped by reactor and HRT (Table 6.1). Process parameter values were normalised or converted to their z-scores using the "*scale*" function in r which uses the formula:

$$z = \left(\frac{X - \bar{X}}{\sigma}\right) \qquad \text{Eq. 6.3}$$

where:

$X =$ process variable value
$\bar{X} =$ the mean of all of the process variable values
$\sigma =$ standard deviation of the process variable values

Table 6.1 Mean values of the physico-chemical and AD process parameters by reactor and HRT used in post-processing

Treatment	Reactor	HRT	Methane Content (%)	Biogas Production L_N/d	Methane Production L_N/d	VS kg/L	TS kg/L	COD g O_2/L	pH	TKN g/L	TAN g/L	FAN mg/L	Total VFA g/L
CS	R1	H1	64.9	868.9	580.3	0.0137	0.0195	33.4	7.60	2.0	1.6	74.21	7.8
	R2	H1	65.9	1237.4	836.3	0.0129	0.0185	29.9	7.62	1.9	1.3	53.13	7.9
60:40	R5	H1	52.0	906.3	508.9	0.0126	0.0182	24.9	7.50	2.7	1.8	80.14	12.3
	R6	H1	56.6	1285.4	786.7	0.0115	0.0315	20.2	7.42	2.3	1.6	64.91	9.3
CS	R1	H2	65.4	1087.1	714.0	0.0168	0.0220	27.5	7.29	1.5	0.8	16.56	1.0
	R2	H2	63.6	1141.5	732.3	0.0162	0.0214	28.5	7.32	1.5	0.9	17.86	1.2
60:40	R5	H2	71.6	1844.8	1336.4	0.0127	0.0178	30.3	7.36	1.8	1.0	26.50	2.6
	R6	H2	67.2	1846.9	1250.4	0.0125	0.0301	27.9	7.34	1.4	0.8	16.50	0.3
CS	R1	H3	66.9	1256.7	836.3	0.0149	0.0207	30.6	7.21	1.3	0.5	10.86	2.6
	R2	H3	64.3	1267.8	811.4	0.0150	0.0207	29.8	7.37	1.2	0.5	9.97	2.7
60:40	R5	H3	68.3	1516.9	1032.0	0.0136	0.0183	24.9	7.20	1.3	0.5	8.84	1.1
	R6	H3	66.0	1565.2	1034.9	0.0146	0.0192	25.0	7.23	1.1	0.4	6.45	0.6

A total of 3.2 million reads were processed and a breakdown of the RSV taxonomic assignment is given in Table 5.2.

Table 6.2 Classification of RSVs at the kingdom taxonomic rank

Bacteria	Archaea	Eukaryota	Unassigned
4,711	82	8	8

Reads classified as *Eukaryota* or unassigned were removed prior to downstream analysis. Unassigned reads, classified at the kingdom taxonomic rank, relating to the CS and 60:40 treatments, represented 0.2% and 0.5% of the respective total abundance.

6.4 Statistics

Statistical analyses were conducted using the open source software package R version 3.3.2. KRONA software (Ondov *et al.*, 2011) was used to depict the relative composition of bacteria and archaea within the microbial communities of the CS and CS:GS treatments. It was not possible to validate the relative abundance data with actual counts from real-time PCR due to a limited research budget. However results were validated through comparison with studies where similar substrates were treated (Westerholm *et al.*, 2012a; Wirth *et al.*, 2012; Sun *et al.*, 2013; Theuerl *et al.*, 2015; Sun *et al.*, 2016). Chao1, Shannon and observed diversity indices were used to describe the alpha diversity of the CS and CS:GS treatments. A prevalence filtering threshold was defined to produce the top 15 bacterial phyla present in 2.5% of the total CS and CS:GS samples and was used to indicate changes in relative abundances.

Principal Coordinate Analysis and Non-metric multidimensional (NMDS) scaling with Bray-Curtis (Bray and Curtis, 1957) dissimilarity were used to visualise phylogenetic variation. The top 30 genera in 10% of the samples were considered dominant and were selected for ordination in order to ensure that the ordination plots were legible and inferences relating to intra- and inter-species interactions can be made. Ribosomal sequence variants for dominant archaeal or bacterial genera were reported using the NCBI sequence ID and percent similarity of the RSV sequence to the sequence identified in the NCBI database.

Ribosomal sequence variants were only reported if their relative abundance within a sample was greater than 0.1%.

Sample counts were transformed to relative abundances to ensure variances were similar across all samples and z-scores of the process variables were used for ordination analysis (Ramette, 2007) since the AD process variables (Table 6.1) were measured in different units and vary in magnitude (Daniel *et al.*, 2011). The function "bioenv" from the *vegan* R package (Oksanen *et al.*, 2017) facilitated the selection of the process variables for use in determining their influence on the dynamics of the microbial community using Constrained Coordinate Analysis (CCA). Significance testing for CCAs was conducted by using Monte Carlo with 1000 permutations and correlations were considered significant at a p-value < 0.05.

6.5 Results

The range in sequence depth, 142,722 for the CSTR samples was much smaller than that for the batch experiment samples analysed in Chapter 4 (218,940) with the largest variation occurring between biological replicates of the CS treatment on day 75. The effect of this variation was minimised by summing the reads of biological replicates together prior to conducting downstream analysis.

6.5.1 Characterization of the microbial community by treatment

The CS and 60:40 RSV classification profiles were different with regards to the top four abundant phyla. Taxonomic profiles for both treatments comprised of 93% bacterial and 7% archaeal microorganisms. *Firmicutes* (46%), *Bacteroidetes* (26%), *Atribacteria* (3%) and *Actinobacteria* (3%) were the most abundant bacterial phyla while *Euryarchaeota* (6%) was the most abundant archaeal phyla for the CS treatment (Fig. 6.3). *Euryarchaeota* was also the most abundant archaeal phyla for the 60:40 treatment with 6%.

However the top four abundant bacterial phyla for the 60:40 treatment were *Firmicutes* (37%), *Bacteroidetes* (30%), *Parcubacteria* (5%) and *Tenericutes* (3%). The relative abundance of the phylum *Bacteroidetes* was higher in the CS:GS (30%) treatment than the CS (23%) treatment throughout the experiment. This was due in part to differences between the CS and CS:GS treatments in the relative abundance of phylotypes within the phylum *Bacteroidetes* belonging to *Proteiniphiium spp*. (0.9%, 4%), *Dysgonomonas spp.* (1%, 4%) and *Petrimonas spp.* (4%, 6%) respectively. As steady-state conditions approached, the relative abundance of the phylum *Bacteroidetes* was significantly higher in the CS:GS (26.3 ± 1.08%) treatment than the CS (19.0 ± 2.29%, $p < 0.01$) treatment and the relative abundance of *Firmicutes* was higher in the CS-only treatment (44.0 ± 2.15%) than the CS:GS treatment (36.3 ± 1.14%, $p < 0.01$).

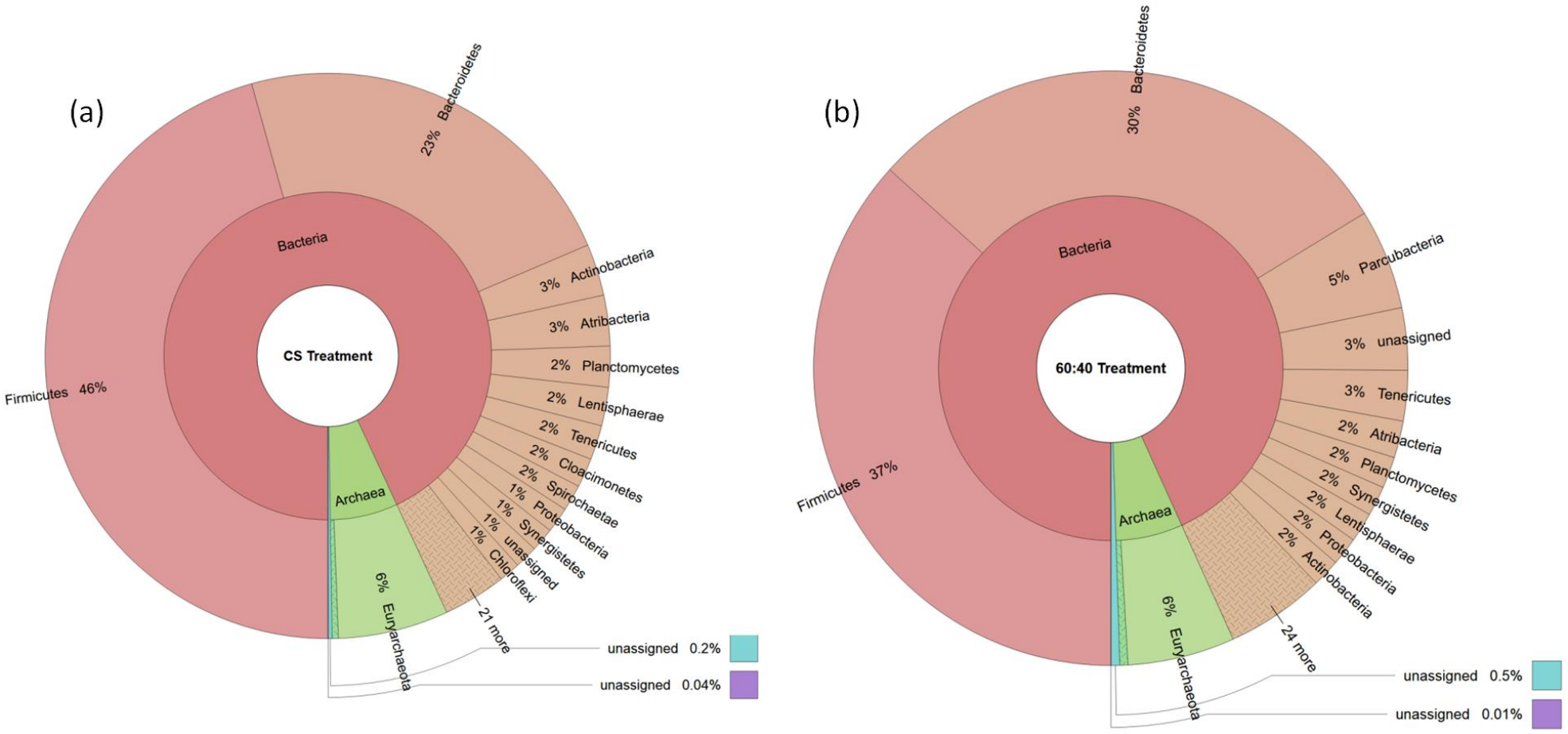

Fig. 6.3 The combined relative composition of taxa in (a) CS and (b) 60:40 treatments at the phylum level

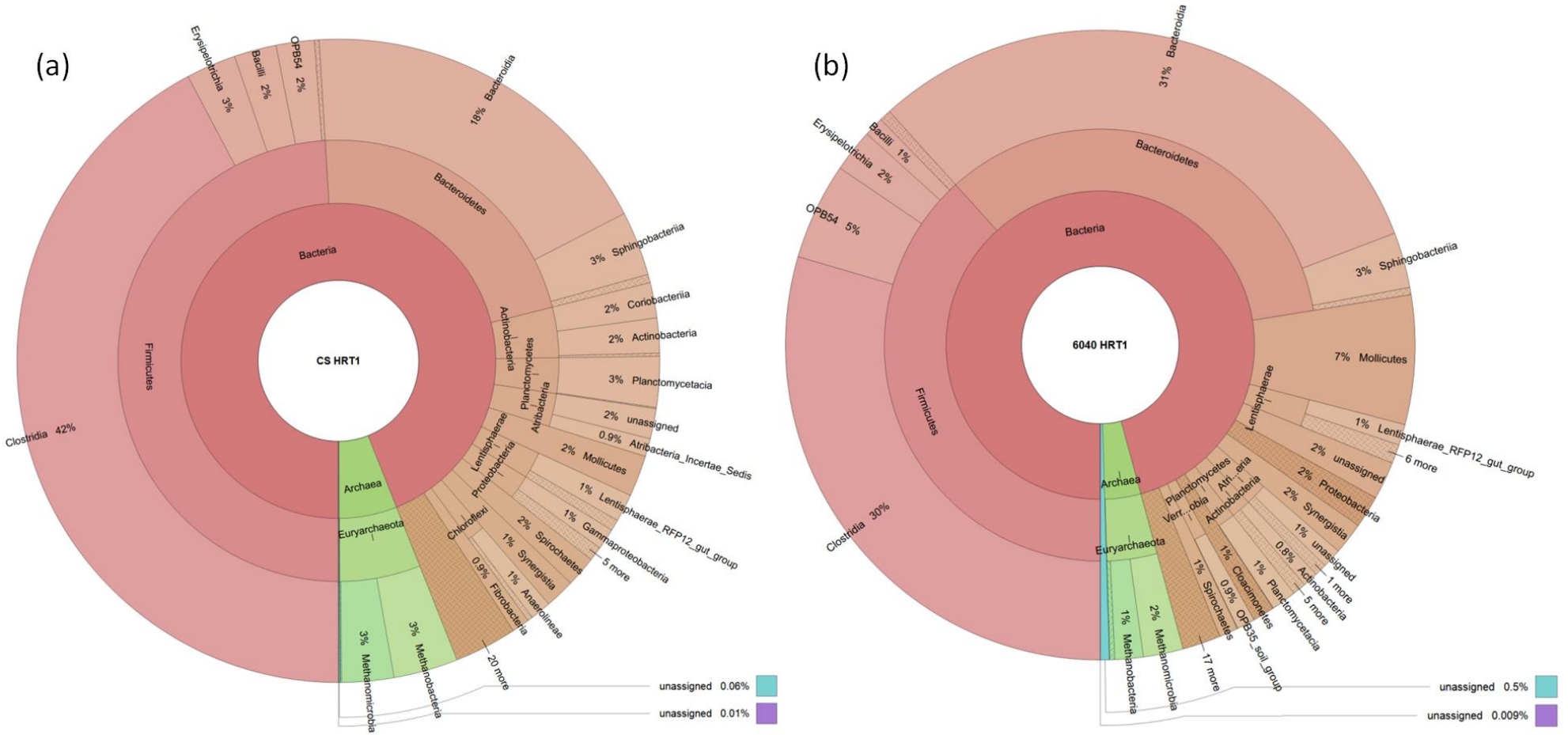

Fig. 6.4 Relative composition of taxa on day 20 in (a) CS (b) 60:40 treatments at the order level

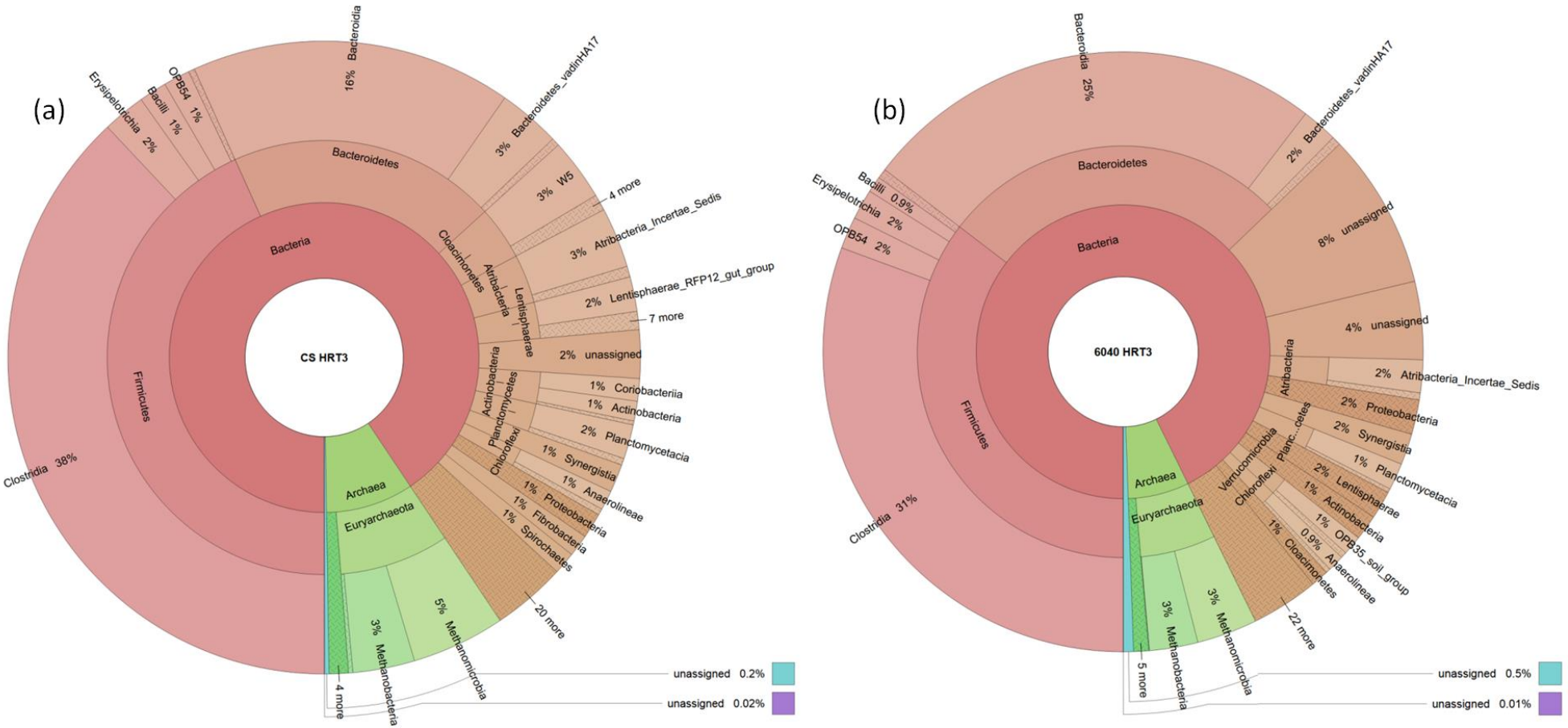

Fig. 6.5 Relative composition of taxa on day 75 in (a) CS (b) 60:40 treatments at the order level

The relative abundances of the taxa within the CS treatment classified at the Order of *Clostridia* (42%) and *Bacteroidia* (18%) were different to those observed for the 60:40 treatment, i.e. *Clostridia* (30%) and *Bacteroidia* (31%) for samples taken on day 20 (Fig. 6.4). Phylogenetic differences were also observed between the CS and 60:40 treatment samples taken on day 75 (Fig. 6.5). Taxa of the Order *Clostridia* and *Bacteroidia* had relative abundances of 38% and 16% respectively in the CS and 31% and 25% respectively in the 60:40 treatment samples. There was therefore evidence of phylogenetic differences within treatments based on the day the samples were taken. Archaea in both treatments were dominated by the phylum *Euryarchaeota* and mainly consisted of the class *Methanomicrobia* (51%, 63%) and *Methanobacteria* (47%, 36%) for the CS and 60:40 treatments, respectively.

The phylum *Parcubacteria* was observed and had a mean relative abundance of 5% in the CS:GS treatment and 0.5% in the CS-only treatment (Fig. 6.6). *Parcubacteria spp.* was only detected in HRT2 and HRT3 of the CS:GS treatment samples; indicating the growth rate of the *Parcubacteria spp.* may be relatively slow. The relative abundance of phyla within the CS-only treatment sample taken on days 25 and the CS:GS treatment sample taken on day 65 of experimental run C2-02, during the period of process imbalance, is illustrated in Appendix 11.

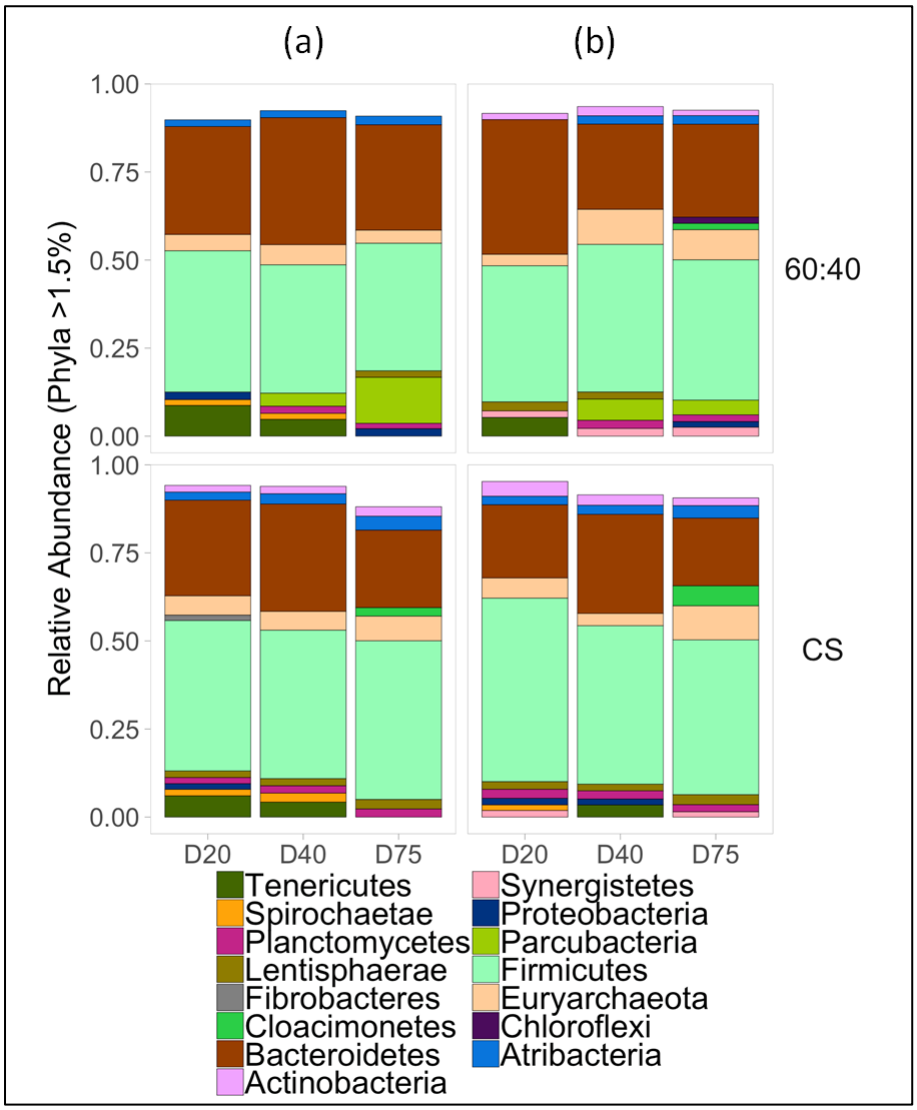

Fig. 6.6 Relative abundance of phyla present in more than 1.5% of the CS and CS:GS samples in CSTR Experiment (a) C2-01 and (b) C2-02.

Although RSVs belonging to the phylum *Parcubacteria* clearly indicated phylogenetic differences between the CS and CS:GS treatments, the sequences were not classified to the lower taxonomic rank of genus by the Silva 123 reference database and were therefore excluded from the downstream beta diversity analysis. Taxa belonging to the phylum *Cloacimonetes* had a significantly higher relative abundance during steady-state conditions in the CS-only treatment (4.0 ± 0.79%) than the CS:GS treatment (1.1 ± 0.45%, $p < 0.05$). Approximately 35% of the reads for both the CS and CS:GS treatments were not characterised to the genus level.

6.5.2 Assessing the alpha diversity within and between treatments

There was no significant difference for the Chao1 index between the fermenting bacteria populations for the CS and CS:GS treatments. Wittebolle *et al.* (2009) note that high levels of evenness within the microbial community promotes good functionality and states that highly uneven microbial communities are more susceptible to environmental perturbations. The bacterial community for the CS treatment exhibited more evenness than the CS:GS treatment ($p < 0.05$) (Fig. 6.7). This implies that the microbial community for the CS:GS treatment had one or a few dominant species whereas the abundance of bacterial phylotypes within the CS microbial community was more balanced.

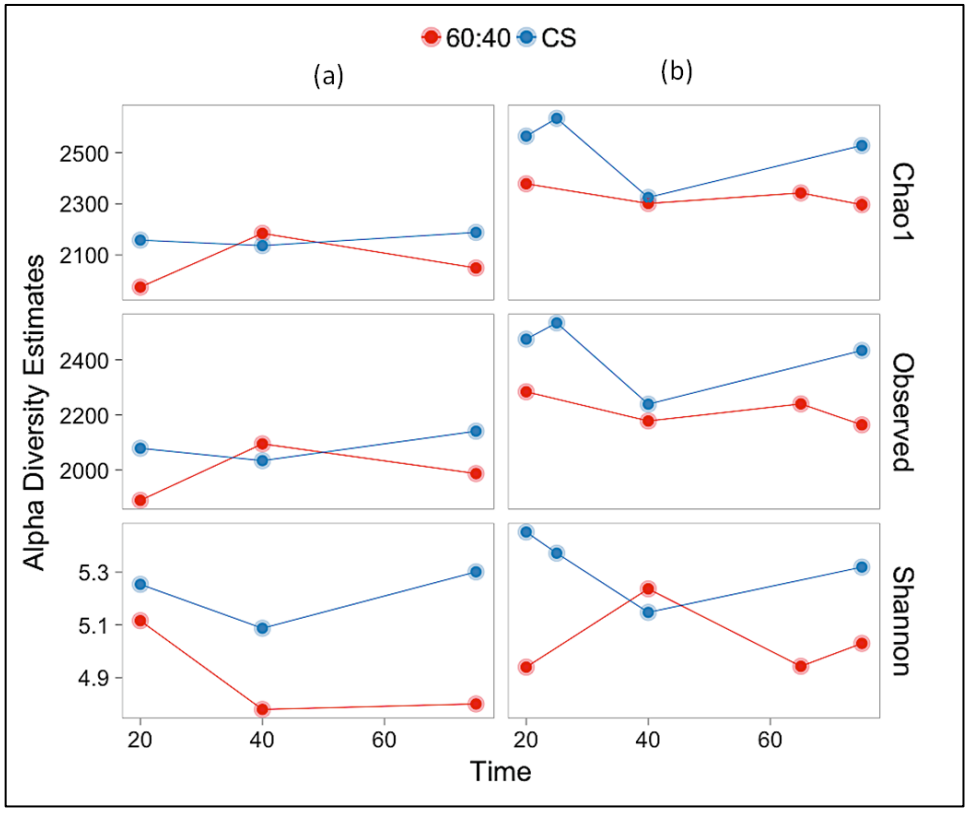

Fig. 6.7 Bacterial observed richness, Chao1 richness and Shannon diversity estimates versus time for the 60:40 and CS treatments for experimental runs (a) C2-01 and (b) C2-02. The 60:40 treatment is depicted in red and the Cattle Slurry only treatment in blue.

In run C2-02, evenness within the CS:GS bacterial community decreased from day 40 to 65, but began to increase towards the end of the run.

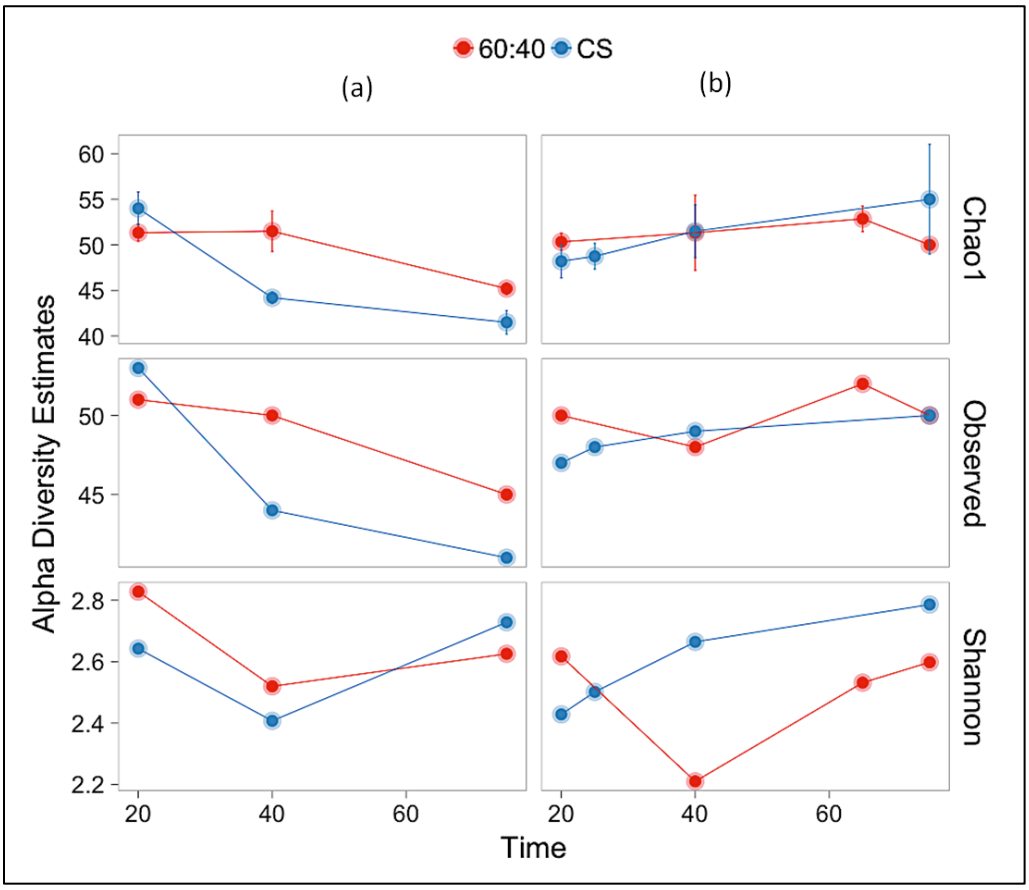

Fig. 6.8 Archaeal observed richness, Chao1 richness and Shannon diversity estimates versus time for the 60:40 and CS treatments for experimental runs (a) C2-01 and (b) C2-02. The 60:40 treatment is depicted in red and the Cattle Slurry only treatment in blue.

There was an overall general trend of increased evenness and decreased richness within the archaeal community for both treatments as the AD process entered steady-state conditions from HRT2 (Fig. 4.4). This implies that the archaeal sub-population evolved in a manner where some species were likely outcompeted for substrate by other dominant species over time.

6.5.3 The archaeal microbial community

The relative abundance of the archaeal genera indicate that evenness within the CS and 60:40 microbial communities at the beginning and end of the experimental run was similar, with a reduction in evenness occurring on day 40, the period of instability (Fig. 6.8 and Fig. 6.9). This is consistent with findings from Wittebolle *et al.* (2009) who state that increased levels of evenness is an indicator of reactor stability.

The process imbalance during the second HRT for both runs described in Chapter 5 may have affected the microbial community within the CS treatment to a lesser extent than the community digesting the 60:40 treatment. The relative abundance of archaeal genera within the CS-only treatment sample taken on days 25 and the CS:GS treatment sample taken on day 65 of experimental run C2-02, during the period of process imbalance, is illustrated in Appendix 10.

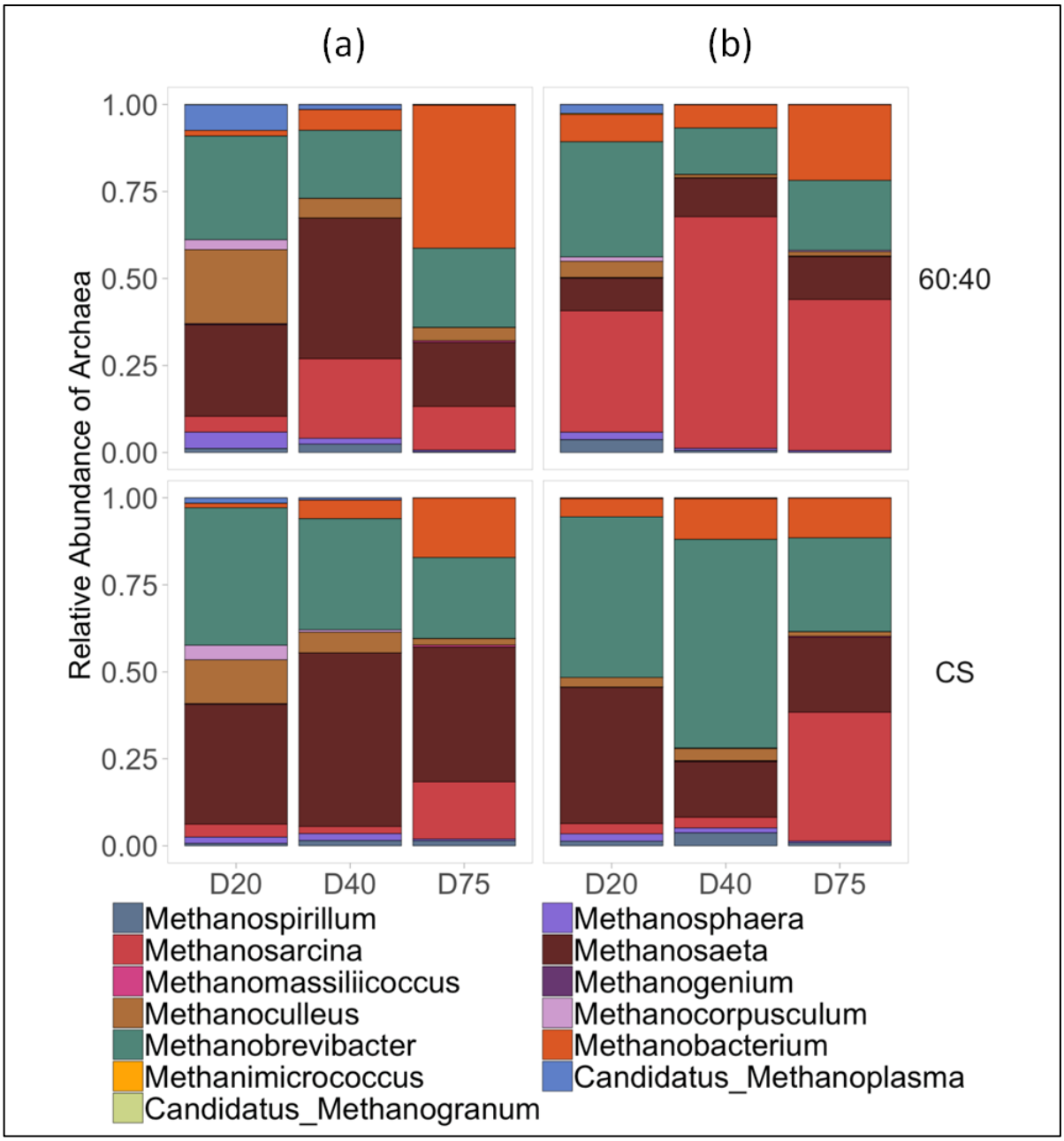

Fig. 6.9 Relative abundance of archaeal microbial community for the CS and 60:40 treatments in CSTR Experiment (a) C2-01 and (b) C2-02

The main archaeal genera found in both treatments for both runs were *Methanosaeta*, *Methanobrevibacter*, *Methanoculleus*, *Methnanobacterium* and *Methanosarcina* with both communities being dominated by hydrogenotrophs. The relative abundance of *Methanoculleus spp.* in the microbial community for both treatments decreased as steady-state conditions were established. *Methanobacterium spp.* however increased in relative abundance over the same period from 4% to 12% in the CS-only treatment and 5% to 23% in the co-digested treatment. The microbial community digesting CS had higher relative abundances of *Methanosaeta spp.* (30%) than the co-digested treatment (18%). *Methanosarcina spp.* generally increased in relative abundance from 3% to 25% in the CS-only treatment and 19% to 32% in the co-digested treatment. The relative abundance of *Methanosaeta spp.* and *Methanobrevibacter spp.* within the microbial communities for both treatments generally decreased, but remained dominant as the system approached steady-state conditions. There was however a momentary increase in the relative abundance of *Methanosaeta spp.* between days 40 and 65 of run C2-02, when the reactors digesting the 60:40 treatment were recovering from the process imbalance described in Chapter 5.

6.5.4 The bacterial microbial community

The bacterial genera *VadinBC27* remained dominant with a relative abundance of 7% for both treatments during both experimental runs (Fig. 6.10). This result was similar to that of the Batch Experiment in Chapter 4 where *VadinBC27 spp*. comprised 6% of the microbial community within the CS-only and 60:40 treatments. *VadinBC27 spp*. have never been cultured therefore very little is known about its role in the AD process. The relative abundance of bacterial genera within the CS-only treatment sample taken on days 25 and the CS:GS treatment sample taken on day 65 of experimental run C2-02, during the period of process imbalance, is illustrated in Appendix 11.

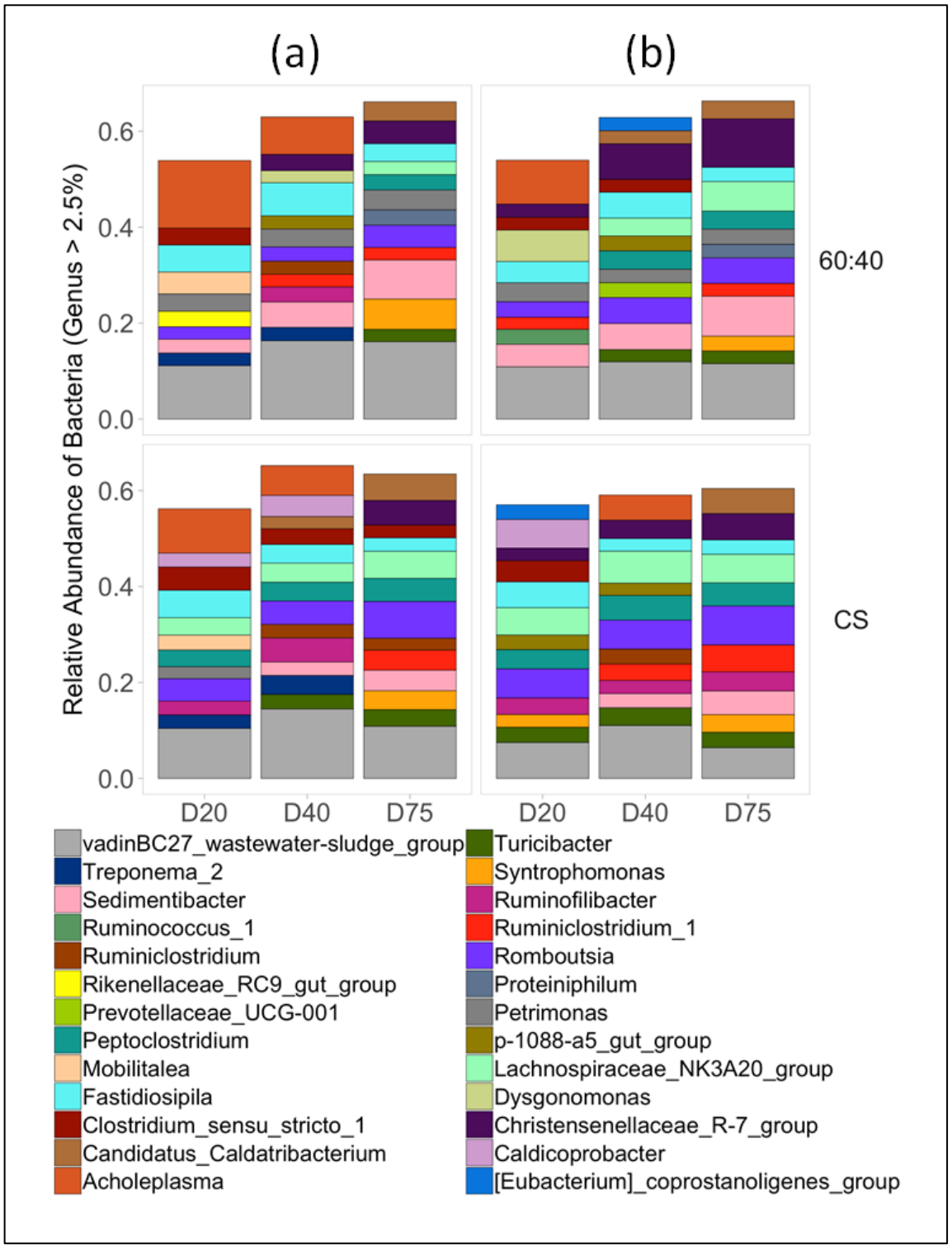

Fig. 6.10 Relative abundance of bacterial genera present in more than 2.5% of the CS and CS:GS samples in CSTR Experiment (a) C2-01 and (b) C2-02.

The presence or absence of genera such as *Rikenellaceae_RC9_gut_group*, *p-108-a5_gut_group* and *Ruminofilibacter* indicate the existence of phylogenetic differences between and within the CS and CS:GS treatment samples taken during run C2-01. Similarly, the presence or absence of genera such as *Ruminofilibacter*, *Ruminococcus_1*, *Caldicoprobacter* and *Dysgonomonas* also highlight phylogenic differences between and within the CS and 60:40 treatments for run C2-02. *Dysgonomonas spp.* were only present in the first two HRTs of the co-digested treatment with a relative abundance of 1% while *Acholeplasma spp.* (3%) were only present in the first two HRTs of both treatments; suggesting that phylotypes of these genera were most likely outcompeted for substrate by other microorganisms.

6.5.5 Assessing beta diversity between treatments

Principal Co-ordinate Analysis (PCoA) using the Bray-Curtis distance matrix explained 80% of the variation between samples with the first three principal co-ordinates (PCs), as shown by the scree plot (Fig. 4.8a). A PCoA plot of the latter indicates distinct, temporal phylogenetic differences in the samples (Fig. 4.8b). When considering a 2D plot of PC1 and PC3, Adonis test results indicate three distinct phylogenetic groupings (Fig. 4.8c), discriminated based on HRT in which each sample was taken ($p < 0.001$). PC2 explains 32% of the observed variation and clearly indicates phylogenetic differences between the CS and 60:40 treatments ($p < 0.001$).

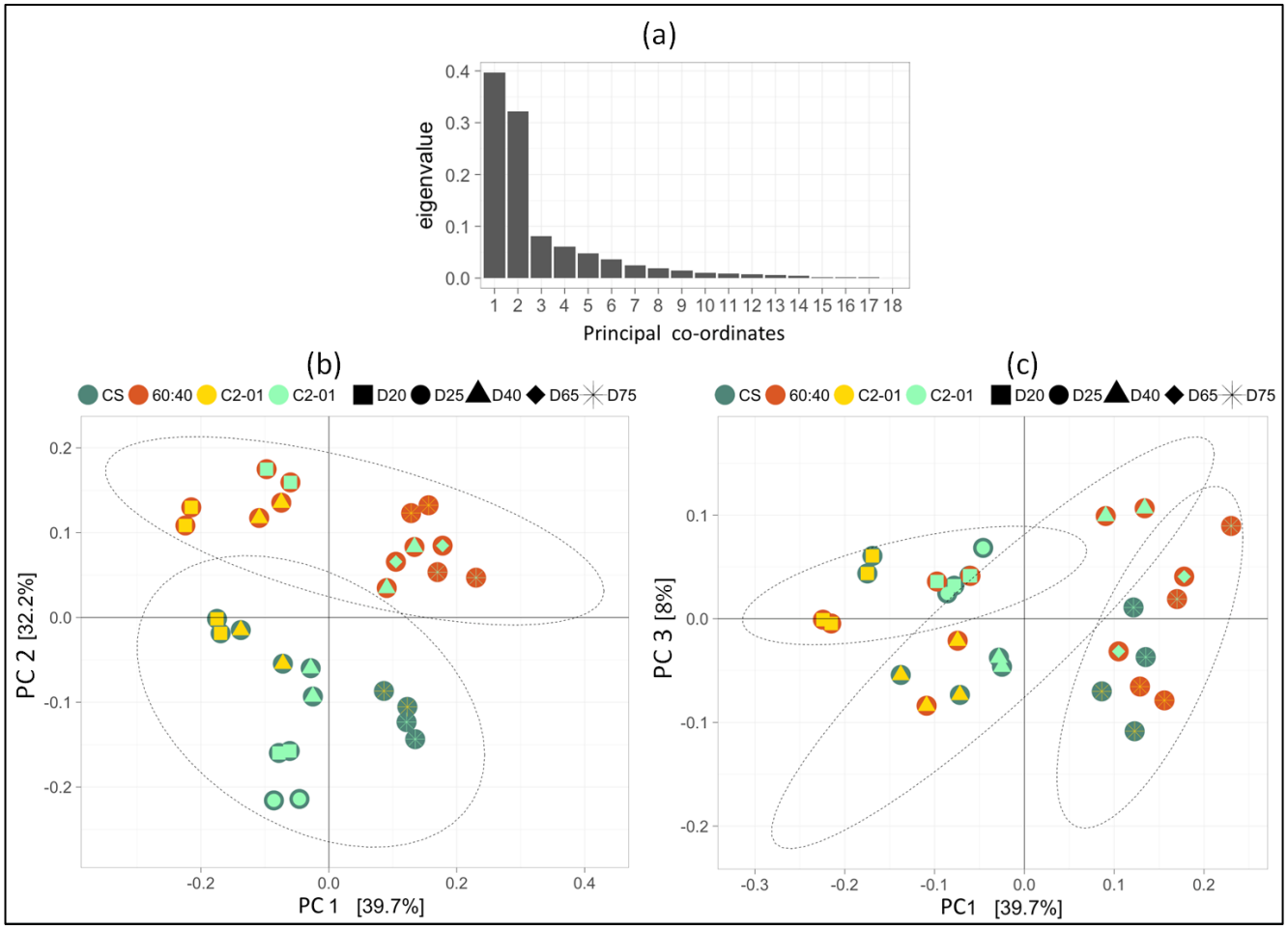

Fig. 6.11 PCoA with Bray-Curtis dissimilarity matrix of CS and 60:40 microbial communities showing axes (a) scree plot of ordination (b) PC1 vs PC2 with sub-samples grouped by experimental run along with 95% normal confidence areas and (c) PC1 vs PC3

Testing for homogeneity of group dispersion did not yield any significant results hence the null hypothesis of variance homogeneity across groups could not be rejected. The PCoA results therefore support previous observations asserting the existence of phylogenetic differences between the CS and 60:40 treatments and their respective microbial communities as they evolve from reactor start-up to attaining steady-state conditions in the CSTRs.

6.5.6 Assessing microbial community correlations with process variables

Volatile solids destruction, pH, TAN, total VFA (TVFA) and methane production were selected as the best combination of process variables to use in CCA ordination. There was a significant correlation of the canonical axes with the chosen explanatory variables ($p < 0.001$). The first two axes of the CCA plot (Fig. 4.9), constrained by the chosen process variables explained 80% of the phylogenic variation within the unconstrained plot. The top 30 genera observed at least once in 10% of all samples were dominated by bacteria of the order *Clostridiales, Bacteroidales*, *Spirochaetales*, *Erysipelotrichales*, *Acholeplasmatales* and *Planctomycetales*. Archaeal genera of the order *Methanosarcinales* and *Methanobacteriales* were the most prevalent.

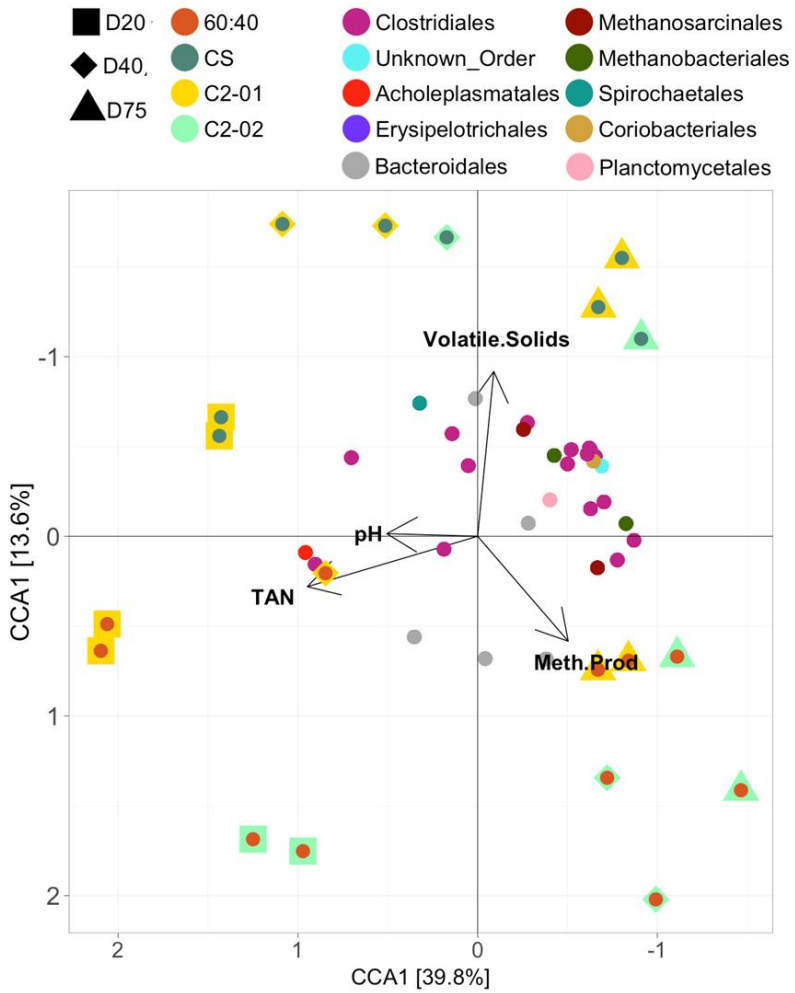

Fig. 6.12 CCA tri-plot of samples and the top 30 genera coloured by order with vectors showing the correlation of process variables. TAN = Total Ammonia Nitrogen, Meth.Prod = Methane Produced

Cattle slurry samples taken on day 20 contained bacterial phylotypes belonging to *Caldicoprobacter spp.* which had a maximum relative abundance of 4% within the microbial community. However the microbial community within samples of the CS:GS treatment taken on day 20 were associated with peaks in relative abundance of bacterial phylotypes belonging to *Mobilitalea spp.(*2%), *Acholeplasma spp.* (6%) and *Dysgonomonas spp.* (2%). Peaks in relative abundance of *Caldicoprobacter spp., Mobilitalea spp., Acholeplasma spp.* and *Dysgonomonas spp.* were associated with mid to high levels of pH, TAN and TVFA (Fig. 6.12) (Appendix 12).

The process imbalance in both treatments, described in Chapter 5, occurred during the second HRTs of CSTR Experimental runs C2-01 and C2-02 and was associated with mid to low levels of TAN. Microbial communities related to CS samples taken during the second HRT on day 40 were associated with peaks in relative abundance of bacterial phylotypes belonging to *Treponema_2 spp.* (2%), *Clostridium_sensu_stricto_1 spp.* (2%), *Turicibacter spp.* (2%) and *Ruminofilibacter spp.* (3%). The microbial community structure of CS:GS treatment samples taken on day 40 consisted of sub-populations belonging to *Fastidiosipila spp.* (4%) and *VadinBC27 spp.* (7%). Therefore fermentative bacterial phylotypes belonging to *Treponema_2 spp., Clostridium_sensu_stricto_1 spp., Turicibacter spp., Ruminofilibacter spp., Fastidiosipila spp. and VadinBC27 spp.* were likely to have been associated with the VFA accumulation observed during days 25-50 of both experimental runs.

The CS samples were associated with mid to high levels of VS while the CS:GS samples were associated with decreasing levels of VS as illustrated by samples taken on day 75 for both treatments (Fig. 6.12). This supports the findings in Chapter 5 where the CS:GS treatment was more biodegradable than the CS-only treatment as lignin, a recalcitrant non-VS, accumulates within the reactors. Mid to high levels of VS and decreasing pH, TAN and TVFA were associated with peaks in relative abundances of fermenting bacteria of the order *Clostridiales, Coriobacteriales*, *Spirochaetales* and *Bacteroidiales* and bacteria of the phylum *Saccharibacteria*.

Fermentative bacterial phylotypes that were associated with day 75 CS-only samples were *Lachnospiraceae_NK3A20_group spp.* (4%), *Candidatus_Caldatribacterium spp.* (4%), *Romboustia spp.* (4%) and *Ruminiclostridium_1 spp.* (3%) while *Sedimentibacter spp.* (4%), *Christensenellaceae_R-7_group spp.* (2%) and *Syntrophomonas spp.* (2%) peaked in relative abundance within the CS:GS treatment as steady-state conditions approached. Peaks in the relative abundance of fermentative bacteria associated with CS and CS:GS samples taken on day 75 occurred with decreasing TAN, TVFA and pH levels which is indicative of an active hydrolysis stage as observed in the batch experiment in Chapter 4. Mid to high levels of methane production were associated with the CS:GS treatment day 75 samples and peaks in relative abundances of fermenting bacteria of the order *Bacteroidetes* and *Clostridiales*. The archaeal community of the day 75 samples for the CS-only and CS:GS treatments primarily consisted of the obligate acetotrophic methanogens belonging to *Methanosaetaceae spp.* (24%, 13%), hydrogenotrophic methanogens belonging to *Methanobacteriaceae spp.* (52%, 46%) and mixotrophic methanogens belonging to *Methanosarcinaceae spp*. (9%, 25%) respectively (Fig. 6.12 and (Fig. 6.13)

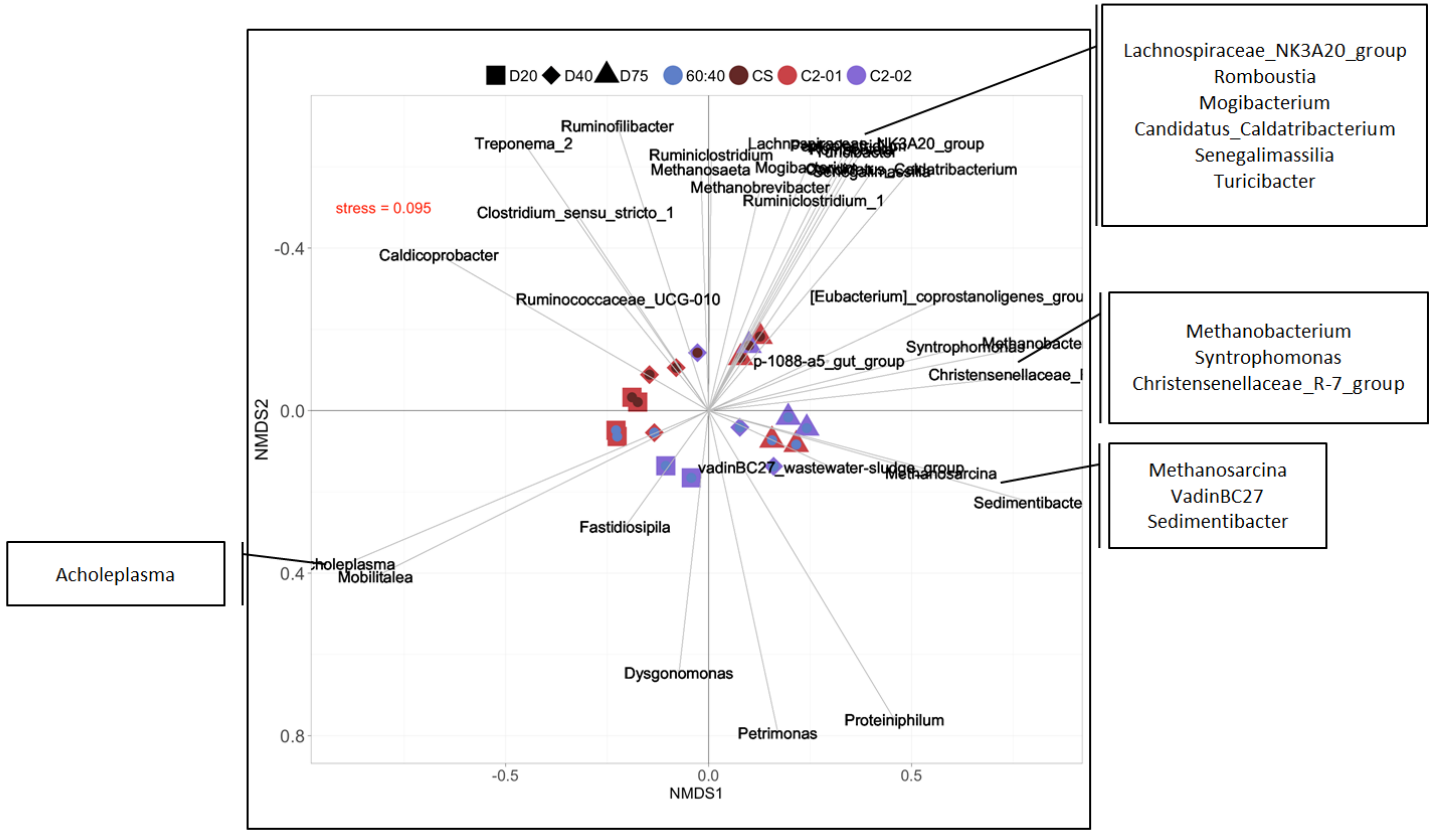

Fig. 6.13 Non-metric dimensional scaling (NMDS) of CS and 60:40 treatments and the top 30 genera observed at least once in 10% of all samples

Apparent coupling was observed between fermenting bacteria and archaea observed which is consistent with findings in the Batch Experiment in Chapter 4. The mono-digested CS treatment samples taken on day 75 were associated with more diverse bacterial and archaeal microbial sub-populations (Fig. 6.12) and (Fig. 6.13). For the CS-only treatments samples taken on day 75, peaks in relative abundance of bacteria such as *Ruminiclostridium spp*., *Turicibacter spp.* and *Mogibacterium spp.* (Appendix 12) were concomitant with peaks in the relative abundance of archaea belonging to hydrogenotrophic *Methanobrevibacter spp.* methanogens and obligate acetoclastic *Methanosaeta spp.* methanogens. However, unlike the previous findings in the batch experiment in Chapter 4, the apparent coupling of bacterial and archaeal partners differed between treatments. Peaks in relative abundance of fermentative bacteria belonging to *Syntrophomonas spp.* and associated with co-digested treatment samples taken on day 75, coincided with peaks in the relative abundance of archaea belonging to mixotrophic *Methanosarcina spp*. (25%) methanogens and hydrogenotrophic *Methanobacterium spp*. (54%) methanogens.

6.6 Discussion

Firmicutes and *Bacteroidetes* had the highest relative abundances in the microbial communities digesting the CS and CS:GS treatments. These observations re-affirm previous findings from the batch experiments in Chapter 4 and existing literature (Wirth *et al.*, 2012; Sun *et al.*, 2013; Ziganshin *et al.*, 2013). Sun *et al.* (2015) characterised the microbial community digesting cow manure (CM) and steam exploded wheat straw at temperatures of 37°C, 44°C and 52°C and observed *Firmicutes*, *Bacteroidetes* and *Cloacimonetes* to be the dominant phyla at 37°C. *Firmicutes* within the CM only treatment had a higher relative abundance i.e. (56-74%) than in the reactors digesting CM and straw (32.6-49.5%). In this study, the relative abundance of *Firmicutes* for the CS-only treatment (44.0 ± 2.15%) was significantly higher than that observed in the CS:GS treatment (36.3 ± 1.14%). Hanreich *et al.* (2013) and Sun *et al.* (2015) reported increased abundances of the phylum *Bacteroidetes* in microbial communities co-digesting wheat straw and hay. A similar trend was also observed in this study where the phylum *Bacteroidetes* was significantly lower in the CS-only treatment (19.0 ± 2.29%) than the CS:GS treatment (26.3 ± 1.08%).

Sun *et al.* (2015) was unable to definitively provide a reason for the higher relative abundance of *Bacteroidetes* in the co-digested treatment and noted that more research in the cellulolytic abilities of the phylum is required. Henderson *et al.* (2015) conducted a microbial community analysis of samples from ruminants across 35 countries and reported that bacteria belonging to the class *Bacteroidales* and *Clostridiales* comprise the dominant bacteria in the digestive tract of ruminants. The need for more research into phylum *Bacteroidetes* is also supported by this study since 35% of phylotypes within the CS:GS treatment belonging to the class *Bacteroidales* were unassigned compared to 29% within the CS treatment. Henderson *et al.* (2015) also state that some dominant rumen bacteria belonging to the poorly characterised phylum *Bacteroidetes* are purported to degrade cellulose and their genomes encode for a wide range of plant polysaccharide degradation. The relative abundance of the phylum *Bacteroidetes* was therefore likely higher in the CS:GS treatment due to the greater availability of easily degradable plant material.

Relative abundances of dominant phyla in the CS treatment were consistent with findings from Wirth *et al.* (2012), Sun *et al.* (2015) and Wang *et al.* (2010a), which highlight *Firmicutes* and *Bacteroidetes* as the typical dominant phyla with reactors digesting manure. St-Pierre and Wright (2014) observed similar lower relative abundances for the minor phyla *Actinobacteria*, *Proteobacteria* and *Synergistes* in mesophilic AD systems digesting manure. The relative abundances of these phyla for both the CS and CS:GS treatments in this study were low and ranged from (1-3%), which is consistent with the literature. Sun *et al.* (2015) stated that the higher relative abundance of the bacterial phylum *Cloacimonetes* in the CM only sample than the co-digested treatment contributed to the phylogenetic differences between the two treatments. A higher relative abundance of *Cloacimonetes spp.* (0.8%) observed in the CS-only treatment taken on day 75 as opposed to the CS:GS treatment (0.007%) also indicates the existence of phylogenetic differences between the CS and CS:GS treatments.

In addition, this study found that the presence of phyla *Parcubacteria* also contributed to phylogenetic differences between treatments (Fig. 6.6). The phylum *Parcubacteria* featured prominently in the CS:GS treatment (6%) but not in the CS treatment for the CSTR Experiments. *Parcubacteria spp*. are obligate fermenters of simple sugars and complex carbohydrates such as cellulose and chitin and possess genes linked to respiratory nitrate reduction (Nelson and Stegen, 2015; Dai *et al.*, 2016; León-Zayas *et al.*, 2017).

The relative abundance of lactic acid bacteria (LAB) belonging to *Lactobacillus spp.* was 70 times higher in the CS:GS than the CS-only treatment (i.e. 0.5% for the CS:GS versus 0.007% for the CS-only treatment). This likely resulted from effects of the ensiling process and was consistent with the trend observed in the batch experiments in Chapter 4. *L. buchneri* had the highest relative abundance in the CS:GS samples (i.e. 0.7%) and is purported to convert lactic acid to acetic acid under anaerobic conditions at temperatures between 15° – 37°C and pH ranging from 3.8 – 5.8 (Oude Elferink *et al.*, 2001). A mean AD process pH for the laboratory CSTR experiments ranging from 7.4 – 7.7 therefore likely inhibited the growth of LAB.

6.6.1 The role of dominant bacteria

Bacteria of the phyla *Firmicutes* and *Bacteroidetes* are typically the dominant bacteria in AD plants treating agricultural waste. They have been reported to be efficient degraders of complex carbohydrates derived from agricultural waste (Wirth *et al.*, 2012; Ziganshin *et al.*, 2013; Mata-Alvarez *et al.*, 2014; St-Pierre and Wright, 2014). Bacteria of the order *Clostridiales* and *Bacteroidales* comprised (39% and 19%) and (30% and 26%) of the CS and CS:GS treatments respectively in this study. Hydrolysis was associated with bacteria that peaked in relative abundance concomitant with mid to high levels of pH, TAN and total VFA. Such bacteria were of the order *Clostridiales*, *Bacteroidales* and *Acholeplasmatales*. Bacterial RSV that peaked in relative abundance within the CS:GS treatment in day 20 and day 40 samples were mainly associated with carbohydrate degradation.

For example, *Dysgonomonas capnocytophagoides* (Sequence ID NR_113133.1, identity 91%) was recently isolated from a bacterial consortium anaerobically degrading alginate under mesophilic conditions and uses complex carbohydrates as a carbon source to produce propionic, lactic and succinic acids without any gas production (Hofstad *et al.*, 2000; Kita *et al.*, 2016). *Mobilitalea sibirica* strain P3M-3 (Sequence ID NR_134091.1, identity 95%) metabolises mono, di- and polysaccharides to produce acetate, ethanol H_2 and CO_2 and was also reported in the batch experiments in Chapter 4 (Podosokorskaya *et al.*, 2014). Bacterial RSVs belonging to *Fastidiosipila spp.* which peaked in relative abundance in the day 40 CS:GS treatment samples were BLASTed against the NCBI database. Ribosomal sequence variants obtained from the current study were 97% similar to an uncultured strain of *Fastidiosipila spp.* that was obtained from an AD plant recovering from increased salinity levels (Sequence ID LT625359, Identity 97%).

Theuerl *et al.* (2015) reported *Petrimonas spp.* in an agricultural AD system treating CS and whole crop maize silage at 43°C and noted the ability of phylotypes belonging to the family *Porphyromonadaceae* to degrade mono and disaccharides, holocellulose and pectin. Ribosomal sequence variants classified as belonging to *Petrimonas spp.* were 98% similar to the bacterium *Petrimonas sulfuriphila* strain BN3 (Sequence ID NR_042987.1, Identity 98%). However hits from the NCBI database identified the RSV as novel species of a new genus *Fermentimonas caenicola* strain ING2-E5B (Sequence ID NR_148809, Identity 100%) which was isolated from a mesophilic digester co-digesting pig and cattle manure with maize silage (Hahnke *et al.*, 2016). *Fermentimonas caenicola* strain ING2-E5B (Sequence ID NR_148809, Identity 100%) identified in this study peaked in relative abundance in the day 75 samples of the CS:GS treatment. It ferments complex proteinaceous substrates and carbohydrates and shows slightly enhanced extracellular enzyme activity against arabinoxylan and xylan (Hahnke *et al.*, 2016). The relative abundance of *Fermentimonas spp.* within the bacterial community of the CS:GS treatment remained at 2% from day 40 to day 75 but decreased from 2% to 0.7% within the CS-only treatment over the same period.

Syntrophus aciditrophicus strain SB (Sequence ID NR_102776.1, Identity 100%) and *Cryptanaerobacter phenolicus* strain LR7.2 (Sequence ID NR_025757.1, Identity 95%) previously identified in Chapter 4 as possible degraders of lignin were also found within the microbial communities for the CS-only and CS:GS treatments.

Dominant bacteria whose peak relative abundance was associated with day 20-40 in the CS treatment samples were also mostly associated with carbohydrate metabolism and were of the genera *Treponema_2* (2%), *Ruminofilibacter* (3%), *Senegalimassilia* (0.7%) and *Caldicoprobacter* (2%); all of which were previously reported in the batch experiments in Chapter 4. Results from the NCBI database revealed that an RSV belonging to the genus *Treponema_2* closely resembled the *Treponema zuelzerae* strain DSM 1903 (Sequence ID NR_104797.1, identity 99%). *T. zuelzerae* metabolises glucose, mannose, galactose, arabinose, xylose, trehalose, cellobiose and maltose to produce CO_2, H_2 and acetic lactic and succinic acids (Veldkamp, 1960).

Heeg *et al.* (2014) noted that OTUs isolated from AD treating wheat straw had 95-97% similarity to the *T. zuelzerae* strain DSM 1903. *R. xylanolyticum,* a species of genus *Ruminofilibacter*, is known to degrade xylan (Nissilä *et al.*, 2012) and was previously isolated from an AD digesting GS operating at 45°C. *R. xylanolyticum* was also found in a commercial AD system treating maize silage, rye and liquid manure (Weiß *et al.*, 2011). Bacterial RSVs BLASTed against the NCBI database highlighted an uncultured *Caldicoprobacter spp.* clone S1_A3 (Sequence ID KX672185.1, Identity 93%). The RSV was also similar to the thermophile *Caldicoprobacter algeriensis* strain TH7C1 (Sequence ID NR_117466.1, Identity 89%) which is known to have xylanolytic properties (Bouanane-Darenfed *et al.*, 2011). Unlike the other bacterial phylotypes that peaked in relative abundance at day 40 in the CS-only samples, *Senegalimassilia anaerobia* strain JC110 (Sequence ID NR_125595.1, identity 92%) is asaccharolytic. *S. anaerobia* strain JC110 is associated with arginine dihydrolase production and nitrate reduction (Lagier *et al.*, 2013).

Bacteria belonging to the genus *VadinBC27* were once again dominant and present in both CS and CS:GS treatments just as in the batch experiments.

Bacteria assigned to this genus are described as cellulose and hemicellulose degraders (Morrison *et al.*, 2017), but remain uncultured. *VadinBC27 spp*. peaked in relative abundance (7%) on day 75 in the CS:GS community most likely because of the availability of more biodegradable substrate due to increased lignin breakdown.

Concomitant peaks in relative abundance were also observed in *Sedimentibacter spp.* (4%), a degrader of pyruvate that produces acetate and butyrate as the main metabolites (Breitenstein *et al.*, 2002). Some of the bacterial phylotypes that peaked in relative abundance within the CS-only treatment at day 75 were of genera *Ruminiclostridium_1 spp.* (3%), *Mogibacterium spp.* (1%), *Turicibacter spp.* (2%) and *Romboustia spp.* (5%). *Ruminiclostridium [Clostridium] cellulolyticum* strain H10 (Sequence ID NG_041947.1, identity 95%), identified in this study was also reported in the literature for its ability to degrade cellulose to acetate (Tracy *et al.*, 2012; Wirth *et al.*, 2012). The *Turicibacter sanguinis* strain MOL361 (Sequence ID NR_028816, identity 99%) is mainly a lactate producing gram-positive bacteria that utilises Maltose and 5-ketogluconate (Bosshard *et al.*, 2002). St-Pierre and Wright (2014) also identified *T. sanguinis* from an agricultural AD co-digesting dairy waste and fish oil.

6.6.2 Propionate and Butyrate Oxidizing Bacteria

In Chapter 5, the plot of mean total VFA in the reactor effluent indicated that there was an accumulation of propionate in the CS:GS treatment during days 20 to 35. Two purported propionate oxidising syntrophs were identified in this current CSTR experiment namely, the *Smithella propionica* strain LYP MOL361 (Sequence ID NR_024989.1, identity 94-97%) and *Pelotomaculum schinkii* strain HH (Sequence ID NR_119207.1, identity 95-97%). Both organisms degrade propionate in syntrophy with hydrogen and/or formate utilising methanogens as a partner (De Bok *et al.*, 2005; Stams and Plugge, 2009; Ahlert *et al.*, 2016). The *S. propionica* strain LYP comprised 10.5 ± 3.7% and 1.2 ± 0.55% of the phylum *Proteobacteria* in the day 75 samples of the CS and CS:GS treatments respectively. Qiao *et al.* (2013) reported *S. propionica* comprising 60% of the phylum *Proteobacteria* in a 500L AD reactor treating CS and corn stalk, operating at a daily OLR of 1.2 kg TS /m^3 and sludge retention time of 40 days.

P. schinkii strain HH utilises a randomising methylmalonyl-CoA pathway to convert propionate to acetate in partnership with *Methanospirillum hungatei* (De Bok *et al.*, 2005) a strain of which *M. hungatei* strain JF-1 (Gunsalus *et al.*, 2016), was identified within this study (Sequence ID NR_074177.1, identity 100%). However, the *S. propionica* strain LYP utilises a non-randomising pathway that dismutates propionate to butyrate and acetate in partnership with *Methanospirillum hungatei*. Butyrate is then converted to acetate via β-oxidation (De Bok *et al.*, 2001). The identification of *P. schinkii* strain HH along with *S. propionica* strain LYP indicates the presence of redundant metabolic pathways for propionate oxidation similar to those observed in Chapter 4. However, the evidence suggests that propionate oxidation may have been performed by other microbes given the low relative abundance of *S. propionica* strain LYP (≤ 0.1%) and *P. schinkii* strain HH (≤ 1%) in the CS-only and CS:GS samples. Nobu *et al.* (2015) suggest that *Atribacteria spp.* and *Cloacimonetes spp.* encode propionate metabolism and express methylmalonyl-CoA pathway genes with 52-71% and 51-57% homology to genes expressed by *Pelotomaculum thermopropionicum* strain SI, a known thermophilic syntrophic propionate degrader. Nobu *et al.* (2015) note that syntrophic propionate oxidation by *Atribacteria spp*. may involve either inter-species hydrogen or formate transfer. BLASTing an RSV related to phylum *Cloacimonetes* and genus *Candidatus Cloacamonas* against the NCBI database identified an uncultured bacterium clone zqB013 (Sequence ID KU589096.1, identity 100%) linked to research conducted by Zhao *et al.* (2016) on improving biogas quality by increasing the syntrophic propionate and/or butyrate oxidation by using ethanol to stimulate direct inter-species electron transfer (DIET).

During the period of imbalance observed between days 20 to 50, *Proteiniphilum spp.*, a member of phylum *Bacteroidetes*, peaked in relative abundance and was 10 times higher in the CS:GS treatment (5.8 ± 0.22%) than the CS-only treatment (0.6 ± 0.04%). Ribosomal sequence variants belonging to *Proteiniphilum spp.* that peaked in relative abundance in the CS:GS day 75 samples were 95% similar to the *Proteiniphilum acetatigenes* strain TB107 (Sequence ID: NR_043154.1, identity 95%) which was isolated from an AD system treating brewery wastewater.

The strain was purported to accelerate the rate of propionate oxidation from 0.75 to 1.25 mM per day in tri-culture with *Syntrophobacter sulfatireducens* DSM 16706 and *Methanobacterium formicicum* DSM 1535T (Chen and Dong, 2005). Hahnke *et al.* (2016) emended the description of the isolate provided by Chen and Dong (2005) subsequent to isolating the bacterium *Petrimonas mucosa* strain ING2-E5A from a mesophilic laboratory-scale continuous digester treating a mixture of maize silage, pig and cattle manure. *Petrimonas mucosa* strain ING2-E5A was also 95% similar to *Proteiniphilum acetatigenes* strain TB107. The RSV identified in the current study was 98% similar to *Petrimonas mucosa* strain ING2-E5A (Sequence ID: NR_148808.1, identity 98%) which is reported to degrade both peptone and complex carbohydrates and shows enhanced extracellular activity against lactose (Hahnke *et al.*, 2016). This suggests that *Petrimonas mucosa* strain ING2-E5A may have played a role in the oxidation of propionate that accumulated during the process imbalance that occurred during HRT2 in both experimental runs. This purported ability to accelerate propionate oxidation ultimately to methane therefore may have also contributed to the 26% more methane produced by the CS:GS treatment reported in Chapter 5.The difference in taxonomic classification is due to differences in the Silva123 and RDP reference databases (Yilmaz *et al.*, 2014).

The species *Syntrophomonas wolfei* strain Goettingen G311 (Sequence ID: NR_122058.1, identity 93-96%), *S. bryantii* strain CuCal (Sequence ID: NR_104881.1, identity 97%) and *S. zehnderi* strain OL-4 (Sequence ID: NR_044008.1, identity 95%), syntrophically degrade butyrate, 2-methyl butyrate and long chain VFA to acetic acid and/or propionate via syntrophic pathways involving hydrogenotrophic methanogens (Wu *et al.*, 2006; Sousa *et al.*, 2007; Stams and Plugge, 2009; Mathai *et al.*, 2015; Narihiro *et al.*, 2016); these organisms were previously identified within both the CS and CS:GS treatments in the batch experiments reported in Chapter 4.

They were dominant members of *Syntrophomonas spp.* in the CSTR experiments and were closely associated with the day 75 samples where they comprised 5.3 ± 0.22 % and 6.5 ± 1.25 % of *Firmicutes* within the CS and CS:GS treatments respectively. *S. bryantii* strain CuCal and *S. zehnderi strain OL-4* partner with hydrogenotrophic methanogens such as *Methanobacterium spp.* via inter-species hydrogen (IHT) and/or formate (IFT) electron transfer to metabolise VFA (Sousa *et al.*, 2007; Mathai *et al.*, 2015). However *S. zehnderi strain OL-4* is more versatile than the *S. bryantii* strain CuCal since it is capable of metabolising longer chain VFA in addition to the substrates both species share in common (Sousa *et al.*, 2007).

6.6.3 Acetogenic Bacteria

Sedimentibacter saalensis (Sequence ID: NR_025498.1, identity 97%) is associated with the oxidation of polypetides, amino acids and pyruvate to produce acetate and butyrate (Breitenstein *et al*., 2002). Bacteria belonging to this genus were identified in the batch experiments in Chapter 4 and have been reported to be in higher abundance in AD co-digesting LB biomass as opposed to manure only treatments (Sun *et al.*, 2015; Theuerl *et al.*, 2015). *Aminobacterium colombiense* strain DSM 12261 (Sequence ID NR_074624.1, identity 100%) metabolises threonine, serine, glycine and pyruvate to produce acetate and hydrogen, or propionate in the case of α-ketoglutarate (Chertkov *et al.*, 2010). Substrate utilisation for *A. colombiense* is improved in the presence of *Methanobacterium formicicum* strain DSM 1525, a hydrogenotrophic syntrophic partner methanogen.

Another RSV identified in this study, the *Pelotomaculum isophthalicum* strain JI (Sequence ID NR_041320.1, identity 98%), metabolise contaminants such as phthalate isomers and a number of aromatic compounds namely benzoate and hydroquinone in partnership with hydrogenotrophic methanogens to produce acetate (Qiu *et al.*, 2006). Acetate is a product of the anaerobic fermentation of glucose in the presence of hydrogen consuming methanogens (Stams and Plugge, 2009).

The concomitant increase in the relative abundances of cellulolytic fermenters such as *Ruminococcus albus* strain 7 along with *Methanobrevibacter spp.* and *Methanosaeta spp.* within the day 75 samples of the CS-only treatment highlight the co-operation between these microbes that resulted in methane production. *Methanosaeta spp*., obligate acetotrophic methanogens, convert the acetate produced from glycolysis to methane and *Methanobrevibacter spp.* also produce methane by utilising the hydrogen produced from the glycolysis process to reduce carbon dioxide.

6.6.4 The role of dominant archaea

This study's finding that archaea comprised 6% of the microbial population across both CS and CS:GS treatments supports existing literature that states archaeal populations in AD comprise 10% of the microflora (Garcia *et al.*, 2000; Wirth *et al.*, 2012). Sun *et al.* (2015) stated more than 99.6% of archaeal reads across all samples could be assigned at the phylum rank with 98.1% belonging to the phylum *Euryarchaeota*. In this study, 93.4% and 94.4% of archaeal reads were assigned to the phylum taxonomic rank across the CS:GS and CS samples respectively; 93% of which were assigned to the phylum *Euryarchaeota* and 6% assigned to the phylum *Miscellaneous Crenarchaeotic Group*. The predominance of *Euryarchaeota* is consistent with findings from the batch experiments (Chapter 4) and the existing literature (Ritari *et al.*, 2012; Wirth *et al.*, 2012). Very little is known about archaea assigned to the *Miscellaneous Crenarchaeotic Group* (MCG). However, current research involving ^{13}C assimilation into RNA using stable isotope probing has been examining their association in relation to the anaerobic oxidation of methane (AOM) in low sulfate iron rich environments (Weber *et al.*, 2017). Further research is required to examine the function of this archaeal phylum since increases in relative abundance for the CS-only and CS:GS treatments from 0.2% at day 20 of the experiment to 9% and 11% respectively on day 75 were observed in this study.

Archaea found across all samples were assigned to the taxonomic orders *Methanobacteriales*, *Methanomicrobiales*, *Methanosarcinales* and *Thermoplasmatales*. Archaea assigned to the *Thermoplasmatales* were previously identified along with archaea of order *Methanomicrobiales* and *Methanosarcinales* from the sludge of the on- farm AD at Cockle Park Farm treating pig and cattle slurry (Wade, 2012). *Thermoplasmatales spp*. have been isolated from wood-feeding cockroaches (Hara *et al.*, 2002), termite guts (Friedrich *et al.*, 2001; Miyata *et al.*, 2007; Paul *et al.*, 2012) and the digestive tract of ruminants such as cattle (Tajima *et al.*, 2001). Walter *et al.* (2016) identified the archaeal genera *Methanosarcina*, *Methanobrevibacter*, *Methanoculleus* and *Methanosphaera* within a mesophilic AD co-digesting CS and industrial paper waste. They noted the dominance *Methanosarcina spp.* within the archaeal population and observed that *Methanobrevibacter spp.* was the second most dominant. In this study the genera *Methanosarcina* (35.3 ± 2.22%, 25.5 ± 9.17%), *Methanobacterium* (12.8 ± 2.36%, 26.8 ± 4.39%), *Methanobrevibacter* (22.5 ± 1.75%, 18.3 ± 0.74%) and *Methanosaeta* (26.3 ± 5.21%, 12.8 ± 3.51%) were the dominant archaeal communities present in the CS-only and CS:GS samples respectively taken on day 75. This was consistent with most of the archaeal genera identified in the batch experiments in Chapter 4. This study's finding regarding the archaeal population composition of hydrogenotrophic, acetotrophic and mixotrophic methanogens is supported by previous research (Wirth *et al.*, 2012; Ziganshin *et al.*, 2013; Sun *et al.*, 2015; Walter *et al.*, 2016).

Hydrogenotrophic methanogens maintain low levels of formate and H_2 partial pressure in order to facilitate the oxidation of intermediates such as butyrate and propionate by syntrophic bacteria (De Bok *et al.*, 2005; Stams and Plugge, 2009; Ahlert *et al.*, 2016). Wade (2012) identified archaea of genera *Methanoculleus*, *Methanocorpusculum* and *Methanosarcina* in the effluent of the mesophilic AD plant from which the inoculum for this CSTR Experiment was taken. Two obligate hydrogenotrophs, *Methanobacterium ferruginis* strain Mic6c05 (Sequence ID NR_113045.1, identity 99%) and *Methanobrevibacter thaueri* strain CW (Sequence ID NR_044787.1, identity 99%) which only grow on H_2/CO_2 (Miller and Lin, 2002; Mori and Harayama, 2011) were identified in this study.

The *Methanobrevibacter millerae* strain ZA-10 (Sequence ID NR_042785.1, identity 99%) which utilises H_2/CO_2 or formate/CO_2 (Rea *et al.*, 2007) and *Methanoculleus palmolei* strain DSM 4273 (Sequence ID NR_028253.1, identity 100%) which utilise H_2/CO_2 formate, cyclopentanol/CO_2, 2-propanol/CO_2 and 2-butanol/CO_2 to produce methane (Zellner *et al.*, 1998) were also identified. Summing the relative abundance of *Methanobrevibacter spp. and Methanobacterium spp.* within samples taken on day 75 indicates that archaea utilising only H_2/CO_2 or formate/CO_2 comprise 35.3 ± 2.34% and 45.1 ± 4.45% of the CS-only and CS:GS treatments respectively. The archaeal community within the CS:GS treatment therefore had a higher relative abundance of hydrogenotrophs than the CS-only treatment creating an environment for the syntrophic oxidation of key intermediates such as propionate and butyrate via the IHT and/or IFT pathways. Wirth *et al.* (2012) note hydrogenotrophs of the genera *Methanoculleus*, *Methanoregula*, *Methanosphaerula*, *Methanospirillum*, *Methanoplanus*, *Methanocorpusculum* and mixotrophs of genus *Methanosarcina* were abundant in an AD co-digesting pig manure and plant biomass at 37°C.

Sun *et al.* (2015) noted *Methanosarcina spp.* as the dominant archaeal phylotype in separate mesophilic AD experiments co-digesting CS and straw and CS-only substrates. The uncultured *Methanosarcina spp.* strain 795 (Sequence ID NR_041320.1, identity 98%), identified in this study, displayed significant positive recovery to acetate catabolism and is purported as a possible biomarker for the recovery of acetotrophic methanogens under acidified AD conditions (Town and Dumonceaux, 2016). RSV belonging to the *Methanosarcina soligelidi* strain SMA-21 (Sequence ID NR_109423.1, identity 98%) utilises H_2/CO_2, methanol and acetate to produce methane (Wagner *et al.*, 2013). The identification of three additional RSV belonging to *Methanosarcina spp.* since the BLASTed sequences identified the following five equally likely species and their substrates:

- *M. bakeri* which utilises H_2/CO_2 and acetate (Shimizu et al., 2015)
- *M. spelaei* grows autotrophically on H_2/CO_2 and utilises methanol, mono-, di- and tri-methylamines to produce methane (Ganzert et al., 2014; Shimizu et al., 2015).
- *M. siciliae* is an obligate methylotroph (Shimizu et al., 2011; Ganzert et al., 2014)
- *M. horonbensis* grows on acetate, methanol, dimethylamine, trimethylamine and dimethylsulfide (Shimizu et al., 2011; Ganzert et al., 2014; Shimizu et al., 2015).

- *M. mazei* utilises acetate, monomethylamines and H_2/CO_2 to produce methane (Shimizu et al., 2011; Shimizu et al., 2015).

The archaeal community for both treatments also consisted of obligate methylotrophic methanogens which produce methane from methyl compounds originating from plant material such as pectin (Garcia, 1990; Sprenger *et al.*, 2007; Tracy *et al.*, 2012). The *Methanomassiliicoccus luminyensis* strain B10, *Methanosphaera cuniculi* strain 1R7, *Methanogenium marinum* strain AK-1 were identified in this current study. Antony *et al.* (2012) note that obligate methylotrophic methanogens have an ecological advantage over other methanogens because they utilise (C_1) compounds such as methanol and methylamines; thus avoiding competition with other microbes such as sulphate reducing bacteria (SRB) for acetate. However, their relative abundances were below 0.09%, suggesting that competition for substrate from dominant *Methanosarcina spp.* may have limited their growth.

This current study identified the acetotrophic methanogen *Methanosaeta concilii* strain GP6 (Sequence ID NR_104707.1, identity 99%) which grows only on acetate (Patel and Sprott, 1990). Ziganshin *et al.* (2013) reported *Methanosaeta spp.* and *Methanoculleus spp.* as the dominant archaea in an AD system digesting cattle manure (CM) and maize operating at 37°C at an OLR of 1.5g VS/m^3 per day and an HRT of 25 days. They noted that the relative abundance of *Methanosaeta spp.* in the archaeal community of another reactor operating at a similar temperature but with a varied OLR of 2g to 2.5g VS per m^3/d and HRT of 68.3 days decreased and *Methanosarcina spp.* increased significantly. Differences in archaeal composition related to the genera and species identified in this study with the literature are likely to be due to variations in feedstock, OLR, HRT used as well as AD process parameters (Ziganshin *et al.*, 2013; Mata-Alvarez *et al.*, 2014). The presence of the *Methanosaeta concilii* strain GP6, the obligate hydrogenotrophs *Methanobacterium ferruginis* strain Mic6c05 and *Methanobrevibacter thaueri* strain CW and the presence of the mixotroph *Methanosarcina spp.* of which *M. siciliae,* an obligate methylotroph, was likely present suggests that all three methanogenic pathways were present within both treatments. This is consistent with

findings by Wang *et al.* (2017) who characterised the microbial community within the mesophilic AD treatment of pig manure (PM) and PM co-digested with corn stalk.

6.6.5 The effect of pH, TAN and VFA on the archaeal community

Total ammonia nitrogen, pH, total volatile fatty acids and methane production explained 80% of the observed variation between the phylogenetically distinct groupings. Digester pH ranged from 7.2 – 7.62 for the CS treatment and 7.19 – 7.49 for the CS:GS treatment. The CSTRs for both treatments, therefore, operated within a range of 6.6 to 7.3, which is acceptable for the dominant methanogens detected (Garcia, 1990; Demirel and Scherer, 2008).

Observations in this study indicated that the relative abundance of *Methanosaeta spp.* decreased from day 20 to 75 for the CS-only (33% to 25%) and the CS:GS treatments (18% to 15%) respectively. During days 20 to 75 however, the relative abundance of *Methanosarcina spp.* increased from 3% to 25% in the CS and 19% to 32% in the CS:GS treatments. The dominance of *Methanosarcina spp*. within AD systems treating agricultural waste has been reported in the literature (Wirth *et al.*, 2012; Sun *et al.*, 2015; Walter *et al.*, 2016). *Methanosarcina spp.* is capable of acetotrophic, hydrogenotrophic and methylotrophic methanogenesis while *Methanosaeta spp*. is an obligate acetotroph (Mata-Alvarez *et al.*, 2014; Walter *et al.*, 2016; Westerholm *et al.*, 2016). High TAN concentrations ranging from 1.7 to 14g/L inhibit the growth of methanogens and reduce methane production (Chen *et al.*, 2008; Fotidis *et al.*, 2014a). Demirel and Scherer (2008) and Chen *et al.* (2008) note that *Methanosaeta spp.* are more sensitive to increased TAN levels than *Methanosarcina spp*. while both *Methanosarcina spp.* and *Methanosaeta spp*. have been observed in AD systems treating manure (Demirel and Scherer, 2008; Ziganshin *et al.*, 2013; Wang *et al.*, 2017). The growth rate of *Methanosaeta spp*. in this study may have been reduced due to TAN levels greater than 1.7g NH_4-N/L resulting in an increased concentration of acetate (Conklin *et al.*, 2006; Chen *et al.*, 2008; Walter *et al.*, 2016).

Mean TAN concentrations of 1.4g NH_4-N/L for the CS-only and 1.7g NH_4-N/L for the CS:GS treatments during the first HRT, therefore suggest that the relative abundance of *Methanosaeta spp*. may have been reduced relative to *Methanosarcina spp*.

The accumulation of formate, acetate, propionate and butyrate during process imbalance that occurred in the CS and CS:GS treatments during HRT2 for both experimental runs may have been due to adverse effects of TAN levels on the methanogenic population. This inhibitory condition due to TAN levels, was alleviated when mean TAN levels were reduced by 40% in the CS-only treatment and 47% in the CS:GS treatment (to a level of 0.9g NH_4-N/L in both treatments) between HRT1 and HRT2.

Biomass present in both the CS-only and CS:GS treatments is high in carbohydrates (Lehtomäki *et al.*, 2007; Wang *et al.*, 2010a; Osborne Homeky *et al.*, 2016) which quickly degrade under anaerobic conditions (Boe *et al.*, 2010; Weiland, 2010). Hydrogen is produced along with intermediate metabolites such as VFA, propionate, butyrate, alcohols, lactate and methyl compounds (Saha, 2003; Chen *et al.*, 2008). Hydrogen partial pressures are maintained at low levels by sulphate reducing bacteria (SRB), acetogenic bacteria and methanogens (Demirel and Scherer, 2008; Aymerich *et al.*, 2013; Fotidis *et al.*, 2014b; Ahlert *et al.*, 2016). The oxidation of intermediates such as propionate and butyrate becomes sufficiently exergonic when the reducing equivalents such as formate and hydrogen are kept low (Stams *et al.*, 2003; Zhao *et al.*, 2016). A shift towards hydrogenotrophic methanogenesis therefore facilitates the metabolism of intermediate substrates such as propionate, butyrate and lactate via IHT and/or IFT with a partner hydrogen and/or formate –utilising methanogen (Stams and Plugge, 2009; Ahlert *et al.*, 2016; Zhao *et al.*, 2016). A shift from *Methanosaeta spp.* to *Methanosarcina spp.* due to TAN levels greater than 2.8g NH_4-N/L has been reported for AD systems treating manure via a 2-step pathway involving the oxidation of acetate to H_2 and CO_2 by syntrophic acetate oxidising bacteria (SAOB) which is then converted to methane by hydrogenotrophic methanogens (Westerholm *et al.*, 2012b; Fotidis *et al.*, 2014a; Mata-Alvarez *et al.*, 2014; Walter *et al.*, 2016).

However, no evidence of syntrophic acetate oxidation (SAO) was found in this study since the relative abundances of mesophilic SAOB such as *Tepidanaerobacter acetatoxydans* strain Re1 (Sequence ID NR_116298.1 Identity 93%) and *Syntrophaceticus schinkii* strain Sp3 (Sequence ID NR_116297.1 Identity 99%) were very low (<0.01%) in both CS and CS:GS treatments and *Clostridium ultunense* was not detected.

The AD process disturbance which led to the accumulation of VFA during the second HRT of both CSTR Experimental runs for the CS-only and CS:GS treatments was likely attributed to high levels of TAN inhibiting the methanogens. The AD fermentation products H_2, CO_2, formate and acetate were not consumed fast enough and the consequent increase in formate and possible increase in H_2 partial pressure resulted in a decoupling of the propionate and butyrate oxidation process.

6.6.6 Conclusions

The salient points from this chapter are as follows:

- Evenness of the bacterial and archaeal population at the beginning and end of experimental run remained somewhat constant. However evenness within the archaeal population decreased during the period of instability characterised by reduced levels of CH_4 production.
- The CS:GS treatment had a higher relative abundance of phylum *Bacteroidetes* than the CS-only treatment which may have contributed to the increased methane production of the co-digestion system. As both AD systems approached steady state, the CS:GS had three times more *Fermentimonas spp.* and two times more *Dysgonomonas spp.*, which are known carbohydrate degraders, than the CS-only treatment. The CS:GS treatment also had 10 times more phylotypes belonging to *Petrimonas spp.*, a strain of which, *P. mucosa* strain ING2-E5A, is reported to increase the rate of propionate oxidation and therefore methane production.
- Co-digestion improved the archaeal population by increasing the relative abundance of hydrogenotrophic methanogens by 1.27 times (45.1 ± 4.45%) compared to the CS-only treatment (35.3 ± 2.34%) thereby creating an environment for the syntrophic

oxidation of key intermediates such as propionate and butyrate via the IHT and/or IFT pathways.

- The presence of the *Methanosaeta concilii* strain GP6, the obligate hydrogenotrophs *Methanobacterium ferruginis* strain Mic6c05 and *Methanobrevibacter thaueri* strain CW and the presence of the mixotroph *Methanosarcina spp.* of which *M. siciliae*, an obligate methylotroph, suggests that all three main methanogenic pathways were present within both treatments.
- The presence of *Smithella propionica* strain LYP MOL361 and *Pelotomaculum schinkii* strain HH provides evidence of the occurrence of redundant syntrophic metabolic pathways for propionate oxidation in these reactors. However the evidence suggests that other uncultured syntrophic propionate degraders such as *Atribacteria spp.* and *Cloacimonetes spp.* might have also performed a similar role.
- Several microbes of interest were identified in this study where further research is needed to identify their role in the AD process e.g.:
    - The uncultured *Methanosarcina spp. 795* for its ability to catabolise acetate and recover acetotrophic methanogenesis under acidified AD conditions;
    - The syntrophic cohort of *Tepidanaerobacter acetatoxydans* strain Re1 and *Syntrophaceticus schinkii* strain SP3 for their role in hydrogenotrophic methanogenesis via the SAO-HM pathway.
    - Uncultured archaea belonging to the *Miscellaneous Crenarchaeotic Group* since they are purported to perform functions such as AOM, homoacetogenesis as well as cellulose and benzoyl degradation that directly impact on biogas production and quality.
    - The bacteria *Syntrophus aciditrophicus* strain SB, *Pelotomaculum isophthalicicum* strain JI, *Cryptanaerobacter phenolicus* strain LR7.2 because they are purported to degrade phenol and benzoate which are lignin-derived compounds.
    - *Petrimonas mucosa* strain ING2-E5A and *Fermentimonas caenicola* strain ING2-E5B for their enhanced hydrolytic abilities and therefore their potential to improve AD process efficiency.

Chapter 7 General Discussion

There is increased interest in research focused on the optimisation of the AD process, particularly with reference to the microbial degradation of lignocellulosic biomass in agricultural AD plants. Organic overloading, accidental addition of toxic substances and variation in operating conditions frequently occur and can upset the delicate balance leading to inefficient process conversions and ultimately process failure. On-farm AD plants treating animal slurries typically operate at high levels of ammonia and are reported to function inefficiently (Ward *et al.*, 2008). Co-digesting animal waste such as cattle slurry (CS) with plant material such as grass silage (GS) increases biogas production and methane yield. The influence of environmental variables on biochemical interactions taking place within the reactors, adds to the complexity of the AD process and makes process control of AD plants challenging. Additionally, process parameters provide a poor indication of process stability.

The environmental impact of H_2S levels within the biogas during the course of the batch and CSTR experiments was not a problem due to the low protein content of the CS and CS:GS treatments. This is beneficial for on-farm AD plants using similar feedstock since biogas may cause air pollution complaints if H_2S is present even at low levels. Therefore, co-digesting with other slurries having higher protein content than CS such as pig slurry, may result in air pollution complaints. Under such circumstances, operators of on-farm digesters may treat the slurry with ferrous sulphate to immobilise the sulphide within the liquid phase by precipitating FeS (Chen *et al.*, 2008). However this must be done with caution as heavy metal toxicity can inhibit methanogenic growth and adversely impact biogas and CH_4 production.

7.1 Effects of co-digesting cattle slurry with grass silage on methane yield

Increased methane yields were observed from the co-digestion of CS with GS in the Batch and CSTR experiments. In both experiments, the co-digested treatment with the highest proportion of GS had significantly higher methane yields than the CS-only treatment. This is partially attributed to a C:N ratio of 20:1 for the co-digested feedstocks being significantly higher than the C:N ratio for CS-only treatment and within the optimal range of 16:1 – 33:1 reported in the literature (Mata-Alvarez *et al.*, 2014).

The increased C:N ratio in the co-digested treatments along with higher SMP indicate that the GS co-substrate provided an additional carbon source for methane synthesis. Both CS and GS provide trace elements required for functions inclusive of bacterial growth and the production of the enzyme cofactor F_{430} used by archaea in the synthesis of methane via the Wood-Ljungdahl Pathway (Weiland, 2010).

Proximate analysis results of the mono-digested feeds highlighted that CS had a significantly higher concentration of lignin than the GS. It has been widely reported that feedstock lignin content is negatively correlated to specific methane production (SMP) because lignin acts a barrier limiting microbial access to holocellulose (Triolo *et al.*, 2013). No synergistic effects were observed between the co-digested substrates. The highest SMP observed in this study was achieved by GS followed by the 60:40 co-digested treatment. This trend supports previous findings reported in the literature (Lehtomäki *et al.*, 2007; Wall *et al.*, 2013). The SMP of the 80:20 treatment was not significantly different to that of the CS-only treatment in the both the Batch and CSTR experiments because it was limited by the lignin content within the feedstock.

Operators of on-farm AD plants can improve biogas production and quality by co-digesting CS with GS. However, the level to which the biogas is enhanced is dependent upon the lignin content of the feedstock and how this can be modified through increased HRT and/or the use of a range of pre-treatment options. The use of ensiling as a pre-treatment has been reported to enhance methane yield as long as there is no net loss of dry matter during the process (Herrmann *et al.*, 2011). However this pre-treatment method does not remove lignin and its effectiveness also varies with the duration of ensiling, the type of grasses ensiled and their maturity at harvest. Therefore lignin content must be periodically evaluated to determine the minimum level of GS to be added in order to enhance biogas production.

7.2 The effect of Process parameters on AD process efficiency

Results from the batch experiments indicate that free ammonia (FA) is a key parameter to monitor since it was negatively correlated with methane concentration and biogas production. This is consistent with the literature since FA inhibits methanogens by diffusing into their cell membrane, causing a proton and/or K^+ deficiency, while ionized ammonium is reported to directly inhibit enzymes involved in methane synthesis (Sprott *et al.*, 1984; Kayhanian, 1999). Initial levels of FA may have temporarily suppressed growth within the methanogenic population. Temporary process imbalances were observed in the CS treatment during Batch Experiment 2 that led to the momentary accumulation of propionate which has often been associated with process imbalance in other studies (Marchaim and Krause, 1993; Ahlert *et al.*, 2016). However propionate levels subsequently reduced without any notable adverse consequences to the AD process efficiency. This is consistent with findings from Labatut *et al.* (2011) and Xie *et al.* (2011) who note that product inhibition is likely reversible in batch systems and levels of accumulated intermediates will begin to reduce once thermodynamic conditions become favourable.

The biomethane efficiency for the CS, 80:20 and 60:40 treatments in the CSTR experiment ranged from 0.70 – 0.80, indicating that the specific methane production (SMP) reported for continuous mode operation was 20 -30% lower than the results obtained from the BMP test. The treatment having the lowest level of lignin had the highest biomethane efficiency. The period of imbalance observed during days 31 to 50 for both runs in CSTR experiment 2 also contributed to reduction in biomethane efficiency since degradable VS was washed out of the reactor This is in line with Schlattmann *et al.* (2004) who suggested that dilution due to hydraulic overloading of slow-growing bacteria as well as the production of inhibitory acids can cause a 15% difference in methane yields between the two batch and continuous operating modes.

The 60:40 treatment produced 26% more biogas and 31% more methane than the mono-digested CS feedstock at both operating OLR levels, confirming that co-digesting CS with GS in a continuous mode increased biogas production and quality.

Despite operating at a low OLR level of 1g VS per m^3/d, there was a transient reduction in biogas production and an accumulation of propionate, butyrate, acetate and formate for the 60:40 treatment. This was as a result of a decoupling of the oxidation of propionate and butyrate by syntrophic bacteria and hydrogenotrophic methanogens due to methanogenic inhibition. This is supported by Ito *et al.* (2012) who note VFA conversion, in particular propionate is also rate-limiting in addition to the hydrolysis rate.

Proper monitoring and control strategies of on-farm AD plants treating agricultural wastes with high levels of TAN such as CS and/or CS co-digested with GS is required in order to improve process stability and efficiency. The overload caused process imbalances at a very low loading rate (ca. 1.0g VS/m^3 per day) during this study whereas Weiland (2006) noted that most AD plants treating animal slurries co-digested with energy crops in Germany operate at low loading rates between 1 – 3 kg VS/m^3 per day. Increasing the loading rate from 0.5g/m^3 per day in CSTR Experiment 1 to 1.0g/m^3 per day in CSTR Experiment 2 led to AD process imbalances likely due to the increased ammonia levels. This observation is consistent with findings from Fotidis *et al.* (2014a) who indicate inefficient operation due to ammonia inhibition is a major challenge for agricultural biogas plants leading to suboptimal performance. They studied eight agricultural biogas plants and reported that the composition of the archaeal community was influenced by operating TAN levels.

7.3 Correlation of microbial community structure with process parameters

Advances in high throughput next generation sequencing (NGS) and molecular fingerprinting tools, within the last 20 years have provided alternative methods for time series analysis of microbial community responses within the AD process. This enables a greater understanding of the correlations between physico-chemical and microbial community changes.

There were no phylogenetic differences were observed between the CS-only and the co-digested treatments. However, temporal changes were observed with day 0 samples being phylogenetically different to days 6-24 samples. Over 90% of the phylogenetic variation observed within the microbial communities for the CS and CS:GS treatments were explained by variations in methane production, effluent total VFA, pH and TAN.

Increasing levels of TAN did not have an effect on the temporal phylogenetic variations observed but indicated that there were phylogenetic differences between the batch experimental runs. This may pose some challenges as to generalising inferences from this study since two clearly distinct microbial communities were obtained. The phylogenetic difference between runs may be linked to the preparation of the inoculum for the Batch Experiment B2-02 where cattle slurry was added to the inoculum to increase the TS content. The addition of CS would have altered the phylogenetic composition of the microbial community as it contains bacteria and archaea (Sun *et al.*, 2015).

Firmicutes and *Bacteroidetes* were the dominant bacterial phyla within the microbial community across all treatments in the batch studies. Bacteria belonging to the phylum *Firmicutes* are efficient degraders of cellulose while bacteria belonging to the phylum *Bacteroidetes* appear to specialise in the digestion of polysaccharides, and both are ubiquitous in anaerobic digesters treating plant derived feedstock (Hanreich *et al.*, 2013). This is consistent with the proximate analysis results in Chapter 3 where the CS, 80:20 and 60:40 feeds consisted of carbohydrates ranging between 76.2 – 77.8% TS.

Decreasing levels of pH and total VFA clearly influenced temporal phylogenetic differences within each treatment, indicating the occurrence of hydrolysis and the subsequent AD process steps. Samples taken on day 0 clustered separately from samples taken between days 6-24. The evidence from Chapter 4 seems to suggest that the sampling regime chosen in this study lacked sufficient resolution to capture the rapid changes that may have occurred between days 0 – 6 of the batch tests. Day 0 samples had high relative abundances of bacteria that were of the classes *Clostridia*, *Gammaproteobacteria*, *Bacteroidia*, *Erysipelotrichia* and *Planctomycetacia*. Ribosomal Sequence Variants (RSV) identified that bacteria from the NCBI database (NCBI Resource Coordinators, 2016) were closely associated to bacteria of genera *Petrimonas spp.*, *Turicibacter spp.*, *Mariniphaga spp., Pseudomonas spp.*, which are reported to be associated with hydrolysis of carbohydrates, proteins and lipids (Bosshard *et al.*, 2002; Grabowski *et al.*, 2005; Xiao *et al.*, 2009; Iino *et al.*, 2014).

During the batch experiments, species richness and evenness decreased within the first 6 days and remained fairly constant thereafter as sub-populations of dominant archaea and bacteria became established. The increased levels of volatile fatty acids (VFA) originating from microbial degradation of holocellulose were quickly converted into methane. This was indicated by the time taken to produce 50% of the methane in the batch experiment, which ranged from 3-5 days across all treatments (treating Batch Experiment B1-01 as an outlier) and the daily methane production curves. Therefore, sampling at a rate of every 12 hours within the first six days of the batch test may be sufficient to conclusively determine whether phylogenetic differences in the microbial communities exist due to treatment. The quick change within the microbial community relative to the duration of the Batch experiment indicates that process controls must respond quickly to control key process parameters in AD systems operating in batch mode to ensure biogas production and quality.

The bacterial community continued to be dominated by carbohydrate degraders namely *Sedimentibacter spp*., *Fastidiosipila spp*. and *Syntrophomonas spp*. from class *Clostridia* during days 6-24, indicating that the degradation pathways for carbohydrates in the latter stages of the batch involved the syntrophic oxidation of butyrate and/or propionate with a hydrogenotrophic methanogen (De Bok *et al.*, 2001). The presence of *Syntrophomonas spp*. was also reported by Hanreich *et al.* (2013) who conducted a batch study treating carbohydrate-rich feedstock and noted that members of the phylum *Firmicutes* seemed to be mainly involved with cellulose degradation while members of phylum *Bacteroidetes* seemed to be involved in carbohydrate degradation.

Acetoclastic, hydrogenotrophic and methylotrophic pathways were the main mechanisms for methane production for both treatments. Archaea phylotypes belonging to mixotrophic methanogens *Methanosarcina spp.* (6%) and hydrogenotrophic methanogens *Methanobrevibacter spp*. (42%) peaked in relative abundance during the days 0 samples in both treatments while the obligate acetoclastic methanogens *Methanosaeta spp.* (38%) peaked in relative abundance between days 6 - 24 (Appendix 7).

Increased methane yields occurred during days 6-24 when propionate and/or butyrate oxidising bacteria belonging to *Syntrophomonas spp.* peaked in relative abundance, indicating the importance of the syntrophic relationship between propionate and butyrate degraders and hydrogenotrophic methanogens. These findings were supported by Ahlert *et al.* (2016) who identified the propionate degrading bacteria from samples taken from agricultural biogas plants along with their partner methanogen. The presence of redundant metabolic pathways for propionate oxidation in the form of the bacteria *Smithella propionica* and *Pelotomaculum schinkii* is indicative of a robust and stable AD process. Obligate acetoclastic methanogens belonging to *Methanosaeta spp.*, in particular *M. concilii* strain GP6, increased in relative abundance from 20% on day 0 to 43% on day 24 as substrate became limited in both treatments. This is consistent with Demirel and Scherer (2008) who note that *Methanosaeta spp.* become dominant in environments with low levels of acetate and total ammonia nitrogen (TAN). Therefore, the difference in methane production observed between the CS-only and CS:GS treatments was due to a net surplus of nutrients supplied by the GS co-substrate since there was no phylogenetic difference between treatments.

Phylogenetic differences were observed within and between the CS-only and CS:GS treatments in the CSTR time-series experiment. The microbial community for each treatment exhibited three distinct temporal transitions, as the AD process approached steady state which explained 40% of the total variation observed. An additional 32% of the variation observed was due to phylogenetic differences between treatments. Therefore substrate composition did influence microbial community dynamics in this study as has previously been reported. Over 86% of the phylogenetic variation observed between treatments as well as the temporal differences within treatments for the CSTR experiment could be explained by the variations in VS, TAN, methane production and pH process parameters, suggesting that these AD process variables are key AD process parameters to monitor during the optimisation of biogas production and quality.

Results from this study indicate that the relative abundance of bacteria belonging to the phylum *Parcubacteria* were 12-fold higher in the CS:GS (6%) than the CS-only (0.5%) treatment samples. Bacteria belonging to this phylum peaked in relative abundance in the CS:GS treatment on day 75 as the reactor approached steady state conditions. CS:GS samples taken on day 75 are associated with increased methane yields. *Parcubacteria spp.* are associated with carbohydrate metabolism but more research is required into understanding the role they play in improving methane yield during co-digestion.

The phyla *Firmicutes* and *Bacteroidetes* were again dominant across all treatments in the continuous digester operation, supporting the evidence that these two phyla contain bacteria that are efficient degraders of holocellulose. The relative abundance of phylum *Bacteroidetes* was 1.4 times higher in the CS:GS (26.3 ± 1.08%) treatment than the CS-only (19.0 ± 2.29%) treatment. In the current study, bacteria of genera *Proteiniphilum*, *Dysgonomonas* and *Petrimonas* contributed to the phylogenetic differences observed within the phylum *Bacteroidetes*. Bacteria belonging to the phyla *Dysgonomonas spp.* and *Petrimonas spp.* were associated with the CS:GS treatment samples and peaked in relative abundance during mid to high levels of TAN and methane production with decreasing reactor effluent VS. The correlation the microbial community with the samples in the CSTR Experiment shows that a peak in relative abundance of *Dysgonomonas capnocytophagoides peaked in relative abundance* within the first HRT in the CS:GS treatment. *D. capnocytophagoides* produce acids inclusive of propionate but do not produce any gas (Hofstad *et al.*, 2000). Therefore the growth of hydrogenotrophic methanogens was likely limited either by a lack of substrate and/or increased TAN levels which limited their ability to syntrophically oxidise propionate, resulting in propionate accumulation.

The peak in relative abundance of *Petrimonas spp.* was associated with mid to low levels of TAN, decreasing reactor effluent VS and mid to high levels of methane. This suggests that the AD process was operating efficiently since reactor effluent VS was decreasing (Angelidaki *et al.*, 2005).

Petrimonas spp. was 10 times higher in the CS:GS treatment than in the CS treatment as the digesters approached stead state. Ribosomal sequence variants that were identified as *Proteiniphilum spp.* in the Silva123 reference database were 98% similar to *Petrimonas mucosa* strain ING2-E5A, a degrader of peptone and complex carbohydrates. *P. mucosa* strain ING2-E5A also has slightly enhanced extracellular enzyme activity against lactose. *Fermentimonas caenicola* strain ING2-E5B, a novel bacterium which degrades proteinacious substrates and carbohydrates was isolated from a mesophilic laboratory-scale mesophilic reactor treating pig and cow manure and maize silage along with *P. mucosa* strain ING2-E5A and shows slightly enhanced extracellular enzyme activity against arabinoxylan and xylan (Hahnke *et al.*, 2016). The enhanced hydrolytic capabilities of *F. caenicola* strain ING2-E5B and *P. mucosa* strain ING2-E5A resulted in peaks in relative abundance during decreasing VS within the reactor effluent.

The CSTR experiment results also indicated the presence of redundant metabolic pathways for propionate oxidation in the form of *Smithella propionica* strain LYP MOL361 and *Pelotomaculum schinkii* strain HH (De Bok *et al.*, 2005). Both CS and CS:GS treatments featured syntrophic relationships with *Syntrophomonas spp.* and *Methanobacterium spp*. confirming the importance of syntrophy to the AD codigestion process. The relative abundance of butyrate oxidising syntrophs *Syntrophomonas spp.* peaked within the CS:GS treatment and the relative abundance of *S. propionica* strain LYP MOL361 was 10.5 ± 3.7% and 1.2 ± 0.55% for the CS:GS and CS-only feeds, respectively. This 10-fold difference between treatments infers that the GS co-substrate influenced the growth of more propionate and butyrate oxidisers, possibly due to the CS-only treatment being less biodegradable (40%) than the CS:GS treatment (50%).

7.4 Possible bio-indicators

Dysgonomonas capnocytophagoides can be used as an indicator of possible propionate accumulation, especially during start-up operations of an AD plant co-digesting CS and GS because of the potential of this bacterium to produce propionate without the H_2 and/or CO_2 generation. AD plant operators must ensure that increases in relative abundance of *Dysgonomonas spp.* are concomitant with increases in hydrogenotrophic methanogens in order to ensure that propionate oxidation occurs at a rate that will maintain low levels of propionate within the AD reactor.

7.5 Conclusion

The present study was designed to improve biogas production and quality in an anaerobic bioreactor co-digesting CS and GS. The recalcitrance of lignocellulose limits the degradation of feedstock for on-farm digesters. Both Batch and CSTR experiments have illustrated the value of co-digesting cattle slurry with grass silage where 34% and 26% more methane was obtained, respectively, compared to the manure only treatment. Nutrient addition using GS as a co-substrate is advantageous to the extent that it provides an additional carbon source for methane synthesis, although no synergistic effects were observed between the CS and GS co-substrates.

Co-digestion of GS and CS at low organic loads is still susceptible to AD process imbalances. Process parameters such as pH, TAN and total VFA should be monitored as they are able to cause shifts within the microbial community during the co-digestion process. Culture independent molecular techniques along with high throughput NGS of the V3-V4 region of 16S rRNA amplicons have proven useful to characterise the microbial community in order to assess the purported function of the various micro-organisms identified.

The use of multivariate statistics to correlate the physico-chemical changes with shifts in the microbial community provides more information regarding putative causes for microbial community shifts as they relate to variations in AD environmental parameters.

Such insight enables AD plant operators to take informed corrective measures and facilitates more comprehensive monitoring and control of the AD process.

The addition of the GS co-substrate resulted in an increase in relative abundance of bacteria belonging to phylum *Bacteroidetes*, in particular *Petrimonas spp*. *P. mucosa* strain ING2-E5A and *Fermentimonas caenicola* strain ING2-E5B. These bacteria are purported to enhance the hydrolysis step and were associated with increased methane and biogas production as well as higher VS degradation efficiencies.

The results obtained indicate that the DADA2 bioinformatics workflow produced inferred RSV with sufficiently high resolution to identify bacteria such as *Petrimonas mucosa* strain ING2-E5A, *Fermentimonas caenicola* strain ING2-E5B and *Dysgonomonas capnocytophagoides*. Correlating the physico-chemical parameters with shifts in the microbial communities characterised by the DADA2 workflow also provided a likely cause for the process imbalance that occurred during the continuous operation of the laboratory digesters. The DADA2 algorithm was used to identify previously undetected variants of *Lactobacillus crispatus* from vaginal samples taken from a cohort of pregnant women (Callahan *et al.*, 2016a). However the extent to which the DADA2 workflow is better than the existing bioinformatics pipelines and OTU binning methods within the context of the characterisation of AD microbial communities remains to be seen since a validation of the method could not be done due to time constraints.

7.6 Future Work

Further work is required to validate the results obtained using the DADA2 bioinformatics software with other bioinformatics pipelines. It is also important to investigate whether the inferences made regarding the microbial communities digesting the CS-only and the CS:GS treatments using DADA2 will be similar to those obtained using OTU clustering at a higher resolution such as 99.5% instead of the traditional 97%.

The hydrolysis step, the syntrophic relationship between propionate and/or butyrate oxidisers and hydrogenotrophic methanogens as well as the diversity and population of the archaeal community were key factors that influenced CH_4 and biogas production. *Petrimonas mucosa* strain ING2-E5A and *Fermentimonas caenicola* strain ING2-E5B likely enhanced the hydrolysis of the co-digested treatment, which resulted in higher levels of methane production than the cattle slurry (CS-only) treatment. Therefore an experimental design that samples both the liquid phase and particulate biomass within the batch and continuous reactors should be conducted to determine whether the relative abundance of bacteria within the liquid phase differs from the composition of bacteria immobilised on the surface of particulates. This would also highlight the micro-organisms associated with the disintegration/hydrolysis step of the AD process since it has been reported that hydrolytic bacteria attach themselves to the particulates prior to extracellular enzyme hydrolysis.

The counts obtained for microbes of interest such as *Petrimonas mucosa* strain ING2-E5A, *Fermentimonas caenicola* strain ING2-E5B and *Dysgonomonas capnocytophagoides* should be verified using qPCR and the appropriate primers to validate the sequence counts obtained. Optimisation experiments should be designed to treat the co-digested (CS:GS) feedstock to increase CH_4 production and investigate the effect of *D. capnocytophagoides* on AD process stability during reactor start-up. This can be accomplished by using inoculum, enriched with varying concentrations of *Petrimonas mucosa* strain ING2-E5A and/or *Fermentimonas caenicola* strain ING2-E5B as well as *Dysgonomonas capnocytophagoides*, to treat the CS:GS feedstock.

The inability to identify some bacteria at the genus and/or species level was a limitation within this current study. Therefore research is required to identify more syntrophic propionate and butyrate oxidisers since these intermediates limit the AD process and therefore can adversely affect the biogas and CH_4 production of an AD plant treating agricultural waste. Focus should be placed on isolating and culturing bacteria belonging to phyla *Atribacteria* and *Cloacimonetes* that were identified in this study in order to define the syntrophic relationships that are purported to exist with hydrogenotrophic methanogens. Bacteria belonging to phylum *Parcubacteria* and archaea belonging to the phylum

Candidatus Bathyarchaeota also need to be isolated and cultured to better understand their role in the AD process itself and whether these functions can be harnessed to improve AD process stability and efficiency.

Results from the CSTR experiment in this study indicate effluent volatile solids (VS) increased within the reactors treating CS, which had the highest levels of lignin. Further research is therefore required to isolate and culture the archaea belonging to the phylum *Candidatus Bathyarchaeota* along with the bacteria such as *Syntrophus aciditrophicus* strain SB, *Pelotomaculum isophthalicicum* strain JI, *Cryptanaerobacter phenolicus* strain LR7.2 identified in this study. These microorganisms are purported to play a role in the degradation aromatic compounds such as lignin-derived aromatic compounds. This may lead to the production of a biological pre-treatment for lignocellulosic biomass, resulting in higher methane and biogas production due to the increased exposure of holocellulose to microbial attack.

Investigating potential biological pre-treatment solutions for lignocellulosic biomass and conducting enrichment experiments to increase CH_4 and biogas production by enhancing the hydrolysis step ultimately leads to scaling up equipment. Future research should therefore involve long-term time series analysis of an on-farm AD plant such as the one situated at Cockle Park Farm. Baseline parameters can be established by measuring the physico-chemical parameters such as those taken during this study. Monitoring of the diversity and population of the microbial community can be done concomitantly with similar laboratory-scaled CSTR experiments to determine the degree to which the laboratory results obtained can be used as a guide for operators of on-farm AD plants treating similar feedstock.

The ability to predict AD process efficiency from information obtained from laboratory experiments as well as long-term time series studies of on farm AD installations will be an invaluable tool for the installation and operation of AD facilities in the UK as well as small island states within the Caribbean such as Antigua and Barbuda. Research is required to assess the biomethane potential of waste feedstocks available in Antigua and Barbuda, inclusive of sargassum and cruise ship waste. Research can then be conducted to optimise the co-digestion of existing feedstocks with sargassum and cruise ship waste which are

seasonally available and to ensure odour control by minimizing H_2S levels in the biogas. The quantification of the environmental and financial impact of diverting the waste from landfill and creating useable products for the energy and agricultural sectors must also be a key objective of any future research since current waste streams are sent to landfill. Molecular analysis must also be conducted to assess the efficiency of the AD process by determining the changes in the diversity and population of key microbes present within the community over time. This current study provides a template for both batch and CSTR laboratory experiments that can be used to adequately assess the energy potential of the mono and co-digested waste streams available in Antigua and Barbuda and conduct the requisite microbial community analysis.

Research is also required to investigate the feasibility of not only using the calorific content of biogas i.e. CH_4 within the electrical, domestic cooking and transport sectors in Antigua and Barbuda, but also using the CO_2 produced for dry ice production or use in greenhouses within the agricultural sector. This research would need to include the financial viability of using additional equipment to strip CO_2 from the biogas for dry ice production and/or for use in green houses.

The constant 30°C climatic conditions, relatively small annual energy demand of 320 GWh and small population implies that the installation of AD plants will make a significant positive impact on the energy and agricultural sectors within Antigua and Barbuda.

Appendices (On accompanied thumb drive)

Appendices for Chapter Three

Appendix 1 Pseudo code for BMP Analysis

Appendix 2 Model normality assumption testing for the Modified Gompertz and Linear Mixed models

Appendix 3 Determination of hydrolysis constant by linear regression for each treatment by experimental run

Appendices for Chapter Four

Appendix 4 Forward and reverse read quality score plots of batch test samples before and after the quality filtering process

Appendix 5 Database of ribosomal sequence variants obtained from the Batch Experiment

Appendix 6 Composition of the microbial community within the (a) CS-only and (b) CS:GS treatments for the Batch Experiment

Appendix 7 Coordinate analysis plots (unconstrained and constrained) for the Batch Experiment

Appendices for Chapter Five

Appendix 8 Individual reactor plots of gas phase parameters versus time

Appendices for Chapter Six

Appendix 9 Database of ribosomal sequence variants obtained from the CSTR Experiment

Appendix 10 Relative abundance of archaeal phyla present in the samples for CSTR experimental by treatment

Appendix 11 Relative abundance of dominant phyla present in more than 1.5% of the samples in the CSTR Experiment by treatment

Appendix 12 CCA tri-plot of samples and the top 30 genera coloured by genera with vectors showing loading of process variables

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