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ANAEROBIC MICROBIAL HYDROLYSIS AND FERMENTATION OF FOOD WASTE FOR VOLATILE FATTY ACID PRODUCTION



A Thesis Submitted to the National University of
Ireland for the Degree of Doctor of Philosophy

By

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to answer my queries. Finally thanks to my kids Nathan and Megan for being so comprehensive.

DECLARATION OF AUTHORSHIP

I Corine Nzeteu hereby certify that this thesis entitled:

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has been generated by me and is based on my own work, unless stated otherwise. No other person’s work has been used without due acknowledgement in this thesis. All references and verbatim extracts have been quoted, and all sources of information, including graphs and data sets, have been specifically acknowledged.

Signed:

Corine Nzeteu

ABSTRACT

Landfill, incineration, composting and anaerobic digestion (AD) are the principal food-waste (FW) treatment methods used in the European Union. Because of the EU landfill directive and waste-management policies on organic wastes, however, the landfill approach is no longer a sustainable strategy. The incineration of FW is generally perceived to be energy demanding and inappropriate because of the high water content (>70%) of FW. Composting and AD both fit well in the “3R” waste management hierarchy and are therefore the most appropriate strategies for FW treatment. AD is more attractive than composting, however, due to its ability to stabilise FW and to generate valuable end-products such as organic acids, biogas and fertilisers. The use of FW as sustainable feedstock for the production of these valuable products through AD processes would contribute to reduce the green house gas emission and could enable to meet the EU 2020 renewable energy target; it could also enable an increase in chemical supply. However when dealing with mixed feedstock such as FW, AD process for methane production as the sole beneficiary product is usually less attractive. An alternative approach to the anaerobic digestion of this type of biomass is to aim for production of organic acids which have higher added value than methane. The sustainability of this approach depends on the extent of FW stabilisation, however, as well as on the yields, rates and profiles of the organic acids that are generated. Although FW is generally regarded as being readily biodegradable because of its high volatile solid fraction (90% of total solids), its hydrolysis is still perceived as a rate-limiting step. The enhancement of the hydrolysis step during anaerobic digestion could improve the rate and yield of organic acid accumulation and shorten the solid retention time required for biodegradation. Furthermore, there is a need to uncover the microbial groups involved in the hydrolytic-acidogenic stage as this could help in selecting the best operational parameters for their growth, which in turn could improve the rate of the processes. The objective of this thesis was to investigate and optimise the accumulation of organic acids from restaurant food waste (RFW) AD.

The first phase of this study (Chapter 2) was conducted to evaluate and optimise the biodegradation efficiency of RFW using biomethane potential assay; the effect of the FW composition (fat, protein, hemicellulose and cellulose) on biodegradation rates was assessed. In addition, a bioaugmentation strategy was used to enhance the hydrolysis efficiency of the RFW components. The RFW biodegradation efficiency was enhanced by 10 to 15% using the bioaugmentation approach, which consisted of supplementing the primary inoculum with enriched culture developed on pure substrates. The hydrolysis rate constant for the different fractions of the RFW indicated that hemicellulose fraction was easily hydrolysable, while fat was the most recalcitrant. Hemicellulose and cellulose were the two fractions of the RFW enhanced as the result of enriched cultures supplementation. *Bacteroides graminisolvens* and species affiliated with *Porphyromonadaceae* were identified as potential cellulose and hemicellulose hydrolysers (respectively) using 16S rRNA profiling. The data obtained suggested a fourteen-day solid retention time for maximum biodegradation of RFW and the possibility of shortening this time through a bioaugmentation strategy.

In the second phase of this study (Chapter 3), three leach-bed reactors fed with RFW and initially inoculated with granular sludge were operated at 37°C in a semi-continuous mode. Based on the results obtained in Chapter 2, the solid retention time of fourteen days was applied; a ratio of 1:4 (inoculum:RFW) was chosen to favour the rapid accumulation of organic acids inside the reactors. Stable bioprocess performance was demonstrated, with volatile solid (VS) efficiency above 60%. The hydrolysis of the components of the RFW was believed to be efficient over the initial two days of the incubation, as indicated by the maximum soluble chemical oxygen demand (sCOD) accumulation over the same period. Leachate analysis revealed the accumulation of up to 49 g l⁻¹ of volatile fatty acids (VFAs), of which circa 35% was butyric acid and 25% acetic acid. Microbial communities identified from 16S rRNA-based Illumina sequencing analysis of leach-bed reactors identified *Enterococcus* as potential hydrolysers. The important fermentative groups (identified as

Lactobacillus, *Clostridium* and *Bifidobacterium*) were likely responsible for the production of lactic acid, butyric acid and acetic acid, respectively. The results gathered in this second phase suggested that it is feasible to biodegrade the RFW over short periods (two days) while at the same time accumulating organic acids.

In the final phase of this study (Chapter 4), process optimisation strategies were investigated in terms of promoting VFAs accumulation; the feasibility to selectively produce caproic and butyric acid from RFW was also assessed. Based on the data generated in Chapter 3, which showed that maximum hydrolysis efficiency was achieved in two days, the solid retention time (SRT) in the leach-bed reactors was reduced from fourteen- to seven days (Chapter 4). Increasing the recirculation regime (frequency) from once to three times per day and reducing the starting liquor VFAs' concentration from 15 to 6 g COD l⁻¹ resulted in a 55% improvement of VFAs production. With these parameters, VS removal efficiency of over 70% was achieved; caproic acid at the concentration of 21.86 g COD l⁻¹ was the highest VFA produced in the leach-bed reactors. The composition of VFAs was influenced by hydrolysis rate, pH, loading rate and the depletion rate of short chain volatile fatty acids (SCVFAs). The selective production of caproic acid from RFW leachate at the rate of 3 g l⁻¹ d⁻¹ was demonstrated in this study by using hydrogen or the combination of hydrogen and ethanol supplementation. Butyric acid accumulation was observed in the presence of ethanol. Microbial community analysis based on the 16S rRNA sequencing suggested the implication of *Clostridium* and *Peptoniphilus* in the generation of butyric acid, while *Lactobacillus reuteri* could play a role in the accumulation of caproic acid. This study has set some basis for the selective production of caproic and butyric acids from FW.

This thesis demonstrates the feasibility of biodegrading RFW while promoting the accumulation of valuable organic acids (mainly VFAs) using leach-bed reactors. The combination of bioprocess monitoring with molecular analyses provided several valuable insights into the complex hydrolytic-acidogenic microbial communities which underpin these processes.

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CHAPTER 1

INTRODUCTION

1.1 Food waste and management strategies

Food waste (FW) may be viewed as the portion of the food that is discarded or lost at any step of the food-supply chain. According to the Food and Agriculture Organization (FAO) of the United Nations, one third of the food produced today for consumption is wasted, lost or discarded; the vast majority of the waste in developed countries occurs at the consumer and retailer level, while in developing countries waste is mostly generated during production, harvesting and processing (Gustavsson *et al.*, 2011). In Ireland, over 1,000,000 tonnes of food waste is thrown away every year, of which 60% belong to the avoidable waste category and 40% to the unavoidable waste category (Creedon *et al.*, 2010; EPA, 2013). The vast majority of the FW generated is usually disposed of in landfills along with other organic solid waste, thereby causing various environmental problems, including: i) the possibility of ground water contamination as the result of leachate generation during the degradation process; ii) the possibility of greenhouse gas emissions; iii) land usage issues; and iv) poor recovery of valuable resources. Due to the environmental implications of the landfill method of disposal of organic solid waste, a landfill directive was implemented among EU member states in 1999. In Ireland, the landfill directive requires that by 2013 biodegradable municipal waste (BMW) going to landfills was to be reduced to 50% of its 1995 tonnage, then to 35% of its 1995 tonnage by 2016 (McCarthy *et al.*, 2010). Various regulations, policies and waste-management strategies have been implemented in many countries across Europe in order to meet the requirements of this directive. In Ireland, the waste-management strategies are based on the EU waste-management hierarchy commonly known as 3R strategies, which consist of waste reduction through prevention, recycling and resources recovery (Carey *et al.*, 2008).

Despite the increasing number of FW prevention campaigns that aim to reduce the amount of avoidable FW that is generated, concerns still remain about the unavoidable fraction of the waste. In addition, it is clear

that a successful prevention campaign will only manage to reduce the avoidable waste to a certain degree. Recycling or reuse can only be applied to certain solid materials (glass, plastic, paper and metal) and are therefore not relevant to the organic solid waste stream. Resource recovery involves the conversion of rich organic waste through composting and anaerobic digestion techniques into valuable products (compost, biogas and biochemical; (Cheremisinoff, 2003). This strategy is believed to bring various solutions not only to the environmental pollution caused by the landfill-disposal method, but also in playing an important role in the bio-economy. In Ireland, food waste disposers (FWDs), composting and anaerobic digestion systems are the primary methods that are used to help in the diversion of large tonnages of food waste and other bio-waste sent to landfills (Carey *et al.*, 2008; Department of Environment Community and Local Government(DECLG), 2006).

1.1.1 Food waste disposers

A FWD is a device that is installed directly under the kitchen sink and connected to a home's sewage pipe. The electrically powered device shreds FW into small pieces, which are then flushed with water through the sewage pipe and transferred to the wastewater treatment plant (WWTP) (Evans, 2007; Marashlian & El-Fadel, 2005). The FWDs use a wastewater system while composting and AD use a solid organic waste system (Fig 1.1). FWDs were first introduced in the 1930s in the United States in an attempt to minimise the solid organic waste going to landfills and to reduce the associated management costs (Marashlian & El-Fadel, 2005). The technology was quickly adopted by many others countries in which landfill space had become a major problem. In many European countries, for example, waste management strategies in which waste charges were applied constituted one factor that triggered the market penetration of such devices. For instance in Ireland, a significant increase in the sale of FWDs was reported in the early 2000s compared to the introduction period of the mid-1990s as a result of the introduction of waste charges which follow the 'polluter pays principle' (Carey *et al.*, 2008). Although the FWD does

provide a few solutions to the diversion of organic waste from the solid waste stream, its implementation as a waste management strategy is still not fully accepted because FWDs are often perceived as devices that transfer existing problems from the solid waste management stream to the waste water system (Carey *et al.*, 2008). By minimising the organic waste that goes to the solid waste management stream, FWDs increase the pressure on the wastewater management system, which then is required to be more robust in order to cope with the increasing organic loading rate that now goes through the system (Iacovidou *et al.*, 2012).

In addition, other issues such as water and energy consumption, sewage system blockage, wastewater characteristics, treatment capacity, and the quantity and quality of sewage sludge and biogas need to be considered. These growing concerns over the use of FWDs have slowed its market penetration in Europe, where its uses are completely banned in some countries and regulated in others (Carey *et al.*, 2008). In Ireland, for example, the use of FWDs is regulated by the local authorities. In some counties its uses is banned, while in others where the WWTPs are believed to be robust and efficient, ‘food and catering establishments’ (FCEs) are required to obtain a Trade Effluent Licence, under which nutrient and solids discharges are limited. The use of FWDs is generally regarded as being similar to the disposal method rather than recovery, and is therefore found not to be in line with the national strategy of biodegradable waste which prioritises 3R strategies (Carey *et al.*, 2008).

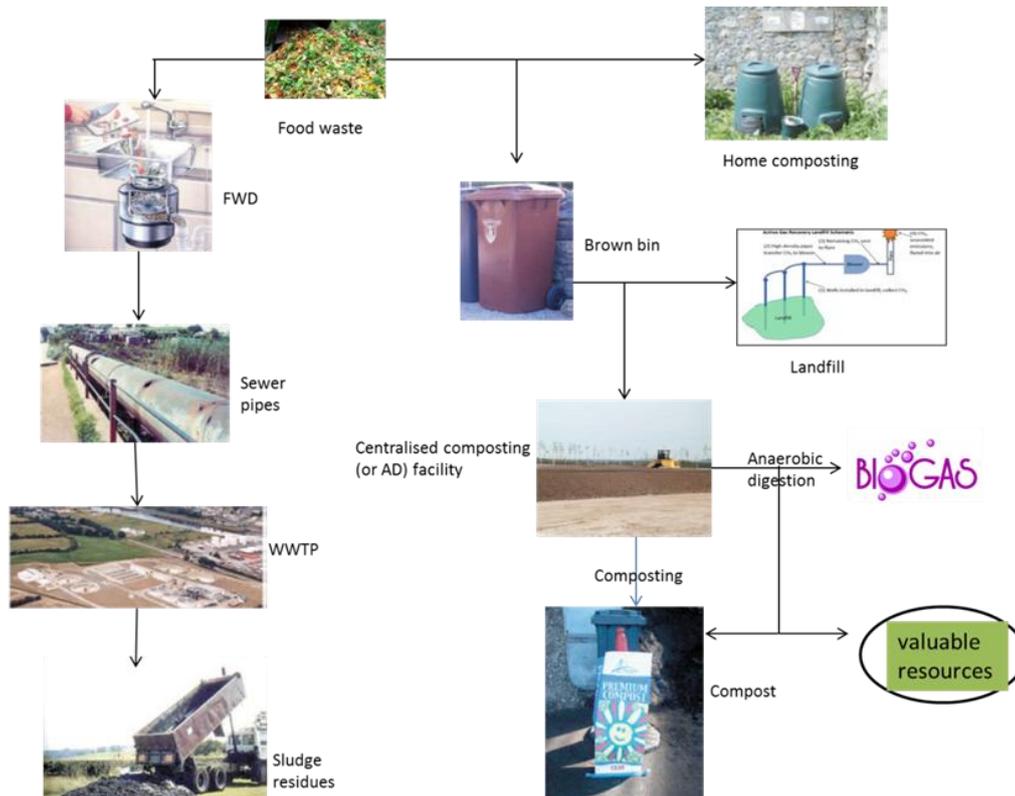


Fig 1.1 Food waste disposal alternatives in Ireland. Adapted from Carey *et al.* (2008).

1.1.2 Composting

Unlike FWD systems, composting is generally perceived as one of the best options in the solid organic waste-management strategy; the landfill strategy is the worst (Department of Environment Community and Local Government(DECLG), 2006). Composting is a biological process in which the organic content of waste is decomposed in the presence of oxygen. It is a form of waste reduction that simultaneously generates usable product, which is often referred to as ‘humus’ or ‘compost’. In addition, composting contributes to waste stabilisation, the removal of pathogenic microorganisms and the elimination of phytotoxic substances. The final end-product of the composting known as ‘compost’ can be used to improve the soil’s physical property for better plant growth. Applying compost on soil can also help achieve better drainage, higher water-holding capacity and increased infiltration. The number of composting facilities in the Republic of Ireland has been reported to have continuously increased from fewer than ten facilities in 2000 to over forty-five in 2012 (rx3, 2012). The repartition

of these composting facilities across the country since 2012 may be seen in Fig 1.2.

The reason for the rapid development of composting facilities in the past decade on the Island of Ireland (including both the Republic of Ireland and Northern Ireland) might be associated with the rising cost of landfills, the polluter pays strategy, and the need to improve environmental protection and to recover resources in accordance with the waste management hierarchy. Despite the rapid increase of composting sites across the country, composting as a waste-management strategy was reported to account only for 3–5% of the biodegradable municipal waste (BMW) during the period 2008–2010 (European Environment Agency, 2013). In 2011, 17–20% of the BMW was biologically treated (via composting and anaerobic digestion) (rx3, 2012; Zavodska *et al.*, 2014). Such low level of composting of the BMW was believed to be due to several factors that influence the process, as well as heavy regulation. Composting is a microbially mediated process, which means that optimum conditions such as aeration, temperature, moisture content and mixing need to be applied to achieve a successful process. These requirements are generally viewed as being time and energy consuming and labour intensive. In addition, the requirement for composting facilities to comply with the animal by-product regulation is reported to increase the cost of composting facilities, thereby making the BMW waste-management strategy unattractive (Zavodska *et al.*, 2014). Very few composting facilities across the country process food waste, as this is believed to contain animal by-products, which in turn contribute to higher gate fees compared to other forms of biodegradable waste such as garden waste. The lack of a quality standard for compost has contributed to the limitation of its usage within the agricultural sector. The fear of contamination of compost by pathogens or heavy metals, or the lack of certain important nutrients such as nitrogen (which is necessary for plant growth), have slowed market penetration (rx3, 2012).

Although composting is by far the most used waste-management strategy for food waste and other biowaste, only a little fraction of this

waste is processed with this technology, which leaves a large fraction of biodegradable solid waste which must then be dealt with by other means.



Fig 1.2 Map of organic waste management facilities on the Island of Ireland. Blue pins represent composting facilities, green pins represent anaerobic digestion facilities and yellow pins represent mushroom compost production facilities. This map is taken from the Market Report on Irish Organic Waste Management and Compost Use, prepared by rx3 (rethink recycle remake) 2012.

1.1.3 Anaerobic digestion

Anaerobic digestion is a microbially mediated process in which complex organic molecules are converted to simple monomers, which are further

converted to biogas in the absence of oxygen. AD naturally occurs in places or environments where oxygen is limited or non-existent (e.g. sediments, soil, landfills, lakes and the gastrointestinal track). The natural occurrences of the process in landfills where municipal solid waste (MSW) is discarded is generally perceived as being a burden to the environment, because the biodegradation and decay of the organic content of the waste release biogas into the environment and produce contaminated liquor that is likely to leach into the ground water. Biogas produced from landfills is mostly composed of methane (60%), carbon dioxide (40%) and traces of others gases (McCarthy *et al.*, 2010). Methane and carbon dioxide are two potential greenhouse gases (GHGs) that have the highest impact on climate change. Most developed countries have adopted the controlled landfill system, in which the biogas is either recovered and used to produce energy or flare to reduce the impact of biogas with a high GHG emission factor (e.g. methane, NO and CO₂;) (Beylot *et al.*, 2013; M Caine, 2000; McCarthy *et al.*, 2010).

Although the controlled landfill system has considerably reduced the emission of toxic landfill gases into the environment, it has not provided a solution to the leachate problem. The process in which AD takes place in landfills is affected by the complexity of the waste, in which the naturally occurring microorganisms do not have easy access to the organic content. This usually leads to a partial decay of waste along with the generation of unstable liquor and low methane content in biogas. In addition, the lack of process control could favour the development of potential pathogens in liquor that may leach and contribute to the contamination of any surrounding water. Landfill treatment of biodegradable waste is classified as the worst in the waste management hierarchy, mainly due to the limitation of resource recovery, lack of a recycling strategy and its environmental implications (Creedon *et al.*, 2010).

In order to overcome the various issues of the landfill treatment method of waste, the AD process has been mimicked in closed systems called ‘anaerobic digesters’ or ‘anaerobic reactors’. These systems provide numerous advantages over the landfill system, in the sense that the process can be optimised, only biodegradable waste is treated, the production of

biogas is controlled, valuable by-products can be recovered, and digestate and leachate can be monitored. The in-vessel AD process is one technology that could provide what is called a 'zero waste' strategy for organic waste. This technology could shift the view of organic waste from environmental burden to valuable resources. Anaerobic digesters can treat different kinds of biodegradable waste in which the end-products (e.g. biomethane, digestate and leachate) in all cases are the same. Similarly to composting systems, the number of AD facilities in Ireland (Fig 1.2) has increased in the past decade but remains low compared to the number of composting facilities (rx3, 2012). The investment cost for AD facilities appears to be very high, which may explain the technology's slow development compared to composting facilities. The simplicity of the composting process makes it easy to operate even at home, while the implementation of the AD process requires a certain level of expertise. While both composting and anaerobic digestions fulfil the requirements of the waste management hierarchy, anaerobic digestion appears to be the most promising technology because of its diverse end-products and applications. The methane produced from the anaerobic digestion process could help in meeting the EU 2020 renewable energy targets (European Commission, 2015). The majority of anaerobic digestion systems in Ireland are on-farm digesters operated by farmers that process mainly agricultural residues, animal slurry and manure. Food waste is usually not accepted in AD facilities because of the animal by-product regulation, which compliance to, tend to increase the already high cost of AD facilities.

Compared to most other EU countries, Ireland has one of the lowest levels of biogas production (Fig 1.3), which is associated with the nation's small number of AD facilities (Fitzduff & Burke, 2014). Large-scale AD plants for the processing of the organic fraction of MSW and industrial waste are inexistent. Two possible reasons for the low level of development of the AD sector in Ireland include the lack of economic viability (which tends to discourage investors) and the presence of numerous regulations, which are sometimes perceived as being time consuming and costly (Fitzduff & Burke, 2014). The low level of government incentives could

also explain the slow increase in the number of AD facilities in Ireland. The REFIT scheme (Renewable Energy Feed in Tariff), which is the government's support for renewable energy, sets the highest price of electricity from biogas plants at €0.15, while in other EU countries where AD is developing rapidly the same price is set at €0.28 for both Germany and Italy and €0.25 for the United Kingdom (Fitzduff & Burke, 2014).

Apart from biogas production, some by-products of AD such as organic acids and minerals which until now have received very little attention could play a key role in the viability of this technology (Kleerebezem *et al.*, 2015). These valuable resources (which are usually synthesised chemically or are derived from petroleum sources) could be environmentally friendly when produced at high concentrations in digesters. These resources reportedly have a high market value and could (along with biogas) make the anaerobic digestion technology very attractive (Kleerebezem *et al.*, 2015).

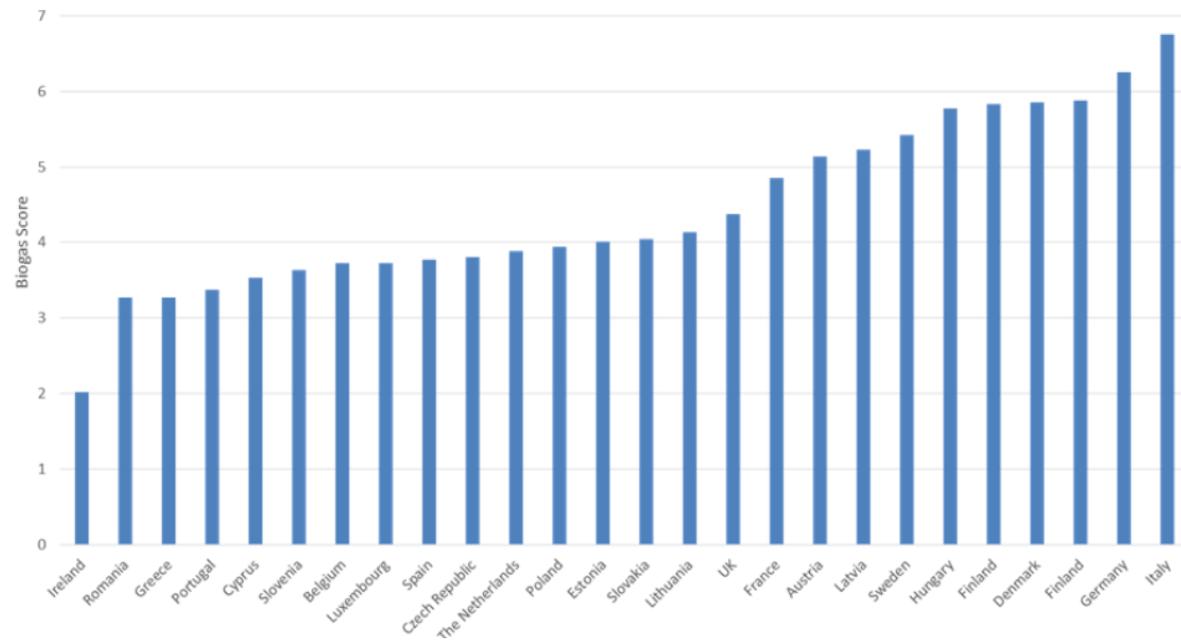


Fig 1.3 Comparison of European Union biogas potential (Fitzduff & Burke, 2014).

1.2 The anaerobic digestion process and microbiology

Anaerobic digestion is the consequence of a sequence of metabolic interactions among various groups of microorganisms, resulting in the generation of valuable end-products. The process of AD usually occurs in four stages, including hydrolysis (stage 1), acidogenesis (stage 2), acetogenesis (stage 3) and methanogenesis (stage 4) (Lastella *et al.*, 2002; Park *et al.*, 2005). Different groups of microorganisms are involved in these stages (de Bok *et al.*, 2004) and work in sequence, with the products of one group constituting a substrate for the next (Fig 1.4).

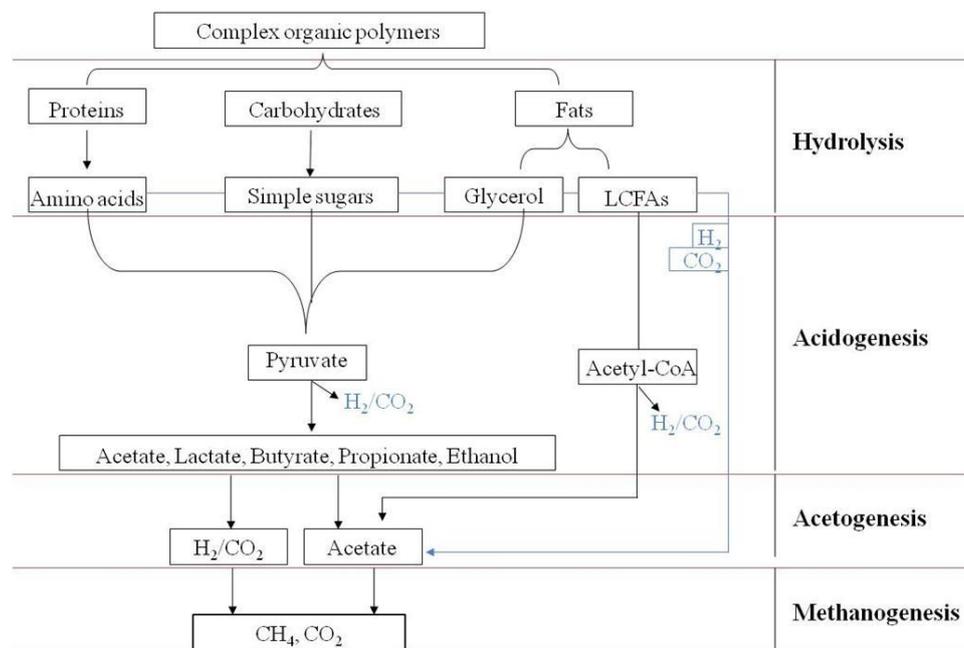


Fig 1.4 Stages of the anaerobic digestion process; LCFAs: long chain fatty acid. Diagram adapted from Niu *et al.* 2015.

1.2.1 Hydrolysis

Hydrolysis is the breakdown of complex organic molecules (protein, carbohydrate and fat) into simple soluble molecules (amino acid, simple sugar and fatty acid). It is usually the first step in the AD process and is catalysed by hydrolytic bacteria. These bacteria secrete enzymes (lipase, protease, amylase, etc.) which are responsible for the solubilisation of the corresponding complex molecules. Hydrolysis is reported to be the rate-

limiting step during the AD of feedstock with high mixed solid organic content (Mata-Alvarez *et al.*, 2000; Veecken & Hamelers, 1999) such as food waste. Several factors, including the type and size of feedstock, temperature, pH and hydrolytic communities, are believed to have an impact on the rate of hydrolysis. Wang *et al.*'s study (2010) on the AD of grass silage suggested that the solid attachment of bacteria to the solid organic material was likely to increase the hydrolysis rate. Similarly, the solid surface adsorption of hydrolytic enzymes has been reported to facilitate the hydrolysis (Sanders *et al.*, 2000; Veecken & Hamelers, 1999). Hydrolytic retention time, temperature and pH have also been found to have an impact on the hydrolysis step of solid waste (Christ *et al.*, 2000; Veecken & Hamelers, 1999). A number of bacteria groups, including *Acetivibrio*, *Clostridium*, *Bacteroides* and *Thermotoga*, have been reported to be involved in the hydrolysis stage of the AD process (Cirne *et al.*, 2007a; Liebl, 2001; Sträuber *et al.*, 2012).

1.2.2 Acidogenesis

Acidogenesis, the second step in the AD process, involves the conversion by fermentative bacteria of soluble compounds generated during hydrolysis into short- and medium-chain volatile fatty acids, hydrogen, lactic acid, alcohol and carbon dioxide (Michael H, 2003). The principal products of this group of bacteria are propionic acid, butyric acid, acetic acid, formic acid, lactic acid, ethanol and methanol, all of which constitute a major substrate for the next group of bacteria (acetogens and methanogens) (Kim *et al.*, 2009). While the fermentation of carbohydrate and nitrogenous compounds (such as proteins and amino acids) has been reported to occur in the acidogenesis phase, the fermentation of fat, on the other hand, is believed to take place simultaneously alongside the volatile fatty acids during the acetogenesis and methanogenesis phases (Eastman & Ferguson, 1981). Acid-forming bacteria are known to grow faster in the presence of soluble substrates, which are rapidly converted to organic acids. Fast acidification in digesters is generally perceived as being problematic because of the inhibitory effect on both hydrolysis and methanogenesis.

Similarly to hydrolysis, a number of factors, such as pH, feedstock composition, microbial community composition, temperature, hydraulic retention time and volatile acids concentration, have been found to influence acidogenesis (Yang *et al.*, 2004; Zhang *et al.*, 2005). Acidogenic bacteria have been found among *Bifidobacterium*, *Lactobacillus*, *Anaerolinaceae* and a few thermophilic bacteria (Balk *et al.*, 2002; Dong *et al.*, 2000; Stiles & Holzapfel, 1997; Yamada *et al.*, 2006).

1.2.3 Acetogenesis

The third stage of the AD process, known as acetogenesis, involves the metabolism of the product of the second phase into acetic acid, carbon dioxide and hydrogen by the action of obligate syntrophic interactions with certain methanogens (Plugge *et al.*, 2010). The obligate syntrophic relation occurs because neither the methanogens nor the acetogens alone can degrade the specific organic soluble compounds which result from acidogenesis (Stams & Plugge, 2009). Both microbial communities are therefore essential. For example, acetogens are obligate hydrogen producers and are inhibited by high hydrogen pressure. Thus in a syntrophic interaction the hydrogen-consuming (methanogenic) organisms contribute to lowering the hydrogen pressure and to making the overall substrate conversion thermodynamically favourable (Plugge *et al.*, 2010). During the syntrophic interaction, the physical distance between the hydrogen-producing and the hydrogen-consuming organisms is an important factor. A few researchers have suggested that the diffusion distance for metabolite transfer between syntrophic communities should be as short as possible in order to allow for efficient interspecies electron transfer (Ishii *et al.*, 2005; Stams & Plugge, 2009). Previous reports have demonstrated that intermicrobial distances influence biodegradation rates and specific growth rates (Stams *et al.*, 2006; Thiele *et al.*, 1988), which results in bacteria- and archaea-forming compact aggregates. In addition to the close proximity with methanogens, syntrophic acetogens require stable neutral pH and are sensitive to even low concentrations of inhibitors (e.g. NH₃, H₂S) (Lv Wen *et al.*, 2010).

1.2.4 Methanogenesis

Methanogenesis is the last stage of the AD process, during which biogas (mostly composed of methane) is generated. This stage is catalysed by groups of microorganisms known as methanogens. Because they can grow only with a few simple substrates (H_2 and CO_2 , formate, methanol and acetate), they depend on other microbial groups that degrade more complex organic compounds for their substrate supply (Stams & Plugge, 2009). Methanogens can be broadly classified into two groups: autotrophic methanogenic archaea and heterotrophic methanogens. The former utilise carbon dioxide and hydrogen, while the latter convert acetate, formate and a few other compounds to support their metabolic functions (Plugge *et al.*, 2010). During AD processes, two-thirds of all methane produced is derived from the conversion of acetate, which is the absolute key intermediate (Plugge *et al.*, 2010); the other third is produced from the reduction of carbon dioxide by hydrogen or formate (Ferry, 1992; Liu & Whitman, 2008). Methanogenic communities are known to be obligate anaerobe, have a narrow pH range (6.7–7.8) and are characterised by low growth rates (Ali Shah *et al.*, 2014; Mshandete *et al.*, 2008). Because the overall substrate conversion rate of the anaerobic reactor directly depends on the number and activity of the microorganisms, reactors that are able to retain a high number of microbes will favour the development of organisms that grow slowly. The microbial communities of such digesters have been generally reported to operate in dense aggregates, which prevent them from being washed out of the system (Mshandete *et al.*, 2008).

1.3 Anaerobic digestion of food waste

Food wastes are considered to be readily biodegradable because of their high volatile fraction of almost 90% of total solids (Zhang *et al.*, 2007). Their relatively high moisture content makes them more suitable for AD (Selvam *et al.*, 2010; Zhang *et al.*, 2007). Indeed, there are various sources of food waste, including uneaten food and food preparation leftovers from residences, commercial establishments such as restaurants, institutional

sources such as school cafeterias and industrial sources such as factory lunch rooms. Since the sources of food waste vary, their organic contents may also vary depending on the type of food activities that are carried out in a specific site. Because the rate of AD is reportedly influenced by the type, availability and complexity of the substrate, it is important to determine the composition of the food waste in terms of its carbohydrates, lipids, proteins and fibre contents (Lesteur *et al.*, 2010). This allows for estimating both the methane potential and the design of the most efficient AD system. Many factors reportedly affect the design and performance of the AD processes of FW; some of these factors are related to feedstock characteristics, reactor design and operational conditions (Zhang *et al.*, 2007)

1.3.1 Impact of the composition of food waste on anaerobic digestion

The biomethane potential of the FW depends on the concentration of its four main components, including proteins, lipids, carbohydrates and cellulose. Neves *et al.* (2008) reported that a feedstock with excess lipids may lead to the reduction of the rate of hydrolysis because lipids and long-chain fatty acids may be absorbed onto the solid surface, thus reducing the accessibility of the enzyme attack of other compounds. In addition, the excess lipids in an AD system may also hinder the access of simple substrates such as acetate, which in turn delays the methanogenesis stage. It has been reported that FW with an excess of carbohydrate and protein contents may present hydrolysis rate constants higher than waste with an excess of lipids and cellulose (Neves *et al.*, 2008). Though the ammonia released during the degradation of protein is known to have an inhibitory effect, this effect has been observed to be reversible over time (Neves *et al.*, 2008).

1.3.2 The effect of pre-treatments on the hydrolysis of main FW components

Given the solid nature and the high organic content of food waste, the hydrolysis stage has been reported to be a rate-limiting step (Mumme *et al.*, 2010; Park *et al.*, 2005). As such, various physical, chemical and enzymatic pre-treatments have been applied to increase the feedstock solubility and to

accelerate their biodegradation rates (Khalid *et al.*, 2011; Mata-Alvarez *et al.*, 2000). Among the various pre-treatment methods, mechanical pre-treatment seems to be the most economically viable technique. This involves the size reduction of the feedstock via maceration, grinding or homogenisation. Size reduction reportedly contributes to increasing the amount of feedstock that can be loaded into the reactor, which thus may increase the yield of the desired end-product (Palmowski & Muller, 2000), although Babaee and Shayegan (2011) reported that overloading the digester could result in low biogas yields. This is because an initial high concentration of organic compounds may cause the number of acidogenic bacteria to increase, thus increasing the production of organic acids; this then leads to a drop in pH and the inhibition of methanogenic bacteria.

1.3.3 pH

The pH value is a pivotal factor in the AD of food waste because methanogenic bacteria are very sensitive to acidic conditions and their growth and methane production are inhibited in acidic environments. On the other hand, acidogenesis can also be inhibited by a pH over 8. Although it has been proven that the optimal range of pH for obtaining maximal biogas yield in AD is 6.5–7.5, different stages of the AD process have different optimal pH values, which will vary according to substrate and digestion techniques (Veeken *et al.*, 2000).

1.3.4 Anaerobic digestion systems

Anaerobic digestion systems have been classified into 3 categories: i) one stage continuous systems (represented by low-solids or "wet" and high-solids or "dry"), ii) two-stage continuous systems (represented by dry-wet and wet-wet) and batch systems (either one stage or two stage) (Vandevivere *et al.*, 2002). One-stage digesters are mostly used in Europe and represent about 90% of the installed AD capacity in Europe (Naik *et al.*, 2013; Rapport *et al.*, 2008). This is due to the fact that they are simple to design, build, operate and are generally less expensive. Depending on the total solid concentration in the digester, one-stage continuous systems are

operated as high solids/dry or low solids/wet. Digesters with less than 15 percent total solid (TS) at the beginning of operation are considered wet systems and digesters with TS greater than 15-20 percent are considered dry systems (Bolzonella *et al.*, 2003; Guendouz *et al.*, 2010). Due to the water constraint and the high level of pre-treatment required for wet systems, dry systems have become attractive in Europe and constituted about 60% of the one-stage continuous digesters capacity installed (De Baere, 2006; Rapport *et al.*, 2008). The most commonly used commercial-scale single-stage dry continuous systems in Europe include the Dranco process, Kompogas process and Varlorga process. All three systems are operate as plug-flow digesters (materials added on one end of the digester push older materials toward the opposite end) and deal with organic solid waste. The only pre-treatment required with these systems is the removal of large pieces and minimal dilution with tap water to keep the solid content in the desire range. The challenge of the dry system is usually encountered in the handling, mixing and pumping the solid stream rather that the biochemical reaction (Rapport *et al.*, 2008).

Single-stage wet AD systems represent about 40% of one-stage AD systems capacity installed in Europe. This digester configuration was one of the first to be build and was commonly used for the treatment of wastewater known to have very low TS content (Baere & Mattheeuws, 2012; Rapport *et al.*, 2008). Single-stage wet AD was also one of the first system used for treatment of the organic fraction of the municipal solid waste (OFMSW). This reactor type required less than 15% TS material in the tank. Examples of commercial single-stage wet continuous AD systems in operation in Europe include the Waasa system, Entec, Biostab, etc. (Rapport *et al.*, 2008). The organic loading rate (OLR) of single-stage continuous digesters (dry or wet systems) is limited by the ability of methanogenic organisms to tolerate the sudden decline in pH that results from rapid acid production during hydrolysis (Naik *et al.*, 2013).

As previous studies have shown, researchers have put considerable effort into overcoming the problems caused by the high organic loading rate and the accumulation of organic acids during one-stage continuous AD

processes. Mata-Alvarez *et al.* (2000) proposed that the use of a two-phase AD process could permit much higher loads in the digester. A two-stage system is considered to be a promising process for treating organic waste with high efficiency in terms of degradation yield and biogas production (Pohland & Ghosh, 1971). In this system the hydrolysis and the acidification stages take place in one reactor, while the methanogenesis occurs in a separate reactor. In this way, not only is the inhibition of the methanogen by the accumulation of VFAs avoided, but different operating conditions (pH, temperature, mixing, etc.) can also be applied in order to maximise the yield of each stage. The number of multi-stage systems throughout Europe was expected to rise due to their ability to handle higher loading rates, improved process stability and flexibility. However less than 10% of installed AD capacity in Europe are multi-stage systems (Baere & Mattheeuws, 2012; Rapport *et al.*, 2008). This is likely due to the complexity and high cost of building and operating such systems. Nevertheless, the potential of the multi-stage systems to improve process performance has encouraged much research which led to the successful development of commercial multi-stage digesters among which Biotechnische Abfallverwertung GmbH & Co. KG (BTA), Linde-KCA-Dresden GmbH and WEHRLE Umwelt GmbH (Biopercolat).

Batch systems represent a smaller portion of all AD systems capacity installed in Europe. This system can handle high organic loading concentration ranging from 30 - 40% total solid. Batch systems are reported to be better at treating recalcitrant biomass such as lignocellulosic material which required longer retention time (Naik *et al.*, 2013). The largest market share of batch digesters is owned by the German company Bekon. Other companies have also provided components and full scale systems to many batch OFMSW digesters, most notably Biocel and Enki Energy. Advantages of the batch system include simplicity of the reactor, low maintenance requirement, and minimal capital cost (Li *et al.*, 2011). The primary disadvantage of batch digesters is the lack of control over the biological processes which can cause uneven gas production and process instability. In batch AD system, a lag phase usually occurs as organic polymers are

hydrolysed followed by a sudden drop in pH as organic acids are produced from the hydrolysate. Methanogenesis can be inhibited under severe pH drop. Attempts to mitigate these disadvantages have been addressed by mixing the incoming feed with digestate from a previous batch, using aerobic pre-treatment, adding buffers, changing the inoculation rate, and altering the leachate recycling rate (Brummeler *et al.*, 1991; Brummeler & Koster, 1990). Sequential and phased batch (SEBAC) digesters and anaerobic phase solid (APS) digesters systems have also been developed in an attempt to surmount the limitation of the batch AD systems. They consist of two- or three-batch, leach-bed reactors with leachate recirculation by sprayer. Unlike the conventional batch system, the SEBAC and APS digesters are loaded in sequence such that leachate can be transferred between reactors.

1.4 Prospects for anaerobic digestion

In the biorefinery concept, the value of each waste stream must be maximized during the treatment. For this reason, nearly all the installed AD systems in Europe have adopted the zero waste strategy resulting in the entire waste being converted into valuable products (Fig 1.5). For single-phase AD systems the attractive product is the methane while for multiphase and batch AD systems, in addition to the methane other attractive product such as carboxylates and ethanol could be generated. The final residue known as digestate generated by both AD system is usually dewatered; the resulting water is recycled through the system while the dewatered digestate is treated through composting process for the production of compost (Rapport *et al.*, 2008). This recycling strategy has generated new ideas to insert anaerobic digester into already existing composting plants. Since AD systems usually required low surface area, the existing composting site and equipment will be used; therefore the investment needed will be reduced and anaerobic digestion will constitute the most economically attractive upgrade of the facility. The composting plant with the inserted anaerobic digester will become an energy producer instead of only an energy

consumer. Example of integration of an anaerobic digestion into an existing composting plant is the Anaerobic digestion plant in Hengelo (The Netherlands) (Baere & Mattheeuws, 2012). Most full-scale AD systems (especially batch and multistage systems) required post-treatment aeration stations, for which the existing composting site could be used without further modification (Kannengiesser *et al.*, 2016; Rapport *et al.*, 2008). The addition of oxygen is usually performed during the early stage of the process (hydrolysis and primary fermentation stages) to improve the solubilisation rate of recalcitrant substrates such as lignocelluloses and also to avoid oxidation as well as methanation reactions (Kannengiesser *et al.*, 2016; Rouches *et al.*, 2016). Although no significant methane yield improvements have been measured for anaerobic digestion of biomass with micro-aeration, this strategy could be beneficial for the generation of MCFA using a two-stage system. The introduction of oxygen in the first stage will allow to reduce the solid retention time and to produce short chain carboxylate which will be further process in the second stage (under strict anaerobic condition) for the generation of MCFAs (Kannengiesser *et al.*, 2016).

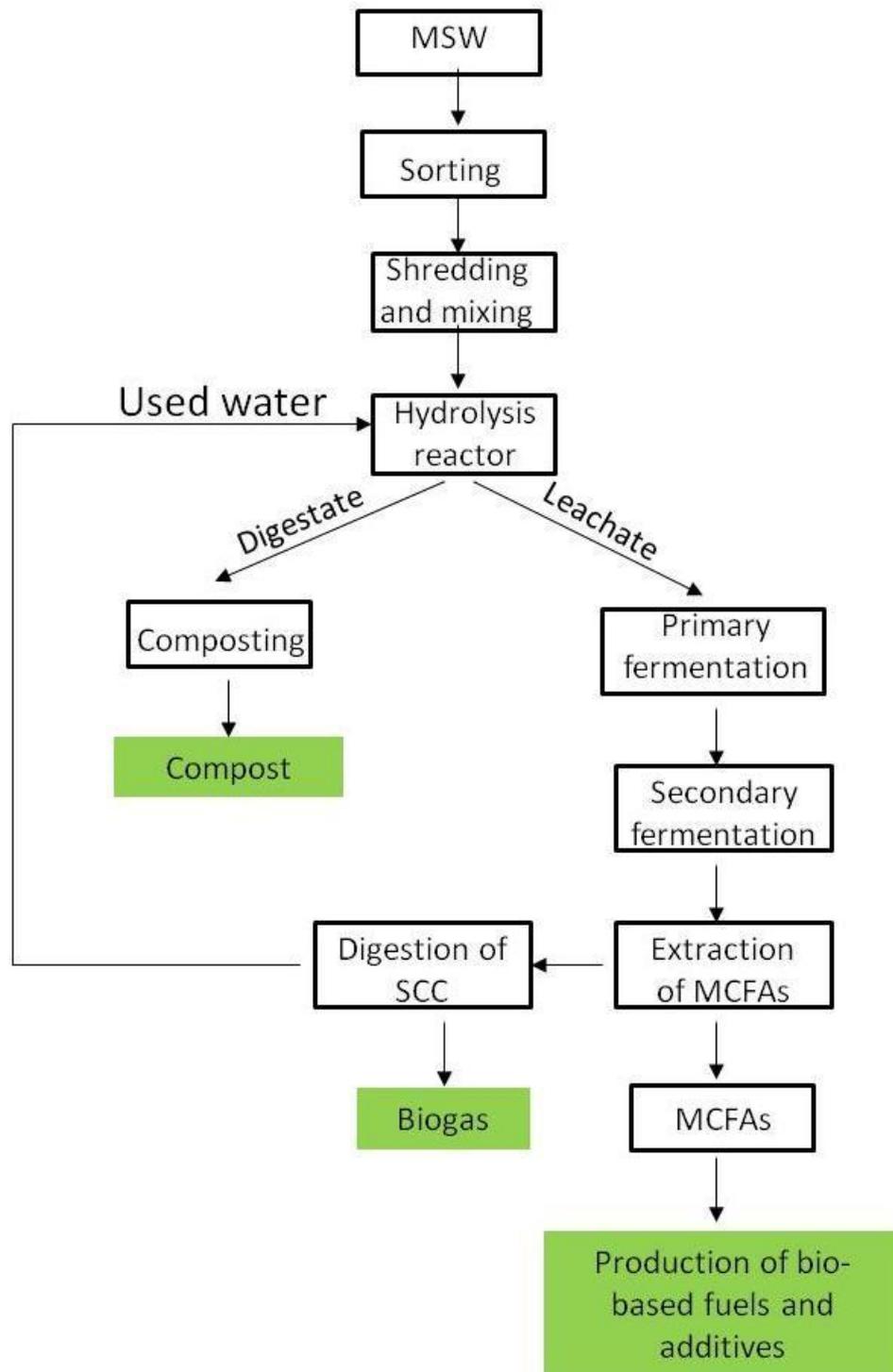


Fig 1.5 Municipal solid waste treatment process (MSW) for maximal recovery of valuable resources. The valuable end products are highlighted in green. SCC and MCFAs stand for short chain carboxylate and medium chain fatty acid respectively.

1.5 Anaerobic production of carboxylates

Carboxylates are dissociated organic acids containing at least one radical COO (carboxyl group). They are key intermediates within anaerobic

digestion processes; they are usually generated as the result of the primary (acidogenesis) and secondary fermentation (acetogenesis) of simple monomers provided from the hydrolysis of complex molecules (protein, cellulose, fat etc.) (Wang *et al.*, 2014).

The carboxylates from primary fermentation are referred to as short-chain carboxylates and include mainly acetate, propionate, lactate and n-butyrate while carboxylate from secondary fermentation are known as medium chain carboxylate or medium chain fatty acids (MCFAs) and include mainly valerate, caproate and caprylate (Agler *et al.*, 2011). Both types of carboxylates have wide applications in the biorefinery industry, where they constitute feedstock for various bio-based products such as biofuel and biochemicals (Wang *et al.*, 2014). They can also be used for nutrient removal and as potential candidates for the fabrication of biodegradable polymers in plastics production (Demirer & Chen, 2005). Several applications may also be found in the food and beverages industry, as well as in the pharmaceutical and synthetic chemistry fields. While short chain carboxylates could constitute valuable resources when separated from fermentation medium, the lack of separation techniques has mostly directed their application towards the production of methane.

Recent studies have envisioned another very promising application for these short chain carboxylates, in which they are used as a precursor for MCFAs through secondary fermentation (Agler *et al.*, 2011; Ding *et al.*, 2010; Steinbusch *et al.*, 2011; Weimer *et al.*, 2015). Because these MCFAs have a wide range of applications and have higher market value than methane, their production from different organic feedstocks has been the subject of significant interest. MCFAs have five to twelve carbon atoms and can be easily separated from fermentation broth because of their low oxygen-to-carbon ratio (Steinbusch *et al.*, 2011). They are usually produced through chain elongation of existing short chain carboxylates during secondary fermentation (Agler *et al.*, 2011; Ding *et al.*, 2010; Steinbusch *et al.*, 2011). Although a wide range of carboxylates might be obtained during secondary fermentation, the yield of each component depends on the operational conditions; for instance, acetate will constitute the main product

of this stage if the hydrogen pressure is low. At high hydrogen pressure, however, acetate will be reduced to either ethanol, butyrate or caproate (Agler *et al.*, 2011). Steinbusch *et al.*'s work (2011) reported a high yield of butyrate, caproate and caprylate using acetate as substrate and H₂ as the electron donor. They also established that the combination of H₂ and ethanol as electron donor could contribute to increasing the yield of caproate and caprylate. In their study, pH 7 appeared to be ideal for the formation of these MCFAs when compared to pH 5.5. In contrast, Ding *et al.* (2010) concluded that caproate generation was hydrogenogenic rather than hydrogenotrophic; they demonstrated that caproic acid could be produced through the secondary fermentation of acetic or butyric acid, both using ethanol as the electron donor. Weimer *et al.* (2015) reported a similar result during the fermentation of cellulosic material. Table 1.1 highlights the production rate and yield for caproate (MCFAs) that have been reported in previous studies. It is clear that high hydrogen partial pressures are required to drive the chain elongation process. However, under this condition, the propionate is likely to accumulate as it can only be oxidised when the hydrogen partial pressure is extremely low (Agler *et al.*, 2011). The accumulation of undissociated propionic acid at low pH has been reported to be toxic for microbial cell during the treatment of industrial waste using pure cultures (Colomban *et al.*, 1993). To alleviate the effect of propionic accumulation on the microbial communities, *in situ* extraction of the propionic acid have been applied; however the polar nature of the propionate makes it difficult to recover from broth (Ozadali *et al.*, 1996). The other option will be to further process the propionic acid (short chain carboxylate) for the methane production as indicated in Fig 1.5.

The optimum pH range for MCFAs production (notably caproic acid) is between 6.5 and 7, which is also optimum for the growth of methanogens; the methanogenic community must therefore be inhibited or removed from the process in order to maximise the yield of the end-products of the secondary fermentation. Several previous studies have used bromoethane sulfonic acid to inhibit the methanogens (Aguilar *et al.*, 1995; Steinbusch *et al.*, 2009; Steinbusch *et al.*, 2011), but this chemical is very

expensive and its uses on a large scale remain out of reach. The two other alternatives for inhibiting the methanogens in the system include heat shock (Ding *et al.*, 2010; Steinbusch *et al.*, 2009) or low pH (~5.5). The former method consists of eliminating all non-spore-forming microorganisms which include methanogens by applying a heat-shock treatment with temperatures ranging from 80–121°C. Not only is this method generally considered to be energy demanding, but it also might eliminate important anaerobic degraders that are non-spore forming; this might occur when the low pH is associated with a high concentration of VFAs. It is also important to point out, however, that under such acidic conditions (~5.5), a vast majority of reactions are thermodynamically unfavourable and microbes are selected (Agler *et al.*, 2011).

Table 1.1 Formation of caproate by the anaerobic fermentation of selected substrates

Substrates	pH	Caproate (g l ⁻¹)	Rate (g l ⁻¹ d ⁻¹)	inoculum	References
Acetate, EtOH, H ₂	7	8.2	0.49	Enriched mixed culture	(Steinbusch et al., 2011)
Acetate, EtOH, H ₂	5.5	0.12	NR ^a	Enriched mixed culture	(Steinbusch et al., 2011)
Cellulosic biomass, EtOH	6.8 ^b	4.9–6.1	1.7–3.1 ^c	Mixed ruminal microflora + <i>Clostridium kluyveri</i> 3231B	(Weimer et al., 2015)
OFMSW ^e , EtOH	UC ^d	2.7	NR	None	(Grootscholten et al., 2013)
EtOH, Acetate	6.8 ^b	12.8	4.2	<i>C. kluyveri</i> 3231B (pure culture)	(Weimer & Stevenson, 2012)
Lactate, Yellow Water	6.0–6.5	23.41	2.97	Mixed culture	(Zhu et al., 2015)

a = Not reported

b = Measured starting pH (overall pH of process was not controlled)

c = Rate reported for active period of caproate production

d = Uncontrolled pH

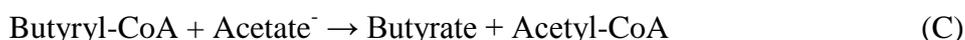
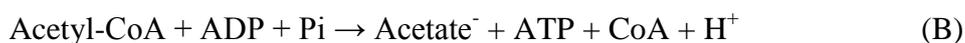
e = Organic fraction of the municipal solid waste

Undefined mixed microbial cultures are reportedly ideal for the conversion of organic feedstock to VFAs using hydrolysis and fermentation, because the different members of the mixed community are able to use diverse metabolic pathways to convert complex molecules into a wide range of products. Several previous studies have successfully produced caproate and butyrate using undefined mixed cultures (Ding *et al.*, 2010; Steinbusch

et al., 2011; Weimer *et al.*, 2015), although these studies also established that caproate was the product of *Clostridium kluyveri*. It is still not clear whether different metabolic routes could be involved in the formation of caproate. Zhu *et al.* (2015) have recently reported on the formation of caproic acids from lactic acids during the synthesis of caproate from lactate. Further investigation is thus still necessary to establish the different metabolic routes involved in the production of MCVFAs.

1.6 Biological chain elongation

Biological chain elongation occurs via the reverse β -oxidation pathway (Angenent *et al.*, 2016). This process which has been up to now mainly described for *Clostridium kluyveri* is a cyclic process involving the addition of a two-carbon acetyl-CoA derived from ethanol to a carboxylate, elongating its carbon chain length with two carbons at the time. As described by Ding *et al.* (2010) and Angenent *et al.* (2016), the formation of butyric and caproic acid by *Clostridium kluyveri* through β -reverse oxidation pathway, start with an oxidation step during which ethanol are oxidised by NAD^+ via acetaldehyde to acetyl-CoA (A), one of which are further converted to acetate by substrate-level phosphorylation for the synthesis of one ATP (B). The second part of the chain elongation process is a reductive part consisting of a series of coupled redox cycles leading in the synthesis of ATP through electron transport phosphorylation. In this reductive part, some of the acetyl-CoA from the oxidation part enters the redox cycle and reacts with additional acetyl-CoA to form butyryl-CoA which subsequently reacts with acetate to form butyrate (C). Similarly, caproate is formed from the reaction between caproyl-CoA and acetate (D).



Although ethanol have been reported has the main electron donor do elongate carboxylates (electron acceptors), other molecules including, methanol, n propanol, amino acids, pyruvate and some simple sugars have also been used as electron donors for chain elongation into butyric or caproic acid (Angenent *et al.*, 2016). However the concentration of the elongated product reported in these studies were low compare to the studies in which ethanol was used as electron donor. Recently, the studies of Kucel *et al.* (2016) and Zhu *et al.* (2015) on the production of caproate from lactate reported chain elongation into caproate with lactate as main electron donor. The concentrations reported in their studies were comparable with studies using ethanol as electron donor. Zhu *et al.* (2015) suggested that n-caproate production from lactate may be similar to the process of ethanol oxidation/reverse β -oxidation; however lactate and not ethanol are firstly oxidised to form acetyl-CoA.

The vast majority of bacteria that are reported to be able to chain elongate are *Firmicute* in the class of Clostridia (Angenent *et al.*, 2016). Beside *Clostridium kluyveri* which is by far the best-studied pure culture bacterium capable of chain elongation (Weimer & Stevenson, 2012), other bacteria including *Eubacterium pyruvativorans* (Wallace *et al.*, 2003), *Clostridium sp. Bs-1* (Jeon *et al.*, 2010), *Megasphaera elsdenii* (Choi *et al.*, 2013; Elsdén *et al.*, 1956) have also been reported to able to chain elongate.

In other to maximise the selectivity and production rate of MCFAs such as Caproate using reactor microbiome, different approaches have been adopted. The first approach consist of aiding fermentation by supplementing external electron donor (ethanol and lactate) and to operate bioreactors at a pH of 7 without in-line product extraction (Steinbusch *et al.*, 2011). At neutral pH, almost all MCFAs are maintained in the non-inhibiting dissociated form and high concentration can be achieved without the need to extract them. This was demonstrated in the study of Grootscholten *et al.* (2013) where 1000-fold increase in the n-caproate production rate and an elevated n-caproate selectivity of 80% were achieved by maintaining a pH close to neutral and shortening the hydraulic residence time to several hours. With this approach of maintaining neutral pH without MCFAs extraction,

the highest volumetric production rates of 2.2 g n-caproate L⁻¹ h⁻¹ to date has been achieved (Grootscholten *et al.*, 2013b). The other approach is the production of MCFAs under slightly acidic condition ~ 5.5 with in line product extraction. At a lower pH ~ 5.5 the acetoclastic methanogenesis is inhibited and the chain elongation is favoured (Angenent *et al.*, 2016). However at this pH the ratio of undissociated/dissociated MCFAs is high compared to pH 7, therefore in-line extraction is required to achieved maximum MCFAs rates (Agler *et al.*, 2012). The extraction methods that have been used to recovered SCCs and MCFAs from fermentation broth include; liquid-liquid extraction, crystallization, ion exchange and electro dialysis (Spirito *et al.*, 2014). The liquid-liquid extraction method favour the extraction of more hydrophobic compounds, such as undissociated n-caproic acid compared to undissociated n-butyric acid (Agler *et al.*, 2012) while crystallization, ion exchange and electro dialysis extract mostly short-chain carboxylates (Forrest *et al.*, 2010; Huang & Ramaswamy, 2013; Zhang *et al.*, 2011).

1.7 Microbial communities in anaerobic reactors and their process stability implications

Anaerobic bioreactors contain highly complex and diverse microbial communities, which greatly contributes to the stability and performance of the systems. A system is perceived as being stable if it possesses the ability to maintain functional stability in response to perturbations. A few parameters related to these communities (including resistance, resilience and variability) have been used as indices of ecosystem stability (Briones & Raskin, 2003; Pimm, 1984). These indices are commonly applied to describing the microbial communities and process components of an ecosystem. A better understanding of these two components and their interaction could contribute to maintaining process stability and predicting how bioreactors can recover from unstable periods. Nonetheless, the vast majority of studies have thus far failed to take the community component into consideration; they usually measure reactors' performance and stability

in terms of process, which is generally perceived as the flow of matter between the different trophic groups in a bioreactor. In such systems, organic carbon reduction, the accumulation of by-products and the quality of effluents are the main indicators of either system failure or success.

Many studies have reported that the accumulation of propionic acid is often responsible for process failure (Marchaim & Krause, 1993; Wang *et al.*, 2009); some studies have found the build-up of VFAs to cause system imbalance (Akuzawa *et al.*, 2011; Murto *et al.*, 2004). Nevertheless, at the moment there is still no common standard to indicate VFA levels that might cause system failure or instability (Angelidaki *et al.*, 1993). It would appear that the cause of system instability differs from one study to the other: a certain level of VFAs might be reported to be responsible for system failure in one reactor, whereas in another reactor the same level does not constitute an issue. Total ammonia nitrogen concentration between 1,700–1,800 mg l⁻¹ has been reported to lead to a complete failure of methanogenesis (Melbinger *et al.*, 1971; Yenigün & Demirel, 2013), whereas Braun *et al.* (1981) and Van Velsen (1979) reported system stability with total ammonia nitrogen concentrations between 3,075–5,000 mg l⁻¹. Because of the aforementioned discrepancy between systems, it seems logical to deduce that processes are driven by microbial communities; investigating their structure and change in species composition therefore might be used to anticipate changes in ecosystem processes more quickly than direct attempts to measure these processes. In AD systems, processes are strongly influenced by the microbial communities and their complexity. Brione *et al.* (2003), Fernandez *et al.* (2000) and Pimm *et al.* (1984) have all reported that a system with diverse microbial communities is prone to maintaining stable processes owing to the ability of the members of the communities to use different metabolic pathways. We must realise, however, that community diversity per se does not drive reactor stability, which also depends on functional redundancy (McCann, 2000). In other words, reactor stability depends on a community's ability to maintain species or functional groups that are capable of differential responses after a perturbation. It is clear that investigating the relation between community diversity and key

functional groups in reactor systems could help to improve process stability and performance.

1.8 Investigating anaerobic digesters' microbial communities

Traditionally, our knowledge about the microbiota of reactors depended on cultivation-based studies. At present, it is widely recognised that cultured microorganisms represent a small portion of the reactors' microbiota due to the reactors' limited growth conditions (Amann *et al.*, 1995). As a result, any information gained from cultivation-based approaches only provides a partial picture of the microbial ecology of a reactor. With the advancement and development of molecular biological tools, cultivation-independent techniques have become suitable methods for gaining knowledge about microbial ecology of anaerobic digesters (O'Flaherty *et al.*, 2006). These techniques have been categorised into two groups: i) partial microbial community approaches based on *16S rRNA* gene analysis and ii) complete microbial community approaches (Rastogi & Sani, 2011). The former approach reveals the bacterial and archaeal diversity structure, while the latter approach reveals the whole microbial diversity structure and functions.

1.8.1 Partial microbial community approach based on *16S rRNA* gene

The *16S rRNA* gene is commonly used as a molecular marker for the identification of bacterial and archaeal communities; the gene is highly conserved but contains nine hypervariable regions (Van de Peer *et al.*, 1996). It was chosen as the marker gene for bacteria and archaea for several reasons: i) its presence in almost all bacteria and archaea, ii) it is large enough (roughly 1,600 bp) to reveal the phylogenetic information needed to distinguish different communities and iii) its function over time has not changed (Janda & Abbott, 2007; Patel, 2001). Because of the widespread utilisation of *16S rRNA* as the marker for phylogenetic studies of microbiomes, public databases containing *16S rRNA* gene sequences have been constructed, including 'greengenes' (<http://greengenes.lbl.gov/>),

SILVA (<http://www.arb-silva.de/>), the Ribosomal Database Project (RDP; <http://rdp.cme.msu.edu/>) and GenBank. The structure of both the hypervariable and the conserved regions of the *16S rRNA* gene allow the development of the polymerase chain reaction (PCR)-based method, where total nucleic acids extracted from reactor samples are used as a template for the characterisation of microorganisms. PCR enables the use of universal and domain-specific primers that are complementary to the conserved regions of the *16S rRNA* gene to amplify the variable regions. Several bacteria- and archaea-specific primers have been designed using sequences retrieved from the aforementioned databases (Chassard *et al.*, 2007; Grosskopf *et al.*, 1998; Hogg & Lehane, 1999). A universal primer that targets the V4 region of the *16S rRNA* gene for both bacteria and archaea was recently designed (Walters *et al.*, 2011) and applied successfully in several studies (Bates *et al.*, 2011; Bergmann *et al.*, 2011; Caporaso *et al.*, 2011b). PCR-based *16S rRNA* gene amplification enables the development of molecular techniques that are capable of revealing bacterial and archaeal community structure and diversity; these techniques can be classified into two major categories: i) low-throughput methods and ii) high-throughput methods, both described below.

1.8.1.1 The low-throughput PCR-based approach

Low-throughput PCR-based methods of investigating bacterial and archaeal diversity structures and the composition of AD systems include mainly the cloning/sequencing approach and various fingerprinting techniques. The cloning/sequencing method has been widely used to determine microbial community composition of anaerobic digesters. Using this approach, several studies have demonstrated that bacterial communities are more diverse than the archaeal community present in AD systems (Collins *et al.*, 2006; Leclerc *et al.*, 2004). On the other hand, fingerprinting methods are commonly used to compare microbial communities' profiles from different systems, or to follow the dynamics of communities from one system over time (Muyzer, 1999; Stahl & Capman, 1994). Among fingerprinting techniques, most studies have employed DGGE (Bialek *et al.*, 2011; Kim *et al.*, 2009; Miura

et al., 2007; Roest *et al.*, 2005; Rosa *et al.*, 2009; Trzcinski *et al.*, 2010; Yan *et al.*, 2014) and T-RFLP (Wang *et al.*, 2010) (Collins *et al.*, 2003; McHugh *et al.*, 2006) to study the microbiota of anaerobic digesters. One common problem when assessing the microbial diversity of complex systems such as anaerobic digesters when using the abovementioned (cloning/sequencing, DGGE and T-RFLP) approaches is the underestimation of community diversity. Nevertheless, despite their limitations, both cloning/sequencing and profiling methods (DGGE and T-RFLP) are commonly used to obtain snapshots of the microbial communities from large numbers of samples in order to identify representative samples to be further analysed by high-throughput sequencing technologies (DeSantis *et al.*, 2007; Neilson *et al.*, 2013).

1.8.1.2 The high-throughput PCR-based approach

Sanger sequencing was traditionally the main technology employed in studying the microbial community (Rastogi & Sani, 2011). One of the biggest limitations of this technique, however, lies in the fact that it is still not possible to generate large quantities of sequences per sequencing run (Caporaso *et al.*, 2011b), and therefore coverage of the diversity of complex environments is poor. In addition, because of the cost and time involved with Sanger sequencing, most researchers generally sequence a low number of clones (100–300), which is believed to capture only the most abundant microbial groups within a system (Rastogi & Sani, 2011). For instance, it has been reported that only 50% coverage of the diversity in soil samples is achieved when sequencing over 40,000 clones (Dunbar *et al.*, 2002); it is therefore likely that very rare microbial groups are not revealed by the use of Sanger sequencing. The rare microbial populations that remain largely unexplored by Sanger sequencing techniques may constitute highly functionally diverse components in almost every environmental system (Lauber *et al.*, 2009), which could be investigated by the use of high-throughput sequencing, also known as next-generation sequencing (NGS), technologies.

The development of high-throughput sequencing technologies makes it possible to almost completely characterise the phylogenetic composition and functional potential of complex communities (Albertsen *et al.*, 2013; Bragg & Tyson, 2014) by employing massive parallel sequencing. NGS technologies can produce millions of sequencing reads in a single instrument run. The two most popular NGS technologies currently in use include 454 pyrosequencing on the FLX Titanium platform (Roche) and Illumina sequencing on the HiSeq and MiSeq platforms (Illumina).

The first commercially available high-throughput sequencing platform was 454 pyrosequencing, introduced in 2004. This platform uses pyrosequencing technology, which relies on the detection of pyrophosphate molecules released during nucleotide incorporation. To date, several studies have applied 454 sequencing on complex microbial communities such as soil (Lauber *et al.*, 2009), activated sludge (Zhang *et al.*, 2012), lakes (Shade *et al.*, 2012) and in anaerobic digesters (Kim *et al.*, 2014; Sundberg *et al.*, 2013). In one molecular investigation, microbial communities of sponges from the Red Sea off the coast of Saudi Arabia were explored by targeting the V5 and V6 hypervariable regions of the *16S rRNA* gene using T-RFLP and barcoded pyrosequencing technique (Lee *et al.*, 2011). Sponge specimens and sea water from four different locations representing a wide range of ecosystems from across the Red Sea were collected and analysed using T-RFLP and pyrosequencing techniques, respectively. A total of 164 T-RFs of different sizes were obtained, and roughly 140,000 high-quality reads with average read lengths of 290–300 bp were generated from the sponge and seawater samples. The result suggested a higher number of bacterial and archaeal phyla when using pyrosequencing (on average 30 phyla) than when using TRFLP (on average 20 phyla) techniques. The authors reported that all of the phyla in the sponges that were not detected by T-RFLP techniques were recorded in very low quantities by the pyrosequencing technique, which indicates that pyrosequencing technology could successfully reveal rare microbial groups in environmental systems where other molecular techniques (such as DGGE, TRFLP and Sanger sequencing) have failed. In Lee *et al.*'s study (2011), although

pyrosequencing was found to be more efficient than T-RFLP at revealing sponges' microbial diversity, the non-asymptotic plot generated by rarefaction analysis of certain samples indicated that true coverage of the microbial community's diversity still had not been achieved. That study demonstrates the need for large-scale sequencing techniques with a high number of reads when investigating the highly diverse microbial communities of environmental systems.

In 2006, Solexa developed the second commercial NGS platform yet developed, known as Genome Analyser (GA), which was later (in 2007) acquired by Illumina (Mardis, 2008). The Illumina platform uses bridge amplification for cluster generation and sequencing by the synthesis approach. Studies that have used Illumina technologies on AD microbial communities have successfully demonstrated the usefulness and power of its applications (Albertsen *et al.*, 2012; Mackelprang *et al.*, 2011). Kim *et al.* (2014) used Illumina HiSeq2000 technology to discover microbial community structure and to develop insights into the dominant functions of microorganisms within full-scale anaerobic digesters treating activated sludge. Li *et al.* (2015) revealed greater microbial richness and evenness in the mesophilic than in the thermophilic solid-state anaerobic reactor treating municipal sludge and food waste by using Illumina MiSeq technology. Compared to 454 pyrosequencing, Illumina sequencing offers the largest output and lowest cost to study the complex microbial communities of diverse ecosystems (Glenn, 2011; Mardis, 2008).

Given the size of the data generated by both 454 pyrosequencing and Illumina sequencing, bioinformatic tools including QIIME (Caporaso *et al.*, 2010), MOTHUR (Schloss *et al.*, 2009), MEGAN (Huson *et al.*, 2011) and MG-RAST (Meyer *et al.*, 2008) have been developed to handle such vast amounts of data. Researchers have processed *16S rRNA* gene-sequencing data via QIIME and MOTHUR, while functional metagenomic sequencing data have been handled by MEGAN and MG-RAST. Before analysing sequencing data through the aforementioned software programmes, however, we should be aware of the possibility of biases generated from the different steps involved in the sequencing process to the data processing and

analysis. These biases can be generated from the DNA preparation steps, including DNA extraction, primer selection and PCR amplification. Both sequencing platforms (Illumina and 454) reportedly produce large amounts of artificial sequences which could be perceived as novel microbial groups (Caporaso *et al.*, 2011b; Niu *et al.*, 2010). Because different programmes and parameters are used to process sequencing data, some biases are also expected at this stage. For instance, the same sequence can be assigned to several OTUs depending on the applied threshold.

1.8.2 Complete microbial community analysis approaches using whole-genome molecular techniques

Although researchers commonly use the *16S rRNA* gene analysis approach to investigate the microbial communities of anaerobic systems, this approach (which completely depends on the PCR amplification of the *16S rRNA* gene) does have some drawbacks: i) PCR-based analysis of *16S rRNA* genes does not provide functional information, ii) the highly conserved nature of the *16S rRNA* gene sequence makes it difficult to differentiate species and strains (Konstantinidis *et al.*, 2006), and iii) PCR primer mismatches may lead to preferential or differential amplification of the *16S rRNA*, which may result in some genotypes being hindered completely (Zhou *et al.*, 2010). All of these challenges have been addressed by the development of techniques that generate important functional information through the analysis of the whole genome. Among these techniques, metagenomics (Chouari *et al.*, 2005), metatranscriptomics (Haroon *et al.*, 2013; Luo *et al.*, 2014) and metaproteomics (Abram *et al.*, 2011; Lu *et al.*, 2014) approaches have commonly been used to investigate the potential functions of microbial consortia in AD systems as well as their implications in different processes. A thorough analysis of the microbial community using whole-genome molecular techniques is beyond the scope of this research but should be taken into consideration for further study.

1.8.3 Statistical analysis within microbial ecology

High-throughput sequencing technologies generate vast amounts of data, which on their own cannot provide answers to fundamental questions such as what lives with what, and why? Researchers have thus developed a set of statistical techniques to facilitate the visualisation and representation of sequencing data and to relate species abundance in the community to environmental conditions. Community ecologists have used diversity indices and multivariate statistical analysis to evaluate the environmental parameters that govern microbial community changes in various ecosystems (Miura *et al.*, 2007; Ritchie *et al.*, 2000).

Species diversity is an important indicator of ecosystem stability and is the primary indicator of community functioning and dynamics in systems. For instance, shifts in the diversity of bacterial communities might be an indicator of potential process failure among bioreactors. Species diversity is measured by taking the concept of alpha, beta and gamma diversity into consideration (Whittaker, 1972). Alpha diversity refers to the richness and evenness of species within a community, while beta diversity measures the ‘nestedness’ and turnover of species between two sites in terms of gain or loss of species; finally, gamma diversity is the overall diversity of a defined region (Moreno & Rodríguez, 2010; Veech & Crist, 2010). Some commonly used indices for describing alpha diversity include the Shannon–Weaver (H'), evenness (E) and Simpson (D) indices (Hughes & Bohannan, 2004). In Gagliano *et al.*'s study (2015) on the temperature-phased anaerobic digestion of sludge, the H' and E diversity indices calculated using fluorescence in situ hybridization (FISH) data demonstrated a lower species richness under thermophilic than mesophilic conditions and a similar evenness in both conditions.

Beta diversity is commonly investigated by using several indices, including Bray-Curtis dissimilarity, the percent similarity index (PSI) and the Jaccard index (Hewson & Fuhrman, 2004; Sinclair *et al.*, 2015). Beta diversity is calculated as the distance between a pair of samples. The Bray-Curtis and Unifrac methods have been used to generate distance matrices

that serve to compare communities between samples. The Bray-Curtis method takes the abundance of species into account, while the Unifrac method generates distance matrices based on phylogenetic trees constructed from the species present in the communities.

Multivariate data analyses are powerful statistical tools which are widely used in microbial ecology studies to help elucidate microbial diversity patterns and the environmental conditions responsible for such patterns (Ramette, 2007). Some of the more common multivariate methods used among microbial ecology studies for the visualisation and exploration of complex data sets include cluster analysis, principal component analysis, multidimensional scaling and nonmetric multidimensional scaling (nMDS). All of these analysis types aim to reduce the amount of data to enable better visualisation and interpretation; all have been applied to studies on microbial communities within AD systems.

Although multivariate analysis is a powerful tool for visualising microbial patterns and for elucidating the correlation between microbial communities and environmental factors, extra caution should be taken when interpreting the results because the synthetic variables, axes or clusters generated during analysis (e.g. PCA, nMDS) do not necessarily have biological meanings (James & McCulloch, 1990). In addition, it is also recommended that statistical significance tests (analysis of similarity and multivariate analysis of variance) should be performed on the observed clusters or groups before drawing any conclusions.

1.9 Thesis scope

Waste stabilisation through the anaerobic digestion process is generally perceived to be one of the most appropriate and sustainable approaches for dealing with the large amount of food waste generated in Europe. Not only does this approach help in addressing the environmental issues caused by the landfill disposal of food waste, but it also allows for the recovery of valuable resources from food waste. The classical example of the resources

that may be recovered from food waste through the AD process is methane production, which most observers consider to be the most attractive feature of AD technology, in the sense that it can be used to produce both heat and electricity, which in turn can serve to operate the waste-treatment facility itself. The excess electricity can be sold to generate profit or put into grid energy storage. However, because of the necessity to reduce the dependence on fossil carbon sources for both chemical and fuel production, a move to a value-cascade model (production of chemical) from feedstock such as FW is gaining more attention. This situation has contributed to the development of alternative processes that generate higher-value end-products than methane. Many researchers in recent years have focussed their studies on the production of carboxylates, using the anaerobic processing of biomass. These carboxylates (namely lactic acids and VFAs) that are produced during the second stage of the AD process have higher economic added value than methane. A certain number of studies in the literature are available on the production of carboxylates from food waste; however, the vast majority of them have mentioned the need for process optimisation to achieved better yield and production rates. For instance, the accumulation of higher levels of carboxylates could be induced if: i) hydrolysis of the different components of the FW is improved, ii) optimum parameters (e.g. pH and temperature) that promote the growth of acidogens are provided, iii) the methanogenic activities are inhibited and the produced carboxylates are continuously extracted from fermentation broth. In addition, fermentation process needs to be steered toward the production of specific carboxylate to facilitate the downstream processing.

This study thus aims to investigate and optimise the hydrolytic-acidogenic phase of the anaerobic processing of FW collected in restaurant facilities. The specific objectives of this study are as follows: i) to promote the accumulation of VFAs by inhibiting the methanogenesis stage and enhancing the hydrolysis of the different components of the FW, ii) to investigate the bacterial groups responsible for hydrolytic-acidogenic processes and iii) to evaluate the feasibility of using FW fermentation liquid

as a platform for the production of medium-chain fatty acids. These objectives are addressed in different chapters of this thesis, as follows.

Chapter 2 investigates the biodegradability extent of restaurant food waste (RFW) and its major groups of components (proteins, fats, cellulose and hemicellulose) by using biomethane potential assay (BMP). Kinetic analysis was carried out to determine the hydrolysis rate of the FW and its components during the assay. The impact of supplementing certain specific proteins, fats, cellulose and hemicellulose degraders (developed from enrichment cultures) on the solubilisation of the FW and its components was also assessed. The microbial communities that underpin the processes inside leach-bed reactors were investigated using 16S rRNA profiling from DNA and cDNA.

Chapter 3 evaluates the feasibility of the anaerobic processing of FW for organic acid production in leach-bed reactors. In order to induce the accumulation of VFAs inside leach-bed reactors, the methanogenic barrier was addressed by promoting rapid acidification. The hydrolysis of the different components of the FW was optimised by promoting the enrichment of hydrolysers inside the digesters. The hydrolysis and acidification efficiency of the FW inside reactors was monitored in terms of soluble chemical oxygen demand (sCOD) removal, ammonia nitrogen accumulation, volatile solid destruction and organic acids (lactic acids and VFAs) accumulation. The microbial communities responsible for the different processes inside digesters were investigated using the 16S rRNA profiling from DNA and cDNA on the Illumina MiSeq platform; R software was used for the statistical analysis of sequencing data.

Chapter 4 investigates various operational parameters, including loading rate, pH, dilution, bioaugmentation and recirculation regimes required for optimum hydrolysis and maximum accumulation of VFA inside leach-bed reactors. The possibility of selectively producing a short-chain VFA (butyric acid) and a medium-chain VFA (mainly caproic acid) from the FW was also addressed in batch experiments using leachate from leach-bed digesters. The pH parameters associated with hydrogen or ethanol supplementation were

the pivotal factors for the accumulation of butyric and caproic acids. Illumina-based 16S rRNA sequencing using cDNA was conducted to identify the functionally active microbial population involved in the production of butyric and caproic acids.

Chapter 5 presents the main conclusions of this research, along with future recommendations.

CHAPTER 2

HYDROLYSIS OF PROTEIN, CARBOHYDRATE, LIPID AND CELLULOSE FRACTIONS DURING ANAEROBIC DIGESTION OF RESTAURANT FOOD WASTE

Abstract

The high energy content of food waste (FW) makes it an attractive feedstock for anaerobic digestion (AD) process. The recovery of this energy is usually, however, considered challenging for many reasons including variability of its composition, high total solid content, low rate of hydrolysis, type of inoculum and process failure. All these usually tend to negatively impact the yield of the end product and thereby making the overall process less attractive. Therefore, investigation and optimisation of these parameters can be exploited for the development and implementation of robust and efficient process at large scale. In this study, batch degradation of restaurant food waste (RFW) and its major group of components (protein, fat, cellulose and hemicellulose) was investigated by using biomethane potential assays (BMP). Process improvement through bioaugmentation using enriched cultures, adapted to the different component of the RFW, was also addressed. After 61 days of incubation, the overall methane (CH₄) yield of the RFW was 731±7.70 ml CH₄ g⁻¹ volatile solid, 91% of which was obtained after 14 days. A high volatile solid destruction (VS) of 93±0.34% was also recorded at the end of the 61 day period, with 80% achieved within the first 14 days. The hydrolysis rate constant (K_h) of RFW was determined to be 0.35±0.00 d⁻¹ after one day and 0.11±0.01 d⁻¹ after 14 days of incubation. Taken together, these results highlighted the ready biodegradability of RFW and the presence of two fractions, the first easily hydrolysable and the second more recalcitrant. RFW component analysis revealed that hemicellulose appeared to be easily biodegradable, while some of the fat was recalcitrant. The destruction of both protein and cellulose fraction were believed to be affected by their bioavailability. Despite the fact that high removal efficiency was achieved for each major component of the RFW at the end of the batch trial, their degradation rates varied considerably. A VS destruction improvement in the range of 10 – 15% was achieved when the initial inoculum (granular sludge) was supplemented with a series of enriched cultures adapted on skimmed milk, xylan, whatman filter paper, oleic and palmitic acid, respectively. High-throughput

sequencing of *16s rRNA* genes carried out on DNA extracted from cellulose and xylan enriched cultures revealed that *Bacteroides graminisolvens* and species affiliated with *Porphyromonadaceae* were likely responsible for the improvement of hydrolysis rate of cellulose and hemicellulose respectively. This study indicates that, firstly, food waste degradation is influenced by substrate composition; secondly, inoculum development based on enriched cultures supplementation could contribute to improve overall biodegradation rates and therefore reduce solid retention time in anaerobic digestion process of waste with high solid content.

2.1 Introduction

Landfill disposal method of biodegradable waste is changing progressively as the result of the implementation of waste management strategies (Department of Environment Community and Local Government(DECLG), 2006) which aim to comply with regulations and landfill directives. Food waste represents one of the largest portions of biodegradable waste stream previously sent to landfill which now required to be dealt with by other means. Regulation on the source segregation of FW has constituted a booster for the development of alternative treatment methods to landfill (Waste Management (Food Waste) Regulations, 2009). Due to a relative high moisture (between 70% - 90%) and high energy content of FW, anaerobic digestion (AD) represents one of the most promising and viable method for its treatment (Zhang *et al.*, 2007).

AD is a microbiological process which contributes to the stabilisation of waste while at the same time generating source of energy (methane), which can be used as electricity, heat and/or fuel (Lastella *et al.*, 2002). The energy content of food waste is largely influenced by the concentration of its major group of components including carbohydrates, lipids and proteins (Neves *et al.*, 2008). Even though lipids have higher energy content than carbohydrates and proteins, they have been reported to be difficult to breakdown in AD bioreactors and have been found to destabilise digesters when present at high concentration (Fernandez *et al.*, 2005; Girault *et al.*, 2012; Neves *et al.*, 2008). Similarly, some complex carbohydrates (lignocelluloses) and proteins have been found to be rate limiting substrates in anaerobic digestion (Labatut *et al.*, 2011; Neves *et al.*, 2008). The efficiency of AD process depends on feedstock characteristics and also on the interaction of microbial communities with different components of the substrate under favourable conditions. Details on the composition of the FW along with some experimental tools can be exploited to investigate the suitability of a substrate (in term of biodegradability and methane content) and the operational parameters for the implementation of

AD processes. Among the available experimental tools, biomethane potential (BMP) have been mostly applied (Esposito *et al.*, 2012).

BMP is a simple assay used to determine the methane potential of feedstock as well as its biodegradability. BMP assays are also very useful for the determination of the residence time required to achieve maximum degradation (Elbeshbishy *et al.*, 2012). BMP assays are usually conducted in batch mode over short period (Labatut *et al.*, 2011). During BMP assay, the origin of inoculum is perceived as the most important parameter since it brings information on the substrate adaptation, the initial activity of the microbial population and diversity of the microorganisms. The impact of the source and adaptation of the inoculum were investigated previously in the study of Elbeshbishy *et al.* (2012). They established that higher methane production rates and biodegradability efficiency were achieved from BMP of FW when using inoculum from an anaerobic digester treating municipal wastewater sludge compared to inoculum from anaerobic digester treating food waste. They inferred that the high initial soluble chemical oxygen demand (sCOD) in the FW adapted inoculum could have favoured the growth of fermenters over hydrolysers and thereby negatively impact the biodegradability efficiency. Pre-incubation of the inoculum in a blank assay (no substrate added) prior to BMP was also found to speed up methane production (Elbeshbishy *et al.*, 2012). Substrate adaptation to inoculum prior AD could contribute to significantly reduce the length of the process start-up phase. The use of undefined mixed culture in AD process is usually appropriate because of the flexibility of microorganisms to grow on complex and variable substrates (Agler *et al.*, 2011; Elbeshbishy *et al.*, 2012). Specific methanogenic activity (SMA) can also be employed to investigate the efficiency of anaerobic sludge to generate methane from fermentation end-products (Colleran *et al.*, 1992). SMA test can also be used to determine the potential loading rate of anaerobic digestion system (Hussain & Dubey, 2014).

Data on the degradability extent of the food waste with its major components, and methanogenesis rates can determine if pH decreases, accumulation of intermediate compounds and other reactor disturbances will

occur when implementing the process. Although many researchers have used BMP as a tool to predict the methane yield and biodegradability extent of food waste (Cho *et al.*, 1995; Elbeshbishy *et al.*, 2012; Neves *et al.*, 2004), very few of them have investigated the contribution of the different fraction of the food waste to the overall degradability. Given the fact that the energy content and degradation of a feedstock are related to its composition, data on the degradability of the major fraction of the food waste can be used to determine if a component will accumulate in AD process under certain retention time and if biodegradation of each fraction need further optimisation.

The objectives of this study were to establish the extent of biodegradability; and the rates of hydrolysis and methane production from food waste, with a view towards the implementation of semi-continuous laboratory-scale AD process. Supplementation of enriched cultures to improve the biodegradation rates of the major food waste components was also assessed. Finally, the microbial community structure of the enriched cultures developed was also characterised using 16S rRNA deep sequencing.

2.2 Materials and Methods

2.2.1 Restaurant food waste

The restaurant food waste (RFW) was provided by a waste collection company (Mr Binman based in Limerick) straight after a fresh collection. Bones, shell fish and other non-degradable materials were manually removed from the RFW, the particle size of the remaining fraction was reduced considerably and homogenised using a blender. The RFW was then stored at -20°C in sterile bags containing each 5 kg. RFW was defrosted slowly in the fridge prior to use.

2.2.2 Characterisation of the restaurant food waste and digestate

The lipid fractions of the RFW and digestate were determined using the methanol-chloroform method previously described by Folch *et al.* (1957). Hemicellulose and cellulose fractions were determined using a combination of dilute and strong acid hydrolysis at high temperature. Briefly, 4 ml of 6 M sulphuric acid was added to 100 mg of dried RFW or digestate. The mixture was incubated at room temperature (RT) for 30 min followed by the addition of 17.5 ml of water and autoclaving at 121°C (15 min holding). The tubes were centrifuged at $6000 \times g$ for 15 min and supernatants containing hydrolysed sugar derived from hemicellulose were stored at room temperature (RT). The pellet was dried overnight at 60°C before concentrated sulphuric acid (18 M) was added. The tubes were incubated at RT for 30 min followed by addition of 18 ml water and autoclaving at 121°C (with 15 min holding). The tubes were centrifuged and the resulting supernatant containing glucose released from cellulose hydrolysis stored at RT. The resulting sugar solutions were analysed using the phenol-sulfuric method by Dubois, *et al.* (1958) .

Dried RFW and digestate were sent to an external laboratory (Teagasc Food Research Centre, Ashtown, Dublin 15, Ireland) for protein quantification on a LECO FP328 (LECO Corp., MI, USA) Protein Analyser based on the Dumas method and according to AOAC method 992.15, 1990. On the LECO platform, total protein quantification is determined using nitrogen analysis. The sample is combusted with oxygen and the nitrogen released is then converted to protein using a conversion factor.

2.2.3 Inoculum

Granular sludge from a full-scale internal circulation anaerobic digester located at Carbery Milk Products (Ballineen, Co Cork, Ireland) was used as inoculum. The total solid (TS) and volatile solid (VS) of the inoculum were $9.01 \pm 0.09\%$ and $7.85 \pm 0.04\%$ respectively. The production of methane due to the residual substrate present in the inoculum was recorded in negative controls where the inoculum was incubated without any substrate. The

values obtained from this blank assay were used to correct the cumulative methane production values in all the assays.

2.2.4 Specific methanogenic activity

To evaluate the ability of the sludge to digest the soluble intermediate compounds, which could be produced in the reactors, SMA profiling on non-gaseous (acetate, ethanol, propionate, butyrate) and gaseous substrate (H_2/CO_2) was performed (Colleran *et al.*, 1992). The test was carried out in triplicate and involved monitoring of the pressure increase in sealed vials fed with non-gaseous substrates or pressure decrease in vials previously pressurised with gaseous substrates (H_2/CO_2) using a transducer. Anaerobic basal medium composed of 4×10^{-4} g l⁻¹ cysteine-HCl, rezasurin, and 3.05 g l⁻¹ NaHCO₃ was used. The pH of the medium was adjusted to 7.0-7.2 using 8 M NaOH. First, the solution was boiled to make it oxygen free and once the pink colour of rezasurin disappeared, indicating that suitable anaerobic conditions were achieved, it was left to cool down under a continuous flow of N₂ in the headspace. Then, bicarbonate was added, the gas was changed to a mixture of N₂/CO₂ before dispensing the anaerobic medium into vials containing 0.35 g of granular sludge. Each substrate was then added to the vials to a final concentration of 12.5 mM. For gaseous substrate, H_2/CO_2 was injected at 1 bar of pressure for 20 second except for the blank where N₂/CO₂ was used instead. All the vials were incubated at 37°C under agitating conditions. The volume of methane produced was corrected to the standard temperature and pressure (STP) conditions.

2.2.5 Biomethane potential Assay

Batch AD trial was performed over a 61 day period using 500 ml bottles. Each bottle was fed with 10 g VS of RFW per litre. A substrate to inoculum ratio of 1:2 on a VS basis was applied. After flushing the headspace with O₂-free gas, the batch reactors were incubated at 37°C on a shaker. Gas bags were used to collect the biogas produced, and biogas volume was determined using the water displacement method while methane content was analysed using gas chromatography. Bottles were sacrificed in triplicate

at regular intervals (days 0, 1, 2, 4, 7, 14, 28, 61) and their contents were analysed for TS/VS, VSS and sCOD (soluble chemical oxygen demand) to determine the extent of hydrolysis and to monitor the accumulation of intermediate compounds. The solid degradation and hydrolysis rate for the major group of components (protein, Lipid, Hemicellulose and cellulose) of the RFW were also determined throughout the assay as described in section 2.2.2.

2.2.6 Enrichment culture assays

Enrichment cultures were performed using the following selected substrates: whatman filter paper 1 (WP), skimmed milk (SM), xylan, oleate and palmitate as the only carbon source respectively. Briefly, granular sludge was used to inoculate 19 ml of sterile anaerobic mineral medium (Angelidaki & Sanders, 2004) to which sterile stock of substrate was provided to a final concentration of 3 g l⁻¹ except for oleate and palmitate provided at 1 mM . All cultures were incubated at 37°C and agitated. Substrates degradation was indirectly monitored by measuring the biogas accumulated in the headspace of the bottles. Distinct enrichment series were obtained by successive transfers of active cultures (10%) into fresh medium containing the relevant substrate.

2.2.7 Substrate utilisation assays using enriched culture

The ability of each enriched culture to metabolise the substrate more rapidly than the initial granular sludge was investigated by following the same procedure as the enriched culture assays (section 2.2.6). This time, sacrificial vials were set up for the direct monitoring of substrate degradation. The protein concentration of the skimmed milk powder was determined by using the RC DC™ Protein Assay kit (BIO –RAD) which is based on the Lowry protocol. Degradation of WP, xylan, palmitic and oleic acid were established gravimetrically through the reduction of their total solid.

2.2.8 Bioaugmentation assays

In order to improve the degradation rates of the RFW major components, a BMP assay was carried out over a period of 7 days using a combination of granular sludge and enriched cultures as inoculum in the ratio of 1:2 (RFW/inoculum). A 5 ml volume of each enriched culture was centrifuged and the resulting pellet (less than 0.1 g) was combined with the granular sludge and the protocol described in section 2.2.5 was followed. A control assay was also performed in parallel, and this time, only granular sludge was used as inoculum.

2.2.9 DNA extraction from enriched cultures and granular sludge

Aliquots of 20 ml from the 18th generation of whatman filter paper 1 (WP) and xylan enriched cultures were pelleted by centrifugation ($8,000 \times g$, 15 min), immediately flash frozen into liquid nitrogen and stored at -80°C until further use. Only these two enriched cultures were selected for DNA extraction since they both led to substrate degradation rate improvements. Total genomic DNA was extracted from the microbial cultures and from 0.1 g of granular sludge using PowerSoil® DNA Isolation Kit DNA (Mo Bio Laboratories Inc) according to the manufacturer's instructions. Ethanol - acetate precipitation was used to purify genomic DNA which was then directly sent to an external laboratory (Research and testing laboratory, Texas US) for *16S rRNA* gene amplification and amplicon sequencing using MiSeq Illumina platform. The primer pair 515F and 806R was used to amplify the V4 region of the *16S rRNA* gene of both bacteria and archaea (Caporaso et al., 2011a)

2.2.10 Analytical method

TS/VS analysis was performed gravimetrically according to standard methods (APHA, 2005). Chemical oxygen demand (COD) analysis was carried out following the Standing Committee of Analysts recommendations (Standing Committee of analysts, 1985). Biogas composition was analysed using a gas chromatograph (Varian) equipped with a glass column and a

flame ionisation detector. The carrier gas was nitrogen and the flow rate was 25 ml min⁻¹. Volatile fatty acid (VFA) was quantified in a Varian Saturn 2000 GC equipped with CombiPal autosampler and using a flame ionisation detector. Separation was carried out on a BP 21, FFAP Capillary column (SGE analytical science) of 30 m length, 0.25 mm internal diameter and 0.25 µm film. Helium was used as the carrier gas at a flow of 1 ml min⁻¹. The GC oven temperature was programmed as follows: 60°C (10s) to 110°C (20s) at rate of 30°C min⁻¹; from 110°C to 200°C (2min) at a rate of 10°C min⁻¹. The temperatures of injector and detector were 250°C and 300°C, respectively. The volume injected was 1 µl. VFA quantification was achieved by using calibration curves of standard VFAs. Prior to GC analysis, the samples were prepared by adding orthophosphoric acid to a final concentration of 5% and centrifuging in a 2 ml safe-lock microcentrifuge tube in a fixed angle rotor at 18,000 × g for 10 min (Eppendorf 5415 D).

2.3 Results

2.3.1 Maximum biodegradation and methane production from restaurant food waste is achieved after 14 days of digestion

RFW biodegradability and methane content were established by performing BMP assays over a period of 61 days. The concentration of this RFW was found to be 1.45 ± 0.16 g COD g⁻¹ VS. The ratio of VS/TS for this RFW was 0.92 (Table 2.1), indicating that almost all its fractions were organic and therefore biodegradable while only 7% was inorganic. Hemicellulose and fat were the highest fraction of the RFW followed by protein and cellulose (Table 2.1).

Table 2.1 Characteristic of restaurant food waste

	TS	VS	VS/TS	Fat	Protein	Hemicellulose	Cellulose
Food waste (%)	28.19±2.34	25.96±2.08	92.09	27.49±1.45	20.69±1.17	32.58±4.48	2.82±0.95

All values are the mean of triplicates (standard deviation; n = 3)

At the end of the trial, on Day 61, the methane yield of the RFW was 731±7.70 ml CH₄ g⁻¹ VS and the VS destruction reached 93±0.34% (Fig. 2.1

A). However, most of the methane produced (91%) and VS destruction (80%) were obtained within the first 14 days of incubation. The methane content of the biogas varies between 70 - 80%. The COD Converted to CH₄ was found to be 97.74%, calculated by converting the CH₄ produced during incubation (1703 ml CH₄) to COD (0.4 m³ CH₄ produced equals to 1 kg COD removed), and dividing by initial COD (4.36 g COD) of the RFW added. This gave an estimate of the amount of organic matter that was converted to CH₄ during digestion

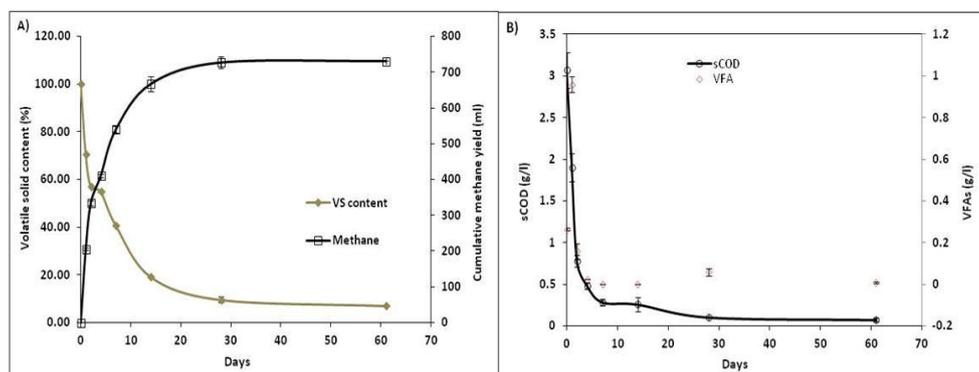


Fig 2.1 Cumulative methane yield and VS degradation of RFW (A); soluble COD and VFA profiles (B) obtained during 61 days of biodegradation assay. Value at each point is the mean of triplicates except for VFAs graph which values are average of duplicates measurements.

An initial sCOD concentration of 3 g l⁻¹ was observed at the beginning of the trial, from which more than 75% was converted to biogas after 2 days of incubation (Fig 2.1 B). This correlated with the high efficiency of the methanogenic community to produce methane from soluble intermediates as highlighted by SMA tests (Table 2.2). Similarly a rapid increase of VFA from 0.26 g l⁻¹ to 0.96 g l⁻¹ during the initial day of the assay (Fig 2.1 B) was observed. This was partly attributed to the fast fermentation of pre-existing simple monomers from the RFW by the microbial communities.

Table 2.2 Specific methanogenic activities (ml of CH₄ (STP) g⁻¹ VSS d⁻¹) of the granular sludge

Substrates	Propionate	Butyrate	Ethanol	Acetate	H ₂ /CO ₂
Methane production rate (ml of CH ₄ (STP)/g VS of waste per day	77.27 ± 6.89	100.06 ± 19.47	114.23 ± 24.79	66.90 ± 24.56	270.94 ± 9.53

All values are the mean of triplicates (standard deviation; n = 3)

Hemicellulose as the largest fraction of the RFW (Table 2.1) was found to be the most biodegradable component with the highest removal efficiency

of 99.08% of which more than 60% was achieved after 1 day of incubation (Fig 2.2). The lowest destruction efficiency of 83.62% was attributed to fat, the second largest fraction of the RFW with 50% degraded within the first day of incubation. Although almost all the protein (96.39%) and cellulose (98.39%) fractions were destroyed at the end of the assay, less than 20% and 10% were destroyed respectively during the first day.

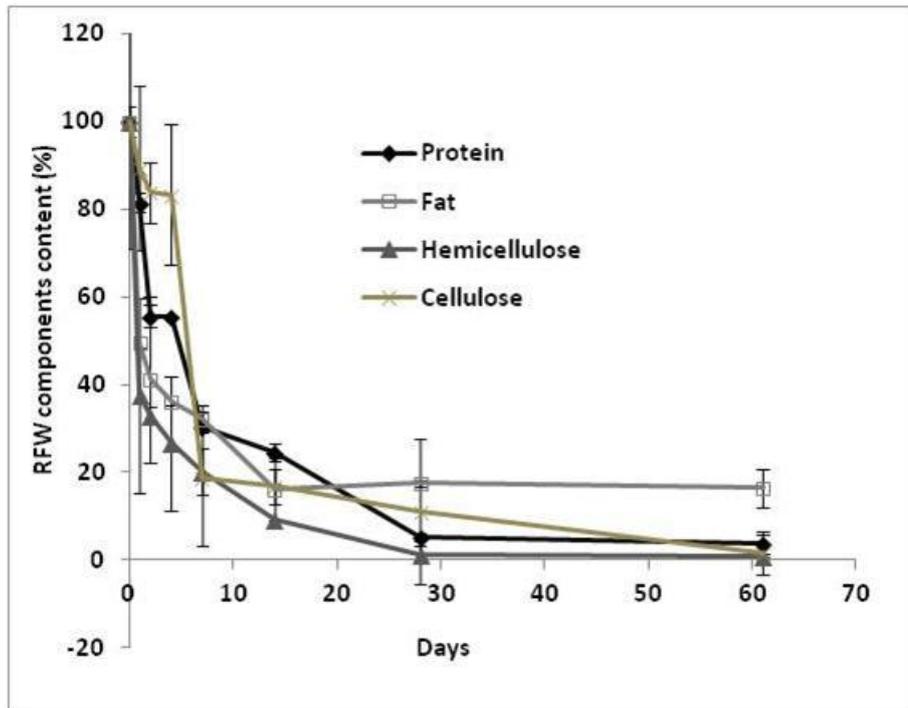


Fig 2.2 Degradation profile of major components of RFW (protein, fat, hemicellulose and cellulose) obtained during the 61 days biodegradation assay. Value at each point is the mean of triplicates measurements.

2.3.2 Hydrolysis rate constants indicate the presence of two fractions within the restaurant food waste, one easily biodegradable and the other recalcitrant

The hydrolysis rate constant of the RFW and its components during biodegradation assay were determined by following the protocol described by Jash & Ghost (1996) and Jiang *et al.* (2005). They established that the solubilisation rate of complex organic matters follows the first order kinetics and is defined as indicated by the following equations:

$$-dS/dt = K_h S \quad (1)$$

$$\ln(S/S_0) = -K_h t \quad (2)$$

Where S_0 and S are concentrations of organic material at time zero and time t respectively. K_h is the hydrolysis rate constant and can be obtained as the slope of the linear curve of $\ln(S/S_0)$ vs t .

Applying the above relationships, the hydrolysis rate constant (K_h) for the RFW biodegradation were found to be $0.35 \pm 0.00 \text{ d}^{-1}$, $0.28 \pm 0.15 \text{ d}^{-1}$, $0.11 \pm 0.03 \text{ d}^{-1}$ and $0.11 \pm 0.01 \text{ d}^{-1}$ on day 1, 2, 7 and 14 respectively. The components of the RFW with high hydrolysis rate after two days of degradation were found to be hemicellulose and fat while the protein fraction and particularly cellulose had a low initial hydrolysis rate (Table 2.3).

Table 2.3 First order hydrolysis rate constants (k_h) of major components of the RFW obtained during BMP assay

RFW components	2 days	7 days
	$K_h (\text{d}^{-1})$	$K_h (\text{d}^{-1})$
Hemicellulose	0.56 ± 0.24	0.18 ± 0.07
Fat	0.44 ± 0.15	0.13 ± 0.06
Protein	0.29 ± 0.05	0.16 ± 0.03
Cellulose	0.09 ± 0.01	0.23 ± 0.07

2.3.3 Inoculum development through enrichment culture processes successfully selected for cellulose and hemicellulose degraders

Enrichment culture assays were performed in order to develop an inoculum capable of breaking down the different components of the RFW faster. Single substrate including whatman filter paper 1 (cellulose source), xylan (hemicellulose source), skimmed milk (protein source), oleate and palmitate (fat source) were used as the only carbon source for each enrichment incubation. A total of 18 generations of whatman filter paper 1 (WP), skimmed milk (SM) and xylan degraders were obtained. The 18th generations from these were used as inoculum for substrate utilisation assays, which were directly compared to that of the initial inoculum. Due to the slow growing nature of fat (oleic and palmitic acid) degraders, only 4 generations were obtained and the 4th generation was used for fat degradation assay. The degradation rate of the skimmed milk, whatman paper and xylan improved when enriched cultures were used as inoculum (Fig 2.3). Similarly, the k_h for WP (0.33 d^{-1}) and xylan (0.14 d^{-1}) were found to have improved when using enriched cultures as compared to the

granular sludge for which K_h for WP and xylan were 0.13 d^{-1} and 0.07 d^{-1} respectively. The K_h for skimmed milk (0.67 d^{-1} and 0.74 d^{-1}) did not show any significant improvement likely due to the previous adaptation of the initial inoculum (granular sludge from dairy waste water facility) to similar substrate. As rates of degradation of oleate and palmitate were very slow, hydrolysis constants were found to be close to zero and were not reported.

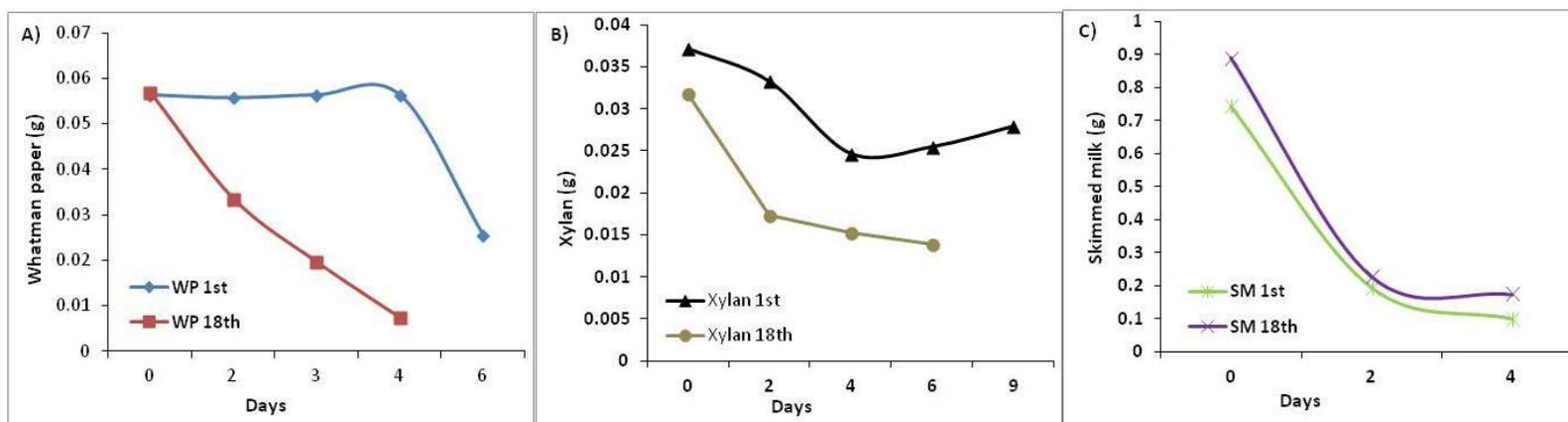


Fig 2.3 Whatman filter paper 1 (WP) (A), Xylan (B) and skimmed milk (SM) (C), degradation using granular sludge and enriched culture as inoculum. 1st and 18th indicate the use of first (granular sludge) and eighteen (enriched culture obtained after 18 sub-culturing) generation of inoculum for the biodegradation of each pure substrate.

2.3.4 Enriched culture supplementation increases the biodegradation rate of restaurant food waste

Two batch degradation assays of the RFW were carried out in parallel: one using granular sludge as inoculum (control) and the other, a combination a granular sludge and enriched cultures (WP, xylan, SM, oleate and palmitate degraders). The addition of enriched cultures resulted in VS destruction improvement; while an overall VS destruction of 35% was achieved in the control assay the enriched cultures supplementation resulted in 50% VS destruction after 2 days of incubation (Fig 2.4). The fraction of the RFW which contributed to the rapid VS destruction observed in the enrichment culture assay appeared to be hemicellulose and cellulose as indicated by their high hydrolysis rate constants of 0.30 d^{-1} and 0.28 d^{-1} respectively (Fig 2.5). The addition of enriched cultures to the inoculum contributed to improve the hydrolysis rate of hemicellulose and cellulose fraction of the RFW by 40% and 6.5% respectively. As expected the hydrolysis constants for protein and fat did not change with the supplementation of enriched culture and were comparable with the control tests in which only granular sludge was used (Fig 2.5).

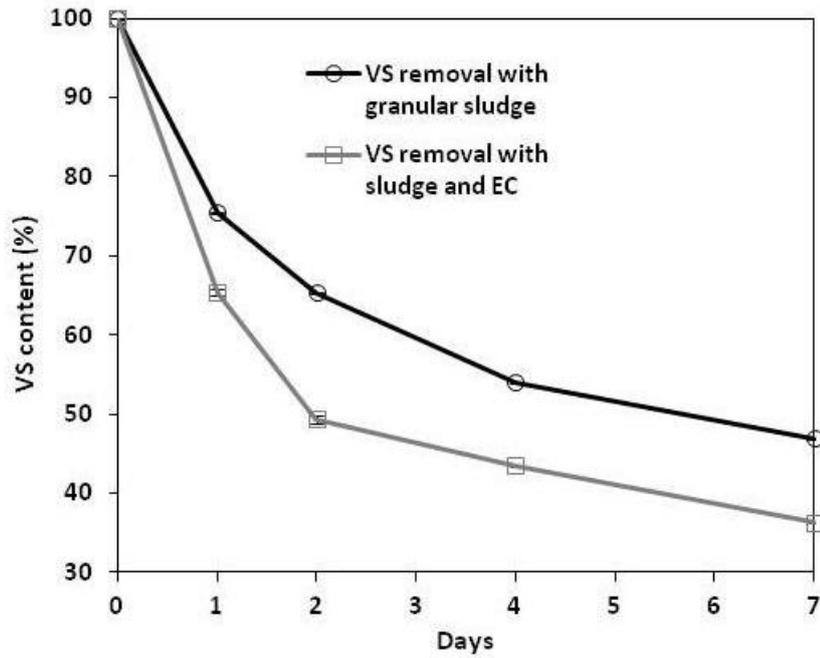


Fig 2.4 Profile of the VS destruction from RFW using granular sludge with and without the addition of enriched cultures (EC); value at each point is the mean of triplicates measurements.

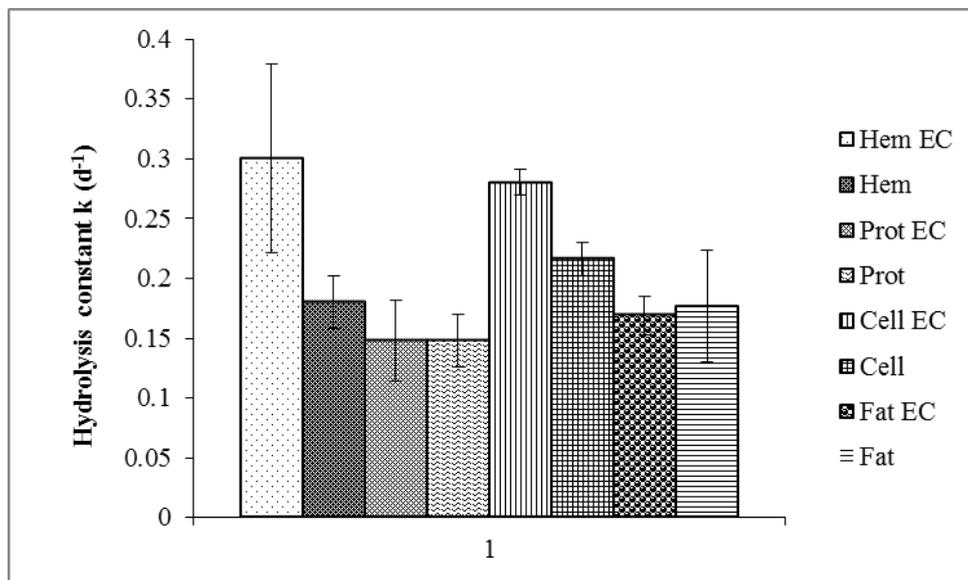


Fig 2.5 First order hydrolysis constant for major component of the RFW established during biodegradation assay with granular sludge in the presence and absence of enriched cultures (EC) Hem, prot, cell correspond to hemicellulose, protein and cellulose, respectively. Data at each point is the mean of triplicate measurements.

2.3.5 Cellulose and hemicellulose enrichments are dominated by species affiliated with *Bacteroides* and *Porphyromonadaceae* respectively

The microbial community composition from the seed sludge and the 18th generation of xylan (hemicellulose source) and WP (cellulose source) enriched cultures were established through *16S rRNA* gene sequencing on Illumina MiSeq platform. While a large number of phlotypes (114) were detected in the seed sludge only 25 and 15 phlotypes were found in the cellulose and hemicellulose enriched cultures respectively, indicating the development of specialised microbial communities (Fig 2.6). The dominating microorganism in the cellulose enriched culture accounting for 73.76% of the community was found to be affiliated with the genus *Bacteroides*. Conversely, 80% of the phlotypes in the hemicellulose enriched culture were associated to the family *Porphyromonadaceae*. Both *Bacteroides* and *Porphyromonadaceae* belonged to the order *Bacteriodales*. *Clostridium* was the second largest community in both enriched cultures with relative abundance of 8.22% for cellulose and 14.51% for hemicellulose. Up to 71.24% of *Bacteroides* were identified as *Bacteroides graminisolvens* while none of the *Porphyromonadaceae* could be classified at the genus level. The relative abundance of archaea was high in the seed sludge compared to the two enriched cultures. *Methanobacterium* was the only archaea identified in the cellulose enriched cultures with a relative abundance of 7.61% while less than 1% methanogenic community was found in the hemicellulose enriched culture.

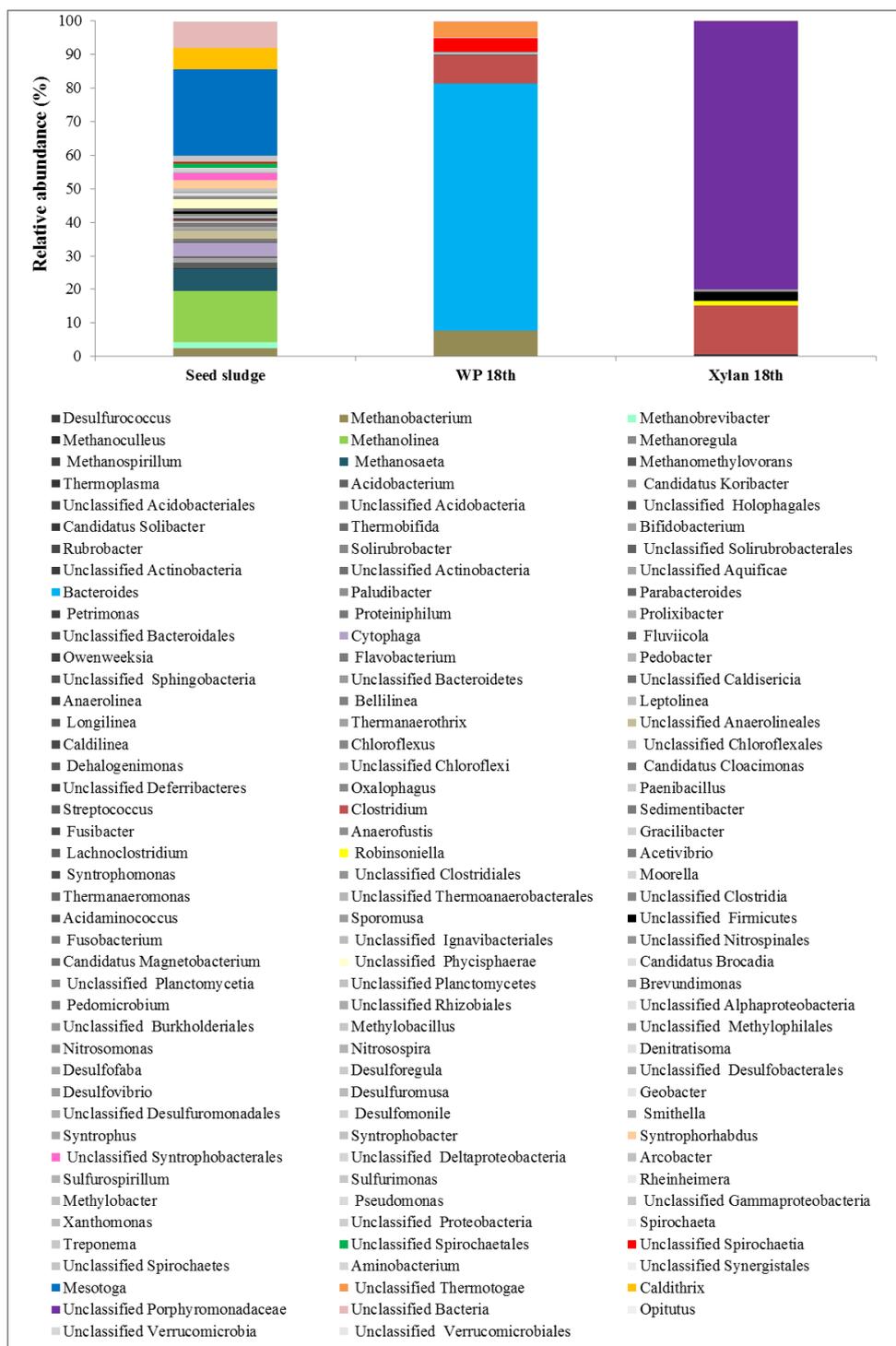


Fig 2.6 Microbial communities profiling from the granular seed sludge, WP and xylan enriched cultures (18th generation) assigned from the *16S rRNA* gene sequencing from DNA samples. WP = whatman filter paper 1.

2.4 Discussion

This study clearly demonstrated that the rate of anaerobic biodegradation of RFW, under batch conditions, depends on the major group of components of the waste (protein, cellulose, hemicellulose and fat) and could be greatly improved if the right pool of microorganisms for degrading specific components within the waste is provided.

2.4.1 Biodegradation rate and methane yield of RFW are influenced by the proportion of the major group of components

The overall VS destruction of 93% obtained during batch degradation of RFW was found to be in the range of previous reported VS destruction from FW. For instance, a VS destruction of 90% was reported for Korean FW (Cho *et al.*, 1995), while Neves *et al.* (2008) established a VS destruction of 94 – 99.6% for batch degradation of RFW at mesophilic temperatures. The values of the methane yield obtained in the present study (667.17±21.60 after 14 days and 731±7.70 ml after 61 days), however, were rather high compared to 400 ml CH₄ g⁻¹ VS reported for FW with excess carbohydrates (Neves *et al.*, 2008) and 472 ml CH₄ g⁻¹ VS added for mixed FW (Cho *et al.*, 1995). Even higher methane production yield of 1400 ml CH₄ g⁻¹ VS corresponding to 87% VS degradation was reported by Elbeshbishy *et al.* (2012) for batch degradation of FW. Herein, the overall VS removed were converted to biogas as indicated by the low concentration of sCOD and VFA towards the end of the incubation period. Similarly, in the study of Neves *et al.* (2008) all the acidified COD corresponding to 94 -99% VS destruction (from RFW) were converted to methane. Taken together, these results indicate that overall methane production from FW is mostly influenced by the feedstock composition.

An overall high VS destruction of 90% was achieved after 28 days of incubation at 37°C. However the different fractions of the RFW were removed at different rates. The destruction efficiency for hemicellulose, cellulose, protein and fat were 99.08%, 98.39%, 96.39% and 83.62% respectively. This indicated the readily degradability of hemicellulose likely

attributed to its bioavailability (highest fraction of RFW) and the presence in the inoculum of microorganisms able to degrade this substrate. A small portion of the Fat (16.38%), however, was found to be recalcitrant, while protein and cellulose degradation was likely limited by their availability as they constituted the smallest fraction of the RFW. The study of Neves *et al.* (2008), which investigated the impact of excess of the different fractions of RFW on BMP, demonstrated that a waste with high fat content was likely to have low rate of hydrolysis and slow methane production. The reverse was observed for waste with excess of protein and carbohydrate fractions (Neves *et al.*, 2008). Labatut *et al.* (2011), however, reported that lipid rich substrates were easily degradable and that their hydrolysis was likely prone to inhibition due to the accumulation of long chain fatty acid (LCFA) during batch degradation of used vegetable oil. It has been suggested that the lipase enzymes, responsible for lipid hydrolysis, require an interface to be activated (Marangoni, 1994; Reis *et al.*, 2009; Rietsch *et al.*, 1977). Poor desorption of long chain fatty acid at the interface has been reported to inhibit lipase activity (Rietsch *et al.*, 1977). Therefore an initial fast hydrolysis could lead to the production of LCFA, which if slowly converted could accumulate thereby reducing the activity of lipolytic enzymes. However, the inhibitory effect due to the accumulation of LCFA has been reported to be reversible over time (Cirne *et al.*, 2007b; Neves *et al.*, 2008; Pereira *et al.*, 2005). Herein, despite the fact that an initial rapid solubilisation of the lipid fraction of the RFW was recorded, no inhibitory effect was observed. The 16.38% of fat remaining from day 14 to day 61 was believed to be recalcitrant.

In the present study, the pattern of VS destruction appeared to match well that of the cumulative methane production (Fig 2.1 A) indicating a balanced digestion where intermediate compounds probably did not accumulate in the bottles. This was supported by the sCOD data, which showed that the soluble intermediates did not accumulate and were rapidly converted to biogas. Slow sCOD conversion over 30 days was previously reported for batch degradation of FW and likely due to the type of inoculum used (Neves *et al.*, 2008). In the present study, the high concentration of

sCOD of approximately 3 g l^{-1} observed at the beginning of the assay (Fig 2.1 B) can be attributed to the initial presence of simple soluble monomers in the RFW. These simple compounds were likely rapidly broken down and consequently contributed to the accumulation of VFAs after 24 hours of digestion (Fig 2.1 B). The rapid doubling time of microorganisms involved in fermentation processes and the typical slow growth observed for Methanogens (Patra et al., 2006; Solera et al., 2002) could explain the build-up of VFAs at the early stage of the process.

The K_h of the RFW of 0.11 d^{-1} established over the period of maximum VS removal (14 days) was found to be in the range of previously reported hydrolysis constants. The work of Elbeshbishy *et al.* (2012) reported K_h of $0.12 - 0.13 \text{ d}^{-1}$ for FW at substrate to inoculum ratio of 0.5 (similar to the ratio used in the present study), while Neves *et al.* (2008) established a hydrolysis rate of 0.12 d^{-1} for RFW with excess of fat. They also observed higher hydrolysis constants of 0.22 d^{-1} and 0.32 d^{-1} for RFW with equal amount of COD (from fat, protein and carbohydrates) and excess carbohydrates respectively. In their study, the hydrolysis rate constant was established over the first 6 days and did not correspond to maximum VS degradation and might thus explain such higher K_h values. In our study, K_h was found to be high when considering the initial 2 days of incubation compare to initial 4, 7 and 14 days of incubation. This observation suggests the existence of two main fractions in RFW; one easily degradable and the other likely recalcitrant. Hemicellulose and fat were believed to be easily degradable as indicated by their relatively high hydrolysis constants K_h of 0.56 d^{-1} and 0.44 d^{-1} , respectively, over the initial two days of digestion (Table 2.3). By contrast, K_h of cellulose of 0.09 d^{-1} was found to be low over the initial two days compared to 0.23 d^{-1} over the initial 7 days of the assay. This supports our hypothesis that cellulose, as the lowest fraction of the RFW, may have been somewhat inaccessible by microorganisms during the early stage of the degradation. The destruction of more than 60% of hemicellulose and 58% fat during the initial two days might have contributed towards increasing the bioavailability of the cellulose in the later stage.

We demonstrated that it is possible to recover almost all the high energy content of RFW in 14 days. Therefore, a solid retention time of 14 days could be applied during bioreactor AD process implementation. This study also suggests that the degradation rate of the different components of the RFW were affected by their bioavailability.

2.4.2 Restaurant food waste degradation rate is improved by enriched cellulose- and hemicellulose-degrading cultures

In this study, a clear improvement in VS destruction in the range of 10% - 15% was achieved for batch degradation of RFW when enriched cultures were added to the initial granular sludge inoculum (Fig 2.4). Specifically, increased degradation of hemicellulose and cellulose fraction of the RFW was observed in the presence of enriched cultures, as indicated by their high hydrolysis constants of 0.3 d^{-1} and 0.28 d^{-1} , respectively (Fig 2.5). In the control assay where only granular sludge was used as inoculum, the K_h for cellulose and hemicellulose were rather low, 0.18 d^{-1} and 0.22 d^{-1} respectively. Enriched culture supplementation therefore contributed to improve hydrolysis constants for hemicellulose and cellulose by 40% and 6.5%, respectively. The degradation of proteins and lipids did not change, however, and were comparable with the control in which only granular sludge was used (Fig 2.5). During the development of the different enriched cultures, the microbial communities' ability to degrade xylan (hemicellulose) and WP (cellulose) increased as indicated by their respective K_h values. This suggests that specialised and/or more active cellulose and hemicellulose degraders accumulated during enrichments, which were likely responsible for the subsequent rapid hydrolysis in the batch degradation of RFW (Fig 2.4). Thouand *et al.* (1995), who investigated the effect of bacterial density on biodegradation of para-Nitrophenol, demonstrated that the concentration of relevant potential biodegraders in the inoculum was key to process success. The authors also reported that inoculum adaptation to relevant substrates could contribute to speeding up biodegradation process. In our study, the granular sludge used to perform biodegradation assays was sourced from a dairy waste water

treatment plant and was, therefore, mostly adapted to animal protein. This may explain the fact that the highest K_h value recorded during the substrate utilisation assay was obtained for skimmed milk as the sole carbon source. However, when supplementing the protein degraders in biodegradation assay of RFW, the K_h of the protein fraction did not change compared to controls, where only granular sludge was used. This was attributed to the fact that some of the proteins from the RFW must be of plant origin and could not have benefited from the microbial adaptation of the enriched culture supplementation. The impact of adaptation of the inoculum to substrate was also addressed in several previous studies (Barkay & Pritchard, 1988; Elbeshbishy et al., 2012; Thouand & Block, 1993; Vazquez-Rodriguez et al., 2011). These authors reported that adaptation of inoculum to the test substrate contributed to: i) decreasing the lag time and increasing the rate of biodegradation; and ii) solving the problem of poor reproducibility observed between biodegradability assays.

The present study demonstrated that inoculum development based on enrichment cultures appeared to be ideal and needs to be considered for rapid start-up of waste biodegradation reactors. Although the supplementation of enriched culture at large scale does not seem practical, a strategy based on the use of digestate to inoculate fresh food waste (Dranco, Kompogas and Varloga approaches) could help in enriching for specific biodegraders.

2.4.3 *Bacteroides graminisolvens* and *Porphyromonodaceas* are implicated in cellulose and hemicellulose hydrolysis, respectively

Bacteroides and *Porphyromonodaceas* were found to be predominant in enriched cultures with WP (cellulose) and xylan (hemicellulose) as sole carbon source respectively (Fig 2.6). During the substrate utilisation assay, the K_h for cellulose was found to be low (0.13 d^{-1}) when using granular sludge (used as initial inoculum for enrichment cultures) compared to K_h of 0.33 d^{-1} at the end of the enrichment procedure (after 18th sub-culturing). A similar observation was made for xylan thereby confirming successful development of cellulose and hemicellulose degraders. Almost 71.24% of

species within the genus *Bacteroides* from the cellulose enrichment cultures were identified as *Bacteroides graminisolvens*. This species have been previously implicated in the anaerobic biodegradation of xylan (Nishiyama et al., 2009). Other studies have also reported that hemicellulose decomposition (including xylan) was mostly carried out by *Bacteroides* species (Chassard et al., 2008; Chassard et al., 2007; Hopkins et al., 2003). To our knowledge, *Bacteroides graminisovens* has never been reported to be involved in cellulose decomposition and yet was found to be the dominating species with 71.24% relative abundance when WP (composed of 98% cellulose and no hemicellulose) was provided as the sole carbon source. This indicated that although *Bacteroides graminisovens* has been reported as being a hemicellulose degrader, they might also possess the ability to degrade cellulose. The attribution of hydrolytic activity to this species was reinforced by the fact that samples for community analysis were taken after 3 days of sub-culturing which corresponded to a high K_h of 0.33 d⁻¹ and 66% cellulose destruction. The enrichment cultures were set up with the intention to enrich for hydrolysers rather than fermenters and methanogens. It was observed that only 8% of the microbial community in the cellulose enriched cultures was affiliated to *Methanobacterium* indicating that the conditions at the time of sampling were likely unfavourable for their growth. In previous studies, cellulose degradation has been mainly attributed to phylotypes within *Clostridium* genus (O'Sullivan et al., 2005; Wang et al., 2010). However only few *Clostridium* species are thought to be able to degrade cellulose (Lynd et al., 2002), while other species within the genus are mainly fermenters (O'Sullivan et al., 2005). In the cellulose enriched cultures, *Clostridium* species were identified, accounting for 8% of the microbial community, and these could be involved both in hydrolysis and fermentation; further investigations would be required to clarify their role *in situ*.

Successful development of xylan degraders was also confirmed by comparing hydrolysis rate constants from the first generation to that of the 18th generation. A 50 % increase in K_h was observed at the end of the enrichment procedure (Fig 2.6) indicating the development of a community

capable of hydrolysing xylan efficiently. Microbial community analysis revealed that xylan degrader's species were mainly distributed among bacteria from the *Porphyromonadaceae* family with relative abundance of 80%. *Porphyromonadaceae* species (order *Bacteroidales*) have been previously reported to possess diverse metabolic properties including the ability to hydrolyse and ferment long chain polysaccharide (Hahnke *et al.*, 2015; Jabari *et al.*, 2012). The 16S rRNA partial sequences generated in the present study were not found to be similar to any sequence at the genus and species level using different classification database (RDP, Silva, and NCBI). Further study will be required to establish whether these species form a novel group within the *Porphyromonadaceae* family. Phylotypes affiliated to *Clostridium* were the second highest microbial group identified in the xylan enriched cultures, accounting for almost 15% of the community. In the initial sludge, used as inoculum for enrichment cultures and for the batch degradation assay, the relative abundance of *Bacteroides* and *Clostridium*, were low (less than 1%; Fig 2.6). From this, it might follow that in the presence of complex polymers, the granular sludge microbial communities would require time to grow, thus negatively affecting decomposition rates. Supplementing active cellulose and xylan degraders for batch degradation of RFW contributed to improve not only the K_h of those two fractions (Fig 2.5) but also the overall biodegradation rates (Fig 2.4). Improving the K_h of the hemicellulose fraction of the RFW might have also contributed to increase the bioavailability of the cellulose since its access is believed to be obstructed by hemicellulose.

The knowledge of microbial groups involved in the hydrolysis of cellulose and hemicellulose could be exploited for example for screening sludge to ensure that cellulose- and xylan-digesting microbes are present and are able to be enriched under appropriate conditions; this could enable for the improvement of rate limiting hydrolysis step during treatment of feedstock with high cellulose and hemicellulose content.

2.5 Conclusions

This study shows that adaptation of the inoculum (through enrichment procedure) to the potential major group of components of the RFW prior to batch degradation could significantly improve biodegradation rates. While RFW was found to be highly biodegradable, the biodegradation rates were found to be influenced by the feedstock composition (fat, protein, cellulose and hemicellulose). It was also found that although some fractions of the food waste are known to be easily degradable; their hydrolysis rate could be low if underrepresented in the substrate. Improving hydrolysis rates for the most abundant fractions of waste could also improve the degradation rates of less abundant fractions, as suggested by this study. We also reported for the first time that *Bacteroides graminisovens* is likely implicated in cellulose degradation. Further study is required to identify species of the family *Porphyromonadaceae* found to be responsible of the hydrolysis of xylan a hemicellulose source. The investigation of the biodegradability of the RFW was believed to be important as the data generated will be used to evaluate the economical viability, the energy efficiency, and the expected solid retention time of the process at large scale.

CHAPTER 3

MICROBIAL COMMUNITY DYNAMICS DURING ANAEROBIC HYDROLYSIS AND ACIDIFICATION OF FOOD WASTE IN LEACHING BED BIOREACTORS

Abstract

Landfill disposal for waste with high organic content, such as food waste, is progressively being reduced as the result of regulation. The ability of anaerobic digestion (AD) to generate bioenergy as well as valuable resources from food waste makes it an ideal technology for its treatment. While AD of food waste has been widely investigated, the hydrolytic-acidogenic phase has been reported as the rate-limiting step either for methane production or for the generation of other high-value products. Therefore, in this study, parameters associated with this phase were investigated. Three leach-bed reactors (LBRs) fed with restaurant food waste (RFW) were operated at 37°C on a semi-continuous mode with 14 days solids retention time (SRT). Volatile solids destruction (VS) in the range of 55% - 75% of substrate total solids (TS) was achieved. The Maximum soluble chemical oxygen demand (sCOD) accumulating in the leachate within the initial 2 days of the 14 days SRT was indicative of efficient hydrolysis of the RFW components over the same period. Up to 47 g l⁻¹ volatile fatty acid (VFA) mix was obtained on day 13 of the last batch, of which circa 35% were butyric acid and 25% acetic acid. High throughput sequencing of *16S rRNA* genes carried out on DNA and cDNA throughout the trial revealed that *Lactobacillus*, *Enterococcus*, *Bifidobacterium* and *Clostridium* were the most Abundant bacterial groups. This study reports for the first time that *Enterococcus* may play a key role in the hydrolysis of some component of RFW. *Lactobacillus*, *Clostridium* and *Bifidobacterium* were found to be involved in the production of lactic acid, butyric acid, and acetic acid respectively. Food waste organic could be greatly reduced in leach-bed reactors over a shorter period of time with *Enterococcus*, *Clostridium*, *Bifidobacterium* and *Lactobacillus* as major players.

3.1 Introduction

Almost one third of the food produced worldwide each year for consumption ends up as unconsumed waste (FAO, 1997). The vast majority of this food waste (FW) generated in Europe and Nord America is landfilled. However this method of disposal is being progressively reduced as the result of its negative impact on the environment including the possibility of ground water contamination and greenhouse gas emissions. In order to prevent or reduce the negative implications of landfill disposal method, the landfill directive was issued and implemented by EU member states in 1999. In Ireland the landfill directive requires that by 2013 biodegradable municipal waste (BMW) going to landfill was to be reduced to 50% of its 1995 tonnage, then to 35 % of the 1995 tonnage by 2016 (McCarthy *et al.*, 2010) . To meet this directive alternative treatment methods need to be developed for the diversion of a large tonnage of biodegradable waste (among which food waste) stream from landfill. Given the high energy content of food waste, an opportunity exists to shift it from pollutant to renewable resource as FW organics can be readily transformed through anaerobic digestion (AD). AD contributes to waste volume reduction while generating a source of renewable energy as methane (Shanmugam & Horan, 2009). AD proceeds through two main phases known as acid-forming phase and methane-production phase, each catalysed by different groups of microorganisms. These two phases are influenced by operating variables, such as feeding type and rate, pH, temperature and reactor's design (Fernández Rodríguez *et al.*, 2012; Lissens *et al.*, 2001). For instance, the optimum pH for hydrolysis and acidogenesis (first phase) has been reported to lie between 5.5 and 6.6 (Arshad *et al.*, 2011; Khalid *et al.*, 2011; Kim *et al.*, 2003), while for methane formation the optimum pH was found to be above 6.8 (Mosey & Fernandes, 1989; Ward *et al.*, 2008).

In one-stage reactors, where the two phases described above occur simultaneously, neutral pH is usually chosen to favour growth of methanogens and thereby production of methane. However for waste with high organic solid content such as food waste, the organic loading rate has

to be maintained below a certain threshold to avoid an excessive build-up of acidification products (such as organic acids and ammonia) with subsequent lowering of the pH and therefore inhibiting methanogenesis. In a two-stage reactor system, the two main phases of the AD process are physically separated in such a way that acid and methane forming stages take place in separate reactors. This approach has been reported as being more stable than a one stage system to treat organic wastes with high solid content (Fezzani & Ben Cheikh, 2010; Khalid *et al.*, 2011). This enhanced performance is reportedly due to the flexibility in process control offered by two-stage systems. Demirer and Chen (2005) reported that, by using a two-phase AD system, it was possible to select and enrich different bacteria involved in each phase. They also reported on increased stability of the process, higher organic loading rates (OLR) and shorter hydraulic retention times (HRT) when using two-stage system by comparison with a single-stage approach. The study of Nielsen *et al.* (2004) established a 6 to 8% higher specific methane yield, and a 9% more volatile solid removal, when using a two-stage reactor compared to a single-stage reactor. Similarly, methane yields were found to increase by 21% during two-stage treatment of cattle manure compared to one-stage reactor (Liu *et al.*, 2006). In addition, the production of volatile fatty acids and organic acids, which constitute the main products of the acid forming stage and precursor for methane formation could be greatly improved through reactor phase separation (Cho *et al.*, 1995).

Although phase separation has been reported to increase the efficiency of AD process for high solid organic waste, concerns still remain on the acid-forming stage, which in contrast with the methane-forming stage is still not well understood and has been reported as the rate limiting step of the process (Palmowski & Muller, 2000).

The proportions of the end-products for this first phase are dependent on substrates, the extent of hydrolysis, environmental conditions, reactor design and the hydrolytic-acidogenic microbial communities. Investigating these parameters would contribute to greatly improve the efficiency and stability of the acid-forming stage. Both efficiency and stability of this phase depend on the microbial communities present in

anaerobic digesters. Hydrolytic and acidogenic bacteria are both involved in the acid-forming stage, with the former involved in the solubilisation of complex organic carbon into simple monomers and the latter involved in fermentation. It is reported that most bacteria active during hydrolysis are also active during fermentation, though the former step does not involve microbial growth and the latter is considered to be growth-related (Carbone *et al.*, 2002; Manyi-Loh *et al.*, 2013). According to Liebl (2001), Cirne *et al.* (2007), Sträuber *et al.* (2012), hydrolytic bacteria are mostly associated with *Acetivibrio*, *Clostridium*, *Bacteroides*, and *Thermotoga*. Acidogenic bacteria include hydrolytic bacteria and fermentative bacteria that do not possess hydrolytic activities. Strictly acidogenic bacteria have been found among *Bifidobacterium*, *Lactobacillus*, *Anaerolinaceae*, and a few thermophilic bacteria (Balk *et al.*, 2002; Dong *et al.*, 2000; Stiles & Holzapfel, 1997; Yamada *et al.*, 2006). Both hydrolytic and acidogenic communities have been reported to be inhibited by high concentration of volatile fatty acids (VFAs) which might penetrate microbial cells causing pH lowering within the cell and therefore acid stress (Pind *et al.*, 2003). According to Pind *et al.* (2003), accumulation of some VFAs may inhibit the anaerobic fermentation process, by causing reactions to become thermodynamically unfavourable.

This research focuses on the hydrolytic-acidogenic phase during AD of restaurant food waste (RFW). The objectives were to i) investigate the hydrolysis and fermentation efficiency of the RFW inside LBRs by monitoring the sCOD accumulation in the leachate and the RFW VS destruction and ii) identify the associated microbial communities responsible for metabolic reactions by using high throughput sequencing of *16s rRNA* genes at both DNA and RNA level.

3.2 Materials and methods

3.2.1 Restaurant food waste

About half a tonne of RFW from a fresh collection was provided by the waste collection company; Mr Binman based in Limerick. The RFW which mainly consisted of meat, tissue papers, potatoes, rice, fruit and vegetable peeling was homogenised after manually removing bones, shells and other non-degradable materials. The homogenised RFW was packed in bags of 5 kg and stored at -20°C. Prior to each experiment, a 5 kg bag of RFW was defrosted slowly in the fridge, the particle size was manually reduced through shredding with a scissor; its physical and chemical characteristics were determined.

3.2.2 Characterisation of the restaurant food waste, inoculum and digestate

The physical and chemical characteristic of the RFW is displayed in Table 3.1. The tCOD and sCOD of the RFW were 1.45 ± 0.016 g COD g⁻¹ VS and 0.11 ± 0.002 g COD g⁻¹ VS respectively. The granular sludge used as starting inoculum was from a full-scale internal circulation (IC) anaerobic digester located at Carbery Milk Products (Ballineen, Co Cork, Ireland). Its total solid (TS) and volatile solid (VS) were $9.01 \pm 0.09\%$ and $7.85 \pm 0.04\%$ respectively.

The methanol-chloroform method (Folch *et al.*, 1957) was used to establish the total fat content of the RFW. Briefly, 8 ml chloroform and 4 ml methanol were added to 50 ml Sarstedt tubes containing 0.5 g of dried RFW or digestate which was then placed on rock roller for 3 hours at room temperature (RT). After this, 2.4 ml distilled water (dH₂O) were added and the tubes were centrifuged at $3500 \times g$ for 15 min resulting in the formation of three layers. The lower chloroform layer containing fats was pipetted into pre-weighed glass bottles and allowed to evaporate overnight under the fume hood. After evaporation, glass bottles were weighed and the lipid content calculated as a % of starting material weight. The assay was performed in triplicate.

Concentration of hemicellulose and cellulose in the RFW and digestate were determined by using the Dubois assay after extraction using a combination of dilute and strong sulphuric acid hydrolysis at high temperature with short retention time as described in chapter 2 (section 2.2.2).

The protein portion of the RFW and digestate were determined by an external laboratory (Teagasc Food Research Centre, Ashtown, Dublin 15, Ireland) as described in Chapter 2 section 2.2.2.

Table 3.1 Characteristic of restaurant food waste

	TS	VS	Fat	Protein	Cellulose	Hemicellulose
Food waste (%)	28.19±2.34	25.96±2.08	27.50±1.45	20.69±1.17	2.82±0.95	32.58±4.48

3.2.3 Reactors design

Three leach-bed reactors (R1 – R3) were operated in a semi-continuous batch mode (batch 1 – 7) with 14 days solid retention time (SRT). A schematic diagram of the anaerobic leach-bed reactor system used for this study is shown in Figure 3.1. The leach-bed reactors had a total and a working volume of 6 and 3 l, respectively. Each reactor was composed of two chambers divided by a wire mesh and the working volume was defined as the volume of the upper chamber. In the lower chamber, a pumice stone bed containing 0.5 kg of water-washed and oven-dried pumice stones was placed above a wire mesh to facilitate the filtering of the leachate and to prevent the clogging of the recirculation line. All three reactors were made of acrylic column and were operated at the constant temperature of 37°C maintained by the water jacket surrounding the column. A pump was used for the recirculation of leachate from the lower chamber to the upper chamber.

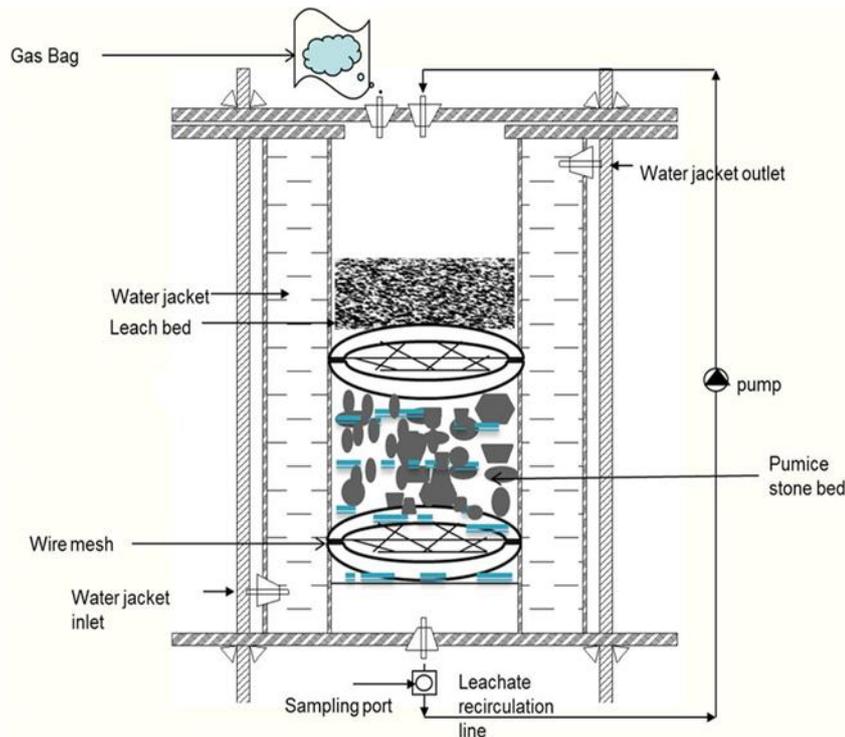


Fig 3.1 Schematic diagram of leach bed reactor

3.2.4 Inoculum acclimation and reactors operation

Initially, the granular sludge used as starting inoculum was mixed to RFW at the ratio of 0.25 on the VS basis and 40 g of sodium bicarbonate (NaHCO_3) was added. The mixture was loaded into the upper chamber above the wire mesh of each reactor. The total organic loading for each reactor was $80 \text{ g VS RFW l}^{-1}$ with 3 l working volume. One litre of water was introduced in the lower chamber and the reactors were closed air-tight with a lid. Reactors were operated for 14 days with one recirculation of 45 min per day. On day 14, the second batch was started by inoculating fresh RFW with the resulting digestate from the first batch at the same ratio of 0.25 and the same loading rate of $80 \text{ g VS RFW l}^{-1}$ was applied. Similarly, leachate from the last day of the first batch was diluted 4 times and pH adjusted to 7 with NaHCO_3 , the resulting mixture was used as starting liquid for the second batch. The third batches were operated as described for the second batch.

The digestate from the inoculum adaptation experiment was used as starting inoculum for the reactors operation. Each reactor was run in the

same way as describes above. During reactor operation, leachates samples were collected for pH, ammonia (NH₃), soluble chemical oxygen demand (sCOD) and volatile fatty acids (VFAs) analyses.

3.2.5 Recovery of microbial cells from samples

Microbial cells were recovered from leachate (pumice stone washed in leachate to ensure removal of biofilm) and digestate samples using the differential centrifugation method by Apajalahti *et al.* (1998). Digestate and leachate fractions of R1, R2 and R3 were sampled in duplicate on day 0, 1, 3, 7 from batch 7 (6 fractions from each reactor). Straight after each sampling 7 g of digestate were suspended in 200 ml of wash buffer (50 mM sodium phosphate buffer [pH 8], 0.1% Tween 80) while 40 ml leachate in 50 ml Sarstedt tubes were centrifuged at $8,000 \times g$ and the pellet resuspended in 30 ml wash buffer. The digestate and leachate suspensions were then shaken for 20 and 10 min respectively on a rock roller platform shaker at $70 \text{ oscillations min}^{-1}$ and were left to decant at RT for 15 min. The supernatants were carefully transferred to a clean and sterilised 250 ml centrifuge tubes (NALGENE Oak Ridge centrifuge tubes) and kept on ice. The solid particles left behind after decantation were again suspended in wash buffer and the differential centrifugation process was repeated for a total of four rounds. The microbial cells in the pooled supernatants were collected by centrifugation at $30,000 \times g$ for 15 min at RT. The resulting pellets which was around 0.50 – 0.6 g for digestate supernatant and 0.10 – 0.25 g for leachate supernatant were immediately resuspended in 2 ml RNAlater® solution (Ambion™) and incubated at RT for 3 hours. The RNAlater suspensions were then transferred into sterilised 2 ml tubes (Eppendorf) which were centrifuged at $8,000 \times g$ for 15 min. The resulting pellets were snapped frozen in liquid nitrogen and stored at -80°C until needed.

3.2.6 DNA, RNA and protein co-extraction

DNA, RNA and protein were co-extracted from 0.2 g and 0.6 g of leachate and digestate microbial cells pellets respectively by using the NucleoSpin

TriPrep kit (Macherey-Nagel). First, the samples were resuspended in 500 μl lysis buffer (from the kit) and 500 μl 1X Tris-EDTA and 5 μl β -Mercaptoethanol ($10 \mu\text{g ml}^{-1}$) were added. To this mixture, 0.5 mm and 0.1 mm diameter zirconia beads (Thistle Scientific) were added and the microbial cells were lysed by bead beating for 10 min using a vortex (IKA Vortex Genius 3). The lysates were recovered after 30 min of centrifugation at $17,000 \times g$ and transferred to clean tubes. Purification of DNA, RNA and protein from the lysate were carried out according to the NucleoSpin TriPreps kit protocol.

3.2.7 Generation of complementary deoxyribonucleic acid

The extracted genomic RNA molecules were DNase treated using the TURBO DNA-free™ Kit (Ambion by Life Technology) in accordance with the manufacturer's instructions. Polymerase chain reaction (PCR) was carried out on DNase treated RNA solution to confirm the removal of any residual DNA. The amplification was conducted on a thermal cycler G-Storm (Eppendorf Mastercycler Gradient) using primer pair 515F/806R (Caporaso et al., 2011a) specific for the bacterial and archaeal *16S rRNA* gene (V4 region). PCR reaction consisted of 25 μl reaction mixture containing 0.2 μM of each primer, 200 μM of each deoxynucleoside triphosphate (dNTPs), 1.5 mM Magnesium chloride (MgCl_2), 1 X ammonium and 5×10^{-2} U/ μl of Taq polymerase (Bioline). Template consisted of 1 μl of neat (DNase treated RNA samples) or 1/5 and 1/10 dilution of neat. The PCR conditions consisted of initial denaturation at 94°C for 30 s, followed by 30 cycles of annealing at 50°C for 30 s, and elongation at 72°C for 30 s. The final extension was carried out at 72°C for 2 min. The resulting PCR products were electrophoresed on a 1% agarose gel containing 5×10^{-4} mg ml^{-1} ethidium bromide and the bands visualised under a UV trans-illumination camera.

Once RNA samples were confirmed to be free from DNA contamination, complementary deoxyribonucleic acid (cDNA) was generated using the superscript reverse transcriptase III (Invitrogen) in accordance with the manufacturer's recommendations.

3.2.8 Polymerase chain reaction for sequencing analysis

Each DNA and cDNA samples was amplified in triplicates. Amplification of the *16S rRNA* gene V4 region of archaea and bacteria using the primer set 515F and 806R was performed in a 25 μl reaction mixture containing 1 x Q5® Reaction Buffer, 200 μM of each dNTPs, 0.2 μM of each primer and $2 \times 10^{-2} \text{ U } \mu\text{l}^{-1}$ Q5 High-Fidelity DNA Polymerase (New England BioLabs_{inc}). The PCR conditions consisted of initial denaturation at 94°C for 30 s, followed by 30 cycles of annealing at 50°C for 30 s, and elongation at 72°C for 30 s. The final extension was carried out at 72°C for 2 min. Bacterial and archaeal DNA were used as positive controls. The resulting triplicate PCR products for each sample were combined into a single volume and purified using NucleoSpin® Gel and PCR Clean-up kit (Macherey - Nagel) in accordance with the manufacturer's instructions.

3.2.9 Amplicon quantification

To determine the concentration of purified amplicons, fluorometric measurements were performed using 1 μl aliquots of purified cDNA or DNA solution with a Quant-iT dsDNA HS assay kit and a Qubit fluorometer (Invitrogen), in accordance with the manufacturer's instructions. Amplicons from all samples were normalised to a final concentration of 20 $\text{ng } \mu\text{l}^{-1}$ and sent to an external laboratory (Research and testing laboratory, Texas US) for *16S rRNA* gene amplicon sequencing using MiSeq Illumina platform.

3.2.10 Analytical methods

Leachate samples from the three reactors (R1 – R3) were routinely analysed for soluble chemical oxygen demand (sCOD) concentrations according to the Standing Committee of Analysts Methods (1985). Samples were analysed for TS and VS using standard methods (APHA, 2005). Ammonia concentrations in leachate samples were measured using HACH methods and test kits (HACK Odyssey). Methane content of biogas was determined by a gas chromatograph (Varian CP-3800) equipped with a glass

column and a flame ionisation detector. The carrier gas was nitrogen and the flow rate was 25 ml min⁻¹. Volatile fatty acid (VFA, C2 – C6) were quantified using a gas chromatograph as described in chapter 2 section 2.2.10. All analyses were carried out in duplicates.

Lactic acid and ethanol assay kits (Megazyme) were used to measure the concentration of lactic acid and ethanol respectively according to the manufacturer's instructions.

3.2.11 Statistical analysis of data

Statistical analysis including non-metric multidimensional scaling (nMDS), clustering, analysis of similarity (ANOSIM), similarity percentage (SIMPER), Kruskal–Wallis test, and Pearson correlation were performed using R software version 3.1.2. The input for each of these statistical tests was a community matrix based on the relative abundance of each group of microorganisms generated from Illumina sequencing data. Bray Curtis calculation was used to determine the distance between each pair of community for nMDS and cluster analysis.

3.3 Results

3.3.1 Solubilisation of restaurant food waste in Leach-bed reactors.

The triplicate reactors (R1 – R3) were operated over a period of 97 days in a semi-continuous batch mode, with a SRT of 14 days. The sCOD in R1, R2 and R3 leachate increased rapidly within the initial two days of the process and did not increase further until the end of the 14-day SRT in each reactor (Fig 3.2 A). The sudden increase in sCOD in the leachate corresponded to a sharp decrease in pH from 7 to 5±0.5 (Fig 3.2 B) within the same period. Unlike sCOD concentration which did not increase from day 3 until the end of the cycle, an increase of the leachate pH was observed toward the end of the 14-day SRT (from day 7 – day 13) (Fig 3.2 B). The initial pH of the mixture FW/inoculum was below 4 and a preliminary experiment under this low pH 4 failed due to the overloading of the system. Therefore, adding the

sodium bicarbonate provided suitable condition for the growth of the hydrolytic-acidogenic community. From economical point of view, adding buffer in system increase the cost of the process; therefore, in other to alleviate the impact of the buffer on the cost of the operation, it could be envisaged for example, in the case of sodium bicarbonate, to recover the CO₂ for further process (methane production through hydrogenotrophic methanogenic process) and to recover the sodium from the stream.

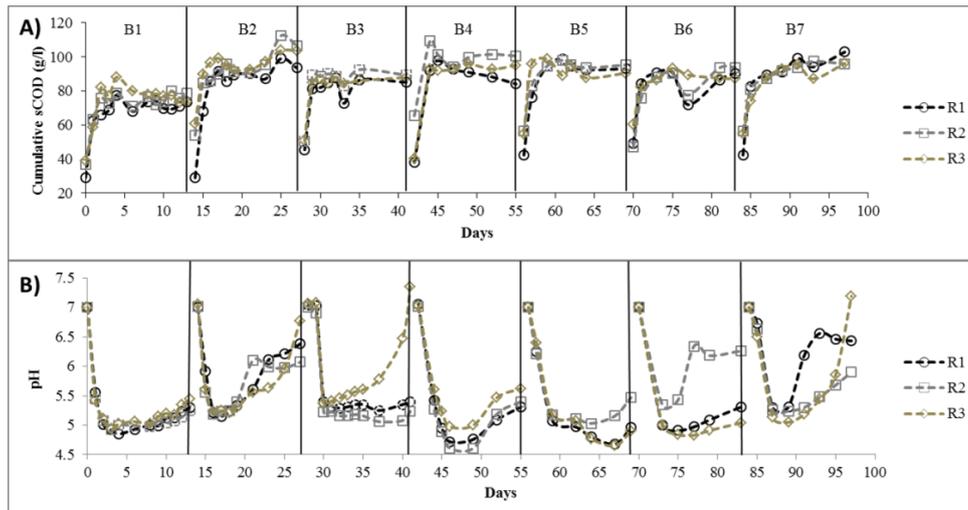


Fig. 3.2 Profile of the sCOD accumulated in the leachate (A) and pH of the leachate (B) during 14 day SRT. B1-B7: batch 1 to batch 7; Data at each point represent average of duplicate measurements.

All three reactor leachates also showed similar trend in ammonia concentrations which first increased sharply from day 1 to day 3, then plateaued and subsequently increased further from day 7 to day 13 (Fig 3.3). Overall, the total ammonia concentration in the leachate samples from R1, R2 and R3 mostly varied from 8 g l⁻¹ – 10 g l⁻¹ with highest concentrations reaching 14 g l⁻¹ in some cases.

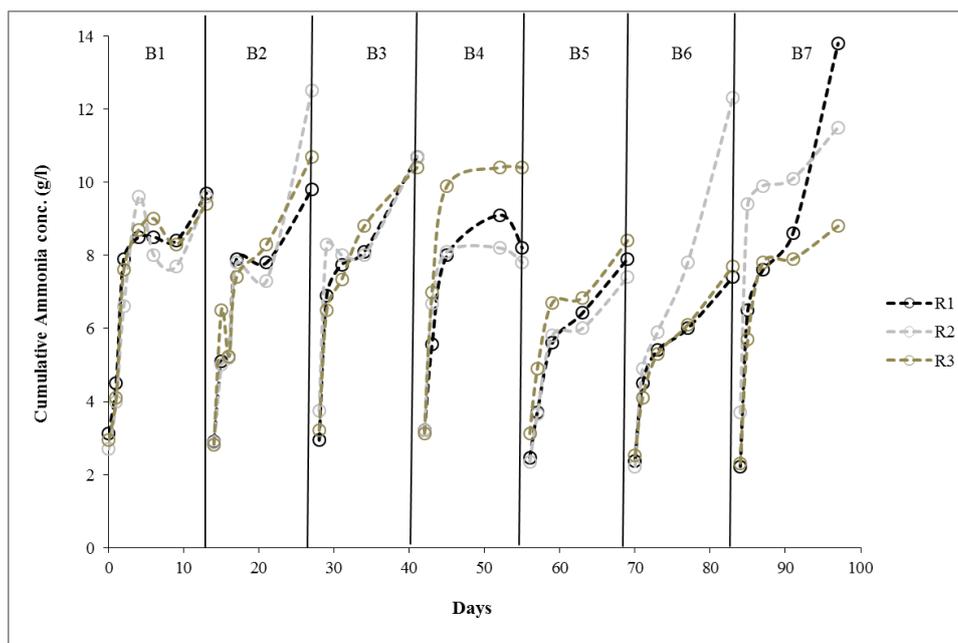


Fig 3.3 Profile of ammonia concentration in leachate samples during 14-day SRT. B1-B7: batch 1 to batch 7. Value at each point is the average of duplicate analysis.

3.3.2 Volatile solid reduction from the triplicate reactors indicated effective hydrolysis.

The physical and chemical characteristics of the RFW used to feed the triplicate reactors during the 97 days trial are shown in Table 3.1. Hemicellulose was the highest fraction of this RFW, followed by fat, protein and cellulose (Table 3.1). At a loading rate of 80 g VS l^{-1} with 14-day SRT, the total VS destruction from R1, R2 and R3 for all batches was between 56% - 75% (Fig 3.4) and the average VS destruction was $65.04 \pm 4.28\%$. This VS destruction was indicative of the solubilisation of the organic matter present in the RFW. The analysis of variance (ANOVA) based on the general linear model revealed that VS destruction efficiency from B1 – B7 were significantly different ($p = 0.009$). However when only B3 – B7 were considered, the ANOVA analysis suggested that VS removal were not significantly different ($p = 0.346$). Since VS destruction from batch 3 – 7 were found to be similar, it was assumed that stable conditions were achieved. Therefore batch 7 was chosen for in-depth investigation of hydrolysis and fermentation with the assumption that the findings were likely applicable to batch 3 - 6. The lowest VS destruction efficiency of

58.81±2.93% and 60.12±3.22% were obtained for B1 and B2 respectively. This was likely due to instability related to the start-up period.

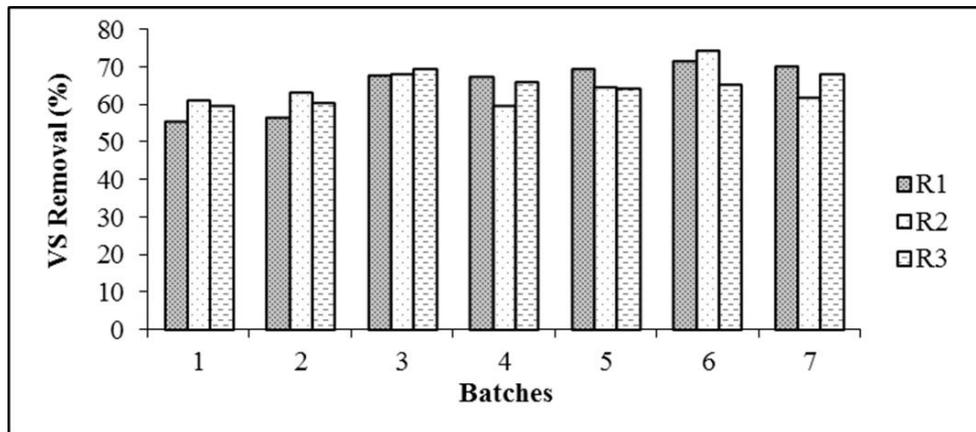


Fig 3.4 Volatile solid (VS) reductions from the restaurant food waste in R1, R2 and R3 operated at SRT of 14 days for each batch. Analysis in each reactor was carried out in duplicate and the average is shown here.

The analysis of the residues at the end of 14-day SRT (batch 7) revealed a destruction efficiency of 99%, 60%, 50% and 20% for hemicellulose, proteins, cellulose and fats respectively (Fig 3.5). This indicated the relatively poor breakdown of fat which would be likely to accumulate in reactors under continuous operation.

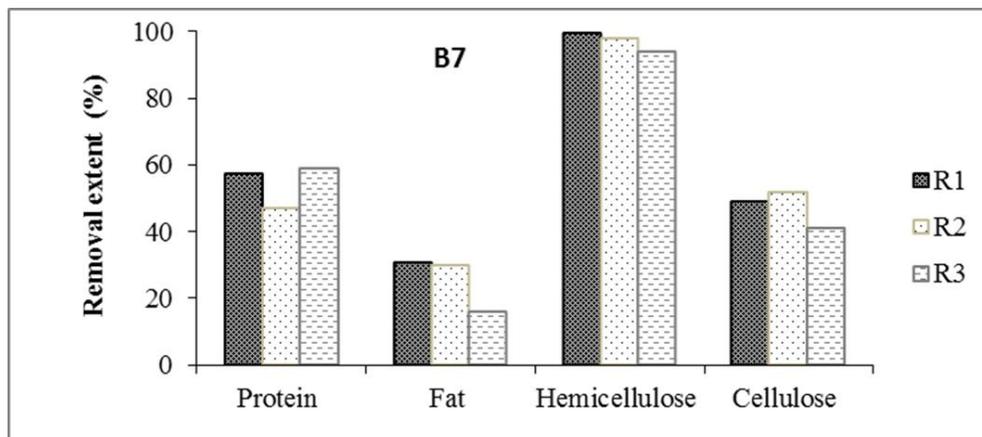


Fig 3.5 Degradation efficiency of the major components of restaurant food waste in R1, R2 and R3 during batch 7 (14-day SRT). All analyses were carried out in triplicates, and only the average is shown here.

3.3.3 Volatile fatty acids and organic acids accumulation in leachate

During batch 7, leachate samples from the triplicate reactors from day 0, 1, 3, 5, 7, 9 and 13 were analysed for VFAs and other organic acids production. The total VFAs yield obtained during batch 7 in R1, R2 and R3

varied between 39 – 47 g l⁻¹ (Fig 3.6 A). However when subtracting the initial VFA concentration from the inoculum on day 0, the actual VFA produced as the result of hydrolysis and fermentation of RFW organic matter was between 24 g l⁻¹ – 27 g l⁻¹. Very low amount of methane were produced from each reactor during each of the 7 batches (Supplementary Fig S3.1). Low pH and probably the high concentration of some VFAs (especially propionic acid) might have contributed to inhibit the methanogens known to be involved in the methane formation process.

Three distinct phases could be distinguished from the pattern of the total VFA production (Fig 3.6 A). Low amounts of VFA were generated during the first phase (day 0 – 3) which corresponded to a lag phase, while a significant increase was recorded during phase two (day 3 – 5) and finally phase three (day 7 – 13) corresponded to a large increase of VFA (Fig 3.6 A). The highest VFA produced in all three reactors during batch 7 was butyric acid (35 % – 41 %) followed by acetic acid (22% - 32%), propionic acid (11% - 22%), caproic acid and valeric acid (7%- 14%)(Fig 3.6 B).

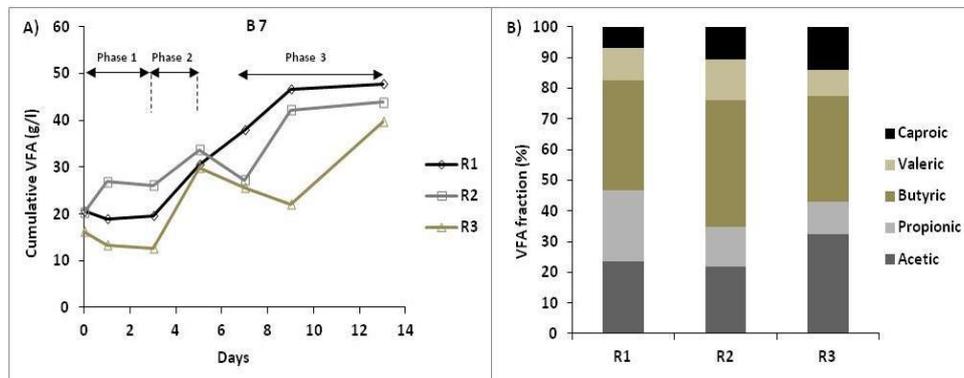


Fig 3.6 Profile of the cumulative VFAs in the leachate of the triplicate reactors R1, R2, R3 (A) and proportion of each individual VFA in the total VFA produced on day 13(B). B7 = batch 7. Value at each point is the average of duplicate measurements.

Butyric acid (Fig 3.7 C), caproic acid (Fig 3.7 D), and valeric acid (Supplementary Fig S3.2), concentrations rapidly increased from day 7 – 13 while acetic acid concentrations increased from day 0 – 7 (Fig 3.7 A). Thus, acetic acid was likely found to contribute to the second phase of total VFA production while butyric acid, caproic acid and valeric acid likely contributed to the third phase (Fig 3.6 A).

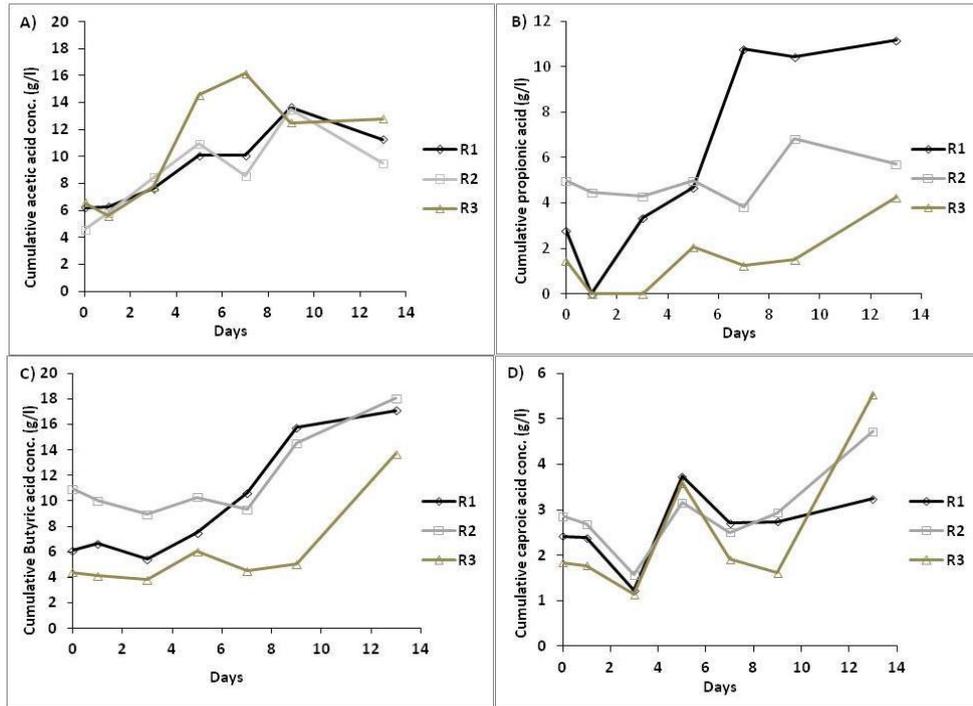


Fig. 3.7 Profile of the major VFAs in the leachate obtained during batch 7. A) Acetic acid; B) Propionic acid; C) Butyric acid; D) Caproic acid. Value at each point is the average of duplicate measurements.

Lactic acid production was observed in R1, R2 and R3 from day 0 – 3 and was subsequently consumed from day 5 – 13 (Fig 3.8 A). On the other hand, the concentration of ethanol rapidly decreased from day 0 to day 1 in all three reactors and subsequently increased from day 1 to day 3 in R1 and R2 and from day 1 to day 13 in R3 (Fig 3.8 B).

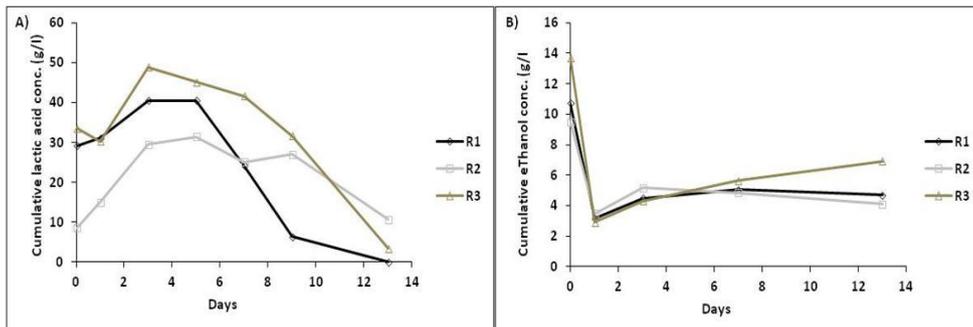


Fig 3.8 profile of the cumulative lactic acid (A) and ethanol (B) produced in triplicate leach-bed reactors (R1 – R3) during batch 7. Value at each point is the average of duplicate measurements.

As methanogenesis was inhibited, acidification products were expected to accumulate in the leachate. The continuous decrease in lactic acid concentrations from day 5 to day 13 suggested that this compound had been consumed. The potential correlation between the consumption of lactic

acid and the production of VFA was investigated using Pearson correlation analysis. The result of the analysis is shown in Table 3.2. Lactic acid appeared to be highly negatively correlated with butyric acid in R1, R2 and R3 with R value of -0.95, -0.41 and -0.85, respectively. This implied that these product pairs did not co-occur in the triplicate reactors, suggesting that one of the compounds was produced while the other was consumed. The profiles of butyric acid (Fig 3.7 C) and lactic acid (Fig 3.8 A) production suggested that lactic acid was likely converted to butyric acid. Caproic acid, valeric acid and propionic acid were also negatively correlated with lactic acid in all three reactors (Table 3.2). However the negative correlation was found to be less strong than that of butyric acid. Most likely however, some of the lactic acid was also converted into these VFAs.

Table 3.2 Pearson correlation coefficient R among VFAs and lactic acid concentration obtained from R1, R2 and R3 leachate samples (batch7).

R1	Acetic acid	Propionic acid	Butyric acid	Valeric acid	Lactic acid	Caproic acid
Acetic acid	-	0.86	0.85	0.62	- 0.67	0.49
Propionic acid	-	-	0.87	0.58	- 0.76	0.40
Butyric acid	-	-	-	0.72	- 0.95	0.46
Valeric acid	-	-	-	-	- 0.61	0.92
Lactic acid	-	-	-	-	-	- 0.33
R2	Acetic acid	Propionic acid	Butyric acid	Valeric acid	Lactic acid	Caproic acid
Acetic acid	-	0.62	0.41	0.27	0.66	0.20
Propionic acid	-	-	0.76	0.64	- 0.07	0.50
Butyric acid	-	-	-	0.89	- 0.41	0.86
Valeric acid	-	-	-	-	- 0.46	0.96
Lactic acid	-	-	-	-	-	- 0.50
R3	Acetic acid	Propionic acid	Butyric acid	Valeric acid	Lactic acid	Caproic acid
Acetic acid	-	0.53	0.33	0.46	- 0.02	0.42
Propionic acid	-	-	0.92	0.77	- 0.74	0.92
Butyric acid	-	-	-	0.63	- 0.85	0.94
Valeric acid	-	-	-	-	- 0.48	0.83
Lactic acid	-	-	-	-	-	- 0.72

The contribution of VFAs, ethanol and lactic acid to the total sCOD from the leachate samples of the triplicate reactors after 14 days of operation is shown on Fig 3.9. Ethanol, lactic acid and VFAs were the principal constituents of the sCOD accumulated in R1, R2 and R3 leachate. They represented between 82.29 – 94.21% of the leachate sCOD obtained during batch 7 (Fig 3.9). However, VFA was the highest fraction and accounted for 66.67 – 77.12% of the accumulated sCOD. In R1 and R3, 14% of accumulated sCOD was not converted to organic acid or ethanol whereas in R2 only 2% of sCOD was not converted. The organic acid content of the sCOD was found to vary over the time. On day 1 and 2, lactic acid was the highest product of fermentation in R1 and R3 (supplementary Fig S3.3 A and B) whereas on day 13 only traces of lactic acid was detected. The VFAs fraction of the sCOD did not show an increase from day 1 – 2, however on day 13 was the highest components. The fraction of sCOD not converted to VFAs or ethanol was the highest (14 – 29%) on day 0 in the replicate reactors.

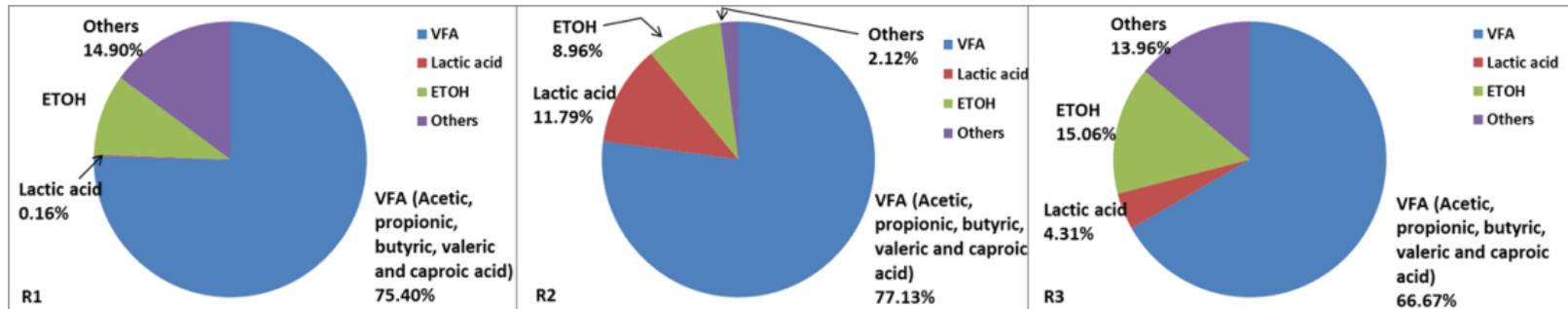


Fig 3.9 Contribution of fermentation products (VFAs, ETOH, lactic acid and others) to the total sCOD accumulated in the leachate of R1, R2 and R3 (batch 7, day 13).

3.3.4 Microbial community dynamics

Digestate and leachate microbial communities profiling from DNA and cDNA on day 0, 1, 3, and 7 during batch 7 were investigated to identify the bacterial and archaeal communities. Digestate microbial communities profiling from cDNA on day 0 was not investigated due to RNA yield issues for this particular sample point.

3.3.4.1 Microbial diversity and species richness

Reactors microbial community profiling from both DNA and cDNA on day 0, 1, 3 and 7 of batch 7 was investigated through sequencing of the V4 region of *16S rRNA* gene on Illumina MiSeq platform. The resulting sequences were clustered into 3368 species-level OTUs (defined as 96% sequence similarity via the USEARCH clustering algorithm). Cluster analysis was carried out to visually compare microbial community composition in different samples. Community distance matrix based on beta diversity between each pair of communities was generated by using Bray Curtis calculation. Cluster analysis indicated that the microbial community composition in technical replicate samples at DNA and cDNA level were similar as indicated by the low beta diversity between 5% - 25% (Fig 3.10). However, the leachate and digestate community composition (at cDNA and DNA level) in biological replicates showed high beta diversity (above 60%), indicating that they were dissimilar (Fig 3.10). Bray Curtis cluster analysis also showed that the microbial communities on day 1 (both in DNA and cDNA) were distant from the communities on other days (day 0, 3 and 7) (Fig 3.10). The overall PERMANOVA test statistic for community difference between days generated R value ranging from 0.55 to 0.64 and p value below 0.05, suggesting that the community composition likely varied between days.

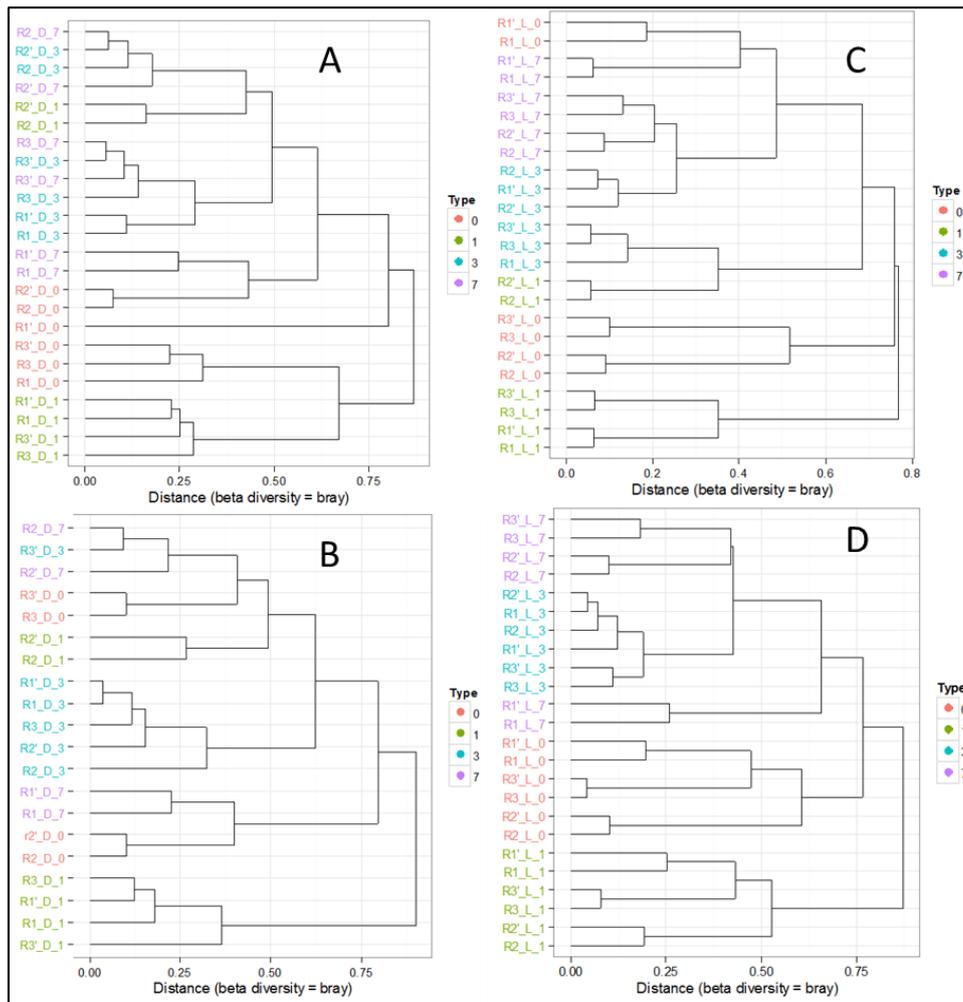


Fig 3.10 Bray Curtis cluster of microbial communities associated with digestate and leachate from R1, R2 and R3. A) DNA from digestate samples; B) cDNA from digestate samples; C) DNA from leachate samples and D) cDNA from leachate samples.

Rarefaction analysis, based on OTUs at 4% dissimilarity, indicated that both cDNA and DNA sequences could well represent the microbial communities as the rarefaction curves were approaching plateaus (Fig 3.11). The number of OTUs was generally higher on day 0 especially in the digestate samples at both DNA and cDNA level (Fig 3.11 A and B).

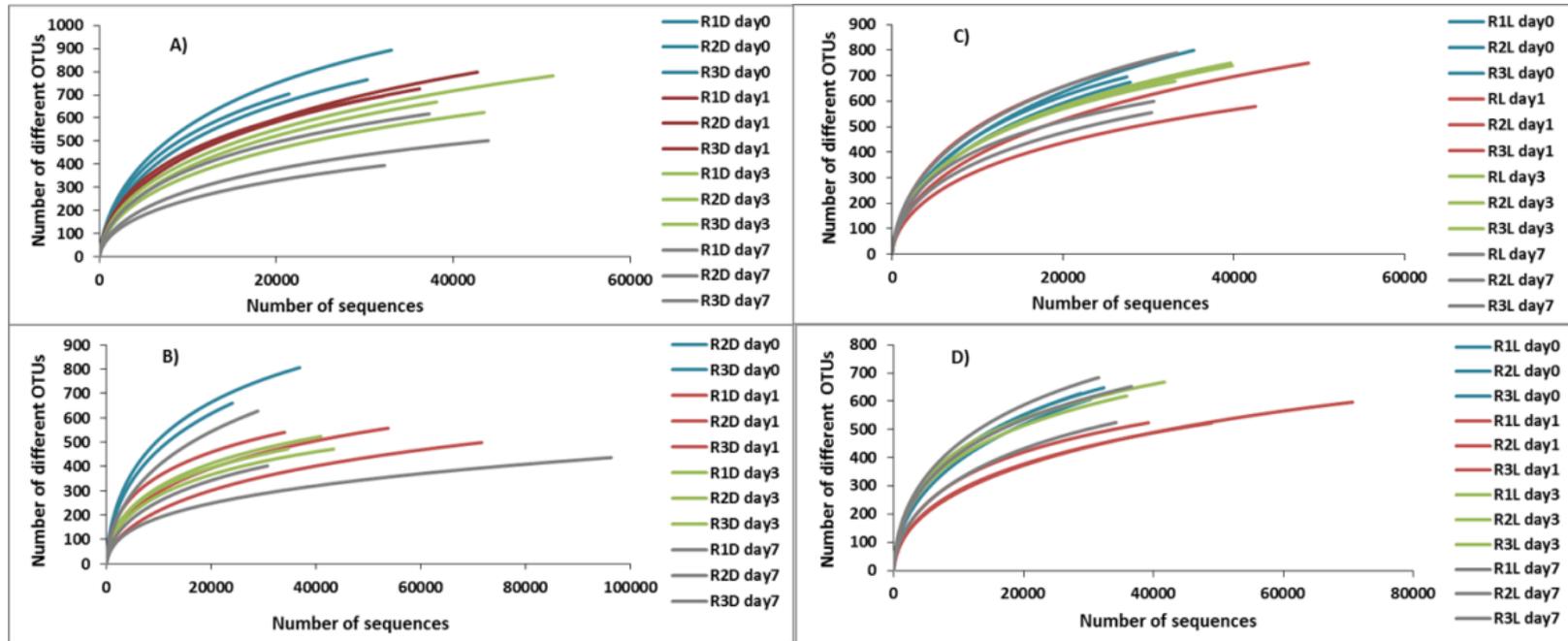


Fig 3.11 Rarefaction curve based on microbial OTUs at 4% dissimilarity associated with digestate and leachate from R1, R2 and R3. A) DNA from digestate sample; B) cDNA from Digestate samples; C) DNA from leachate samples and D) cDNA from leachate samples.

3.3.4.2 Similarity and difference among microbial communities

The difference between day 0, 1, 3 and 7 microbial communities profiling from both DNA and cDNA was investigated by using Non-metric multidimensional scaling (nMDS) analysis. nMDS performed on the community matrix both at DNA and cDNA level revealed that microbial populations in the triplicate reactors clustered as a function of time (day 0 – 7) and as a function of reactor fractions (leachate and digestate) (Fig 3.12 A and B). In both nMDS plots, the stress value was less than 0.3, indicating an acceptable representation of the data using a two dimensional configuration (Clarke, 1993). This was confirmed by the Shepard plot (Supplementary Fig S3.4 A and B) which showed a narrow scatter around the regression line, indicating that the dissimilarities in the 2-D configuration were well preserved.

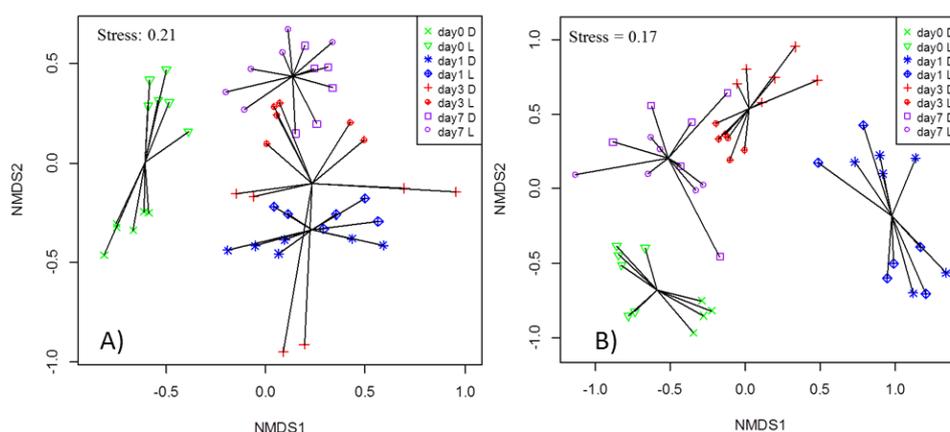


Fig 3.12 nMDS plot illustrating microbial community shifts analysed from DNA (A) and cDNA (B) based 16S rRNA Miseq sequencing. The community profile on the plot at each sampling point is represented by a specific colour (green for day 0, blue for day 1, red for day 3 and purple for day 7) while the community present in each fraction (leachate = L and digestate = D) of the reactors is illustrated by symbols. Cluster significance: $R = 0.77$ and $p = 0.001$ (A); $R = 0.66$ and $p = 0.001$ (B).

A one-way analysis of similarity (ANOSIM) test was performed on both nMDS plots to confirm whether the observed clusters (day 0 – 7) were significantly different from each other. The “no day-to-day” difference hypothesis was tested by permutation of the rank similarity matrix generated by Bray Curtis similarity calculation. The ANOSIM R value for nMDS plot at DNA level (Fig 3.12 A) was 0.66 with a p -value of 0.001 while R value

of 0.77 with p-value of 0.001 was recorded for nMDS plot at cDNA level (Fig 3.12 B). This analysis indicated that the observed clusters on both nMDS plots were significantly different from each other. Microbial communities were likely more similar within clusters than between clusters.

Since the microbial communities in the triplicate reactors also appeared to cluster based on fraction (leachate and digestate), one-way ANOSIM analysis was also performed to establish whether the community in these two fractions were different. On day 0, the communities in the leachate in both DNA and cDNA samples appeared to be significantly different from those in the digestate samples as indicated by the high corresponding R values (Tables 3.3). However on day 1 in the DNA samples, a small R value of 0.32 was recorded (when comparing leachate and digestate) but this was found to be significant (p=0.041) (Table 3.3), indicating that although the community in the leachate and digestate might be different, some overlapping might also be observed. Day 1 leachate and Digestate microbial communities profiling from cDNA were similar (p>0.05) whereas on day 3 and 7 they were significantly different (p<0.05) (Table 3.3)

Table 3.3 Analysis of similarity (ANOSIM) between leachate (L) and digestate (D) fraction from DNA and cDNA-based 16S rRNA Miseq sequencing.

Days	Fraction 1	Fraction 2	R Value (DNA)	P Value (DNA)	R Value (cDNA)	P Value (cDNA)
0	L	D	0.98	0.002	1.00	0.006
1	L	D	0.32	0.041	0.19	0.097
3	L	D	0.59	0.003	0.66	0.002
7	L	D	0.62	0.003	0.53	0.003

3.3.4.3 Determination of microbial groups responsible for samples clustering

The nMDS plot at DNA and cDNA level revealed the presence of 4 distinct clusters (day 0, 1, 3 and 7) (Fig 3.12 A and B) suggesting microbial community shifts occurring over time. Similarity percentage (SIMPER) test was carried out to determine the microbial groups responsible for the

observed clusters. The result of the SIMPER analysis based on the microbial communities profiling from cDNA is displayed in Table 3.4. *Enterococcus*, *Clostridium*, *Corynebacterium*, *Lactobacillus*, *Bifidobacterium* and unclassified *Bifidobacteriaceae* all contributed to 73% of the clustering between day 0 and day 1 with the highest contribution attributed to *Enterococcus* (Table 3.4). Between day 1 and day 3 clusters, 80% of the discrimination was attributed to *Enterococcus* and *Lactobacillus* while *Lactobacillus*, *Clostridium* and *Bifidobacterium* were identified as discriminatory microbial groups between day 3 and 7 clusters (77% contribution) (Table 3.4). Similar observations were also made on the microbial communities profiling from DNA, that *Enterococcus*, *Lactobacillus*, *Bifidobacterium* and *Clostridium* were the most influential species (Supplementary Table S3.1).

The distribution of these influential species over the time was established by using Kruskal Wallis test. Only influential species with significant ($p > 0.05$) distribution were plotted. At cDNA level, the distribution of this six influential groups revealed that *Enterococcus* was only abundant on day 1 while *Lactobacillus* and *Bifidobacterium* abundance increased on day 3 and finally on day 7, *Bifidobacterium*, *Lactobacillus* and *Clostridium* were the most abundant (Fig 3.13). This distribution analysis therefore suggested that *Enterococcus*, *Lactobacillus*, *Bifidobacterium* and *Clostridium* were likely responsible for the microbial community shift over the time. A Similar distribution profile was observed at DNA level except that *Clostridium* distribution was not significant over the time and was therefore not plotted (Supplementary Fig S3.5)

Table 3.4 Cumulative contribution of most influential species responsible for nMDS clustering (DNA and cDNA level). Similarity percentage analysis (SIMPER).

Clusters	Influential microbial groups	Cumulative Contributions
day 0 – day 1	<i>Enterococcus</i>	38
	<i>Clostridium</i>	53
	<i>Corynebacterium</i>	60
	<i>Lactobacillus</i>	64
	<i>Bifidobacterium</i>	70
	<i>Unclassified Bifidobacteriaceae</i>	73
Day 1 – day 3	<i>Enterococcus</i>	46
	<i>Lactobacillus</i>	80
Day 3 – day 7	<i>Lactobacillus</i>	41
	<i>Clostridium</i>	61
	<i>Bifidobacterium</i>	77

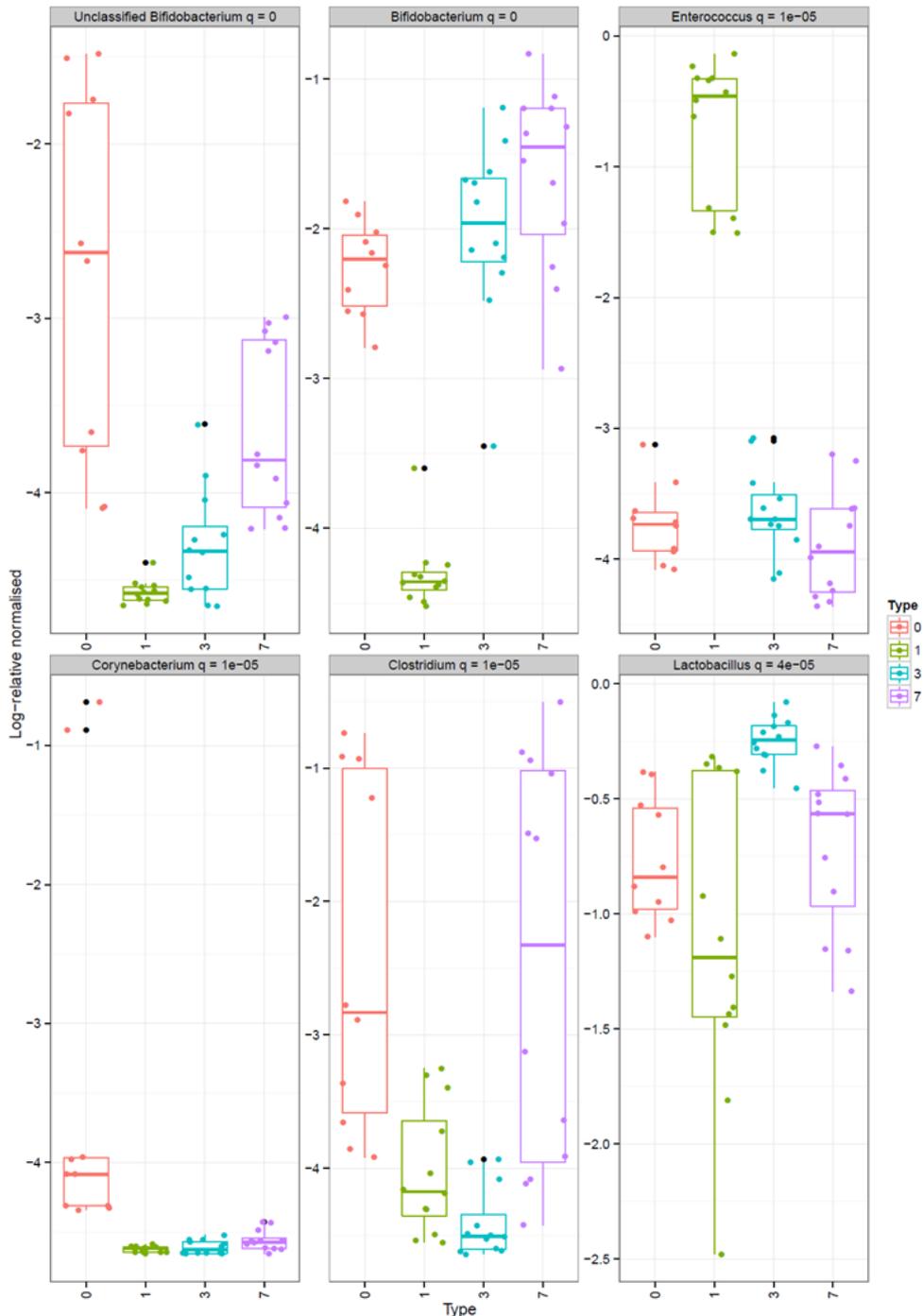


Fig 3.13 Distribution of the most influential species responsible for microbial communities clustering at cDNA level. Numbers on the X axis represent the days

3.3.4.4 Taxonomic diversity in food waste leach-bed reactors

Collectively, the microbial community in the samples contained 288 genera of which only 10% had a relative abundance greater or equal to 1 in at least one sample. The only archaea genera identified with a relatively low abundance ($< 1\%$) was *Methanobacterium*. These major genera with relative abundance greater than 1% at each sampling point accounted for 95% of the

total microbial sequences obtained at DNA level on day 0 and 99% on other days (day 1, 3 and 7). This indicated that communities with relatively low abundance were mostly present on day 0. The bacterial diversity at the genera level in both DNA and cDNA samples were different over time as shown in Figure 3.14. A clear shift of the major microbial groups at cDNA level (digestate and leachate) was observed over the time (Fig 3.14 B). The major shift between day 0 and day 1 was the rapid increase in the relative abundance of *Enterococcus* from less than 1% on day 0 to 55% (on average) on day 1 while the major discriminator between day 1 and day 2 was the high increase in the relative abundance of *Lactobacillus* and the sudden reduction in the relative abundance of *Enterococcus*. Finally, the major communities changed between day 3 and day 7 was the occurrence of *clostridium* species. *Lactobacillus* was the only genus present in each reactor from day 0 to day 7 with relatively high abundance (Fig 3.14). On day 0 species affiliated with *Weissella* were only found in the digestate and not in the leachate fraction, indicating that their original source was likely the food waste rather than the inoculum. In addition, they were mostly found to be active in the digestate fraction on day 0 and day 1, suggesting their implication in the solubilisation and acidification of the RFW during the early stage of the process (Fig 3.14).

The digestate and leachate microbial communities profiling from DNA was compared to that of the cDNA by using nMDS analysis. The results indicated that communities profiling from DNA clustered away from the one from cDNA in both digestate (Supplementary Fig S3.6 A) and leachate (Supplementary Fig S3.6 B) fractions.

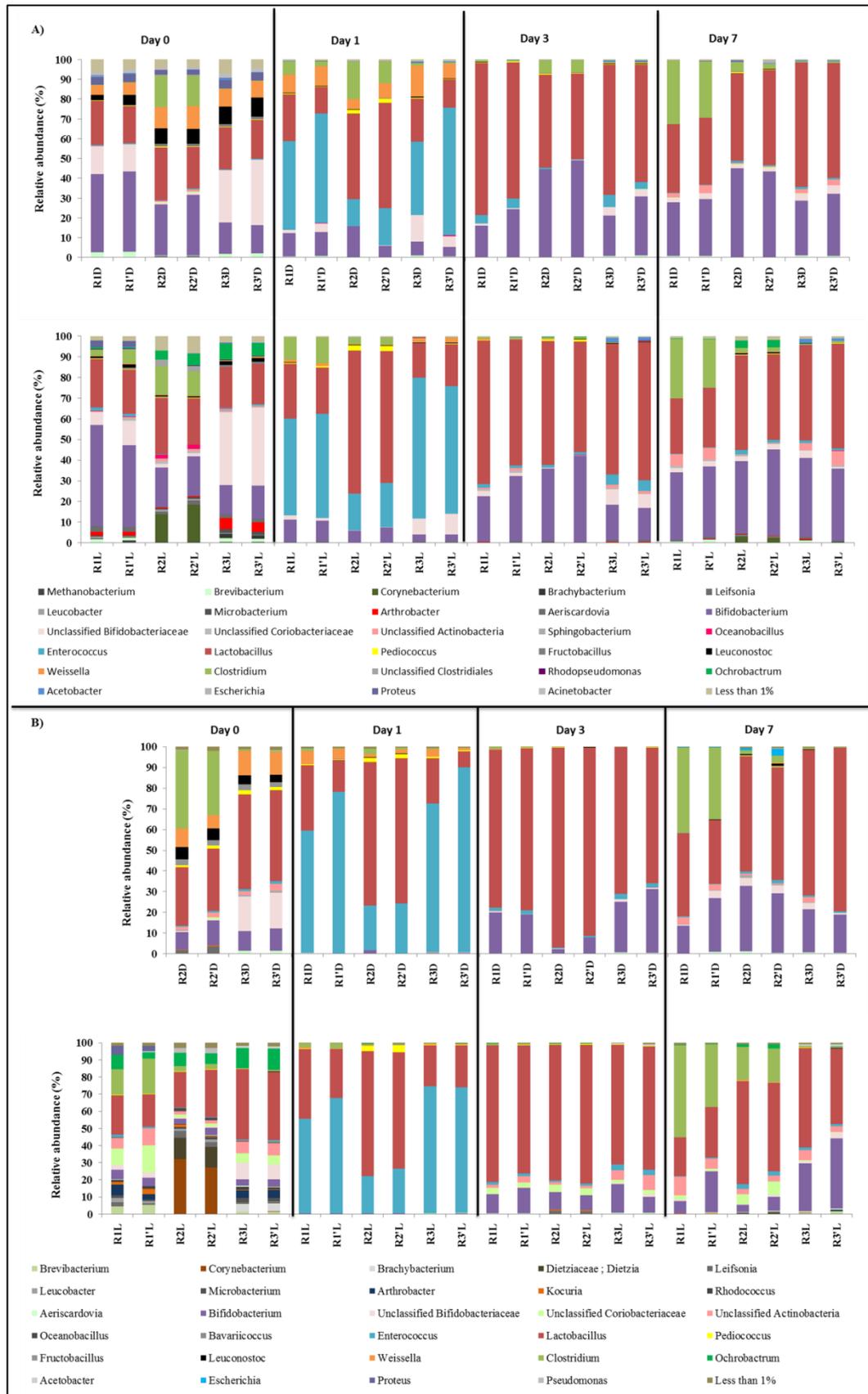


Fig 3.14 Representation of genera identified in the leachate and digestate of R1, R2 and R3 from DNA samples (A) and cDNA samples (B), assigned from the *16S rRNA* gene sequencing. D stand for digestate and L for leachate.

3.3.5 Correlation between influential species and reactor performances

Given the fact that the relative abundance of *Enterococcus*, *Lactobacillus*, *Bifidobacterium* and *Clostridium* in the leachate (at cDNA level) varied over time (Fig 3.14), it was decided to investigate whether they also had an impact on the process performance in term of VFA and organic acid production. Pearson correlation coefficient (r) was measured to determine the strength of a linear association between microbial community's activities and organic acid production over the time. Lactic acid production and *Lactobacillus* distribution displayed a similar trend in all three reactors. The Pearson correlation coefficient revealed r values of 0.92, 0.75 and 0.99 for R1, R2, and R3, respectively, indicating a strong correlation between the production of lactic acid and the detection of *Lactobacillus*. Similarly, the production of butyric acid and the distribution of *Clostridium* species in R1 and R3 appeared to follow the same trend. This observation was confirmed by r values of 0.92 and 0.90 for R1 and R2, respectively. Acetic acid production correlated with *Bifidobacterium* and to some extends with *Clostridium* while some weak correlation was found between caproic acid and *Clostridium* in all three reactors (Table 3.5).

Table 3.5 Pearson correlation coefficient (r) between VFA production and influential species obtained from R1, R2 and R3 leachate.

R1	<i>Bifidobacterium</i>	<i>Lactobacillus</i>	<i>Enterococcus</i>	<i>Clostridium</i>
Acetic acid	0.86	0.00	-0.47	0.78
Butyric acid	0.48	-0.50	-0.17	0.92
Caproic acid	-0.22	-0.96	0.19	0.69
Lactic acid	0.00	0.92	0.00	-0.82

R2	<i>Bifidobacterium</i>	<i>Lactobacillus</i>	<i>Enterococcus</i>	<i>Clostridium</i>
Acetic acid	0.74	0.66	-0.28	0.47
Butyric acid	-0.68	-0.83	0.10	-0.25
Caproic acid	-0.83	-0.72	0.31	0.22
Lactic acid	0.77	0.75	-0.26	0.28

R3	<i>Bifidobacterium</i>	<i>Lactobacillus</i>	<i>Enterococcus</i>	<i>Clostridium</i>
Acetic acid	0.98	0.33	-0.47	0.47
Butyric acid	0.41	-0.42	-0.21	0.90
Caproic acid	0.14	-0.73	0.17	0.71
Lactic acid	0.48	0.99	-0.82	-0.01

3.4 Discussion

3.4.1 Two days maximum hydrolysis of restaurant food waste in leach-bed reactors

Both increase in sCOD concentration and reduction of VS can be used to assess the rate and extent of hydrolysis of the RFW. The sCOD of the FW used for each batch represented only 7% of the tCOD of the FW indicating that the concentration of easy assimilable substrate (soluble components) was low; therefore, an increase of sCOD in the leachate was likely to indicate the solubilisation of the different fraction of the FW. In this study, it was observed that the production of VFA and methane during the initial 2 to 3 days of the process was low; therefore the rapid increase of the sCOD in the leachate of the triplicate reactors during the initial two days of operation were likely due to the accumulation of amino acid, sugar and fatty acid, resulting from the hydrolysis of proteins, carbohydrates and fats (fraction of the RFW), respectively. Since the sCOD concentration in the leachate from day 3 to the end of the process did not increase further, it was concluded

that maximum hydrolysis extent was achieved during the initial two days of reactor operation. In the study of He *et al.* (2012) on the impact of temperature on the hydrolysis of food waste, maximum sCOD production was only achieved after 5 days of operation under mesophilic condition. In the present study, it was believed that reactors design, in combination with the method of inoculation, based on the use of part of the digestate and leachate from previous batch as inoculum for the new batch might have favoured development of specialised community which contributed to achieve maximum hydrolysis in two days. The inoculation strategy used in this study could be comparable to enrichment culture method in which only communities capable of growing under the condition that prevail inside the leach-bed reactors were present over the time.

The increase in ammonia concentration in the leachate was indicative of protein solubilisation. In AD process, proteins are firstly broken down into amino acids, which are subsequently converted into ammonia and different types of organic acids. The extent of protein degradation is usually evaluated by using ammonia nitrogen (Selvam *et al.*, 2010; Wang *et al.*, 2014). For all batches, the concentration of ammonia increased sharply during the initial 3 days of the process and was likely due to rapid solubilisation and acidification of the RFW protein fraction. The increase in the pH of the leachate observed towards the end of each SRT was believed to be due to the existence of a buffering system, resulting from the release of free ammonia during fermentation of protein and amino acids (Forster-Carneiro *et al.*, 2008).

The average VS destruction of $65.04 \pm 4.28\%$, obtained in this study, was lower compare to the VS destruction efficiency of 80% reported for the same RFW in previous study (chapter 2 section 2.3.1). The discrepancy of the VS destruction efficiency was attributed to operating parameters applied during these two experiments. Indeed, during the first experiment where 80% VS destruction efficiency was achieved, methanogenic conditions (pH 7, high ratio of inoculum to RFW and mixing) were favoured while during the second experiment which resulted in 65% VS destruction efficiency, acidogenic conditions (pH < 6 and high loading concentration). It is clear

that the degradation of the fat fraction of this RFW was poor (less than 20%) under acidogenic condition as reported in the present study compare to the methanogenic condition during which 83.62% of the fat were destroyed (chapter 2 section 2.3.1). The VS reported in the present study under acidogenic condition was within the range of VS destruction from FW previously reported (Wang *et al.*, 2002). A higher VS destruction of 76% was, however, achieved by previous study also dealing with AD of food waste (Tampio *et al.*, 2014). In their study, the operational set-up included continuous stirring and trace elements supplementation, which may have contributed to improve the process. In the present study, the reduction of VS from the solid bed was attributed to the breakdown of the RFW which was believed to occur within the initial two days of the process as indicated by the maximum sCOD accumulation. Therefore it was believed that similar VS reduction could have been achieved by applying 2-day SRT.

Hemicellulose, cellulose, protein and fat reduction from the RFW in the replicated reactors during batch 7 was attributed to a net solubilisation. With 99% removal efficiency hemicellulose, which was the highest organic matter in the RFW was likely the preferred substrate for microorganisms. Fat was believed to be recalcitrant as only 20% on average was destroyed. Cellulose degradation was likely limited by their availability as they constituted the smallest fraction of the RFW. The result of this study suggested that 2-day SRT could be envisaged for the solubilisation of FW inside leach-bed reactors.

3.4.2 Effect of fermentation time on volatile fatty acids and organic acids production

During the acid forming stage, the ratio of organic acids (VFAs, alcohols and lactic acids) to sCOD can be used as a proxy for fermentation efficiency, as it shows the amount of soluble products from hydrolysis converted into fermentation products (Jiang *et al.*, 2013; Wang *et al.*, 2014). Higher organic acids/sCOD ratios, of between 85% – 98%, were achieved in the leachate of the triplicate reactors at the end of the retention time (day 13). However when considering only the VFA fraction of the organic acids,

this ratio was between 66% - 77%, indicating therefore that VFA was the highest fraction of the sCOD. This indicated firstly that only 2% - 15% of sCOD in the leachate was not acidified after 13 days of operation and, secondly, that VFAs generation was the preferred metabolic pathway for fermenters. The studies of Wang *et al.* (2014) and Jiang *et al.* (2013) reported similar VFAs/sCOD ratios, however their retention time varied between 20 – 100 days. In the present study, although high ratios of VFA/sCOD were achieved at the end of the process, these ratios varied over time within a batch. Indeed much lower ratios of VFAs/sCOD were found on day 1 and day 3 compared to that of day 13. During day 1 and 3 lactic acid/sCOD ratios were the highest (especially in R1 and R3) indicating that lactic acid-type fermentation occurred during the early stage of the process as confirmed by the profile of lactic acid production. Since hydrolysis occurs before fermentation, the ratio of organic acids/sCOD is believed to increase over time before reaching a stable value. Therefore, the lower ratio of organic acids/sCOD found on day 1 and 3 than day 13 suggested that hydrolysis rate were high in the early stage (day 0 – 3) of incubation compare to the latter stage (day 13) where fermentation rates were high instead.

The end products of fermentation, mainly VFAs, can either be transferred to a methanogenic reactor for methane production or one or more of the VFA can be directly recovered for use in pharmaceutical, food and biorefinery industries as well as in agriculture (Agler *et al.*, 2011). Additionally, VFAs produced during primary fermentation can be subjected to further fermentation for the generation of medium chain VFAs, which are easier to recover from broth and are more valuable than short chain VFAs (Agler *et al.*, 2011; Steinbusch *et al.*, 2011).

The production of acetic acid was observed during the initial 7 – 9 days of reactor operation, while butyric, valeric and caproic acid production was mostly achieved towards the end of the retention time (from days 7 – 13). This suggested that two fermentation stages occurred within the reactors, during which: i) short chain VFAs (acetic acid and propionic acids) were firstly generated; followed by ii) medium chain VFAs (Valeric acid

and caproic acid). High concentrations of short chain VFAs, associated with small amounts of ethanol and high hydrogen pressure, have been reported to induce formation of medium chain VFAs (Agler *et al.*, 2011; Steinbusch *et al.*, 2011). Although the hydrogen pressure was not measured in this study, the production of propionic acid was believed to reflect a high hydrogen pressure as indicated by Agler *et al.* (2011). Acetic acid was likely involved in the process of chain elongation to medium and long chain VFAs as indicated in studies of Wang *et al.* (2010) and Steinbusch *et al.* (2011). Similarly, the decrease in lactic acid concentrations from day 3 – 13 was probably indicative of its implication in the process of medium chain VFAs generation.

The potential for lactic acid oxidation to butyric acid was revealed through Pearson correlation, which displayed negative correlation between these two products in the triplicate reactors (Table 3.2). Lactic acid was also likely oxidised to caproic acid in R2 and R3, while in R1 and R3 it was found to be likely reduced to propionic acid. The studies of Steinbusch *et al.* (2011) and Ding *et al.* (2010), demonstrated that the main precursors for butyric and caproic acid production were acetic acid and ethanol in mixed culture fermentation. Herein we reported that lactic acid could also constitute a precursor for butyric and caproic acid which correlate with previous finding that caproic acid formation could be induced by lactic acid (Zhu *et al.*, 2015).

3.4.3 Microbial communities in leach-bed reactors changes over the time

It has long been documented that during the AD process, the acid forming stage is driven by two groups of metabolically linked microorganisms known as hydrolysers and fermenters. Hydrolysis of solid organic matters has been reported to be facilitated by firm attachment of bacteria to the surface of the substrate (Wang *et al.*, 2010). Fermenters, on the other hand, are mostly found in the liquid phase where they metabolise soluble compounds. Analysis of the solid and liquid reactor fraction has previously identified potential hydrolysers and fermenters (Cirne *et al.*, 2007a; Wang *et*

al., 2010). In this study, the microbial community in the digestate and leachate fraction of leach-bed reactors (R1, R2 and R3) were investigated using 16S rRNA profiling from DNA and cDNA samples in order to identify microbial groups responsible for hydrolysis and fermentation of the different components of RFW.

The archaeal communities in the leachate and digestate profiling from DNA and cDNA represented less than 1% of the total microbiota. They were likely inhibited due to the low pH and accumulation of VFAs in the reactors. The microbial communities from each set of leachate and digestate technical replicates (in DNA and cDNA samples) appeared to share 70 – 95% similarity in their bacterial communities. The 5 – 30% dissimilarity observed is likely attributed to the inherent heterogeneity i) of the samples themselves, ii) in the recovery of microbial cells, iii) in the nucleic acid extraction, iv) amplification and v) sequencing. All these steps are reported to constitute source of biases during molecular analysis (McCaig *et al.*, 1999; v. Wintzingerode *et al.*, 1997). The highest community dissimilarity (30%) was observed between digestate technical replicates and was attributed to its complex nature.

The reactors microbial communities shifts revealed by the nMDS analysis suggested the presence of three metabolic phases; i) the first corresponded to hydrolysis (day 0 – day 1 cluster), ii) the second corresponded to primary fermentation (day 1 – day 3 cluster) and iii) the third corresponded to secondary fermentation (day 3 – day 7 cluster). The relative abundance of *Enterococcus* species (profiling from cDNA) clearly increased during the period of maximum hydrolysis, implying a role in the first metabolic phase. The relative abundance of *Lactobacillus*, *Bifidobacterium* and *Clostridium*, on the other hand, appeared to increase with VFAs production, and therefore these organisms were probably responsible for the second (primary fermentation) and third (secondary fermentation) metabolic phases. These three microbial groups have been found in previous studies to be involved in the acidification of solid organic substrates (Sträuber *et al.*, 2012; Xu *et al.*, 2014a; Xu *et al.*, 2014b)

The analysis of the microbial composition of leachate and digestate (at DNA and cDNA level) revealed a higher number of phylotypes on day 0 than other days (day 1, 3 and 7), which could be explained by the fact that on day 0, the different microbial groups were provided by the inoculum (digestate and leachate from previous batch) and the RFW itself (naturally present in the FW). The decrease of the number of phylotypes from day 0 to day 1 may possibly be attributed to the inability of the indigenous FW microbiota to adapt to the environmental conditions prevailing in the reactors. Species affiliated with *Weissella* were believed to come from the RFW as they occurred in the digestate and not in the leachate samples on day zero. The observed relative abundance (through 16S rRNA detection in cDNA samples) of this microbial group on day 0 digestate sample indicated that some decomposition might have occurred in the RFW during storage. *Weissella* were also detected in cDNA samples on day 1, mainly in the digestate fraction indicating their implication in the degradation of some components of the RFW. This correlated with the study of Xu *et al.* (2014b) in which *Weissella* were also found to be involved in the degradation process of FW.

The high relative abundance of *Enterococcus* on day 1, in both leachate and digestate samples (at cDNA level) appeared to correspond to the rapid increase of sCOD during the same period and we believe *Enterococcus* to be strongly involved in hydrolysis, as indicated earlier by the distribution analysis. The increase in the relative abundance of *Enterococcus* in the anaerobic digesters has not been previously highlighted in the literature. To our knowledge, this may be one of the first studies on AD of FW in which *Enterococcus* have been reported as potential hydrolyzers. Such information could be used for further characterisation and inoculum screening. Among the most influential species, *Enterococcus* was the only microbial group whose dynamics did not seem to correlate with the production of VFAs and lactic acids (products of the fermentation stage). The high relative abundance of *Lactobacillus* in the leachate samples (at cDNA level) was likely attributed to the fact that the highest fraction of the RFW was hemicellulose, which represents a suitable substrate for

Lactobacillus. The study of Sträuber *et al.* (2012) on the anaerobic hydrolysis and fermentation of maize silage, a source of hemicellulose and cellulose, identified species affiliated with *Lactobacillus* as the most abundant.

The consistent correlation between *Lactobacillus* dynamics and lactic acid production in all three reactors indicated a role for this genus in production of lactic acid. *Lactobacillus* species were found to be responsible for the generation of lactate during the fermentation of vegetable wastes (Ye *et al.*, 2007). They were also believed to be implicated somewhat in the production of acetic acid in R2 and R3 as indicated by the positive Pearson correlation coefficient ($0.33 < r < 0.66$). *Bifidobacterium* appeared to be strongly involved in the production of acetic acid, while *Clostridium* was likely involved in the generation of butyric acid and caproic acid in R1 and R3. Several *Clostridium* species in anaerobic process have been reported to produce butyric acid (Kuhner *et al.*, 2000; Ye *et al.*, 2007) and caproic acid (Ding *et al.*, 2010; Steinbusch *et al.*, 2011; Zhu *et al.*, 2015). In addition it was reported that some *Clostridium* species could produce acetate, butyrate, lactate, H₂ and CO₂ from glucose (Kuhner *et al.*, 2000). Similar observations were made in the present study regarding the involvement of *Clostridium* species in the production of butyric, acetic and caproic acid.

Apart from *Weissella* species which was digestate specific on day 0, other major microbial groups were detected both in leachate and digestate. This indicated that the main difference between the microbial communities of these two fractions (leachate and digestate) was mainly based on their relative abundance. It was believed that recirculating the leachate on top of the solid bed contributed to homogenise the microbial community in the reactors washing away some hydrolysers from the solid bed.

Overall, *Enterococcus*, *Bifidobacterium*, *Lactobacillus* and *Clostridium* were likely responsible for most metabolic process in both digestate and leachate fraction inside reactors. This association of microbial communities with reactor's performance was inferred from 16S rRNA profiling from both DNA and cDNA; therefore targeted functional studies

are required to decipher the role and implications of these microbial communities in different processes.

3.5 Conclusion

This study demonstrated that food waste organics could be efficiently converted over a short time in leach-bed reactors with production of various valuable end-products, including butyric acid and caproic acid. Further study should aim to enhance the yield of these products through process optimisation. It was demonstrated that maximum hydrolysis of the RFW components could be achieved within two days of reactor operation by using leach-bed reactors configuration. It was also found that hemicellulose fraction of the RFW was easily degraded and likely contributed to most of the VS removal. In contrast, the fat fraction was recalcitrant and would be prone to accumulation if continuous operation was applied. Fermentation of the RFW hydrolysate occurred in 2 metabolic phases characterised by the production of distinct primary (early stage) and secondary (latter stage) fermentation products which appeared to correlate with specific microbial groups. The *Lactobacillus* lactic acid-type and *Bifidobacterium* acetic acid-type fermentation predominated during the primary fermentation whereas *Clostridium* butyric acid-type fermentation predominated during secondary fermentation. Further studies should investigate the correlation between *Lactobacillus*, *Bifidobacterium*, *Clostridium* and the production of lactic acid, acetic acid and butyric acid respectively. This study also reported for the first time the possible implication of *Enterococcus* in RFW hydrolysis. Further investigations, such as for example those involving metaproteomics, are required to test this hypothesis.

CHAPTER 4

ENHANCED HYDROLYSIS AND VOLATILE FATTY ACID ACCUMULATION DURING ANAEROBIC BIODEGRADATION OF FOOD WASTE

Abstract

Food waste (FW) has been identified as attractive sustainable feedstock for the production of chemicals or fuels. The choice of producing fuels or chemicals depends on the cost of the process, the economic added value and the type of feedstock. Therefore, in recent year, many researchers have focussed their studies on the production of carboxylates mainly volatile fatty acids (VFAs) which value could be high especially from feedstock such as FW when compare to the value of methane. Because the value of VFAs depends on their recovery yields, production of medium chain volatile fatty acids (MCFAs) that are easy to recover from the AD process have received significant attention. However up to now, almost all of the bioprocessing studies with MCFAs production have used substrates other than purely organic waste streams. It could be predicted that heterogeneous substrate such as food waste known to be rich in sugar and proteins could represent great opportunity to generate high yield of MCFAs in anaerobic digestion processes under optimised operational conditions without the need for external electron donor. In this study, anaerobic fermentation of restaurant food waste (RFW) was performed using leach-bed reactors (LBRs) to optimise volatile fatty acids (VFAs) and MCFAs production efficiency. During operation, production of caproic acid (MCFA) as the main fermentation products was also investigated. The accumulation of VFAs in the LBRs was affected by the leachate recirculation regime. An increase in the leachate recirculation regime from one to four times per day (the whole leachate volume from the liquid bed was recirculated on top of the solid bed) combined with the reduction of VFAs concentration in the starting liquid from circa 15 to 6 g COD l⁻¹ resulted in a 55% increase of the VFA yield in LBRs. Under these conditions, the concentration of caproic acid produced (21.86 g COD l⁻¹) was the highest recorded during this trial. Volatile fatty acids composition was not affected either by the recirculation regime or by the low VFA concentrations in starting liquid; however it was influenced by hydrolysis rate, pH, loading rate and short chain volatile fatty acids (SCFAs) depletion rate. Caproic acid concentration as high as 23.91 g

COD l⁻¹ (11 g l⁻¹) at a maximum rate of 3 g l⁻¹ d⁻¹ was achieved in batch trials using LBRs leachate. The highest butyric acid yield was achieved in the presence of ethanol supplementation. High-throughput sequencing analysis of the active microbial communities profiling from cDNA revealed that *Clostridium* and *Peptoniphilus* were likely both involved in butyric acid formation whereas *Lactobacillus reuteri* was likely implicated in caproic acid production. These findings indicate that it is feasible to steer fermentation of FW toward production of caproic or butyric acid, at high yields. Their continuous production from reactor systems at high yield could therefore be envisaged.

4.1 Introduction

Food waste (FW) constitutes one of the largest fractions of municipal solid waste and is still considered an environmental burden where landfill disposal is employed (Pham *et al.*, 2015). Given the high energy content of FW, an opportunity exists to shift it from pollutant to renewable resource as FW organics can be readily transformed through anaerobic digestion (AD) processes. Conventionally, the main focus of AD was the production of renewable energy in the form of biomethane. However due to the high investment cost associated with the implementation of AD processes, methane as the sole beneficiary product is often considered to be inadequate in generating a return on investment due to its low market value (Zaks *et al.*, 2011). The exploitation of alternative valuable by-products could therefore represent new opportunities for AD technology. Volatile fatty acids (VFAs) also known as low molecular weight (MW) organic acids constitute one such by-product of high added value, primarily produced during the initial phase of AD process known as the hydrolytic-acidogenic phase (Wang *et al.*, 2014). These higher intermediates have wide application in the biorefinery industry where they constitute feedstock for various biobased products such as biofuel and chemicals. They are also used in the food and beverages industry, as well as in the pharmaceutical and synthetic chemistry fields. In addition, volatile fatty acids are considered as a potential platform for the production of biodegradable polymers (Demirer & Chen, 2005).

The end-products of the hydrolytic-acidogenic phase are carboxylic acids, ethanol, lactic acid and to a large extent VFAs. These VFAs are usually referred to as short chain volatile fatty acids (SCFAs) and are generated during primary fermentation of soluble compounds deriving from hydrolysis. They are made of 2 to 4 carbon atoms and mainly include acetate, propionate and butyrate. The studies of Yesil *et al.* (2014) and Cavdar *et al.* (2011) demonstrated the production of SCFAs from the biodegradation of municipal solid waste by using leach-bed reactors. Acetate and butyrate were successfully produced at pH ranging from 4 – 6

in batch fermentation experiment using FW as substrate (Wang *et al.*, 2014). Short chain volatile fatty acids constitute valuable products when separated from fermentation liquid. However separating them from broth has proven difficult due to their high solubility in water (Li *et al.*, 2010; Yesil *et al.*, 2014) and high oxygen to carbon ratio (Steinbusch *et al.*, 2011). These SCFAs can be subjected to further fermentation in the same system or in separate system for the production of medium chain volatile fatty acids (MCFAs). Medium chain volatile fatty acids are composed of 5 to 12 carbon atoms and can be easily separated from fermentation broth due to their low oxygen to carbon ratio (Steinbusch *et al.*, 2011). Their production can be made possible through chain elongation of existing SCFAs during secondary fermentation (Agler *et al.*, 2011; Ding *et al.*, 2010; Steinbusch *et al.*, 2011). Caproate and caprylate (both MCFAs) have been reported to be formed through chain elongation of acetic acid with ethanol as electron donor under high hydrogen pressure (Steinbusch *et al.*, 2011). In contrast, the study of Ding *et al.* (2010) reported that caproate formation led to the production of hydrogen rather than its consumption. Chain elongation of lactate and butyrate to caproate in the presence of acetate and ethanol has also been mentioned in previous studies (Agler *et al.*, 2011; Ding *et al.*, 2010; Steinbusch *et al.*, 2011).

The yield of the SCFAs and MCFAs obtained during primary and secondary fermentation are dependent on the nature of the feedstock, the extent of hydrolysis, environmental conditions, reactor design and microbial consortia. Total VFAs production from acidogenic fermentation of municipal solid waste in leach-bed reactors were improved by increasing the leachate recirculation rate and by diluting VFAs in the leachate (Cavdar *et al.*, 2011; Yesil *et al.*, 2014). Dogan & Demirer (2009) reported that increasing the organic loading rate contributed to increased acetic acid production and decreased butyric acid formation. In the study of Wang *et al.* (2014), food waste hydrolysis was found to be high at pH 4 while VFA production was high at pH 6. Steinbusch *et al.* (2011) demonstrated higher production of MCFAs at pH 7 than pH 5.5.

Productions of VFAs through primary and secondary fermentation using mixed microbial communities have been reported to be both energy-efficient and cost-effective (Lettinga & Pol, 1991). This is due to the fact that the AD microbial consortia can tolerate the complexity and variability of the substrate as well as the operational conditions required for VFA production (pH, temperature, recirculation rate). In addition, mixed microbial community fermentation does not require sterilisation.

Although an important number of studies are available on the production of MCFAs through chain elongation of short chain carboxylates, almost all of them thus far, have used substrates other than purely organic waste streams with supplementation of external electron donor. Some of these studies involved the use of: i) synthetic substrates, such as ethanol and acetate (Steinbusch *et al.*, 2011); ii) valuable organic substrate streams, such as corn fermentation beer that includes the electron donor ethanol (Ge *et al.*, 2015); iii) a combination of procured ethanol and real food and municipal solid waste (Grootscholten *et al.*, 2014); and iv) yellow water (from wheat, sorghum and corn fermentation) and lactate as electron donor (Zhu *et al.*, 2015). The production of MCFAs from complex substrate such as FW is reported to be influenced by the hydrolysis efficiency and the rate of SCFAs accumulation (Grootscholten *et al.*, 2014; Kleerebezem *et al.*, 2015). Therefore optimisation of hydrolysis and acidification stages is critical. In addition, the production cost of MCFA can be substantially improved by using purely organic wastes as substrates without the need to supplement external electron donor such as ethanol and lactate. Furthermore, a significant knowledge gap remains regarding the microbial populations underpinning the process. Therefore, this study aimed to optimise the production of MCFAs especially caproic acid from pure FW stream while also establishing the structure of the microbial communities involved.

4.2 Materials and methods

4.2.1 Restaurant food waste

Restaurant food waste (RFW) and its characteristics were previously described in Chapter 3 sections 3.2.1 and 3.2.2. The total solid (TS) and volatile solid (VS) percentage of the RFW were $28.19\pm 2.34\%$ and $25.96\pm 2.08\%$ respectively. The hemicellulose, fat, protein and cellulose fractions of the RFW were $32.58\pm 4.48\%$, $27.50\pm 1.45\%$, $20.69\pm 1.17\%$ and $2.82\pm 0.95\%$, respectively. The original particles size of the RFW was manually reduced through shredding with scissor to provide a more homogenous material.

4.2.2 Experimental equipment

In this study, 3 identical LBRs operating at 37°C under semi-continuous mode were used. The reactor design was previously described in Chapter 3 section 3.2.3. Briefly, each reactor consists of an acrylic column, heated through a water jacket, with a total volume of 6 l and a working volume of 3 l (schematic diagram of reactor shown in chapter 3 Fig 3.1). Each reactor has a lid that allows it to be sealed to maintain anaerobic conditions within the reactor. The lid has two openings of which the first serves for the output of biogas and the second was used to recirculate the leachate on top of the solid bed and also to take samples. The bottom of the reactor has an opening used for sampling the leachate inside the reactor.

4.2.3 Operational conditions

The solid retention time (SRT) of the replicate LBRs previously operated at 14-day SRT was reduced to 7-day. Initially, the operational conditions were similar to the one at 14-day SRT as described in Chapter 3 section 3.2.5. The leachate from day 14 of the 14-day SRT last batch was diluted 3 times, the pH adjusted to 7 and 1 litre of the resulting liquid was introduced in the lower part of each reactor constituting the liquid bed. Similarly, the corresponding digestate from day 14 of the 14-day SRT last batch was

mixed with RFW at the ratio of 0.25 (on the VS basis) and 40 g NaHCO₃ was added. The mixture was loaded in the upper part of the reactor where it constituted the solid bed. The total organic loading for each reactor was 80 g VS RFW l⁻¹ with 3 l working volume. The anaerobic bioreactors were operated at 7-day SRT and the whole leachate volume (1000 ml) was recirculated on top of the solid bed once a day (each recirculation lasting for 45 min). Next and subsequent batches were operated under similar conditions with the exception that the inoculum used for each batch operation was taken from the last day of the previous batch.

After running several 7-day SRT batches, the leachate recirculation was increased from once a day (phase 1) to 4 times a day (phase 2). Reactor performance was monitored during both phases by measuring the pH, VS removal and VFA production. A third phase consisted of reducing the concentration of VFA in the starting liquid from circa 15 g COD l⁻¹ to 5 g COD l⁻¹ by performing a 15 times dilution of the leachate from day 7 of the previous batch. Phase 4 involved the bioaugmentation of each reactor with microbial community enriched on cellulose, hemicellulose, protein and fat (see Chapter 2 section 2.2.6). Phase 5 consisted of simulating the removal of VFAs in the leachate on day 2 of the retention period. This was performed by removing half of the leachate from the liquid bed of each reactor and replacing it with water, thereby reducing VFA concentration by half. Finally, phase 6 consisted of increasing the RFW loading concentration in each reactor from 80 g VS l⁻¹ to 120 g VS l⁻¹.

4.2.4 Batch degradation assay

Batch degradation trial was performed over a 7 day period using 500 ml bottles. Each bottle was fed with the mixture of inoculum (R3 digestate from day 7 of batch 18) and RFW at the ratio of 0.25 (inoculum/RFW on the VS basis). A liquid consisting of a 15 time dilution of the leachate from day 7 of batch 17 was added to each bottle. The loading concentration was 80 g VS l⁻¹ with 150 ml working volume. After flushing the headspace with O₂-free gas, the bottles were incubated at 37°C. Each bottle was manually inverted 4 times per day to simulate the recirculation applied during

operation of reactors. Bottles were sacrificed in duplicate at regular intervals (days 0, 1, 2, 3, 4, 5, 6 and 7) and their contents were analysed for TS and VS to determine the period of maximal solid removal and hydrolysis.

4.2.5 Bioaugmentation Assay

Bioaugmentation experiment was performed using the cellulose, hemicellulose, protein and fat enriched cultures previously described in chapter 2 section 2.2.6. Five percent of each enrichment culture obtained after several sub-culturing were mixed in 50 ml tubes and were centrifuged at low speed. The supernatants were discarded and the cells pellets were added to 500 ml bottles containing mixture of digestate (from R1, R2 and R3, day 7 of batch 18) and RFW to a ratio of 0.25 (digestate/RFW, on the VS basis). The operational conditions were similar to the one described in section 4.2.4. After 7 days of incubation at 37°C, the content of each bottle was mixed with some digestate from day 7 of batch 19 and the mixture was used to inoculate reactor R1, R2 and R3 (containing fresh RFW) at the ratio of 0.25 (inoculum/RFW on the VS basis).

4.2.6 Selective production of volatile fatty acids

To investigate the selective production of caproic acids in the fermentation broth, batch fermentation assays were performed using 160 ml vials containing 37 ml of reactor R2 leachate from day 2 of batch 30. All the vials were divided into two sets. In the first set, named uncontrolled pH, the leachate pH was not adjusted whereas in the second set, named controlled pH, the leachate pH was increased to 7 by using NaOH. The initial concentrations of ethanol and acetic acid in all vials were 4 g l⁻¹ and 9 g l⁻¹, respectively. The concentration of ethanol in some of the uncontrolled and controlled pH vials was adjusted in such a way that ethanol concentration was twice the concentration of acetic acid. In some of the controlled pH vials with adjusted ethanol concentration, H₂/CO₂ (at the ratio of 80/20) was injected at 0.5 bar of pressure for 5 seconds.

The assay with pH adjustment to 7 was also performed using reactor R2 leachate from day 2 of batch 31. However this time, some of the vials without ethanol adjustment were pressurised with H₂/CO₂. All the vials were sacrificed in duplicate on day 0, 1, 3, 4, day 6, 8 and 11.

4.2.7 Analytical methods

Soluble chemical oxygen demand (sCOD) concentrations in the leachate samples from the replicate reactors were measured according to the Standing Committee of Analysts (1985). Samples were analysed for TS and VS using standard methods (APHA, 2005). Volatile fatty acid (VFA, C2 – C6) were quantified using a gas chromatograph as described in previous study (Chapter 2 section 2.2.10). All analyses were carried out in duplicates. Lactic acid and ethanol assay kits (Megazyme) were used to measure the concentrations of lactic acid and ethanol respectively according to the manufacturer's instructions.

4.2.8 Ribonucleic acid extraction and generation of complementary deoxyribonucleic acid

For each treatment during the assay on the selective production of caproic acids in batch experiment, 30 ml leachate from sacrificial vials (on day 0, 4, 6 and 11) were added into 50 ml tubes which were then pelleted by centrifugation (8,000 × g, 15 min), immediately flash frozen into liquid nitrogen and stored at -80°C until further use. RNA was extracted from each cell pellet by using the method described by Griffiths *et al.* (2000) with modifications. Briefly, 0.5 ml of 10% hexadecyltrimethylammonium bromide (CTAB) extraction buffer and 0.5 ml of phenol-chloroform-isoamyl alcohol (25:24:1) were added to 2 ml Eppendorf tubes containing frozen microbial cell pellets (from -80°C). CTAB extraction buffer was prepared by mixing equal volumes of 10% (wt/vol) CTAB (Sigma, Poole, United Kingdom) in 0.7 M NaCl with 240 mM potassium phosphate buffer, pH 8.0. To each tube, 0.5 mm and 0.1 mm diameter zirconia beads (Thistle Scientific) were added and the microbial cells were lysed by bead beating for 5 min using a vortex (IKA Vortex Genius 3). The top aqueous layer

were recovered after 10 min of centrifugation at $16,000 \times g$ and transferred to clean tubes. To remove residual phenol from previous step, 0.5 ml of chloroform-isoamyl alcohol (24:1) were added and the aqueous layer was recovered after 5 min of centrifugation at $16,000 \times g$ and transferred to clean tubes. Subsequent precipitation of total nucleic acids were performed by adding two volumes of 30% (wt/vol) polyethelene glycol (PEG) 6000 (Fluka BioChemika)–1.6 M NaCl and incubated at 4°C for 2 hours followed by 20 min centrifugation at $16,000 \times g$. Supernatant were carefully discarded and the pellets were washed by adding 1 ml ice cold 70% ethanol followed by 30 min centrifugation at $16,000 \times g$. Ethanol was completely removed and the pellets left to dry at room temperature for 2 min. Finally, the pellets containing DNA and RNA were resuspended in 50 μl RNase.

RNA purification was carried out using the TURBO DNA-free™ Kit (Ambion by Life Technology) in accordance with the manufacturer's instructions. Polymerase chain reaction (PCR) was performed on treated RNA solution to confirm the removal of any residual DNA following the protocol indicated in previous study (Chapter 3 section 3.2.8). Complementary deoxyribonucleic acid (cDNA) was generated from DNA free RNA samples using the superscript reverse transcriptase III (Invitrogen) in accordance with the manufacturer's recommendations.

4.2.9 Polymerase chain reaction for sequencing analysis

Each cDNA sample was amplified in triplicates. Amplification of archaeal and bacterial V4 region of 16S rRNA using the primer set 515F and 806R (Walters et al., 2011) was performed in a 25 μl reaction mixture containing 1 x Q5® Reaction Buffer, 200 μM of each dNTPs, 0.2 μM of each primer and $2 \times 10^{-2} \text{ U } \mu\text{l}^{-1}$ Q5 High-Fidelity DNA Polymerase (New England BioLabsinc). The PCR conditions consisted of initial denaturation at 94°C for 30 s, followed by 30 cycles of annealing at 50°C for 30 s, and elongation at 72°C for 30 s. The final extension was carried out at 72°C for 2 min. Bacterial and archaeal DNA were used as positive controls. The resulting triplicate PCR products for each sample were combined into a single

volume and purified using NucleoSpin® Gel and PCR Clean-up kit (Macherey - Nagel) in accordance with the manufacturer' instructions.

4.2.10 Amplicon quantification

To determine the concentration of purified amplicons, fluorometric measurements were performed using 1 µl aliquots of purified cDNA solution with a Quant-iT dsDNA HS assay kit and a Qubit fluorometer (Invitrogen), in accordance with the manufacturer's instructions. Amplicons from all samples were normalised to a final concentration of 20 ng µl⁻¹ and sent to an external laboratory (Research and testing laboratory, Texas US) for 16S rRNA amplicon sequencing using MiSeq Illumina platform.

4.3 Results

4.3.1 Impact of leachate recirculation, dilution, bioaugmentation and loading rate on restaurant food waste volatile solid removal in leach-bed reactors

Three identical LBRs operated at 7-day SRT under semi continuous batch mode were used for the biodegradation of RFW. The average VS destruction from the solid beds were the lowest (below 70% removal) when leachate in the replicate reactors were recirculated once a day (Fig 4.1; phase 1) and also when the RFW loading concentration was increase from 80 g VS l⁻¹ to 120 g VS l⁻¹ (Fig 4.1; phase 6). The analysis of variance (ANOVA) revealed that the average VS destruction of phase 1 and 6 were not significantly different with $p > 0.05$, however, their average VS destruction efficiency were significantly different ($p < 0.05$) to that of phase 2 – 5 during which higher VS destruction was achieved (average VS destruction $> 70\%$). The average VS destruction of phase 2 – 5 were not significantly different with p values greater than 0.05. The 95% confidence interval (CI) for the mean of the six phases confirmed that mean VS destruction from phase 1 and 6 were similar as their confidence interval overlapped to a greater extend (Fig 4.2). The confidence interval analysis also confirmed that the mean VS destruction of phase 1 and 6 were the

lowest and significantly different from other phases as their interval were distinct from others. The overlapping of the 95% CI of phase 2 – 5 confirmed that the mean VS destruction for these phases were similar.

The highest VS removal, between 85 – 90 %, was achieved for batch 20 during phase 4 (Fig 4.1) which corresponded to the period where reactors were bioaugmented with cellulose, hemicellulose, protein and fat degraders obtained from enrichment cultures. The batches subsequent to the batch in which the bioaugmentation was performed (batch 20) did not achieve similar high VS destruction.

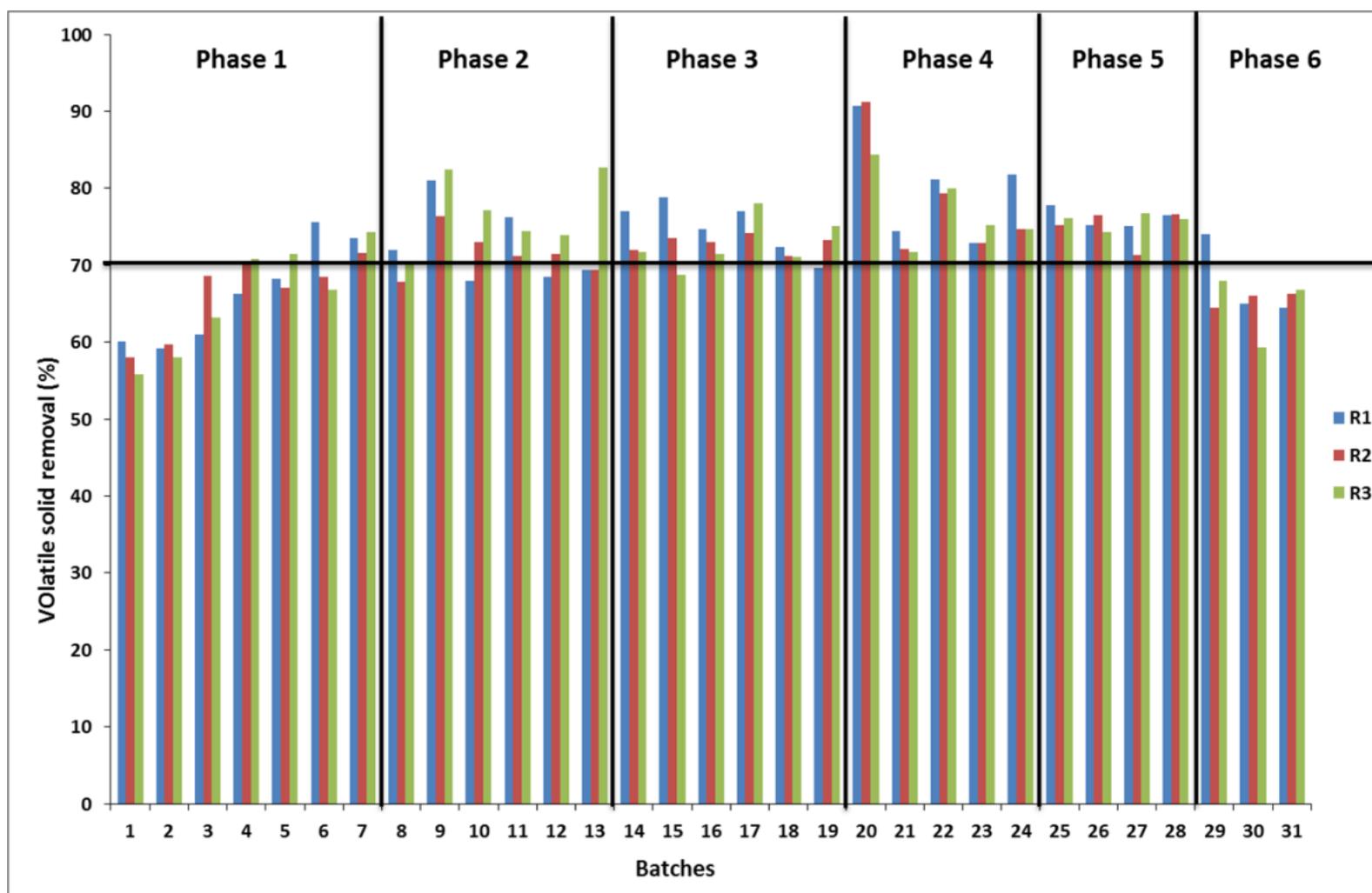


Fig 4.1 Volatile solid (VS) reductions from the restaurant food waste in triplicate reactors R1, R2 and R3 operated at 7-day SRT for each batch. Phase 1: leachate was recirculated on top of solid bed once a day for 45 min; Phase 2: leachate recirculated four times per day; Phase 3: dilution of VFAs in starting liquid; Phase 4: bioaugmentation; Phase 5: dilution of VFA in the leachate on day 2; Phase 6: increase of loading rate. Analysis in each reactor was carried out in duplicate and only the average is represented.

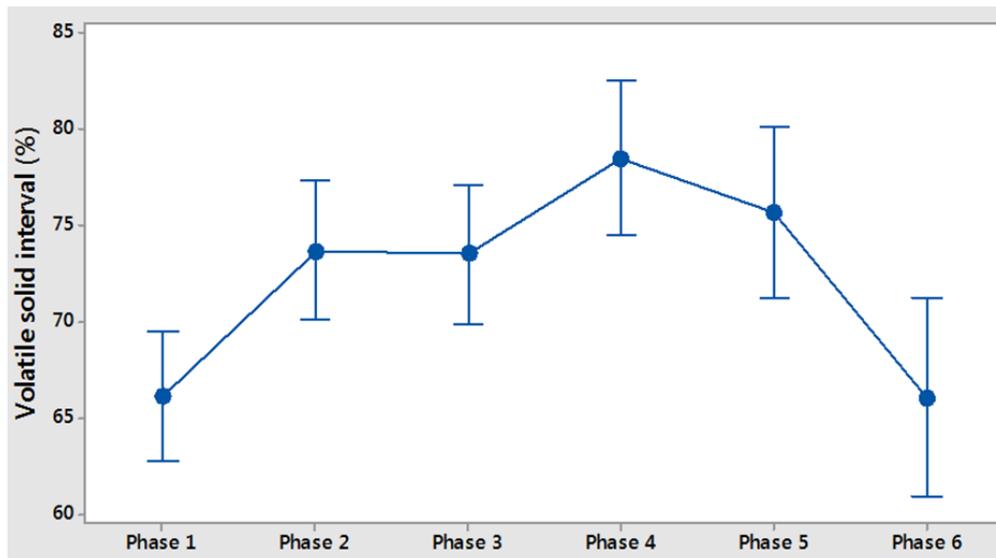


Fig 4.2 Plot of 95% confidence interval for the mean of volatile solid destruction during phase 1 – phase 6.

4.3.2 Impact of solid retention time on volatile solid removal efficiency

Batch biodegradation of the RFW in sacrificial 500 ml bottles using digestate and leachate from reactor R3 on day 7 (batch 18) as inoculum revealed that maximum VS destruction of 70% was achieved after two days of incubation (Fig 4.3). This actually confirmed our previous observations (chapter 3 section 3.3.1) where maximum soluble chemical oxygen demand was achieved within the initial two days of the RFW biodegradation process, which corresponded to maximum VS destruction. The overall average VS destruction from the batch test performed in bottles b1 and b2 (Fig 4.3) was quite comparable to that of reactor R3 (batch 19) inoculated with the same digestate and leachate (Fig 4.1)

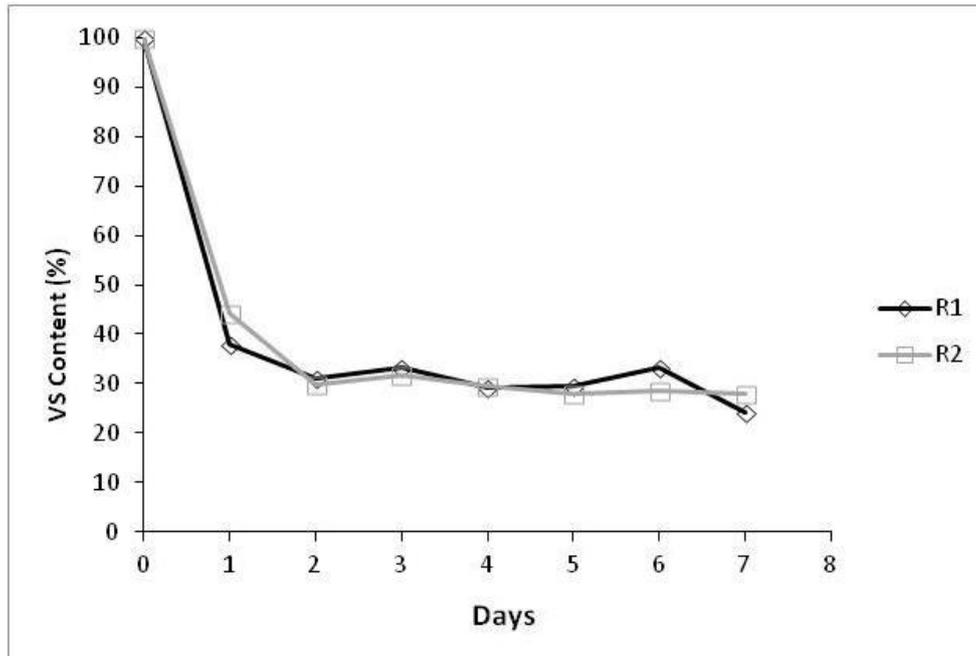


Fig 4.3 Batch degradation of RFW using reactor R3 digestate and leachate from batch 18 as inoculum, with b1 and b2 corresponding to bottle 1 and 2 respectively. Data at each point is the average of duplicate measurements.

4.3.3 Effect of pH, leachate recirculation, substrate concentration and bioaugmentation on VFAs production.

In the semi-continuous batch operation, the yield of VFAs and their composition varied according to the treatment applied. The highest VFA concentration in the range of 72 – 76 g COD I⁻¹ (47 – 48 g I⁻¹) was achieved in phase 3 (Fig 4.4). During this phase, the contribution of caproic, acetic and butyric acids to the total VFAs concentration were 29%, 28 and 24% respectively. These three VFAs represented 80% of the accumulated VFAs, and the remaining 20% was shared between propionic and valeric acids. The lowest VFA concentration between 20 – 33 g COD I⁻¹ was achieved in phase 6. During this phase, acetic and butyric acids constituted the principal individual VFA and contributed to 58% and 34% of the accumulated VFA respectively. Although acetic, propionic, butyric and caproic acid were the principal VFAs produced during the different phases, other VFAs including isobutyric, isovaleric, valeric and isocaproic were also detected; They contributed to 6%, 18%, 20%, 13%, 29% and 8% of VFA accumulated in phase 1, 2, 3, 4, 5 and 6 respectively.

Caproic acid was produced in all phases except phase 6 in which the RFW loading was increased from 80 g VS l⁻¹ to 120 g VS l⁻¹ (Fig 4.4). Caproic acid concentration of 21.86 ± 0.57 g COD l⁻¹ (10 ± 0.26 g l⁻¹), 17.60±2.70 g COD l⁻¹ (8.00 ± 1.22 g l⁻¹) and 12.73±2.11 g COD l⁻¹ (5.77± 0.96 g l⁻¹) were achieved during phase 3, 2, and 1 respectively. During these phases, caproic acid was the highest constituent of the VFA accumulated in the leachate. In phase 4, butyric acid concentration was the highest instead. Acetic acid production dominated in phase 5 and 6 (Fig 4.4).

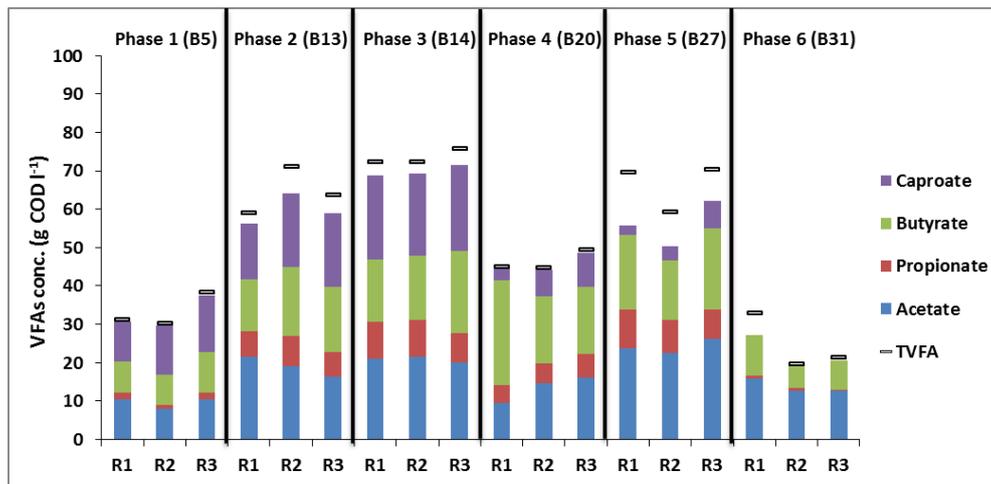


Fig 4.4 Profile of individual and total volatile fatty acids (TVFA) concentrations in leached reactors R1, R2 and R3 under different treatments (phases). Phase 1 (batch 5): leachate was recirculated on top of the solid beds once a day for 45 min; Phase 2 (batch 13): Leachate was recirculated four times per day; Phase 3 (batch 14): dilution of VFAs in starting liquid; Phase 4 (batch 20): bioaugmentation; Phase 5 (batch 27): dilution of VFA in the leachate on day 2; Phase 6 (batch 31): increase of loading rate. Data at each point is the average of duplicate measurements.

During phase 1 – 4 the pH of the three reactors dropped within the initial 3 days of incubation from circa 7.5 to 5.2 and subsequently increases in the range of 5.8 – 6.8 toward the end of the incubation period (Fig 4.5). The decrease in pH was quite pronounced for phase 5 and 6 with values below 5 within the initial day of incubation. Caproic acid concentration was found to be high when the pH reached a value of 6 and above. At low pH < 5.5 caproic acid production appeared to be inhibited and completely ceased at pH < 5.

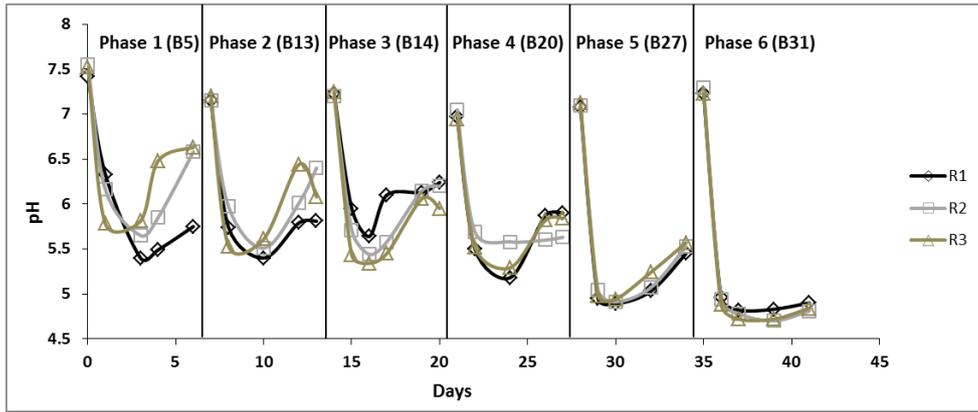


Fig 4.4 pH profile in the leachate of the replicate reactors R1, R2 and R3 for B (batch) 5, 13, 14, 20, 27 and 31.

During phase 2, VFA production rates were high within the initial 3 days of the incubation whereas during phase 3 the production rates were high over the last three day of the process (Fig 4.5). Overall, higher total VFA production was achieved in phase 3 than phase 2 (Fig 4.5).

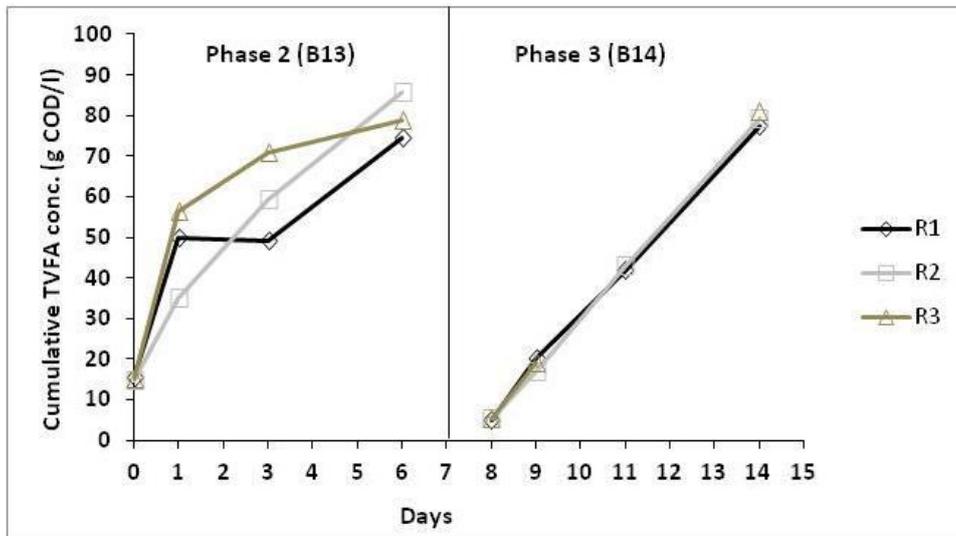


Fig 4.5 Profile of the cumulative VFAs in the leachate of the replicate reactors R1, R2, and R3. Batch (B) 13 and 14 corresponding to high and low VFA in starting liquid respectively. Data at each point represent average of duplicate measurements.

Maximum acetic acid production was achieved within the initial 3 days of the reactor run during both phase 2 and 3 (Fig 4.6 A). Production of propionic, butyric and caproic acids were lower within the initial 3 days of the process during phase 3 than phase 2 (Fig 4.6 B, C and D)

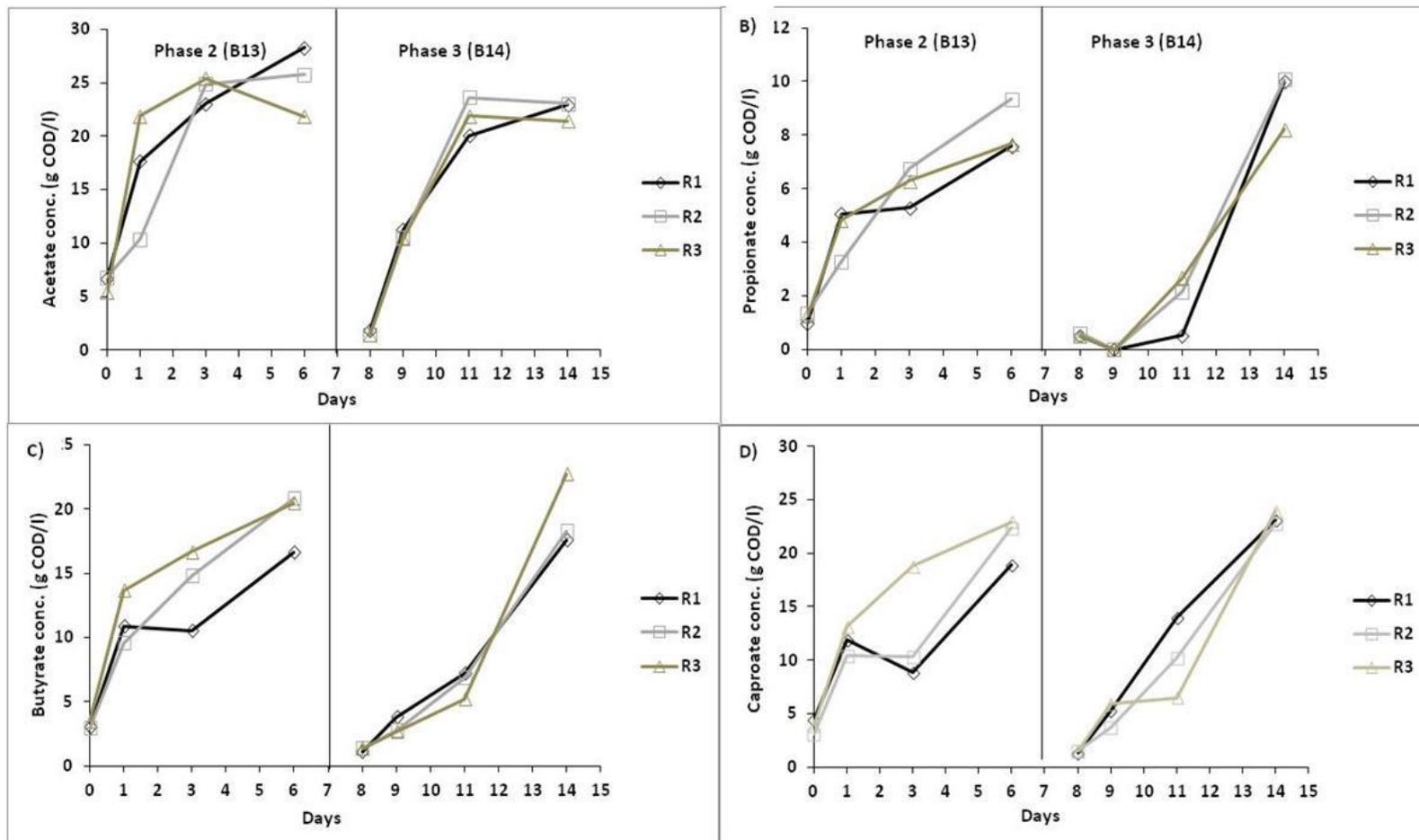


Fig 4.6 Profile of individual VFA production in leach-bed reactors R1, R2 and R3 during Phase 2 and 3. Batch (B) 13 and 14 were selected to represent phase 2 and 3 corresponding to high and low VFA concentrations in starting liquid respectively. Data at each point represent average of duplicate measurements.

4.3.4 Effect of ethanol, hydrogen and pH on caproic and butyric acids production in batch experiment

Batch experiment was performed using 160 ml vials in which 37 ml leachate collected from reactor R2 on day 2 (phase 6, Batch 31) was added. VFA to a chain length of 6 carbon atoms was produced from the different combination applied (see section 4.2.6)

Acetic acid and butyric acid production was observed within the initial day of incubation whereas propionic and caproic acid (Fig 4.7) production showed a lag phase. At the end of the incubation period, acetic and propionic acid concentrations were higher in the control and ethanol supplemented vials than in the H₂ and H₂/ethanol supplemented vials (Fig 4.7 A and B). Butyric acid production reached 17 g COD l⁻¹ (9 g l⁻¹) in ethanol supplemented vials whereas in the control and other treatments only 7 – 9 g COD l⁻¹ (4 – 5 g l⁻¹) was achieved (Fig 4.7 B). Higher caproic acid concentration of 23 g COD l⁻¹ (10 g l⁻¹) were achieved after 6 – 8 days of incubation in both H₂ and H₂/ethanol supplemented vial than in the control vial in which only 10 g COD l⁻¹ (4 g l⁻¹) was achieved (Fig 4.7 D). Caproic acid was formed in the control, H₂ and H₂/ethanol supplemented vials, but was not produced in vials supplemented with only ethanol. At uncontrolled leachate pH of 4.78, the accumulation of acetic acid was comparable to the one at pH 7, however butyric and caproic production was not recorded (Supplementary Fig S4.1 A, B, C and D).

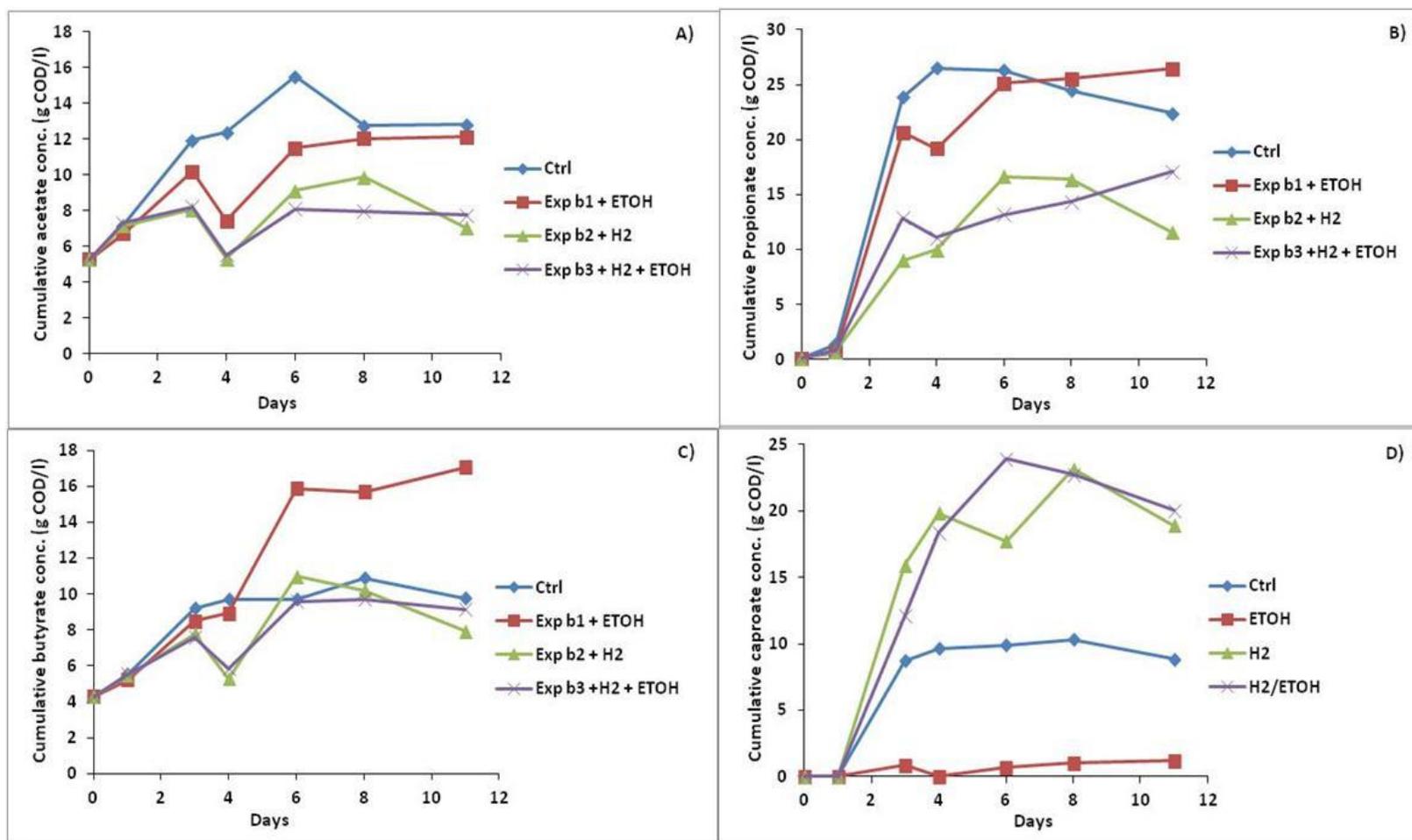


Fig 4.7 Production pattern for acetic (A) propionic (B), Butyric (C), and caproic (D) acid obtained in the control (ctrl), ethanol (ETOH), hydrogen (H₂) and H₂/ETOH supplemented vials (Exp b1 – b3) during batch experiment using leach bed-reactor leachate. Data at each point is the average of duplicate analysis.

Lactic acid was completely depleted within the initial 3 days of the incubation in the control vial whereas in H₂ and H₂/ethanol treated vials lactic acid was depleted by day 4 (Fig 4.8). The lowest lactic acid removal rate was observed in ethanol treated vials (within the initial 6 days) (Fig 4.8). Ethanol on the other hand showed a slight overall increase in all treatments at the end of the incubation period (Fig 4.9).

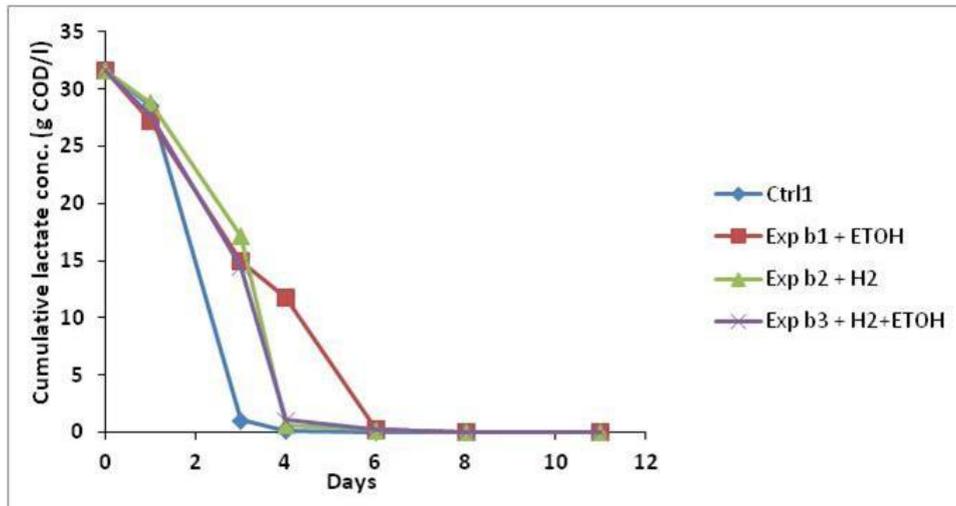


Fig 4.8 Profile of the lactic acid in the control (ctrl1), ethanol (ETOH), hydrogen (H₂) and H₂/ethanol supplemented vials (Exp b1 – b3) obtained during batch experiment using leach-bed reactor leachate. Value at each point is the average of duplicate analysis.

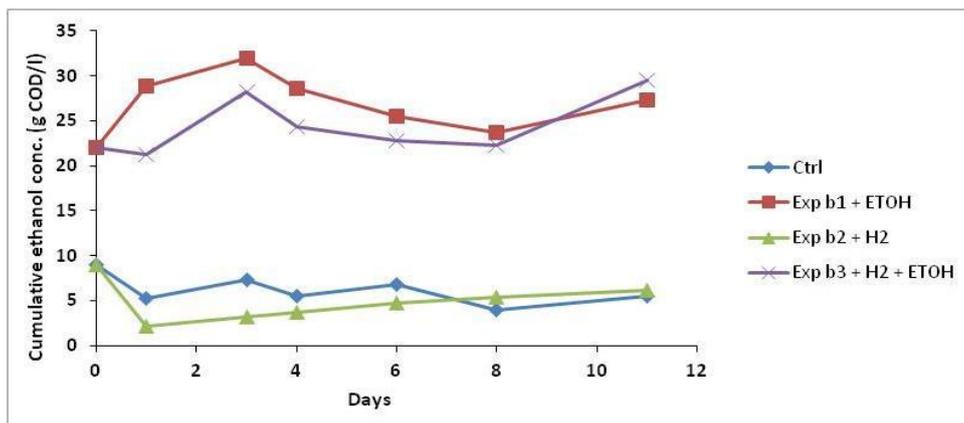


Fig 4.9 Profile of ethanol concentration in the control (ctrl1), ethanol (ETOH), hydrogen (H₂) and H₂/ETOH ethanol supplemented treated vials (Exp b1 – b3) obtained during batch experiment using leach-bed reactor leachate. Value at each point is the mean average of duplicate analysis.

4.3.5 Microbial communities involved in the production of butyric and caproic acids

The different microbial groups involved in the production of butyric and caproic acids were investigated by 16S rRNA profiling from cDNA samples. *Clostridium*, *Lactobacillus* and *Bifidobacteriales* were likely the major active microbial groups on day 0 (Fig 4.10) and therefore are likely to be involved in the production of acetate, butyrate, lactic acid and ethanol which constituted the main fermentation products on day 0 (Fig 4.7, 4.8 and 4.9). *Peptoniphilus* relative abundance rapidly increased from less than 1% on day 0 to 20 – 30% on day 4 in the control, ethanol, and H₂/ethanol supplemented (Fig 4.10 A, B and D) vials. Microbial species within the genus *Peptoniphilus* were identified as *Peptoniphilus stercorisuis* (Supplementary Fig S4.2). In ethanol supplemented vials, *Anaerosalibacter* relative abundance increased from less than 1% on day 0 to 7% on day 4 and subsequently increased to 15% on day 11. However in the control and H₂ treated vials, *Anaerosalibacter* relative abundance was low during the experimental time frame. An increase in the relative abundance of *Anaerosalibacter* was also detected in H₂/ethanol supplemented vials from day 0 – 1. Given the fact that caproic acid production was not recorded in ethanol supplemented vials, *Anaerosalibacter* were likely not involved in the production of caproic acid observed in H₂ and H₂/ethanol supplemented vials. Microbial community members affiliated with *Clostridium* in all treatments were identified as *Clostridium cochlearium* and *Clostridium sp* (Supplementary Figure S4.2). The relative abundance of *Clostridium cochlearium* increased within the initial 4 – 6 days of incubation in all treatment whereas *Clostridium sp* relative abundance only increase from day 6 – 11 in H₂ and H₂/ethanol treated vials. Although *Lactobacillus* relative abundance were decreased over time in all treatments, that of *Lactobacillus reuteri* was found to increase from day 0 – 6 in the H₂ and H₂/ethanol treated vials (Supplementary Fig S4.2 C and D). A small increase in the relative abundance of *Lactobacillus reuteri* was also observed from day 0 – 1 in the control vials (Supplementary Fig S4.2 A). Methanogenic species were not identified in all the treatments Even though pH 7 was favourable

for their growth. This actually suggested that they were not present in the R2 leachate (batch 31) used to set up this batch experiment.

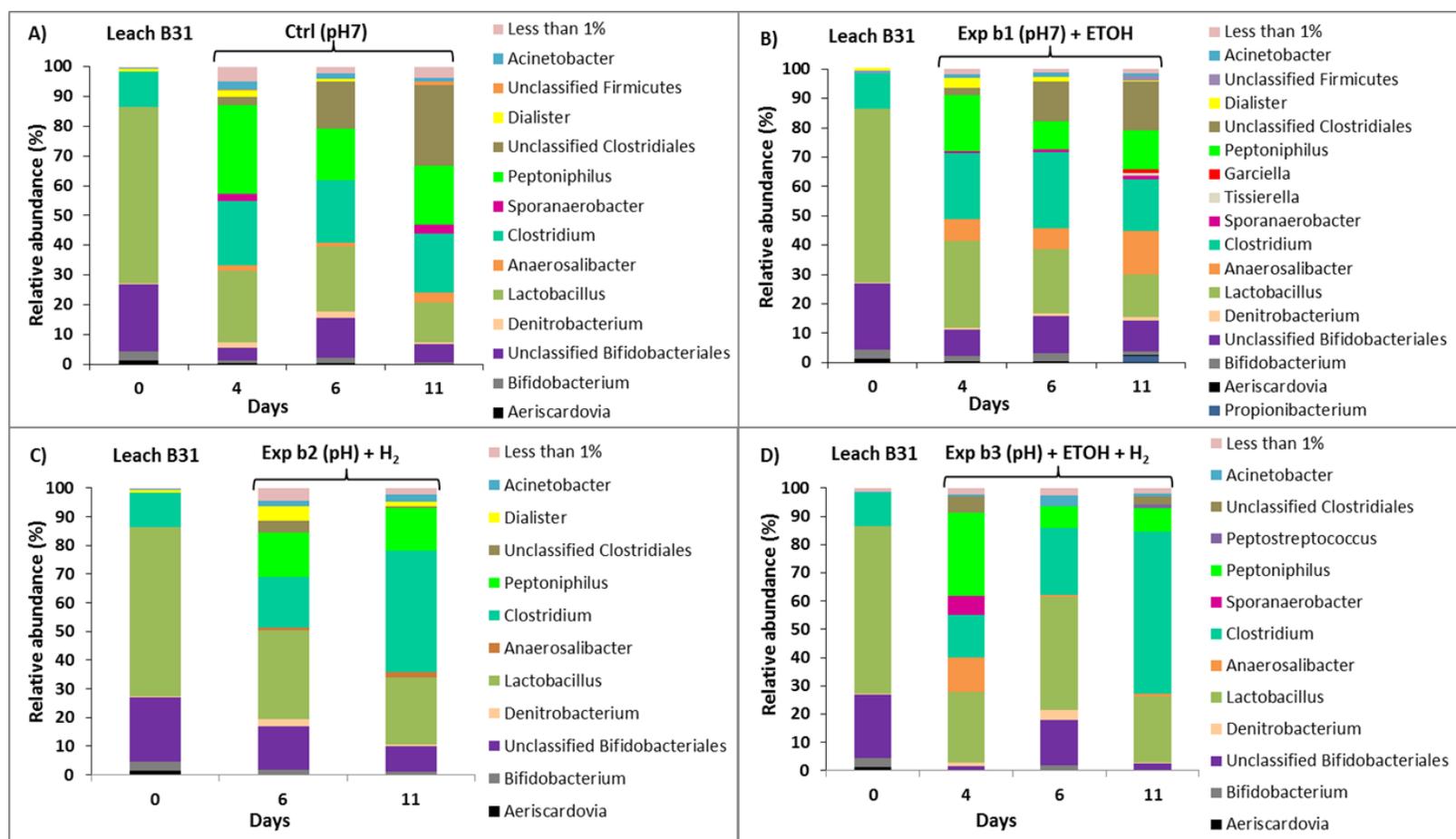


Fig 4.10 Taxonomic classification of the 16S rRNA sequences retrieved from: A) control (ctrl1); B) ethanol (ETOH); C) hydrogen (H₂) and D) H₂/ETOH supplemented vials (Exp b1 – b3) obtained during batch experiment using leach-bed reactor leachate. Leach B31: leachate collected from batch 31 on day 2.

4.4 Discussion

4.4.1 Hydrolytic-acidogenic phase influenced by recirculation rate, dilution and bioaugmentation

In this study, increasing the recirculation regime from once per day to 4 times per day clearly improved the VS destruction by 10% in the replicate leach-bed reactors indicating an increase in hydrolysis efficiency. Leachate recirculation from the bottom to the top of the reactors created a mixing effect in the solid bed thereby likely facilitating the distribution of enzymes and microorganisms throughout the bed. Griffin *et al.* (1998), Stroot *et al.* (2001), McMahon *et al.* (2001), McMahon *et al.* (2004) reported that high mixing conditions in reactors resulted in fast hydrolytic-acidogenic activity and lower methane production. In the present study, the highest VS destruction obtained straight after bioaugmentation (supplementation of enrichment cultures) likely indicated a further increase in hydrolysis efficiency. It was demonstrated in previous work (Chapter 2 section 2.3.4) that supplementing these enriched cultures to granular sludge during batch degradation of RFW contributed to increased VS destruction by 10%. Bioaugmentation with hydrolytic bacteria has proven to be beneficial in several previous studies. Indeed, Cirne *et al.* (2006) reported on the improvement of lipid hydrolysis using bioaugmentation with an anaerobic lipolytic bacterium, isolated from bovine rumen, during anaerobic digestion of lipid-rich restaurant waste. Degradation of manure fibres was also improved by using hemicellulose degrading bacterium as supplement (Angelidaki & Ahring, 2000). Nielsen *et al.* (2007) demonstrated that reactor bioaugmentation with cellulose and hemicellulose degrading bacteria contributed to improve biodegradation of cattle manure. Although bioaugmentation strategies appeared to be a cheap and simple procedure for improving hydrolysis of solid organic materials, the ability of the enriched microorganisms to adapt to the new environment and establish in reactor systems are perceived as poor (Nielsen *et al.*, 2007). In the present study, the reason for the drop of VS destruction efficiency from batch 21 to 24 (Fig

4.1) can likely be attributed to the inability of the enriched microbes to subsist in the reactors. They were likely washed out of the system as only one fifth of the leachate and part of the digestate from batch 20 was used to start up batch 21. The positive effect of bioaugmentation on biodegradation of organic substrates in previous studies lasted for 18 days (Nielsen *et al.*, 2007); these authors reported that washout of supplemented bacteria was likely the cause of the drop in solid degradation efficiency.

The total VFA yields increased on average by 49% when leachate recirculation was increased from once per day to 4 times per day. In addition, a further 6% VFA yield improvement was observed when the VFA concentrations in the starting liquid was reduced from circa 15 g COD l⁻¹ to 6 g COD l⁻¹. This indicated that the acidification stage could be optimised by applying a combination of high leachate recirculation regime and low initial VFA concentrations. Similar findings were reported in the studies of Yesil *et al.* (2014) and Cavdar *et al.* (2011) on the anaerobic acidification of municipal solid waste. In the present study, the order of VFAs accumulated in term of yield was as followed: caproic acid ≥ acetic acid > butyric acid > propionic acid, for phases 1 – 3. This was surprising because caproic acid has never been reported as being the highest VFA constituent from direct fermentation of FW. Previous studies on acidification of organic solid waste, including FW, reported the following VFA accumulation order: acetic acid > propionic acid > butyric acid > valeric acid > caproic acid (Cavdar *et al.*, 2011; Wang *et al.*, 2014; Yesil *et al.*, 2014). In our previous study involving biodegradation, of the same RFW, in leach-bed reactors operated at 14-day SRT (leachate recirculation once a day), butyric acid yield was found to be the highest followed by acetic acid, propionic acid and then caproic acid (Chapter 2 section 3.3.3). VFA composition with caproic acid as the highest VFA reported in this study was believed to be mainly due to a combination of parameters including: i) FW composition; community composition; and iii) operating parameters (pH, recirculation regime and inoculation method). Given the fact that the only difference between phase 1 and the previous reactors operation was the solid retention time, it was suggested that SRT might likely have an impact on the

composition of VFAs generated during acidification of RFW in leach-bed reactors. The average VFA yield obtained in the previous study at 14-day SRT was comparable with the one achieved in the present study at 7 day SRT (phase 1: one recirculation a day). However, with 4 times leachate recirculation per day and low initial VFA concentrations, the VFA yield was twice higher than the one achieved with 1 recirculation per day.

The high VS removal achieved for batch 20 (phase 4, Fig 4.1) did not really translate to high VFA production. Indeed, the VFA yield obtained in phase 4 was 1.6 times lower than the one obtained in phase 3 and yet the opposite was observed for VS destruction which was higher for phase 4 than phase 3. This suggested that high hydrolysis activities due to the bioaugmentation might have somewhat negatively impacted acidification. The VFA yields and VS destruction achieved for phase 6 (high loading) were the lowest on average 24.70 g COD l⁻¹ and 66% respectively. This was likely due to an initial rapid release of soluble particulate from the RFW which contributed to lower the pH to a value below 5 and consequently inhibit the hydrolysis and acidification processes. In the study of Doğan & Demirer (2009) on the acidification of the organic fraction of municipal solid waste, low solubilisation efficiency due to effect of low pH value on hydrolysis was observed. The high VFA yields and VS destruction obtained in phase 5 despite the dropped of leachate pH below 5 were attributed to the simulation of VFA removal from the leachate (by replacing half of the leachate with distilled water). This might have contributed to remove most of the acids from the system and thereby increase the buffering capacity as showed by the pH increase after leachate dilution. It was suggested in previous studies that optimal pH for better hydrolytic-acidogenic activity is comprised between 5.5 and 6 (Bouallagui *et al.*, 2004; Doğan & Demirer, 2009). Herein, it was found that at pH value below 5.5 the formation of caproic acid was inhibited whereas its production completely stopped at pH value below 5. Simulating the removal of VFA from leachate by dilution (via water addition) enhanced the total SCFAs production by removing higher order VFAs from the leachate. In phase 5 where half of the leachate was replaced with water, acetic acid was the highest individual VFA produced

follow by butyric acid and propionic acid. At the light of the aforementioned, it was suggested that SCFAs accumulation could constitute a platform for the generation of MCFAs such as caproic acid.

Looking at individual VFA accumulation during phase 2 and 3 (Fig 4.6), it was deduced that high VFA concentrations in the starting liquid (phase 2) contributed to boost the production of propionic, butyric and caproic acids during the initial 3 days of the process whereas with low VFA concentrations in the starting liquid (phase 3) a lag phase was observed. Caproic acid was mostly produced in both phases during the last three days of the incubation implying that favourable conditions ($\text{pH} > 5.5$ and high SCFAs yields) prevailed in the leachate during this period. This study is one of the first to reported high concentration of caproic acid, $21.86 \pm 0.57 \text{ g COD l}^{-1}$ ($10 \pm 0.26 \text{ g l}^{-1}$) from the anaerobic processing of pure solid organic waste without the supplementation of electron donor (ethanol and lactate) under 7-day SRT. Grootscholten *et al.* (2014) reported caproic acid production of 12.6 g l^{-1} during two-phase anaerobic processing of municipal solid waste with ethanol supplementation using similar SRT (7 days). Much more lower concentration of caproic acid of 2.7 g l^{-1} was reported during batch anaerobic processing of municipal solid waste using 28-day SRT (Grootscholten *et al.*, 2013b). The VFA yields ($47 - 48 \text{ g l}^{-1}$) achieved in the present study under optimised conditions (phase 3) was 2.7 times higher than the one reported by Yesil *et al.* (2014) and 1.7 times higher than the yields reported by Cavdar *et al.* (2011) during anaerobic acidogenic digestion of the organic fraction of municipal solid waste.

4.4.2 Successful production of caproic and butyric acids as principal restaurant food waste fermentation products

This study clearly demonstrates that it is feasible to produce butyric and caproic acids as the main VFAs constituent from anaerobic processing of RFW. The concentration and production rate of caproic acid found in this study were higher than reported in several previous literatures (Agler *et al.*, 2011; Ding *et al.*, 2010; Steinbusch *et al.*, 2011). In the study of Steinbusch *et al.* (2011), mixed culture fermentation of acetate with ethanol or

hydrogen and combination of both was used to promote the accumulation of caproic acid; they reported concentration of 8.17 g caproic acid l⁻¹ and the highest production rate was 25.6 mM C caproate per day. Similarly, the work of Ding *et al.* (2010) reported caproate concentration of 11 – 23 mM (1.3 – 2.7 g l⁻¹) by using undefined mixed culture fermentation of glucose. In the research of Agler *et al.* (2012), in-line extraction was used to enhance the production of caproic acid from chain elongation of ethanol. The authors reported caproate production rate of 108.3 mM C per day (2.1 g l⁻¹ per day). In the present study, the highest caproic acid concentration of 10 g l⁻¹ and production rate of 3 g l⁻¹ per day obtained in H₂ and H₂/ethanol supplemented vials were higher than the aforementioned studies. Unlike the aforementioned researches, ethanol did not appear to be necessary for the formation of caproic acid which was in fact inhibited at high ethanol concentrations (Fig 4.7 D). The inhibition of caproic acid formation due to high ethanol concentration was found to be beneficial for the production of butyric acid as the principal fermentation product. This suggested that some of the caproic acid was likely formed through chain elongation of butyric acid. In the control vials, when all the lactic acid was consumed on day 3, maximal caproic acid was also achieved indicating the possible implication of lactic acid in the formation of caproic acid. Similarly, the depletion of lactic acid appeared to correlate with the production of caproic acid in H₂ and H₂/ethanol supplemented vials. Caproic acid formation from lactate has been observed in the study of Zhu *et al.* (2015) on the synthesis of caproate from lactate. The authors suggested that n-caproate production from lactate may be similar to the process of caproate formation from ethanol (oxidation/reverse β -oxidation). Lactate instead of ethanol is firstly oxidised to acetate which elongates with lactate to form butyric acid which in turn elongates with another lactate to form caproate. During the process, 3 moles of lactate is consumed to form 1 mole of caproate. Although Caproic acid yields of 23.41 g l⁻¹ reported in their study was more than twice higher than the one reported in the present study, their maximum production rate 2.97 g l⁻¹ d⁻¹ was comparable. The high caproate yields achieved in their study was likely due to the periodic lactic acid supplementation; trace elements and vitamin supplementation might have also played a role. Herein high caproic

acid yields could have been possibly achieved if lactic acid was supplemented after its complete depletion on day 4. High caproic acid yield were achieved only in vials where H₂ was supplemented, indicating that H₂ also contributed to some extent to the formation of caproic acid. High hydrogen partial pressure have been reported to be necessary to drive chain elongation reaction (Agler *et al.*, 2011). Under low hydrogen partial pressure, the carboxylate and ethanol will be oxidised to form mainly acetate and hydrogen (Grootscholten *et al.*, 2014; Metje & Frenzel, 2005). The yields of caproic acid production in the control vials (H₂ not supplemented) were lower than in vials supplemented with H₂, suggesting that caproic acid from lactate was likely not the only route involved. A plausible explanation to the higher caproic acid concentration in vials supplemented with H₂ than the control vials would be that part of the supplemented H₂ was likely used in another route for the production caproic acid (Steinbusch *et al.*, 2011).

The finding of this study that H₂ and lactic acid but not ethanol were necessary for the formation of caproic acid contrasted with the findings of Grootscholten *et al.* (2014), Ding *et al.* (2010), Steinbusch *et al.* (2011) and Agler *et al.* (2012) in which formation of caproic acid was demonstrated using ethanol as electron donor. The authors reported that caproic acid formation was possible through chain elongation of acetic acid and ethanol or ethanol with butyrate. Although Steinbusch *et al.* (2011) also observed caproic acid formation through chain elongation of acetate with H₂ as electron donor the yields obtained were lower than the ones obtained with ethanol as electron donor. In the present study, chain elongation of lactic acid and acetic acid was believed to form the vast majority of the butyric acid which in turn elongated with acetic acid to form some of the caproic acid. The other part of caproic acid was likely formed through chain elongation of acetic acid with H₂ as electron donor. It was also believed that some of the lactic acid was likely reduced to propionic acid (Chen & Wolin, 1977; Steinbusch *et al.*, 2011) especially in the control and ethanol supplemented vials.

The yields of caproic acid achieved in this study were in the range of maximum solubility which is reported in the literature to be 10 – 11 g l⁻¹ (Angenent *et al.*, 2016; Spirito *et al.*, 2014; Steinbusch *et al.*, 2011). Consequently some caproic acid might have precipitated out of the solution and were not accounted for during measurement. It is clear that higher caproic acid yields could have been achieved if caproic acid was continuously extracted from the system. In the study of Agler *et al.* (2012) the rate of caproic acid formation was improved by increasing the rate of extraction from the fermentation broth.

Although this study demonstrated that fermentation process was directed toward the production of caproic acid under high hydrogen partial pressure, the selectivity calculated as the ratio of final caproic acid concentration to initial sCOD added was found to be low (0.34 g COD caproate/g sCOD added). This low selectivity was mainly caused by propionate production (22%), butyrate production (14%) and acetate production (13%). Higher selectivity above 70% has been reported in previous study using substrates other than purely organic feedstocks. It was believed that a number of parameters including: the continuous process mode, the use of high rate reactor, the continuous extraction of MCFAs from stream and the supplementation of external electron donor (ethanol) contributed to achieved high MCFAs selectivity (Grootscholten *et al.*, 2013a; Grootscholten *et al.*, 2013b; Grootscholten *et al.*, 2014).

Mixed culture fermentation of soluble substrates from hydrolysis of heterogeneous feedstock, such as food waste, could constitute a platform for the selective production of caproic or butyric acids. Their production through several metabolic routes is perceived as beneficial in terms of rate and product yield.

4.4.3 Butyric and caproic acid formation dominated by *Clostridium*, *Peptoniphilus* and *Lactobacillus*

The vast majority of bacteria that have been previously identified as MCVFA producers were found in the phylum *Firmicutes* (Angenent *et al.*,

2016). During the experiment on the selective production of butyric and caproic acids, *Firmicutes* which represent 73 – 87% of the bacterial community was the most relatively abundant microbial group identified in all vials and at all sampling point. This was followed by *Actinobacteria* which represent 11 – 27% of the bacterial community. *Lactobacillus* were the most relatively abundant microbial group on day 0 (leachate from reactor R2, Batch 31). They represented 60% of the microbiome. The best known function of *lactobacillus* is to ferment simple sugars (e.g., glucose) into lactic acid (Zhu *et al.*, 2015). During the batch experiment, the continuous decrease in the concentration of lactic acid indicated that fermentation of simple carbohydrates was not the main activity. Since the relative abundance of *lactobacillus* and concentration of lactic acid were only high on day 0, it was deduced that *lactobacillus* contribution to the fermentation of sugar may have occurred in the early stage of the process inside leach-bed reactors (prior to batch experiment). Strong Pearson correlation coefficient ($R = 0.99$) was found between lactic acid production and increase in relative abundance of *Lactobacillus* in previous study with the same reactor design, RFW and operational parameters (except retention time) (Chapter 3 section 3.3.5). The other two bacterial groups, *Bifidobacterium* and *Clostridium* identified on day 0 might have also make important contribution during the early stage of the process inside reactors. They were likely involved in the production of acetic and butyric acid which concentration was also non negligible on day 0. *Lactobacillus*, *Clostridium* and *Bifidobacterium* identified at the start of batch experiment were likely adapted to acidic environment that prevail inside leach-bed reactor ($\text{pH} < 5$). At this low pH, the range of metabolites inside reactors was limited to acetate, butyrate, lactate and ethanol. Acidic conditions ($\text{pH} < 5.5$) has been reported to be incompatible with the growth requirements for most microbial cells (Agler *et al.*, 2011). In addition, at low pH, an important number of metabolic reactions are thermo-dynamically unfavourable and change in the pathway of certain reaction may occurs (Pind *et al.*, 2003; Yesil *et al.*, 2014). In the present study, after pH adjustment and incubation of the vials at 37°C , the relative abundance of the active microbial groups

increased along with the metabolites in all treatments indicating that environmental conditions were favourable.

The relative abundance of *Peptoniphilus* known to convert peptone to butyrate (Ezaki *et al.*, 2001; Johnson *et al.*, 2014; Supaphol *et al.*, 2011) was mainly high on day 4, 6, and 11 in all treatments suggesting that this substrate deriving from partial hydrolysis of protein was likely not metabolised at low pH. In this experiment, the production of butyric acid was believed to occur through various metabolic reactions. The first reaction likely involve the conversion of simple sugar to butyric acid (Sharmin *et al.*, 2013) at low and high pH, catalysed by *Clostridium* sp. The second reaction involves the formation of butyric acid from peptone at high pH, catalysed by *Peptoniphilus*. The third metabolic reaction likely catalysed by *Clostridium cochlearium*, involve the conversion of amino acids into butyric acid. *Clostridium cochlearium* have been reported in previous studies to be involved in the fermentation of glutamate and L-aspartate to acetate and butyrate (Buckel & Barker, 1974; Laanbroek *et al.*, 1979). The third possible butyric acid formation pathway at pH 7 is the chain elongation of lactic acid and acetic acid in the control and ethanol supplemented vials, probably catalysed by species affiliated with *Clostridiales*. *Anaerobaculum bizertensis* mainly abundant in ethanol supplemented vials was believed to be involved in the production of short chain fatty acids and ethanol. The study of Rezgui *et al.* (2012) demonstrated that *Anaerobaculum bizertensis* was unable to grow on acetate, butyrate and ethanol. Therefore they could not be involved in chain elongation reactions.

Among *Lactobacillus* species, a large increase in the relative abundance of *Lactobacillus reuteri* was observed in H₂ and H₂/ethanol treated vials from day 0 – 6 whereas *Lactobacillus* sp. relative abundance decreased considerably in all treatments. This seems to suggest that the former were likely involved in the production of caproic acid which production was maximal on day 6, while the latter might be involved in the formation of lactic acid. *Lactobacillus reuteri* relative abundance did not appeared to increase in ethanol treated vials, however in the control vials, a small increase was detected from day 0 – 4 and was associated with the

increase in caproic acid production during the same period. In several previous studies, *Clostridium* species have been found to be responsible for caproic acid production (Ding *et al.*, 2010; Zhu *et al.*, 2015). *Clostridium kluyveri* has been reported as the best known species involved in the production of caproic acid from ethanol and acetate (Spirito *et al.*, 2014; Steinbusch *et al.*, 2011). However in the present study *Clostridium Kluyveri* was not identified despite the availability of ethanol and acetate. This simply suggested that ethanol as electron donor was perhaps not the preferred route in the presence of lactic acid. Although the study of Zhu *et al.* (2015) reported the formation of caproic acid from lactic acid, *Clostridium species* were found to be responsible for its production. A previous study on the influence of adjunct culture on Volatile free fatty acids in reduced-fat Edam cheeses reported n-caproic acid as one of the highest VFA produced during maturation of reduced-fat cheese with *Lactobacillus reuteri* (Tungjaroenchai *et al.*, 2004). No other previous study has reported caproic acid as a product of *Lactobacillus reuteri*.

The relative abundance of *Clostridium* was more than twice higher on day 11 than day 6 in H₂ and H₂/ethanol supplemented vials. However the production of all the VFAs in these two treatments did not significantly change from day 6 – 11, suggesting that the increase in the relative abundance of *Clostridium* observed on day 11 was likely not related to the formation of caproic and butyric acid.

In this study, the long-time exposure of the microbial communities to high carboxylic acids concentrations during acidification of RFW in LBRs contributed to eliminate the archaeal community from which members could not be detected at pH 7 during the selective production of caproic acid in batch experiments. In previous studies, methanogens were inhibited either by applying periodic heat shock of the inoculum (Ding *et al.*, 2010; Steinbusch *et al.*, 2009) or by adding bromoethane sulfonic acid (Aguilar *et al.*, 1995; Steinbusch *et al.*, 2011). The former is energy demanding and in addition, might also eliminate some important microbial groups that are not spores forming bacteria. The latter is known to be expensive and might have negative impact on operational cost (Agler *et al.*, 2011). Therefore

exposure of the communities present in the inoculum to high concentration of VFAs appeared to be ideal for the elimination of methanogens from the system.

This study suggests that *Clostridium* species could be major players in the production of butyric acid from carbohydrates whereas *Peptoniphilus* could be responsible for the formation of butyric acid from protein source. Caproic acid from lactic acid with *Lactobacillus reuteri* could also be envisaged.

4.5 Conclusions

The findings of this study could be used to design a process that will allow for the continuous production of selective acids such as caproic and butyric acid from food waste thereby providing an effective solution for resource recovery from waste which fits in the waste management strategy while contributing to the economy. It was demonstrated that maximum hydrolysis of the RFW components was possible in two days. Furthermore, hydrolysis efficiency was improved by increasing the recirculation regime and by using bioaugmentation strategy. Rapid hydrolysis and high loading in LBRs were found to be inhibitory for fermentation due to a rapid drop in pH below 5. A high VFAs production in the range of 72 – 76 g COD l⁻¹ (47 – 48 g l⁻¹) could be obtained during anaerobic acidification of RFW in LBRs under optimised conditions. Volatile fatty acids production was greatly enhanced by using a combination of increased leachate recirculation regime and low initial VFA concentrations. Chain elongation of SCFAs to MCVFAs occurred in the leach-bed reactors as demonstrated by high yields of caproic acid (21.86 g COD l⁻¹). Periodic removal of VFAs from the leachate could contribute to improve the production of SCVFAs and delay the production of MCVFAs. High caproic and butyric acid yields were achieved in batch experiments. Caproic acid was believed to be formed through lactic acid oxidation/reverse β -oxidation and acetate elongation in the presence of H₂ as electron donor. Butyrate was likely produced through primary fermentation from carbohydrate or protein source and through secondary

fermentation of lactic acid and butyrate. *Clostridium* and *Peptoniphilus* species were both believed to be responsible for the production of butyric acid whereas *Lactobacillus reuteri* was likely involved in the formation of caproic acid.

CHAPTER 5

CONCLUSIONS AND FUTURE RECOMMENDATIONS

5.1 Conclusions

This research study set out to investigate the impact of operating conditions (e.g., pH, loading rate, solid retention time, dilution regime and bioaugmentation) on hydrolysis efficiency and to identify the microbial communities that are potentially involved in the hydrolytic-acidogenic stage during anaerobic processing of FW. This thesis has also sought to enhance the accumulation of VFAs during the anaerobic processing of RFW and to investigate the feasibility of using food waste fermentation liquor as a platform for the selective production of caproic acid and butyric acid. Overall, the result in this thesis has demonstrated a two-day maximum hydrolysis efficiency of the RFW and the successful enhancement of its biodegradation efficiency using a higher recirculation regime and a bioaugmentation approach. The results have also demonstrated that acidogenesis (instead of hydrolysis) was the rate-limiting stage. The enhancement of VFA accumulation at high concentrations through the optimisation of operating parameters inside leach-bed reactors has also been demonstrated in this study. In addition, the research has achieved the selective production of both butyric acid and caproic acid.

The hydrolysis of complex organic material, which remains one of the least understood steps in the AD process (Gavala *et al.*, 2003; Miron *et al.*, 2000), has been reported in several studies as a rate-limiting step (Kim *et al.*, 2005; Noike *et al.*, 1985). Enhancement of the hydrolytic reaction during anaerobic digestion is crucial to shorten the solid retention time thus allowing a rapid processing of large amount of food waste and to increase the yield of the desire end product (e.g., VFAs or methane). In Chapters 3 and 4 of this thesis, hydrolysis efficiency was assessed by taking the accumulation of the soluble chemical oxygen demand into consideration, the concentration of which rapidly increases within the initial two days of the process, thereby indicating maximum hydrolysis. This two-day maximum hydrolysis was found to correspond to 70% volatile solid (VS) destruction (Chap 4). This study is the first to report on the optimum

solubilisation of FW in only two days at 37°C; the closest hydrolysis efficiency of FW reported in the literature was achieved in five days at 35°C (He *et al.*, 2012). The impressive hydrolysis efficiency reported in this research study was likely due to the combination of a number of parameters (method of inoculation, recirculation regime and pH) that are known to influence the growth of microorganisms and thus likely to promote the conversion of organic substances (He *et al.*, 2012).

The rapid development of hydrolytic communities in the leach-bed reactors was evident during this study, as demonstrated by the maximum accumulation of soluble compounds over the initial two days of incubation in all trials (Chapters 3 and 4). The method of inoculation, based on the use of part of the digestate and leachate from previous batches to inoculate the new batch (the process of semi-continuous operation), has favoured the development of microbial communities specialised in the hydrolysis and fermentation of the different fractions of the RFW. The inoculation strategy adopted in this study was similar to the method used by the Dranco, Varloga and Kompogas process which are continuous single-stage dry systems with large capacity installed throughout Europe (Rapport *et al.*, 2008). With the Dranco and Kompogas, fraction of the digestate is transferred to the mixing pump where it is blended with fresh feed while Valorga digester uses pressurized biogas for mixing part of the digestate with new material. This method of inoculation, in combination with the pre-acclimation of the microbial communities to the FW, may have contributed to the rapid growth of hydrolysers, which might explain the fast solubilisation throughout reactor trials (Chapter 3 and 4).

This study demonstrated the bioaugmentation strategy based on the supplementation of leach-bed reactors with enriched cultures (Chapter 2 and 4) in order to improve the biodegradation efficiency of the RFW components. Their effect did not last for long inside the reactors, however, which was believed to be due to the washout of the supplemented communities from the reactors. This demonstrated the need to develop a strategy that would maintain the supplemented microbes inside the reactors. The short solid retention time of two days reported in this study for

optimum hydrolysis and VS removal could be beneficial in addressing the increasing amount of FW that is generated on a daily basis, and could help in meeting the EU landfill directive target.

Although the operating conditions inside the leach-bed reactors were believed to be favourable for the biodegradation of RFW in general, they were unsuitable for the degradation of the lipid fraction. Only 20–30% fat degradation was achieved in the leach-bed reactors (Chap 3). Various studies have reported that operating conditions favourable for methanogenic activities best promote the biodegradation of lipids in anaerobic systems (Alves *et al.*, 2009; Cirne *et al.*, 2007b; Ma *et al.*, 2015) because the hydrolysis of lipids mostly generates LCFAs, which are further processed (through β -oxidation pathway) in a syntrophic interaction of acetogens and methanogens (Weng & Jeris, 1976). The degradation of LCFA is strongly influenced by the ability of the hydrogenotrophic methanogens to utilise the hydrogen that is produced during β -oxidation (Cirne *et al.*, 2007b). In the absence of hydrogenotrophic methanogens, the LCFA will accumulate at the lipid–water interface, and consequently the quality of the interface may change. Since the enzyme lipase responsible for lipid hydrolysis requires an interface to be activated, a physical and chemical change of the interface may result in the inhibition of the enzyme activities and consequently the inhibition of lipid degradation (Reis *et al.*, 2009; Rietsch *et al.*, 1977).

This research study reported higher degradation efficiency of the lipid fraction of the RFW using methanogenic conditions (Chapter 2) compared to acidogenic conditions (Chapter 3). Indeed, during the BMP experiments performed in Chapter 2, the operating conditions applied (pH, inoculum and FW: inoculum ratio) were favourable for the development of methanogenic activities; successful methanogenic activity was demonstrated by the absence of sCOD and VFA accumulation and high methane production. Under these conditions, 83.62% lipid degradation efficiency was achieved after fourteen days of incubation. During the anaerobic processing of RFW inside the leach-bed reactors, however, the operating conditions were more favourable for the development of hydrolysers and acidogens. This experiment inside leach-bed reactors sought to promote the

accumulation of organic acids (especially VFAs) and the inhibition of methanogens. The successful inhibition of the methanogenic communities was demonstrated by the high accumulation of VFAs and the presence of trace amounts of methane at a very low concentration. Under this acidogenic condition, the degradation efficiency of the lipid varied between 20–30% after fourteen days of incubation.

The results gathered from these two experiments (Chapters 2 and 3) indicate higher lipid degradation efficiency when methanogenic activities are high. The lipid fraction of the RFW used in this research study represented 27.49%, which was the second-highest fraction after the hemicellulose fraction. Given the considerable RFW lipids fraction, a different setup is required to address its biodegradation when using the anaerobic processing of FW without the methane production approach. The effect of lipid accumulation on hydrolysis and acidification was not perceptible, which was likely due to the operation of reactors in a semi-continuous batch mode. It is evident that if the reactors were to be operated in the continuous mode, the accumulation of lipids could constitute an issue over time. One approach to cope with the lipid accumulation (as reported in the literature) is to physically remove them from FW prior to the anaerobic digestion process (Cho *et al.*, 2012; Gumisiriza *et al.*, 2009). Given the high methane content of lipids (Cirne *et al.*, 2007b), however, the most attractive approach to addressing the lipids issue encountered in this research would be to process the digestate generated at the end of the acidogenic phase in a separate reactor in which methanogenic activity is favoured.

The two trials (Chapters 3 and 4) undertaken in this study achieved a successful accumulation of VFAs in leach-bed reactors processing RFW. Seven days' solid retention time (SRT; Chapter 4) rather than fourteen (Chapter 3) was found to be appropriate for maximum VFA accumulation. Although the maximum hydrolysis efficiency was achieved within the initial two days of the process, maximum carboxylate production efficiency was achieved after seven days. This indicates that with this reactor configuration, the rate-limiting step was the carboxylate production, rather than hydrolysis, as is commonly reported in the literature (Kim *et al.*, 2005;

Noike *et al.*, 1985). It could be anticipated that when using a two-stage process in which hydrolysis and fermentation take place in separate reactor, the carboxylate production rate could be enhanced by using a high rate reactor system. Grootscholten *et al.* (2014), demonstrated that MCFAs production from real OFMSW and (diluted) ethanol in a two-stage system results in higher MCFAs production rates and higher caproate concentrations than in single-stage system (Grootscholten *et al.*, 2013b). Parameters including high recirculation regime, low VFA in starting liquid and dilution of the leachate were found not only to increase the production of VFAs but also to influence their profiles. Indeed, a 49% increase in the leachate VFA concentration was achieved when the recirculation regime was increased from once per day to three times per day (Chapters 3 and 4). A further 6% improvement in VFA production was obtained when the starting liquor VFA concentration was reduced from $\sim 15 \text{ g COD l}^{-1}$ to 6 g COD l^{-1} . The removal of short-chain carbon organic acids (including acetic and lactic acids) mainly produced during the initial two to three days of the process limited the production of caproic acid. This demonstrates that these two organic acids play an important role in the accumulation of caproic acids inside reactors. Steinbusch *et al.* (2011) and Zhu *et al.* (2015) reported acetic and lactic acids (respectively) to be precursors for caproic acid formation. This thesis reported for the first time the production of caproic acid as the highest VFA inside leach-bed reactor-processing FW. The vast majority of studies on the anaerobic processing of FW or organic municipal waste under acidogenic conditions have reported butyric and acetic acids to be the principal VFAs to accumulate (Doğan & Demirer, 2009; Grootscholten *et al.*, 2014; Yesil *et al.*, 2014). In this study, parameters such as low pH range (below 5.5) and high loading rates were found to negatively influence the overall VFA production and to negatively affect in particular the accumulation of caproic acid. Caproic acid formation started when the pH was above 5.5 and short-chain volatile fatty acids (SCVFAs) were available. Apart from caproic acid, other VFAs (including butyric and acetic acids) also accumulated in the reactors at concentrations ranging from $16\text{--}21 \text{ g COD l}^{-1}$ (Chapters 3 and 4). These VFAs could be directed to methanogenic reactor for methane production after the extraction of caproic

acid. The anaerobic processing of FW without methane production could constitute a platform for organic acids, specifically carboxylate production. Furthermore, the organic acids produced from the anaerobic processing of food waste could also constitute a platform for the production of valuable MCFAs. This was demonstrated in this study through the successful production of caproic acid in a batch experiment using FW liquor containing mainly short-chain organic acids (lactic acid, acetic acid and butyric acid). Parameters including hydrogen pressure and pH were the key factors for the production of caproic acid in this batch experiment.

Microbial community analysis using 16S rRNA profiling from DNA and cDNA revealed the presence of few microbial groups involved in FW biodegradation processes. *Enterococcus*, *Lactobacillus*, *Bifidobacterium* and *Clostridium* were the main active microbial groups identified in the leach-bed reactors at the moment of sampling. Their contribution to the clustering of microbial communities (indicated by an nMDS plot) varied from 73–80%, as demonstrated by the similarity percentage analysis. Apart from *Enterococcus*, the other bacterial groups have been reported to be involved in the hydrolysis and fermentation of solid organic waste (Sträuber *et al.*, 2012; Xu *et al.*, 2014a; Xu *et al.*, 2014b). The increase in the relative abundance of *Enterococcus* in the anaerobic digesters has not been previously highlighted in the literature. In this research study, however, the relative abundance of the active members of the *Enterococcus* group was found to rapidly increase over the initial day of incubation and constituted the most abundant microbial group. Because the main process detected on day 1 was hydrolysis, *Enterococcus* was believed to play an important role in the solubilisation of the different fractions of the RFW inside the leach-bed reactors. This may be one of the first studies on the AD of FW in which *Enterococcus* have been reported as potential hydrolyzers.

During the development of cellulose and hemicellulose degraders through enrichment cultures (Chapter 2), species affiliated with *Bacteroidetes* were identified as potential hydrolyzers, while in the leach-bed reactors using the same starting inoculum (granules), *Firmicutes* were likely the hydrolytic communities. The discrepancy between these findings,

despite the use of the same starting inoculum and RFW, certainly lie in the operating conditions that prevailed in these two experiments. Indeed, a methanogenic condition was favoured in the enrichment assay, while an acidogenic condition was promoted in the leach-bed reactors trials. Studies have reported that the stability of anaerobic digesters depends on the functional diversity of the microbial community: in other words, the ability of the different members of the community to generate similar outcomes from different metabolic routes (Briones & Raskin, 2003; Fernandez *et al.*, 2000; McCann, 2000). This could explain the development of *Bacteroidetes* species under methanogenic conditions while *Firmicutes* might well adapt to an acidogenic environment.

Although 16S rRNA gene sequencing and phylogenetic analyses provided detailed information on the microbial community structure inside the reactors and batch trials, the functionality of these communities was not established. The suggestion that *Enterococcus* are involved in hydrolysis (Chapter 3), *Clostridium* and *Peptoniphilus* involved in butyric acid production, and *Lactobacillus* responsible for caproic acid formation (Chapter 4) were all inferred from observed processes. Further molecular analyses are thus required to establish the functionality and to correlate important functional groups with processes.

5.2 Future recommendations

This thesis provides several new insights into the anaerobic accumulation of VFAs in leach-bed reactors processing RFW. Nevertheless, certain areas of the process still need to be addressed. We therefore propose the following recommendations:

- The continuous biodegradation of the FW by using two-phase anaerobic digesters in which the first phase will be optimised for hydrolysis and primary fermentation of the different fraction of FW, while the second phase will serve to promote the accumulation of VFAs of interest.

- The use of mixed microbial communities from different sources as the starting inoculum to promote functional diversity, which will then determine the stability of the reactors. The functional diversity is thought to be beneficial in digesters that process heterogeneous feedstock such as FW because of the ability of the various members of the communities to adapt to the fluctuation of certain environmental parameters.
- The use of support materials to develop biofilm that will help in maintaining important functional microbial groups in the systems.
- The use of function-oriented studies to link processes and community structure. The application of metaproteomics could provide significant insights into microbial activity by linking microbial community structure to functions and providing information about which microorganisms are carrying out which functions. In addition, the use of metabolomics (the study of the intermediate and end-products of cellular processes) could help in identifying the microbial groups responsible for specific processes in reactor systems.
- The design of a system for the continuous production and recovery of caproic acids as the main product from the anaerobic processing of FW.

Finally, because this study found that anaerobic leach-bed systems promoting the accumulation of organic acids from FW was unsuitable for the biodegradation of the fat fraction, another approach (such as the introduction of a third phase under methanogenic conditions) could be used to address this issue. The flow chart of the proposed process that could be used in further studies to maximise the value of the food waste stream is shown on Fig 5.1.

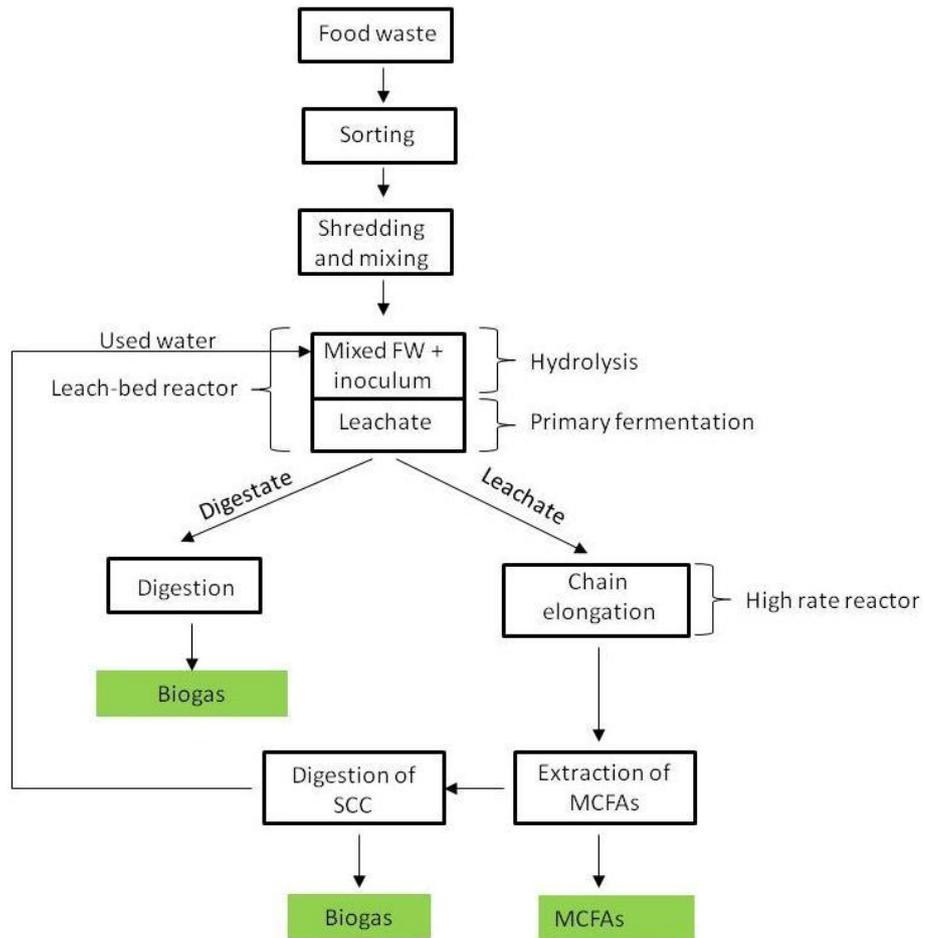


Fig 5.1 Flow chart of food waste resource recovery process

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Supplementary materials chapter 3

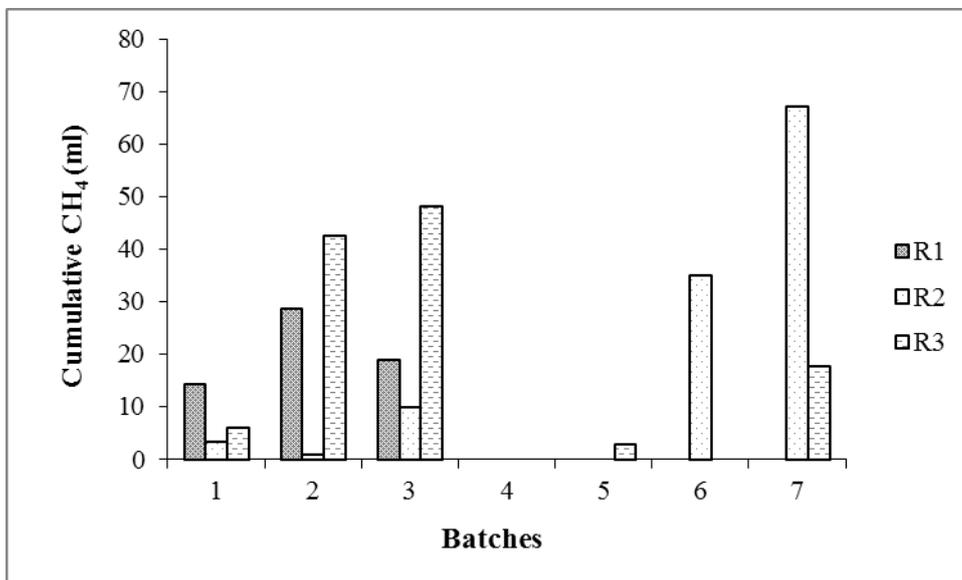


Figure S3.1 Profile of methane production from R1, R2 and R3 leach-bed reactors treating RFW.

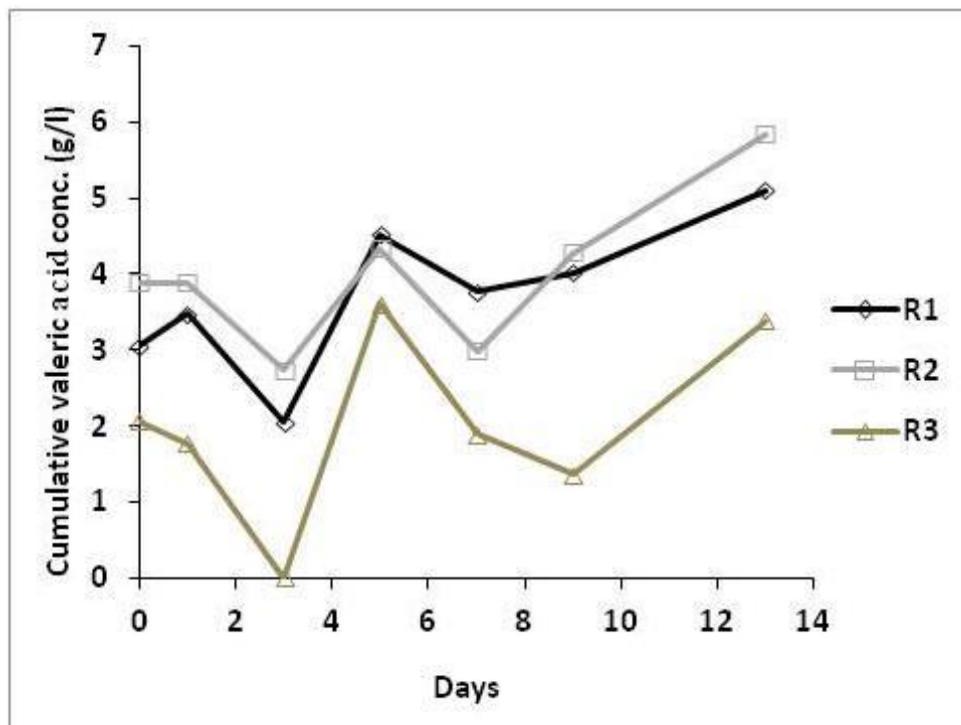


Figure S3.2 Profile of the valeric acid in R1, R2 and R3 leachate obtained during batch 7. Value at each point is the mean of duplicate measurements

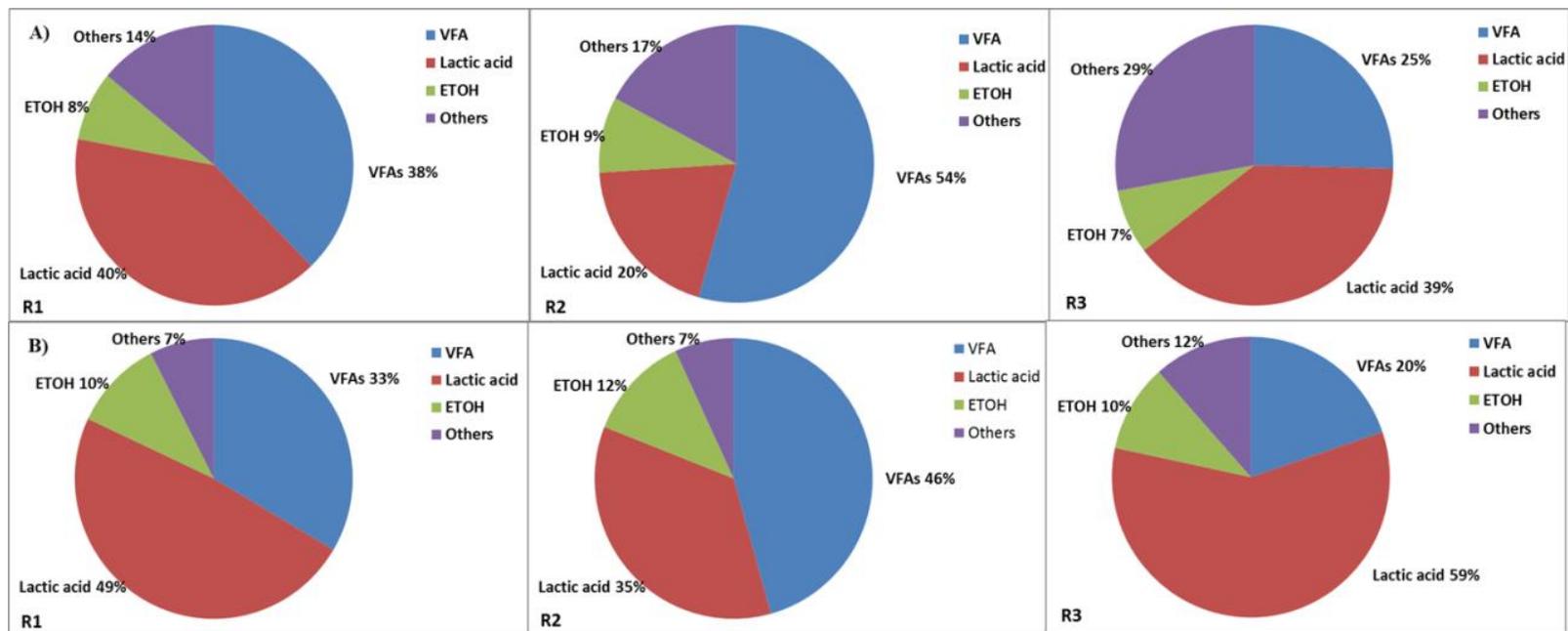


Figure S3.3 Contribution of fermentation products (VFAs, ethanol, lactic acid and others) to the total sCOD accumulated in R1, R2 and R3 leachate on day 1 (A) and day 3 (B) during batch 7.

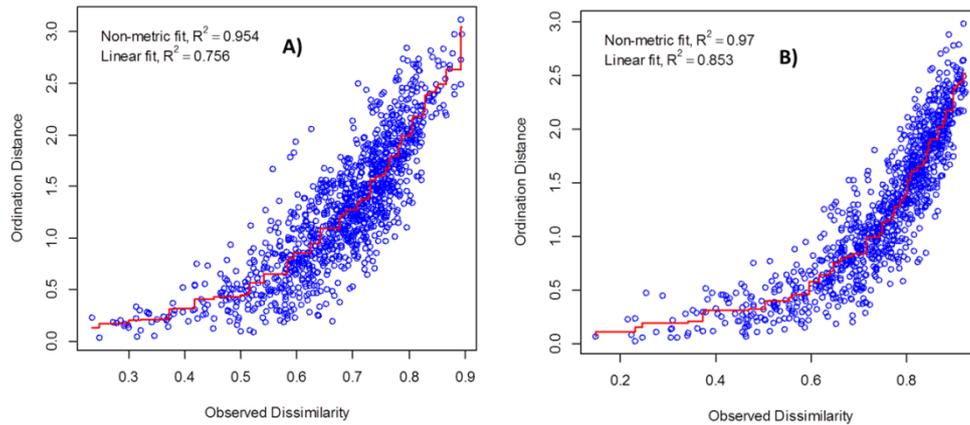


Figure S3.4 Shepard plot showing relationships between rank dissimilarity matrix and rank projected distance using the microbial community matrix profiling from DNA (A) and cDNA (B). The blue circles represent the projected rank order of the distance between each pair of community in the matrix whereas the red line represents the actual dissimilarity between communities. The projected rank order is in agreement with the observed dissimilarity as indicated by the low scatter of points around the regression line. The larger the scatter around the regression line the higher the stress value indicating that the number of dimension has to be increased

Table S3.1 Cumulative contribution of most influential species responsible for nMDS clustering (DNA level). Similarity percentage analysis (SIMPER)

Clusters	Influential species	Cumulative contributions
day 0 – day 1	<i>Enterococcus</i>	33
	<i>Bifidobacterium</i>	48
	<i>Lactobacillus</i>	60
	<i>Unclassified Bifidobacterium</i>	70
Day 1 – day 3	<i>Enterococcus</i>	35
	<i>Lactobacillus</i>	64
	<i>Bifidobacterium</i>	83
Day 3 – day 7	<i>Lactobacillus</i>	36
	<i>Bifidobacterium</i>	58
	<i>Clostridium</i>	77

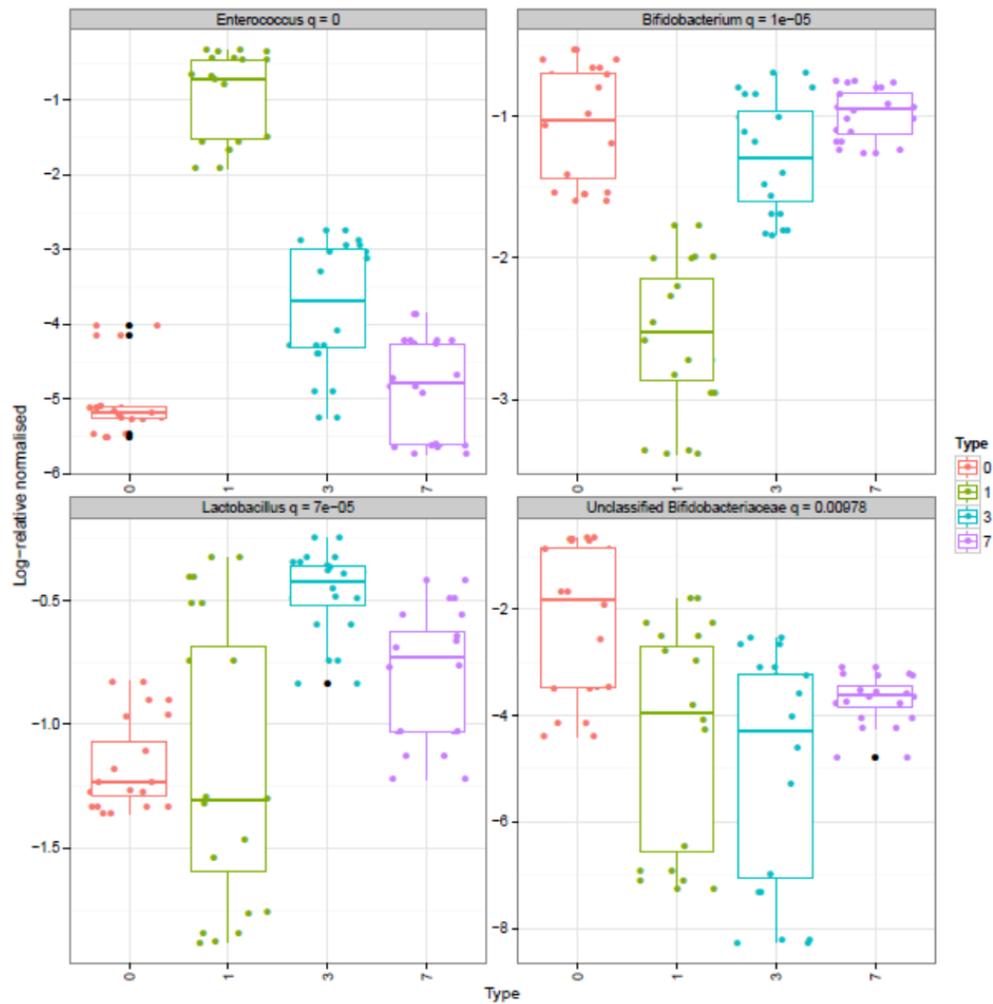


Figure S3.5 Distribution of the most influential species responsible for microbial communities clustering at DNA level. Numbers on the X axis represent the days

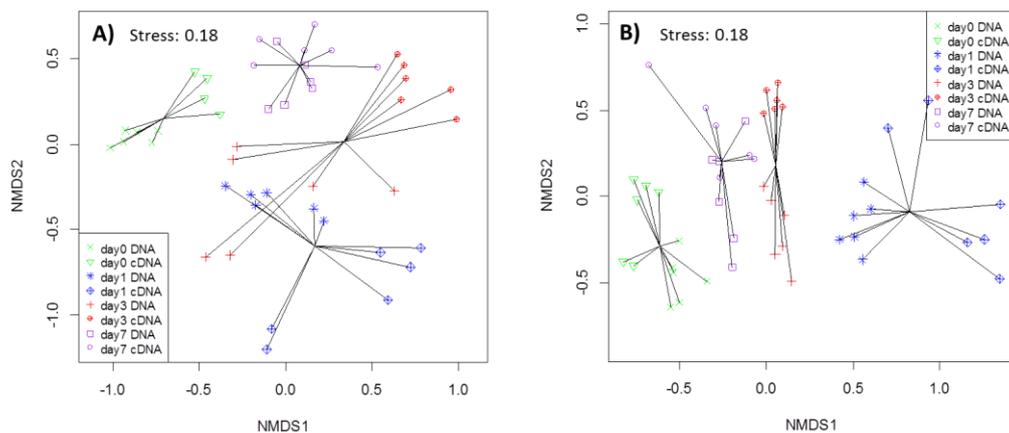


Figure S3.6 nMDS plot illustrating distance between DNA- and cDNA-based digestate (A) and leachate (B) microbial communities. The community profile on the plot at each sampling point is represented by a specific colour (green for day 0, blue for day 1, red for day 3 and purple for day 7) while the community present in each fraction (DNA and cDNA) of the reactors is illustrated by symbols.

Supplementary materials Chapter 4

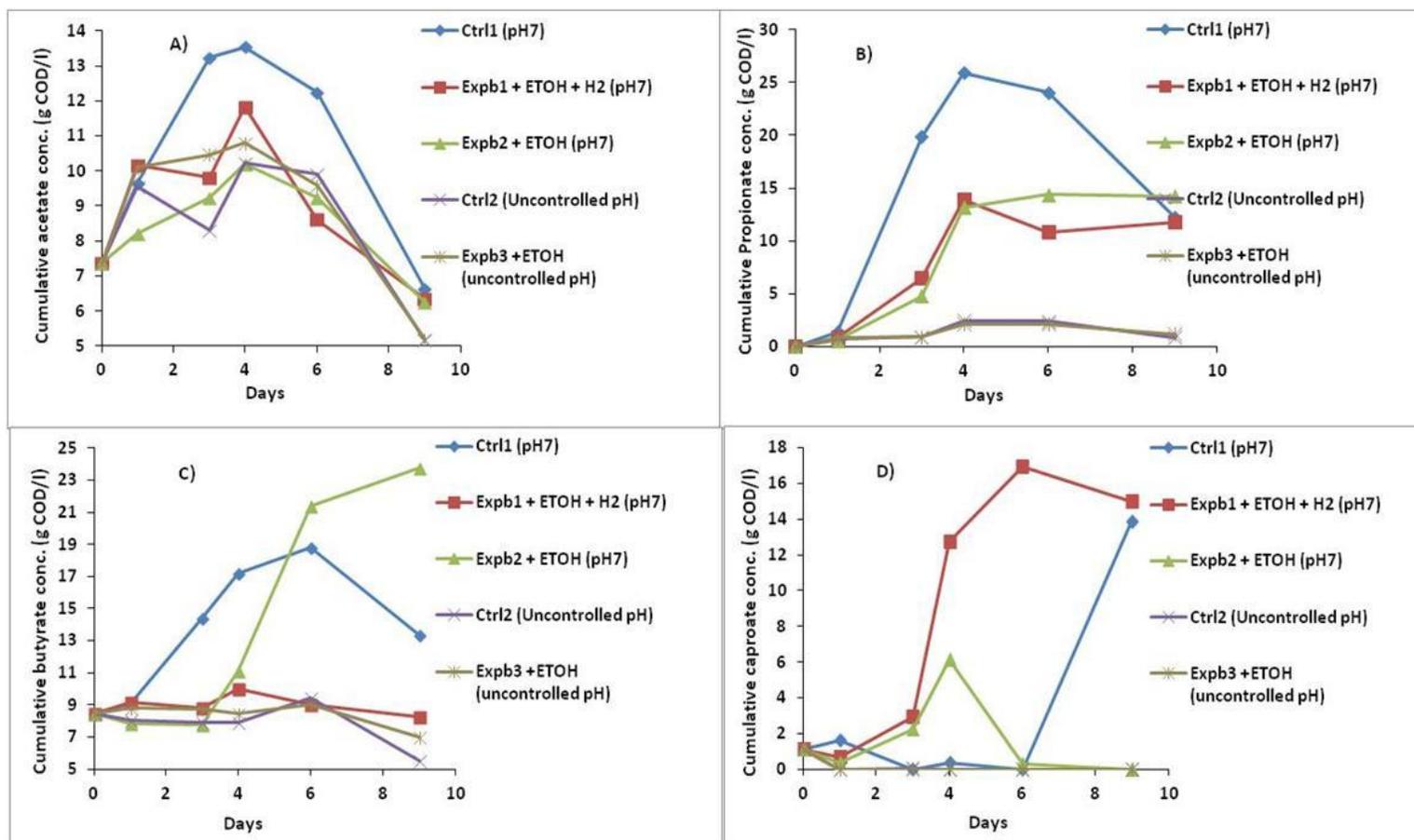


Figure S4.1 Production pattern for acetic (A) propionic (B), Butyric (C), and caproic (D) acid obtained in the control (ctrl), ethanol (ETOH), hydrogen (H2) and H2/ETOH supplemented vials (Exp b1 – b3) during batch experiment using leach bed-reactor leachate at uncontrolled pH. Data at each point is the average of duplicate analysis.

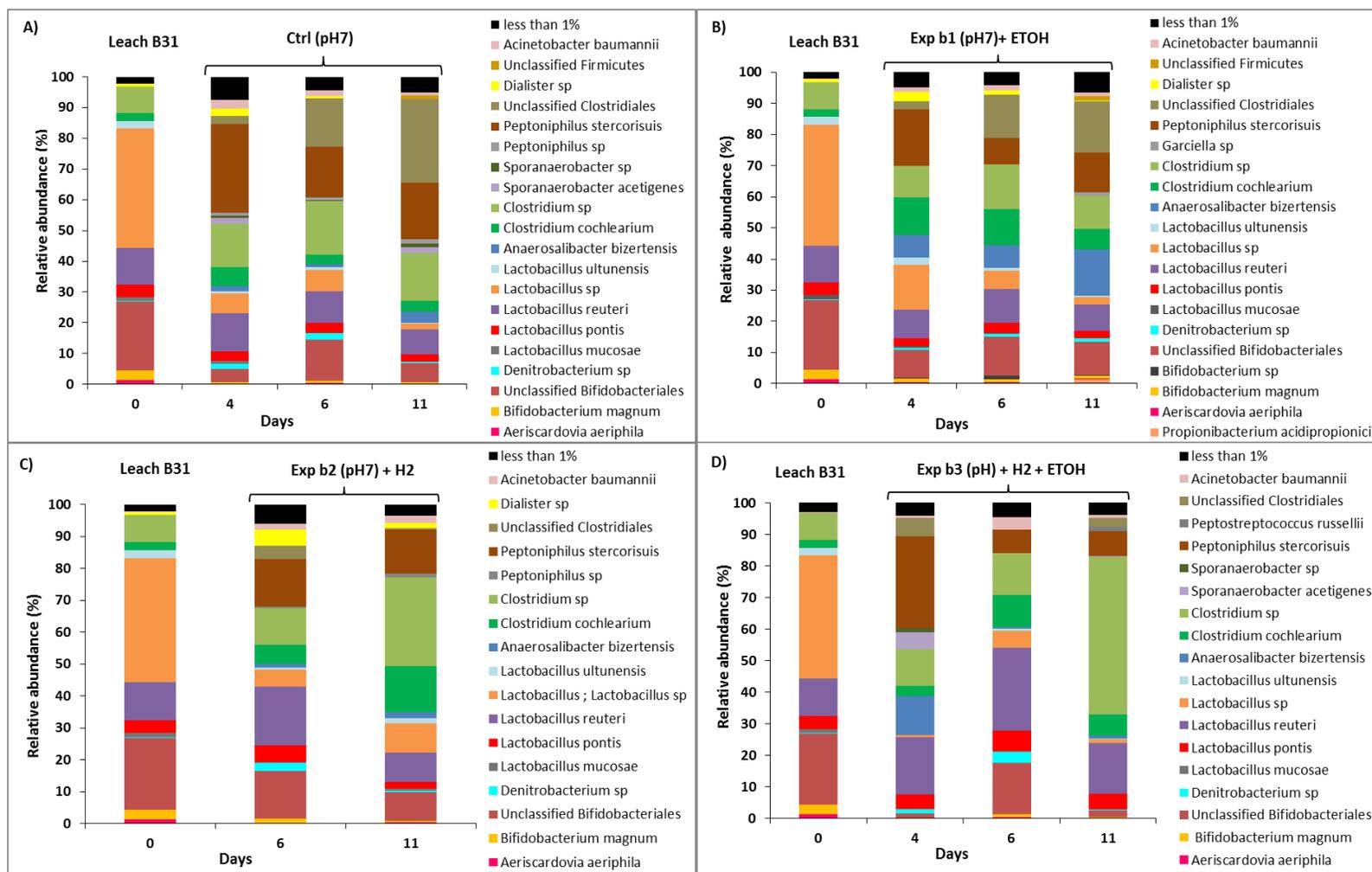


Figure S4.2 Taxonomic classification (at species level) of the 16S rRNA sequences retrieved from: A) control (ctrl); B) ethanol (ETOH); C) hydrogen(H_2) and D) H₂/ETOH supplemented vials (Exp b1 – b3) obtained during batch experiment using leach-bed reactor leachate . Leach B31: leachate collected from batch 31 on day 2.