UNIVERSITÀ DI VERONA

DOCTORAL PROGRAM IN BIOTECHNOLOGY

(XXXII CYCLE)

VOLATILE FATTY ACIDS PRODUCTION FROM URBAN ORGANIC WASTES FOR BIOREFINERY PLATFORMS

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SUMMARY

LIST OF ABBREVATIONS

COD: Chemical Oxygen Demand

HRT: Hydraulic Retention Time

OLR: Organic Loading Rate

PHAs: Polyhydroxyalkanoates

PLA: PolyLactide Acid

sCOD: Soluble Chemical Oxygen Demand

TKN: Total Kjeldahl Nitrogen

TP: Total Phosphorus

TS: Total Solids

TVS: Total Volatile Solids

VFAs: Volatile Fatty Acids

CHAPTER 1: INTRODUCION

1.1 BACKGROUND

In the last years, European Union (EU) has promoted several strong actions to support implementation of european circular economy, a new economic model based on recovery of resources from wastes, principally in form of bio-based products with a high added-value rather than compost and energy. Historically, the main products obtained by bioconversion of bio-wastes are bioenergy, mainly biogas, and soil improvers. Nevertheless, they have cheap market value and rise several environmental issues, related with spreading on soils of residues of their bioconversion (i.e. sludge and anaerobic digestate). Fortunately, several novel technologies are available for full exploitation of residual biomass, which allow to produce bio-based products with a high added-value and higher commercial potential (Ahorsu et al., 2019).

Therefore, the circular economy strategy can be adopted for a sustainable recovery of organic waste originated in the urban environment, mainly residues derived from separate collection of municipal solid waste and sludge from wastewater treatment plants. This new approach is sustainable not only with respect to environmental impacts, but also contributing to support economic growth. The market of high value bio-based products, in fact, exists and it is constantly growing, representing a more profitable way for management and valorization of organic residues, than anaerobic digestion, or composting.

In urban environment, organic fraction of municipal solid wastes (OFMSW) and wastewater sludges (WWS) represent the main waste streams, consequently, a circular approach for their sustainable disposing should be based on cotreatment. Although OFMSW and WWS share a similar volume of organic matter and are both originated from the same area, they usually are separately handled. Even though this separation allowed to limit the ecological impact by their treatment, it is not the most effective strategy.

A viable alternative could be an integrated treatment of WWS and OFMSW, as well as other main urban bio-waste streams in a novel biorefinery platform. This facility would allow a flexible co-treatment of urban bio-waste, bringing advantages in both environmental and economic terms. In particular, a co-treatment of urban organic waste would allow to reach the critical production threshold for economic viability of a novel bio-waste biorefinery platform.

Nevertheless, integration of waste streams treatment in a novel biorefinery can also include other organic wastes that are not usually collected with organic waste, like diapers.

Nowadays, according to data provided by Intergovernmental Panel on Climate Change (IPCC), about 0.48 tons per capita of municipal solid wastes are produced every year in Europe, 32% of which is composed by food waste (Towprayoon et al., 2019). Assuming that, according to last data released by EUROSTAT, the European population is composed by around 500 million citizens, it can be hypothesized that about 78 million tons of food waste are available for biological treatment every year in Europe. This enormous volume of waste stream includes not only the one collected at household level (53%), but even food waste generated by manufacturing industry (30%), food service sector (12%), and retail level 5% (Stenmarck et al., 2016; Battista et al., 2019).

WWS represents another abundant waste stream in urban environment. The implementation of the european legal framework toward a stricter sustainable handling of wastewater led to a strong rise in sludge generation, with an estimate of more than 13 million tons of dry matter in 2020 (Battista et al., 2019; Collivignarelli et al., 2019). Historically, these huge waste streams, when not landfilled or incinerated, have been biologically stabilized by composting or anaerobic digestion processes. Obviously, due to the cheap value of the final products, along with a low reuptake of resources and energy, a boost of research toward more effective and sustainable way of handling of these organic wastes has taken place in the last decade.

1.2 THE CIRCULAR ECONOMY MODEL

One of the greatest challenges our society has to face in the 20th century is to find a way for concealing a constant increasing demand of resources with the fact that a single planet is available to us, with limited reserves and limited capacity to take in our wastes (Clark & Deswarte, 2015).

Since the dawn of Industrial Revolution, a linear economic model has been adopted by our society, based on a "take-make-consume-throw away" pattern. This kind of economic model is based on the erroneous belief that resources are unlimited and easy to find, and that products at their and of life must be throw away as useless wastes. This approach leads to an overuse of resources of Earth, getting dramatically closer the "Overshoot Day" every year (Figure 1) (www.overshootday.org).

Fortunately, a new economic model is emerging worldwide under the pressure of more stringent legislation, known as "circular economy". About the "circular economy

concept", European Environment Agency (EEA) stated that: «*the concept can, in principle, be applied to all kinds of natural resources, including biotic and abiotic materials, water and land. Eco-design, repair, reuse, refurbishment, remanufacture, product sharing, waste prevention and waste recycling are all important in a circular economy*» (EEA, 2016).

In this model, products at their end of life and materials they are composed of are highly valued, unlike in the linear model. The circular economy, in fact, is based on two complementary cycles (Figure 2): (*i*) one is related to biodegradable residues (decomposable by living organisms), (*ii*) and one for "technical" wastes (not biodegradable).

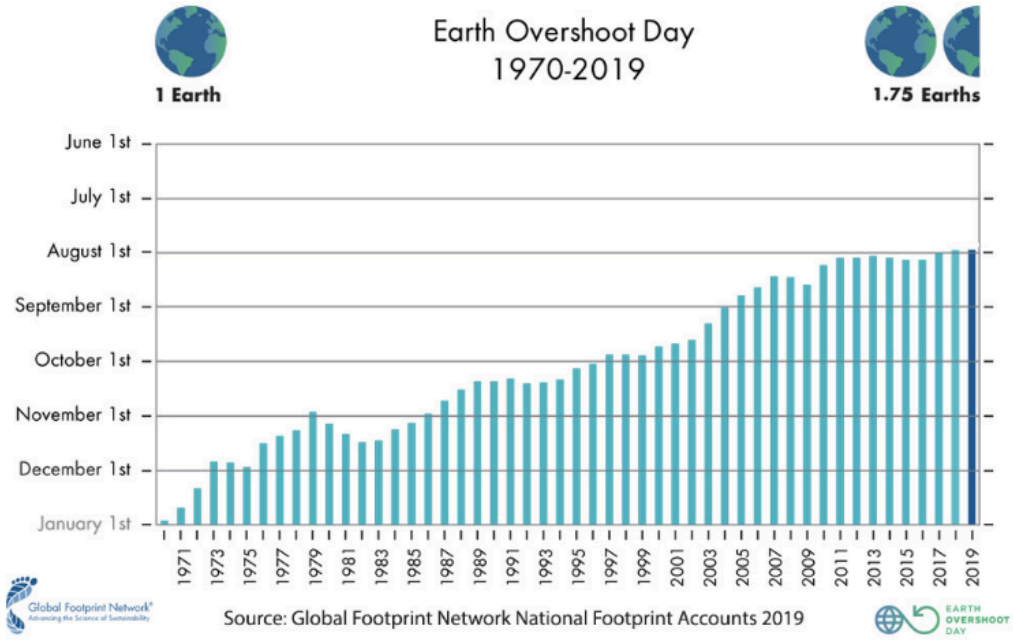

Figure 1. Earth over-shooting day from '70s to nowadays (modified from www.overshootday.org)

In both cases, the goal is to avoid consumption of primary resources and generation of wastes as far as possible, maintaining materials within products at end of life in the economy as possible: they can be used again and again, generating new value. In other words, circular economy model is circular itself, since what used to be considered as "waste" can be turned into a valuable resource by reusing, repairing, refurbishing, and recycling existing materials and products (Bourguignon, 2016).

EU has strongly encouraged a transition of european economy towards a circular sustainable model. In this direction, the first action was the "Waste Framework Directive" (i.e. Directive 2008/98 EC), introduced in 2008. This Directive contains the definitions for activities like prevention, treatment, recycling, reuse, preparing for re-use and recovery of materials and energy.

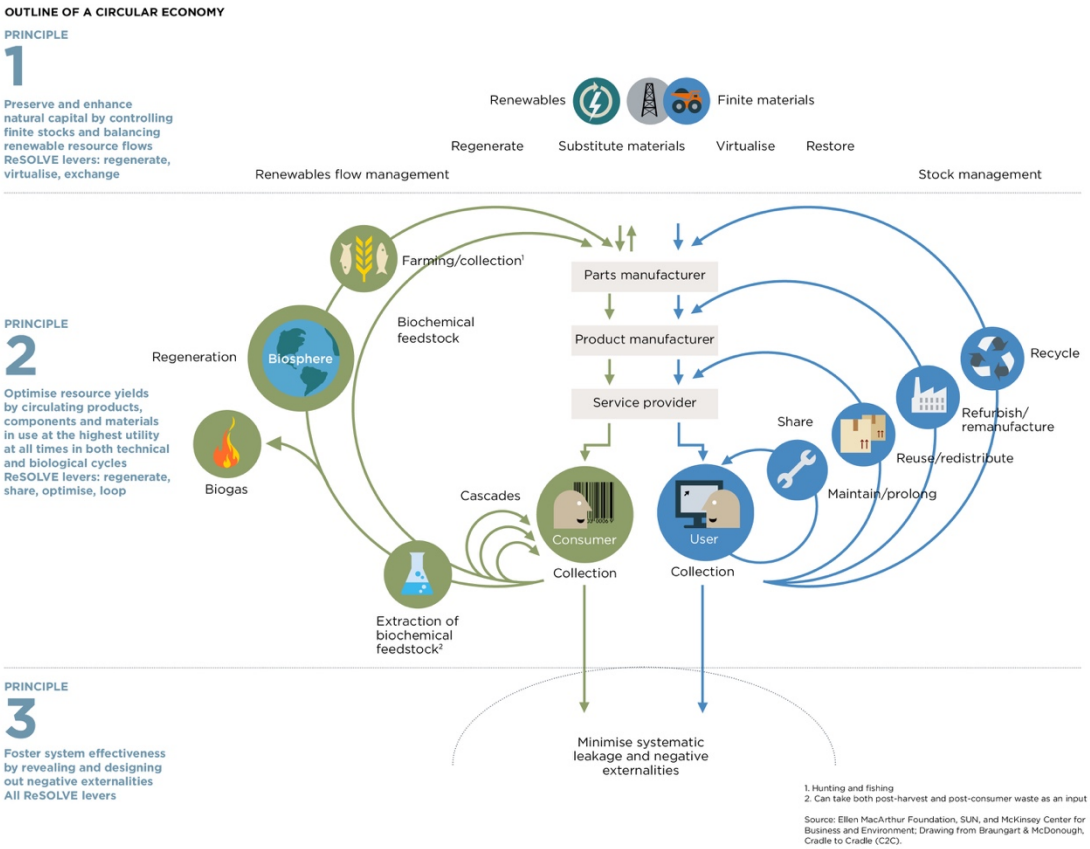

Figure 2. Outline of circular economy (from www.ellenmacarthurfoundation.org)

Figure 3. The waste management hierarchy (from https://ec.europa.eu/environment/waste/framework)

Furthermore, it also stated the so called "waste management hierarchy", which has created the basis for the subsequent legislative updates (Grosso et al., 2010; Hughes, 2017). As shown in Figure 3, the waste hierarchy is focused first on: (*i*) the reduction of wastes; (*ii*) secondly on the reuse of the wastes; (*iii*) thirdly on waste sorting and

recycling; (*iv*) finally on other form of recovering from waste (also forms of energy recovering) and the disposal (Papargyropoulou et al., 2014).

The European Union has recently approved a second "circular economy package" to allows a complete transition towards a linear economic model, based on the "three R" principle (Reduce, Reuse and Recycle) introduced by Directive 2018/851. This Directive contains some amendments to the Waste Framework Directive of 2008, in order to favor and implement the aforementioned economic strategy transition in the Member States within 2020. The latest Directive, in particular, establishes that waste hierarchy must be functional not only to protect the environment and human health, but also to achieve a greater competitiveness of EU. Nonetheless, Directive 2018/851 clarifies the "end-of-waste criteria", i.e. the conditions under which a waste ceases to be waste, introduced for the first time by the previous Directive. In fact, Directive 2018/851 puts a further condition: «*The natural or legal person who: (a) uses, for the first time, a material that has ceased to be waste and that has not been placed on the market; or (b) places a material on the market for the first time after it has ceased to be waste, shall ensure that the material meets relevant requirements under the applicable chemical and product related legislation*». Moreover, the Directive underlines the importance of monitoring the development of national end-of-waste criteria in Member States, and assess the need to develop Union-wide criteria on this basis (Migliore et al., 2019)

The novel circular economy approach is the only economic model capable of ensure a sustainable development of our society. Several reasons are behind sustainability of circular economy, since it allows to (EEB 2014; Blok et al., 2016; Bourguignon, 2016; MacArthur et al., 2016; Schroeder, 2019):

(i) balance the fast-growing demand of resources and energy, due to a constant increase of global population, and facing shortage of primary resources (water, land, and materials) in manufacturing industry. In this way, circular economy defends the security of supply of raw materials, since can mitigate price volatility, availability of resources and import dependency;

(ii) reduce the amount of wastes to dispose of, limiting the negative impact of conventional management of wastes, both on environment and public health, due to pollution and greenhouse gasses (GHGs) emission (with negative consequence on climate change). It was estimated that circular economy practices could reduce up to 7.5 billion tones of CO_2 equivalent, favoring the 1.5°C target stated at the Paris Agreement. Furthermore, it was also estimated that a combination of more stringent actions on recycling of municipal and packaging waste, along with a decrease of landfill in the EU,

could bring a reduction in GHGs emission of about 424 to 617 million tones of CO_2 equivalent by 2035.

(iii) achieve profitable consequences in economic terms. According to EU, in fact, a redirection of european industries towards a circular economic strategy can provide a descrese of production cost related to raw materials. Moreover, an increase of gross domestic product of about 3.9% is expected by creation of new markets, products, and jobs. Nonetheless, a report, published by Ellen MacArthur Foundation in 2015, estimates that by 2030, a shift towards a recovering of resources can reduce net spending cost in the EU by €600 billion annually, improve resource productivity by up to 3% annually, and leading to total net benefits estimated at €1.8 trillion per year.

(iv) create new job opportunity. According to the European Environment Bureau (EEB), a shift toward circular economy in EU can create new employment opportunities, the number of which varies from 634,769 (in a modest scenario) to 747,829 (in an ambitious scenario) by 2025.

On a practical level, a transition to a novel circular economy cannot be established without an application of practices and technologies which fall within the so called "biorefinery" approach. In other words, a new circular economy approach, based on innovative and cost-efficient use of residuals materials, especially starting from biodegradable matter for the production of both bio-based products and bioenergy, needs to be driven by well-established integrated biorefining platforms (De Jong & Jungmeier, 2015).

Next paragraph exploits issues about the different biorefinery generation approaches for bioenergy and bio-based products generation starting from biodegradable biomass, highlighting the advantages deriving from the so called "carboxylate platform".

1.3. BIOREFINERIES FACILITIES

Depletion of fossil resources and increasing of waste generation, due to the constant growth of population, are becoming two more and more hard challenges to overcome for ensuring a bright future for humanity.

In order to limit our dependence on fossil resources, but also to mitigate climate warming, the development of alternative production technologies in energy and chemical sectors are almost mandatory. This requires a shift towards renewable resources, which are not finite and can be easily regenerated. While energy can be

produced starting from various alternative sources (wind, sun, water, biomass, etc.), an alternative market of valuable substances should mainly depend on biomass. This implies the development of facilities for conversion of bio-resources, that, likewise to conventional petroleum-based refineries, are able to produce both energy and chemicals starting from biomass. Therefore, they are the key for access to a sustainable circular economy model of integration of bio-based goods production (food, feed, chemicals and materials) and bioenergy (fuels), maximizing the value of the biomass and minimizing waste and GHGs emissions (Clark & Deswarte, 2015).

Waste generation and management is one of major issue for our society. Approximately 50% of the three million tons per day of wastes produced worldwide are composed by organic material. This includes household, food manufacturing and pre-factory wastes, the rest being paper, plastic, glass, metal and others. All these residual materials are rich of different compounds, most of which have unexploited energetic and economic value (Coma et al., 2017).

Furthermore, the enormous amount of waste globally produced is one of the main sources of GHGs, so recovering renewable energy and bio-based products from organic wastes could be a valuable strategy for limiting emission of GHG in the atmosphere. This is not a secondary aspect, since according to climate models a further increase of GHGs emissions is projected to rise Earth's surface temperature from about 0.3 to 5 °C by the end of this century with respect of 1986 through 2005 temperature levels (USEPA, 2018).

IEA Bioenergy, an organization of International Energy Agency (IEA) with the aim of improving cooperation and information exchange between countries with national program in bioenergy research, in "Task 42" has developed the following definition for biorefinery: «*Biorefinery is the sustainable processing of biomass into a spectrum of marketable products and energy*» (Lindorfer et al., 2019)

This is not a completely new concept, since biorefineries are gaining increased commercial and academic attention in many parts of the world in the last decades. Over the years, different generations of biorefinery have been established, with increasing sustainable features (Figure 4).

Since the 1990's the so called "first generation biorefineries" have become well-established facilities. Essentially, they are facilities specialized in the production of biofuels (i.e. biodiesel, bioethanol, and biogas), characterized either by their capability to be blended with petroleum-derived fuels, combusted in existing internal combustion engines, and distributed through existing infrastructures. The first-generation biofuels have been widely commercialized, with almost 50 billion liters produced annually since about ten years ago (Naik et al., 2010).

Nonetheless, an increase of first-generation fuels market, such as bioethanol, has led to huge disagreements (i.e. "food vs. fuel" debate), since these fuels are produced starting from sugar-rich and oleaginous biomass, which it is usually used for human consumption.

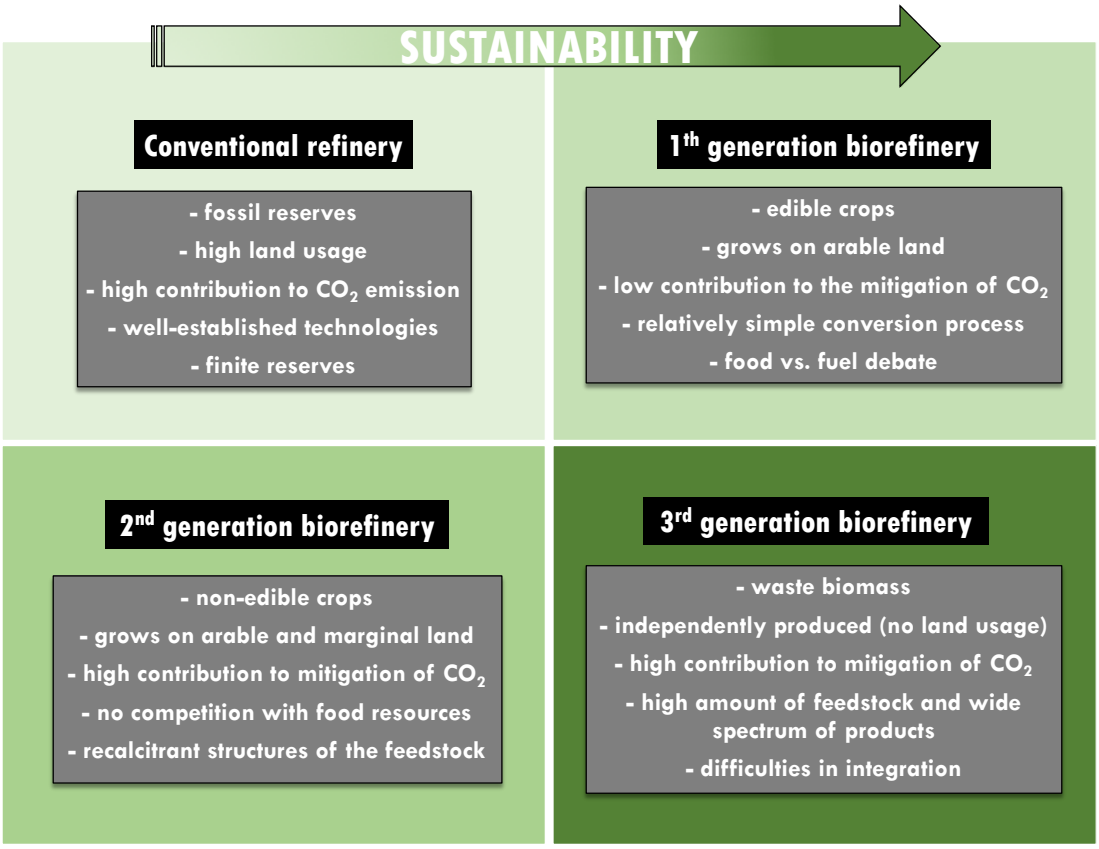

Figure 4. Comparison among conventional refinery and 1th, 2nd, and 3rd generation biorefineries

The food versus fuel debate intensified as food prices rose. For instance, between 2005 and 2010 world grain prices strongly increased, from $106 per ton to $277 per ton, and this was correlated to a tripling of maize-based ethanol production in United States in the same period, because of supportive policies and a volatility of petroleum prices (Ghosh et al., 2019).

Therefore, the growing food versus fuel debate, along with economic production concerns, sustainability policies, promoted the rise of a second generation of biorefinery, based on lignocellulosic biomass feedstocks for production of biofuels and chemicals with high added value (Scoma et al., 2016). Although the second generation biorefineries show more sustainable futures, allowing to overcome the food versus fuel controversy, they are still far from a circular economy approach, due to the consume of resources (e.g. water) and land. Furthermore, the economic feasibility of these plants is vitiated by the low-cost efficiency, mainly due to expansive pre-treatment needed for the exploitation of lignocellulosic biomass (Hassan et al., 2019).

From a sustainable point of view, the best choice is a novel third generation biorefinery, based on residual biomass (multi-feedstock and multi-purpose biorefinery). It derives from a great spectrum of wastes, such as agricultural residues, municipal solid wastes, manure, food waste and so on, along with marine biomass (i.e. micro- and macroalgae) (Chang et al., 2010; Jambo et al., 2016). This novel facility is very promising, in fact, combining multiple feedstocks and processes, it was estimated that third generation biorefineries could satisfy a significant proportion of the overall european demand for chemicals, energy and materials, using residual biomass, by 2030: (*i*) 30% of overall chemical production is expected to be bio-based by this date (for high-added-value chemicals and polymers, the proportion might twofold higher), (*ii*) 25% of energy demanded by Europe's transport sector will be provided by biofuels, and (*iii*) 30% of Europe's heat and power generation will be derived from biomass.

The next paragraph provides a comprehensive discussion on the novel biorefinery approach, based on biological conversion of waste-derived biomass first into short chain carboxylates, which can serve as intermediates for bioenergy and chemicals production. This biorefinery represent the so called "carboxylate platforms", that, by the way, is the primary object of this thesis.

1.4 THE CARBOXYLATE PLATFORM: VFAs AS KEY BIO-PRODUCTS

In a biorefinery based on carboxylate platform, residual biomass is converted, thanks to hydrolysis and fermentation carried out mainly by mixed microbial cultures (MMCs), into short chain carboxylates, which serve as intermediate molecules for different downstream applications (Monti et al., 2015).

Carboxylates are dissociated organic acids, characterized by the presence of at least one carboxyl group. Among them, VFAs represent the most important class of compounds, since they can be used in a several downstream applications, thanks to their chemical nature (Agler et al., 2011). VFAs are linear short-chain aliphatic mono-carboxylate compounds with a saturated carbon chain consisting of C2 (acetic acid) to C6 (caproic acid). Because of their functional groups, VFAs are themselves valuable products, as shown in Figure 5, with a world market size over 13 million tons per year, and a market value of about 8 billion dollars (Kim et al., 2018).

Furthermore, VFAs are extremely useful in biorefinery context, since they represent suitable precursors for production of biopolymers, such as PHAs, reduced chemicals and derivatives (esters, ketones, aldehydes, alcohols and alkanes), as well as biofuels like CH_4 and H_2 (Raganatia et al., 2014).

Conventionally, VFAs are produced biologically via acidogenic fermentation pathways, using pure culture of specific anaerobic bacterial strains, achieving high yields. Nevertheless, to make economically viable the production of VFAs from residual biomass, such as the main organic waste streams produced in urban environment (i.e. food waste and WWS), the use of MMCs is particularly attracting (Bhatia & Yang, 2017; Domingos et al., 2017). Although MMCs performances may lead to lower yields in terms of VFAs, they have several advantages, as non-sterile conditions are needed, and risk of contamination is decreased. At the same time, MMCs can be able to metabolize a wide spectrum of organic wastes, such as agricultural or urban wastes, like wastewater, sludge, and food waste (Jankowska et al., 2015).

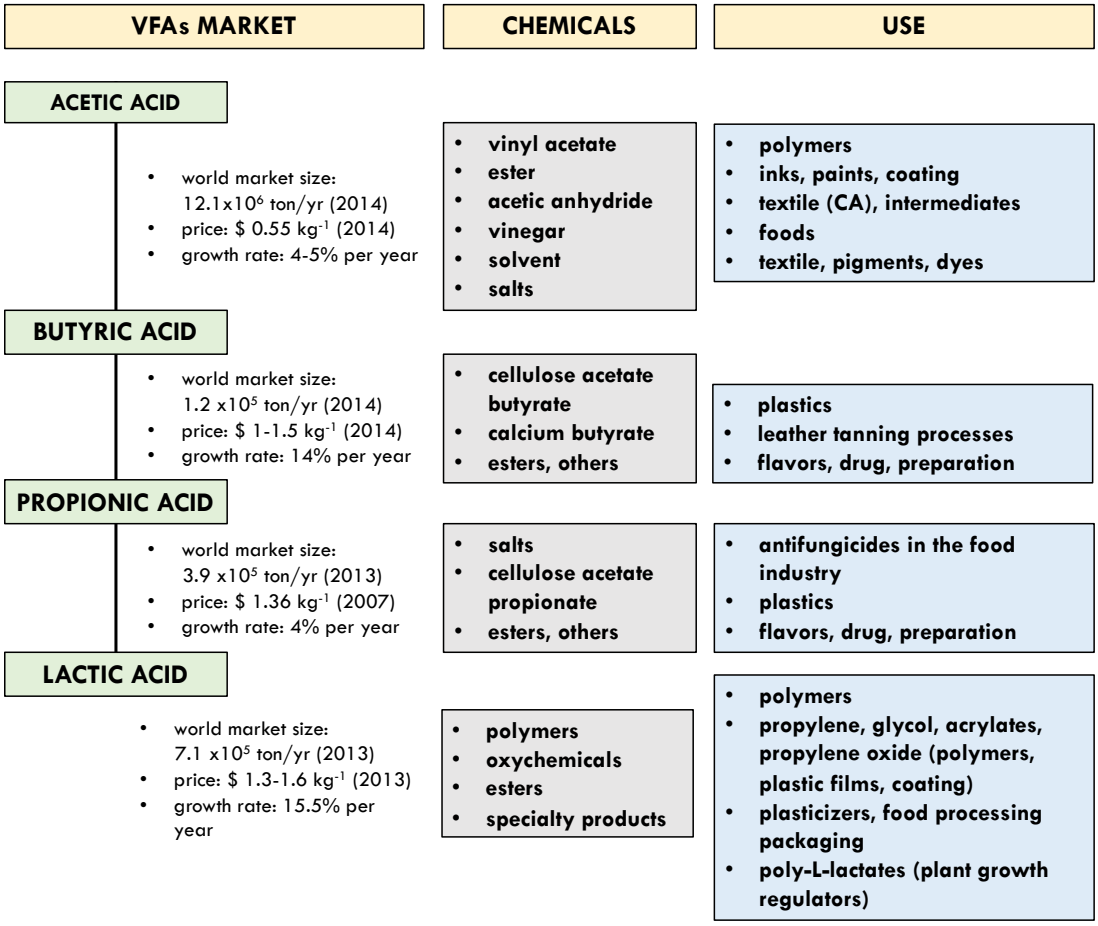

Figure 5. Market size and application of main VFAs (modified from Kim et al., 2018)

However, in order to strengthen the potential of MMCs acidogenic fermentation in terms of VFAs production, it is necessary to pay close attention to process parameters and operational conditions during the experimental setup. In fact, different HRTs, OLRs, temperature, and pH, may lead to fermentation end-products other than VFAs, such as

longer chain fatty acids, alcohols, biohydrogen, biomethane, esters, and other intermediates (Mohan et al. 2016; Strazzera et al., 2018).

The increasing interest in VFAs production is easily explainable as they are the main precursors of interesting added value compounds. Indeed, VFAs can undergo further chemical post-processes, which allow to convert these and other carboxylates to bulk fuels or solvents. These processes consist of esterification, reduction, and ketonization, as shown in Figure 6, by mean of which VFAs can be converted into viable carbonyls, esters, alcohols, alkenes for solvents and fuels production (Agler et al., 2011).

Moreover, VFAs can be used as "starting blocks" for a variety of biological processes, that make them a class of very marketable compounds. Some of these applications are discussed below.

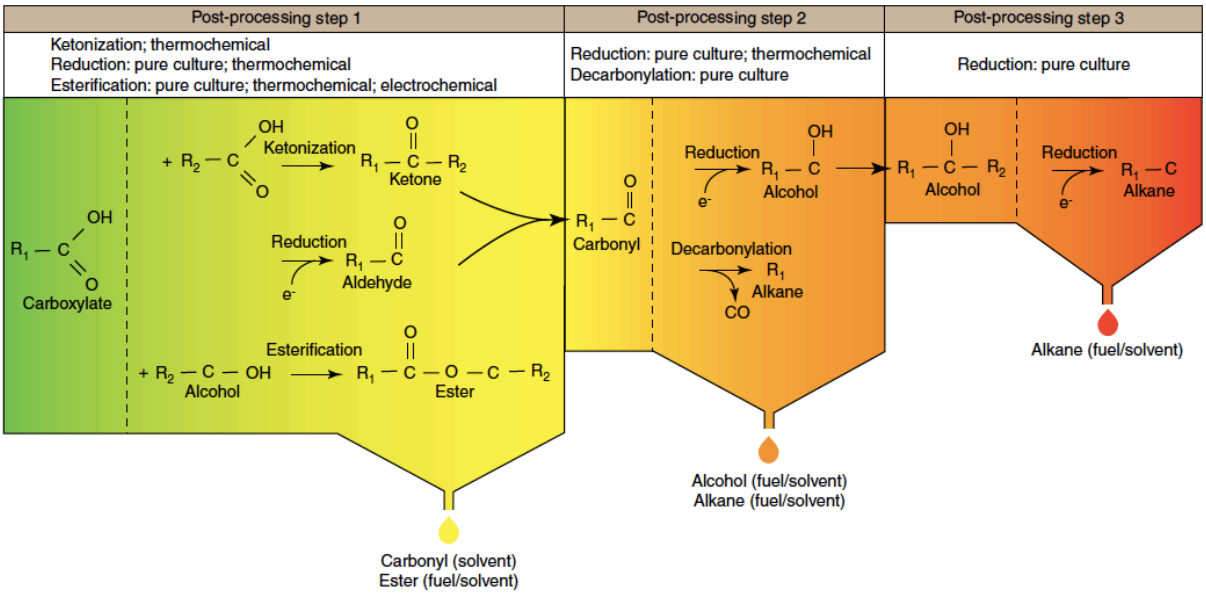

Figure 6. Chemical post-processes that convert carboxylates to bulk fuels or solvents (Agler et al., 2011)

1.4.1 Bioenergy (biogas & biohydrogen)

Historically, VFAs production by acidogenic fermentation was investigated due to its key role in anaerobic digestion (AD) for biogas production. AD is a complex biological process, consisting of the following steps: hydrolysis, acidogenesis, acetogenesis, and methanogenesis (Figure 7). By the end of the process, a mix of gases, called biogas, is produced, which consist of 60%-70% CH_4, 30%-40% CO_2, and traces of other gases such as H_2 and H_2S. The aforementioned steps involve different microbial communities: acidogenes account for hydrolysis and fermentation of monomers to produce VFAs,

which can be further metabolized by acetogenic bacteria into acetate and H_2, subsequentially converted into CH_4 by methanogens. Therefore, VFAs, whose production needs hydrolysis of complex biomass and subsequent acidogenic fermentation of monomers, is the limiting step of the whole AD process (Siddique & Wahid, 2018; Wang et al., 2018). At the same time, when VFAs concentration exceeds the buffering capacity of system, they can show an inhibitory effect on downstream methanogenesis process (Braguglia et al., 2018). A widely adopted solution to avoid inhibition of methanogenic archaebacteria due to VFAs accumulation, is the employment of a two-stage digestion system. Thus, VFAs are partially buffered by recirculation of digestate, rich in ammonia and other buffer agents, from second stage AD (Bolzonella et al., 2018). Even if microorganisms involved in AD are able to convert VFAs into acetic acid, allowing their utilization as carbon source for downstream methanogenic archaebacteria, some of these carboxylic acids are less available for this bioconversion. For example, it was observed that butyric acid generally is accumulated into digester during AD of food waste, as consequence of the obstacle for bacteria to convert it toward biomethane (Braguglia et al., 2018).

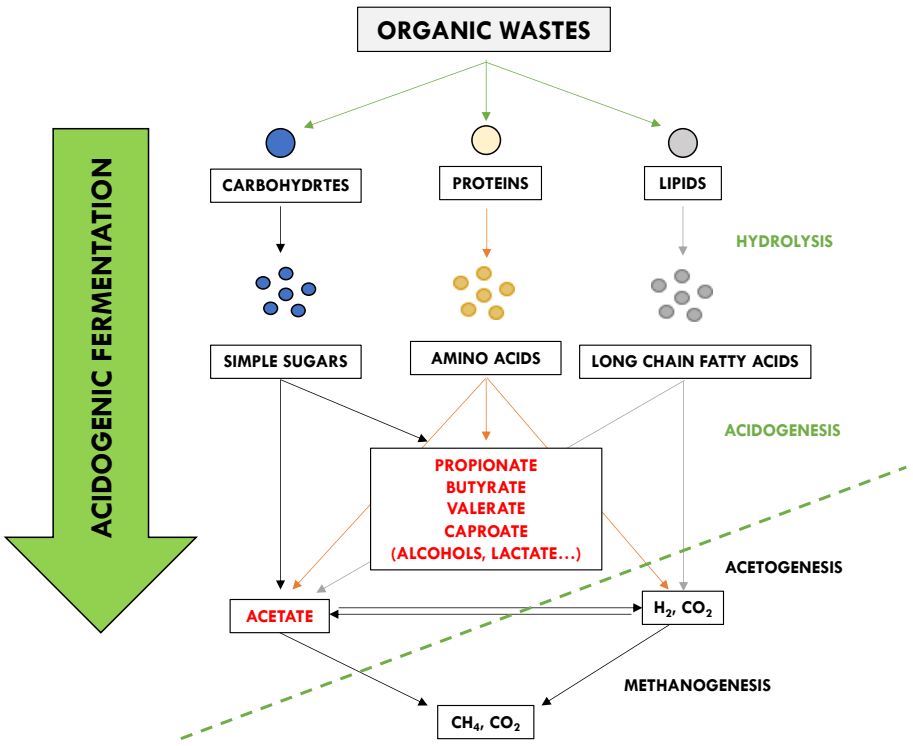

Figure 7. Biochemical stages flow of AD process (modified from Wang et al., 2018)

Another profitable gas, hydrogen, can be obtained during AD by acidogenic fermentation step. Because of its efficiency peculiarity, H_2 is one of the most desirable

form of renewable energy, and it is considered to be the future fuel (Hay et al., 2013; Kim & Kim, 2013; Dutta, 2014; Ghimire et al., 2015). Furthermore, thanks also to its clean intrinsic nature, many researches were carried out to develop biotechnological routes to produce H_2 in an environment friendly manner from residual biomass, mainly by dark fermentation (DF) and photofermentation (PF) (Elbeshbishy et al., 2017).

DF consists of acidogenic fermentation, mainly of sugar-rich material, in absence of light and oxygen, for the production of bio-hydrogen, along with acetic acid, butyric acid, propionic acid, alcohols, such as ethanol, and other by-products like CO_2. In other words, DF coincides with the acidogenic step of AD, which is optimized for maximizing H_2 production (Yasin et al., 2013; Bundhoo, 2019).

PF is a biochemical route, typical of purple non-sulphur bacteria (PNSB), that allows to obtain H_2 using energy driven from light (Ghosh et al., 2017). PNSB, as well as other photofermentative bacteria, produce H_2 in presence of light photoheterotrophically, making use of electrons derived from metabolism of organic compounds. This process involves a nitrogenase, an enzyme that forms H_2 as a byproduct during nitrogen fixation to ammonia, consuming ATP produced by photosynthesis, and the electrons produced from metabolism of organic compounds, such as VFAs (Hay et al., 2013; Morsy et al., 2019). For example, *Rhodopseudomonas spp.* are able to metabolize different fatty acids, while *Rhodobacter spp.* show more difficult to use the heavier VFAs, valerate and caproate, which have an inhibitory effect on growth of these species (Okubo et al., 2005). VFAs are extremely suitable for PF, using specific strain of PNSB bacteria. For example, a specific isolated PNSB, named ZX-5, is able to carry out photofermentation on VFAs, with a conversion efficiency of about 69, 71, and 62% growing on acetic, butyric, and propionic acid, respectively. Moreover, a conversion efficiency of only 12% was obtained growing on valeric acid, while caproic acid resulted to be inhibitory on H_2 production, as consequence of inhibition of cells growth. Surprisingly, conversion efficiency was lower or zero using simple sugars (Tao et al., 2008).

1.4.2 Biological nutrient removal in wastewater treatment

VFAs are commonly used for biological nutrient (e.g. nitrogen and phosphorus) removal in wastewater treatment plants. For example, to ensure a complete denitrification step, due to low carbon concentration in wastewaters, an addition of an external carbon source is required. Usually methanol, ethanol and sodium acetate are used to increase denitrification efficiency (Bolzonella et al., 2001; Shao et al., 2019). Nonetheless, VFAs derived from acidogenic fermentation of organic wastes are a viable organic carbon source for biological nutrient removal, which allow to reduce the overall cost of the process (Zhang et al., 2016; Kim et al., 2017). It was observed, in fact, that VFAs, when

used as denitrification carbon source, can increase denitrification rate, and limit nitrite accumulation comparing to other compounds (Liu et al., 2016; Kim et al., 2016). However, VFAs show a different employment pattern during denitrification process. For example, it was observed that, when a mix of VFAs was used, acetic acid led to a more than double greater denitrification rate with respect to propionic acid, reflecting a preference for acetate. This result is explained considering that denitrifying populations use preferentially acetic acid as carbon source, and only when acetate concentrations began to decline, bacteria consume butyric acid and propionic acid. The last choice was represented by valeric acid, confirming that denitrifying bacteria have a sequential preference for VFAs (Elefsiniotis et al., 2004; Elefsiniotis & Wareham, 2007). With regard to phosphorus, it can be removed by a process known as enhanced biological phosphorus removal (EBPR), which requires an alternation of anaerobic and aerobic conditions. EBPR is extensively used in wastewater treatment plants and allows to remove 80–90% of soluble phosphorus from effluent (Mehta et al., 2015). Since a carbon source is needed during anaerobic stage of EBPR (a C/P ratio of 5 or higher), VFAs are suitable class of compounds for EBPR itself (Rashed & Massoud, 2015). Furthermore, it was proven that VFAs derived from acidogenic fermentation are more effective in promotion of phosphorus removal with regard to synthetic acetic acid. For example, it was observed that a phosphorus removal efficiency of 98% was achieved using the fermentative, while an efficiency of only 71% was reached using pure acetic acid (Tong & Chen, 2007). Nonetheless, it was discovered that different VFAs show a different impact on the overall EBPR process. However, different EBPR performances using different VFAs were reported in literature, leading to dubious conclusions about the optimal acid among VFAs for EBPR process (Hood & Randall, 2001; Oehmen et al., 2005).

1.4.2 Biopolymers

Among the wide range of applications of VFAs and other carboxylic acids from acidogenic fermentation, such as lactic acid, biopolymers production (e.g. PHAs and PLA) is likely one of the most attractive, also driven by the establishing of the novel circular economy approach.

The global production of plastic materials reached about 320 million tons in 2016, and a growing trend in plastic production is expected at least until 2020 (Anjum et al., 2016). Almost the entire production derived from petroleum, posing serious environmental concerns, such as accumulation in the environment, global warming, human health risks or ecosystem toxicity (Rodriguez-Perez et al., 2018).

Biopolymers can contribute to decrease conventional plastics environmental impact, showing, at the same time, similar physicochemical properties as conventional plastics. In particular, PHAs have thermoplastic properties similar to those of polypropylene, good mechanical properties, and excellent biodegradability in various ecosystems (Cinelli et al., 2019).

PHAs are natural thermoplastic polyesters, consisting of 3-, 4-, 5-, and 6-hydroxyalknoic acids, which are synthesized by a variety of microorganism as granular intracellular inclusions for energy storage (Arcos-Hernández et al., 2015). These inclusions are in the form of polyesters, enclosed by both phospholipids and proteins, with a size ranging from 0.2 to 0.5 μm (De Grazia et al., 2017). The most prevalent PHAs are the poly([R]-3-hydroxybutyrate) (PHB) and its co-polyester with [R]-3-hydroxyvalerate (PHB-HV), which is well suited for applications such as food packaging, thanks to its chemical-physical characteristics. Despite their good properties and excellent biodegradability, they have a relatively high cost (about 7 and 12 € per kg, respectively), which limits their use in commodities industry, restraining their use to high-value applications in the medical and pharmaceutical sectors (Cinelli et al., 2019). To reduce the overall production cost of PHAs, different researches have been carried out on PHAs production starting from carbon sources derived from waste materials (Raza et al., 2018). PHAs are produced by at least ninety different genera of both aerobic and anaerobic bacteria, of both Gram-positive and Gram-negative groups. VFAs from acidogenic fermentation of organic wastes are suitable as carbon source for PHAs production, being their direct metabolic precursors (Girotto et al., 2015; Anjum et al., 2016). Furthermore, production cost can be reduced using MMCs, i.e. selected PHAs-storing cultures, since the use of pure cultures is intrinsically more expensive, demanding sterile conditions, complex equipment and control devices, in comparison to MMCs approaches (Koller et al., 2017; Morgan-Sagastume et al., 2019). Within this approach, PHAs are produced by mean of a three-stages process: an acidogenic fermentation step, for VFAs production, is followed by selection, through a feast and famine cycle, and growth of PHA-storing bacterial biomass, and finally a third step of PHAs accumulation (Reis et al., 2011).

One issue that must be addressed when PHAs are obtained using VFAs from acidogenic fermentation of organic wastes is the influence of VFAs distribution on the PHAs produced at end of the process. When PHAs producers grow on a mixture of VFAs a wider fraction of monomers other than PHB is produced (i.e. 3-hydroxyvalerate, 3-hydroxy-2-methyl-valerate or 3-hydroxyhexanoate), with respect to PHAs produced by pure cultures, that are typically fed with refined sugars (Albuquerque et al., 2011). In particular, it was proven that the hydroxyvalerate monomer in the polymer is

positively associated with the abundance of acids with odd carbon atoms such as propionic, *iso*- and valeric acids in the VFA mixture. PHA with a higher hydroxyvalerate content have more strength, with a positive effect on processing performance of the polymer (Huang et al., 2018). In conclusion, it is clear that driving acidogenic fermentation toward VFAs with and odd number of carbons is a key aspect to take into account for the production of PHAs with optimal characteristics for their industrial application.

PLA is one of the most extensively researched and utilized biodegradable and renewable aliphatic polyester. As for PHAs, PLA is a thermoplastic, high-strength, high-modulus polymer, that can be made from renewable resources. Since PLA is produced from lactic acid, which is a chiral molecule, it has stereoisomers, and exist in the form of poly(L-lactide) (PLLA), poly(D-lactide) (PDLA), and poly(DL-lactide) (PDLLA). PLA can be synthetized by different polymerization processes such as polycondensation, ring opening polymerization and by direct methods, like azeotopic dehydration and enzymatic polymerization. PLA is classified as safe (GRAS) by the United State Food and Drug Administration (FDA) and it can be used for both food packaging applications and medical applications (Hamad et al., 2015; Farah et al., 2016; Elsawy et al., 2017).

1.5 AIM OF THE WORK

Thanks to a more stringent legislation policies, especially in Europe, the awareness of public opinion about the depletion of fossil energy resources, the future of energy and its environmental impacts is finally growing up in the last decade. Therefore, these actions provided a basis for the beginning of a transition of our economy towards a novel model, the circular economy.

It was predicted that world population will reach 9.6 billion by 2050, 66% of whom will live in urban areas. Presently, 54% of population live in cities and urban environment cause 70% of the total emissions of carbon dioxide (Angeli et al., 2018). It follows that urban environment is the target for the implantation of sustainable resources management. In this scenario, the overall aim of this work is to investigate the possibility of VFAs production through acidogenic fermentation using as feedstock the main organic waste streams produced in urban areas. Due to the central role in several bioprocess of this class of compounds, the optimization of VFAs production is a basic condition for the implementation of an integrated urban biorefinery.

In Chapter 2, the best operational parameters set up for maximizing VFAs from acidogenic fermentation of a synthetic household food waste was investigated. The long-term experiment was carried out in semi-continuous mode, changing a single

parameter at each experimental run. Specifically, were tested two OLRs, two temperatures, and three different pH conditions, monitoring at each run the overall VFAs production, as well as the relative distribution among fermentation products.

Chapter 3 is focused on the effect of chemical nature of carbon source available to acidogenic bacteria on VFAs production, both in qualitative and quantitative terms. To achieve this result, a mixture representative of real household food waste was divided into five fractions, on the basis of the most abundant form of carbon content (i.e. proteins, lipids, more or less complex carbohydrates), and they were fermented in batch reactors. Furthermore, to compensate the different buffering effect of substrate, the experiment was carried out at three different pH, uncontrolled, *5.5*, and *7*.

Chapter 4 is focused on co-fermentation of three different organic wastes produced in urban environment: household food waste, WWS, and diapers. In particular, a di-fermentation and a tri-fermentation were carried out, fermenting household food waste/WWS and household food waste/WWS/cellulose from diapers, respectively. The best operational parameters found out during the mono-fermentation of household food waste were applied during this experimental run, carrying out the experiment under meso- and thermophilic conditions.

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CHAPTER 2: MAXIMIZATION OF VFAS PRODUCTION VIA ACIDOGENIC FERMENTATION OF HOUSEHOLD FOOD WASTE

2.1 INTRODUCTION

In 2016, about 88 million tones of food waste were generated in EU-28, which equates to 173 kilograms of food waste per person per year. Household food waste (HFW) is the major contributor, accounting for 53 % of total food waste. This amount corresponds to about 300 g of HFW per capita per day, which is equivalent to some 75–90 g of dry matter per person every day (Stenmarck et al., 2016; Battista et al., 2019). This implies that, among organic waste streams generated in urban environment, HFW is one of the most abundant. HFW is the manually sorted organic fraction of wastes collected at domestic level. Although actual composition of HFW strongly depends on period of collection, also on specific municipality or area, it shows a high energetic potential, due to its residual organic matter consisting of 80–90% volatile solids, mainly lipids (10–40%), protein (5–10%) and starch (10–60%). For this reason, HFW is an optimal candidate for use as renewable feedstock in an urban biorefinery system (Alibardi & Cossu, 2015; Battista et al., 2019).

In this scenario, optimization of VFAs production from HFW is a pre-condition for a complete exploitation of HFW, since this class of compounds have a crucial role for downstream bioprocesses within biorefinery, as discussed in Chapter 1.

Nowadays, VFAs are industrially produced mainly through oxidation or carboxylation of chemical precursors, such as aldehydes and alkenes, deriving from petroleum processing (Riemenschneider, 2000). However, a novel biorefinery approach for VFAs production contemplates the acidogenic fermentation process carried out by mixed microbial cultures (MMCs), which are able to face with the heterogeneous composition of HFW in terms of carbohydrates, proteins and lipids (Jankowska et al., 2015; Dai et al., 2017).

In order to maximize VFAs production from MMCs, it is necessary to pay close attention to operational conditions during the experimental setup. In fact, different HRT, OLR, temperature, and pH, influence the VFAs yield, as far as the amount of other fermentation by-products, such as longer chain fatty acids, other carboxylic acids,

alcohols, biohydrogen, biomethane, esters, and other intermediates (Mohan et al., 2016).

The aim of this work is to establish the best operational parameters setup to maximize VFAs production via acidogenic fermentation of HFW. After a complete characterization of HFW employed as substrate, acidogenic fermentation was carried out in semi-continuous mode, without an exogenous inoculum, changing properly the operational condition setup at each experimental run. Therefore, this study investigated the influence of three different operative parameters, pH, OLR, and temperature, on the acidogenic fermentation for VFAs production from HFW. Furthermore, to better understand the metabolic pathways involved, a characterization of microbiota involved during the different experimental runs was carried out.

2.2 MATERIALS AND METHODS

2.2.1 Substrate

To simulate the chemical and physical characteristics of the real substrate, a standardized HFW was used. It was formulated according to data found in literature. In particular, fresh foods, available all the year long, were chosen, to obtain similar carbohydrates, lipids, proteins and fibers contents of the real HFWs collected in Mediterranean Area (Garcia et al., 2005; Matsakas et al., 2014; Alibardi & Cossu, 2016). The theoretical contribution of each chosen food in terms of proteins, lipids, and sugars was valuated using the database on food composition provided by United States Department of Agriculture (https://www.usda.gov).

Thus, the fresh food fractions chosen were mixed and the synthetic substrate obtained was finely homogenized with a professional blender, and stored at -20±1°C, until usage. At the same time, an aliquot was used for the characterization of the feedstock. In particular, the following characteristics were measured: TS, TVS, COD, sCOD, N-NH_4^+, TKN, TP, proteins, carbohydrates, lipids, fibers, cellulose, hemicellulose, lignin, sugars.

2.2.2 Analytical methods

TS, TVS, TKN, TP, COD, and sCOD were measured according to Standard Methods (APHA-AWWA-WPCF, 2005). pH was determined using a portable probe (Eutech pH 700). N-NH_4^+ concentrations were measured by an ion selective electrode (Orion 9512). VFAs concentration was determined by ion chromatography system (Dionex ICS

1100 with AS23 column). Lignocellulosic composition was determined in terms of neutral detergent fiber (NDF), acid detergent fiber (ADF), acid detergent lignin (ADL), and crude fiber (CF) according to Van Soest and Wine (1967). Lipids analysis was carried out following method described by Soxhlet (1879), crude protein was determined by the standard Kjeldahl procedure of the Association of Official Analytical Chemists, (1990). Sugars were determined by mass spectrometry (MS), previous sample filtration at 0.20 µm. Lactic acid was measured using a commercial kit (Megazyme, Bray, Ireland). Grams of VFAs and lactic acids are following reported as grams of COD.
Finally, biogas composition was analyzed with a portable biogas analyzer (BIOGAS 5000 Geotech, UK). This instrument allows to detect methane, carbon dioxide, and oxygen as volumetric percentage, while hydrogen sulfide was detected as ppm. Thus, the daily volumetric hydrogen production was estimated as the total of volumetric biogas production after deduction of aforementioned gaseous products.

2.2.3 Experimental setup

The experiment was carried out in a laboratory scale CSTR, with a working volume of 4.2 L and a headspace of 0.8 L. The reactor was continuously stirred at 16.5 rpm, hermetically sealed, and volumetric biogas production was measured by a Milligascounter (RITTER, Germany). Temperature was controlled by water flowing through an external jacket. The presence of MMCs for the acidogenic fermentation was guaranteed by microorganisms still present in the substrate, rather by the addition of an exogenous inoculum.
The reactor was fed daily, except during the weekend, diluting the substrate with a proper volume of tap water. At that time, pH was adjusted, according to the one applied at each experimental run, using a NaOH solution (30% w/v).
An aliquot of the effluent flux was collected daily, and it was stored at -20±1°C for subsequent monitoring of fermentation performance. The experimental campaign was articulated in five experimental runs, with different operative parameters setup, as shown in Table 1. The effect of three different parameters on fermentation of HFW was investigated: pH (uncontrolled, 5.5, 7), OLR (22 and 11 gTS/Ld), temperature (37°-55°C). The HRT was kept constant at 6 days at each stage, according to experimental evidences from Moretto et al. (2019). During the first run, acidogenic fermentation was tested at uncontrolled pH, and high OLR (22 gTS/Ld) and mesophilic temperature (35°C). Then the pH was set up at 5.5 during second run, adjusting it during the daily feeding. These conditions avoided to reach a steady state of the system. Thus, the OLR was reduced at 11 gTS/Ld keeping constant the pH at 5.5 at third run. Finally,

the pH was increased to 7 at fourth run. OLR and pH were kept at 11 gTS/Ld and 7, respectively, during the last experimental run when the temperature was increased from mesophilic to thermophilic conditions (55°C).

Table 1. Operational parameters setup during each experimental run

	°T (°C)	HRT (d)	OLR (gTS/Ld)	pH
RUN 1	37 ± 1	6	22	N.C.*
RUN 2	37 ± 1	6	22	5.5
RUN 3	37 ± 1	6	11	5.5
RUN 4	37 ± 1	6	11	7
RUN 5	55 ± 1	6	11	7

*not controlled

2.3.4 Data analysis

The total VFAs yield was determined by the ratio between the daily VFAs production rate measured in the effluent from CSTR and the grams of TS fed per day, as follows:

$$\text{Yield}_{\text{totVFAs}} = \frac{\text{gVFAs/Ld}}{\text{gTS fed/d}}$$

The substrate solubilization rate was calculated as the ratio between the net sCOD at time *t*, calculated as the difference between sCOD at time *t* ($sCOD_t$) and sCOD derived from substrate ($sCOD_0$), and the TS of feedstock daily fed, as follows:

$$\text{Solubilization} = \frac{sCOD_t - sCOD_0}{\text{gTS fed/d}}$$

To understand the synergetic effect of the three operational parameters (pH, OLR, and temperature) applied at each experimental run on total VFAs production, principal components analysis (PCA) was performed. PCA allows to reduce the multidimensional space into few components and therefore to study the relationship among variables and objects in the modelled space composed by principal components (PCs), saving data variability. Finally, to find correlation between individual VFAs produced, operational parameters and bacterial populations, a correlation matrix was

constructed on excel, then it was used to draw an heatmap using the software GraphPad Prism 8.

2.2.5 Microbial community structure: high-throughput 16 s rRNA gene sequencing

In order to characterize the populations involved in acidogenic fermentation of HFW at each experimental run, a high-throughput 16 s rRNA gene sequencing test was performed. In particular, two sample were analyzed for each run, one at the beginning of steady state and one at the end for run 1 (40.90 – 80.87 days), 3 (240.83 and 257.80 days), 4 (289.82 and 324.84 days), and 5 (419.08 and 440.12 days). Whereas, for run 2, due to instability of the system, a sample taken at the first maximum (104.13 day) and at the first minimum (131.07) of total VFAs production were used.

Genomic DNA was extracted using about 0.25 g of dry weight of each sample. The extraction was performed with PowerSoil DNA Isolation kit (MoBio, Italy), according to the manufacturer's instructions. DNA was eluted in 100 µL of sterile water and the concentration and purity were determined by NanoDrop (2000c) spectrophotometer (Thermo Scientific, USA). Aliquots were stored at −20 °C for a few days and then used for high-throughput 16 S rRNA gene sequencing. V1-3 variable regions were used as target for procedure to bacterial 16 S rRNA amplicon sequencing, based on Caporaso et al. (2012). Primers adapted from the Human Gut Consortium were used. Ten ng of extracted DNA was used as template and the PCR reaction (25 µL) contained dNTPs (400 nM of each), MgSO4 (1.5 mM), Platinum® Taq DNA polymerase HF (2mU), 1X Platinum® High Fidelity buffer (Thermo Fisher Scientific, USA), and barcoded library adaptors (400 nM) containing V1-3 specific primers: 27 F AGAGTTTGATCCTGGCTCAG and 534 R ATTACCGCGGCTGCTGG. All PCR reactions were run in duplicate and pooled afterwards. The amplicon libraries were purified using the Agencourt® AMpure XP bead protocol (Beckmann Coulter, USA). Library concentration was measured with Quant-iT™ HS DNA Assay (Thermo Fisher Scientific, USA) and quality validated with a Tapestation 2200, using D1K ScreenTapes (Agilent, USA).

The purified sequencing libraries were mixed in equimolar concentrations and diluted to a final concentration of 4 nM. The samples were paired end sequenced (2x301bp) on a MiSeq (Illumina) using a MiSeq Reagent kit v3, 600 cycles (Illumina, USA) following the standard guidelines for preparing and loading samples on the MiSeq. 20% Phix control library was spiked in to overcome low complexity issue often observed with amplicon samples. Bacteria V1-3 forward reads were trimmed for quality using

Trimmomatic v. 0.32, according to Bolger et al. (2014), with the settings SLIDINGWINDOW:5:3 and MINLEN:275 and clipped to a length of 275 bp. The cut down forward reads were dereplicated and formatted for use in the UPARSE workflow (Edgar, 2013). The dereplicated reads were clustered, using the usearch v. 7.0.1090 -cluster_otus command with default settings. OTU abundances were estimated using the usearch v. 7.0.1090 -usearch_global command with -id 0.97. Taxonomy was assigned using the RDP classifier (Wang et al., 2007) as implemented in the parallel_assign_taxonomy_rdp.py script in QIIME (Caporaso et al., 2010), using the SILVA database.

2.3 RESULTS AND DISCUSSION

2.3.1 Substrate characterization

Figure 1 shows the formulation of synthetic HFW used for the experimental campaign. Before storing the substrate, it was characterized, obtaining the results listed in Table 2. The chemical-physical characteristics found out for the standardized HFW were comparable to ones of real substrate (Battista et al., 2019).

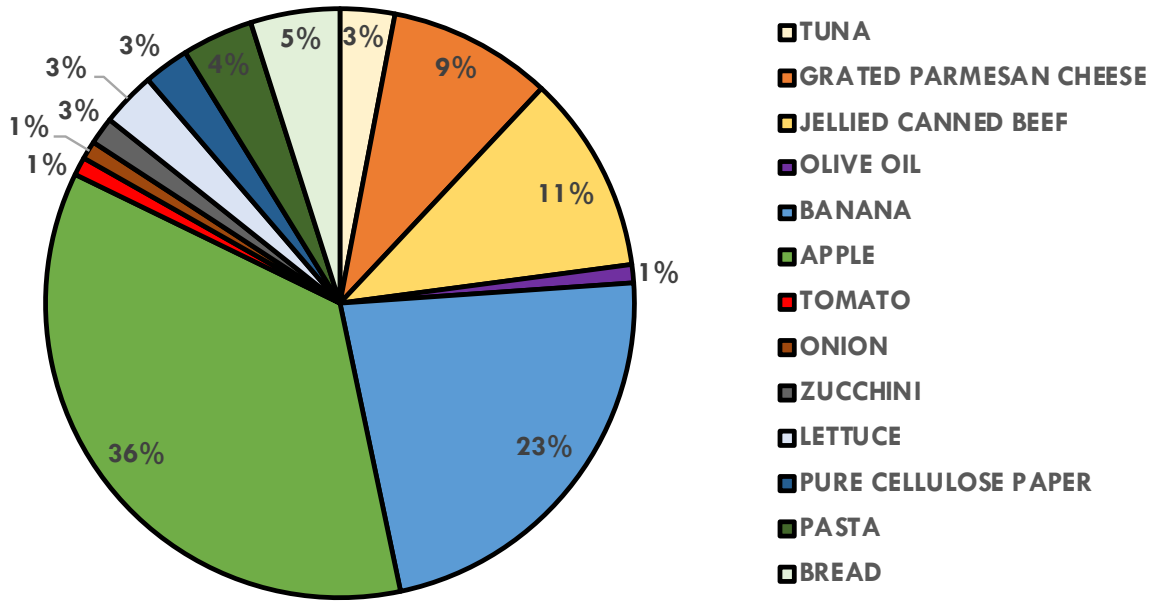

Figure 1. Standardized HFW composition (% w/w)

Table 2. Physic-chemical characteristics and macromolecular composition of synthetic HFW

Parameter	Value	Parameter	Value (%TS)
pH	5.66 ± 0.18	Proteins	23.9 ± 1.0
TS (g/kg)	257.26 ± 13.92	Carbohydrates	21.1 ± 0.6
TVS (g/kg)	250.87 ± 13.03	Lipids	15.6 ± 1.0
COD* (g/kg)	292.42 ± 47.93	Fibers	31.6 ± 0.5
sCOD (g/L)	51.68 ± 8.82	Cellulose	10.7 ± 0.6
TKN* (g/kg)	9. 90 ± 1.95	Hemicellulose	10.4 ± 1.0
TP* (g/kg)	1.45 ± 0.51	Lignin	10.5 ± 1.0
C/N	29	Sugars	30.2 ± 1.0

*on dry basis

2.3.2 Acidogenic fermentation performance

Figure 2 shows the variation of total VFAs production during the whole experimental campaign. At the beginning, when acidogenic fermentation of HFW was tested at an OLR of 22 gTS/Ld, mesophilic condition, and uncontrolled pH, the pH of the system quickly dropped to an average value of 3.6 ± 0.2. Under these conditions, the reactor reached a stable VFAs production rate of around 3.5 g/Ld after twenty days of work. Acetic acid was almost the unique fermentation product, as shown in Figure 3, accounting for about the 90% of total VFAs produced. Furthermore, in this experimental run a very low VFAs yield of 0.03 gVFA/gTVS fed was achieved. These values are in line with results reported by other authors under strong acid conditions (Bolzonella et al., 2005). Therefore, this poor fermentation performance is not surprising. Under strong acid conditions (~ 3) the fermentative metabolism of acidogenic bacteria is inhibited. VFAs are weak acids, having an acid dissociation constant (*pKa*) comprised from 4.76 (acetic acid) to 4.90 (caproic acid), thus, when pH is under a value of 4, VFAs in the medium are undissociated. Undissociated acids are more liposoluble and can diffuse across the plasma membrane. In the cytosol, dissociation of these acids occurs, due to the neutral intracellular pH, decreasing the cytosolic pH, with a subsequent growth-inhibiting effect on microorganisms (Palmqvist, E., & Hahn-Hägerdal, 2000). Finally, an average solubilization rate of 0.30 gCOD/gTVSd was measured in the efflux, but only the 5% of which consisted of VFAs. This low solubilization ratio, along a small VFAs/sCOD ratio, is a further confirmation of a low metabolic activity toward VFAs.

Nonetheless, an average production rate of 9.38 g/Ld of lactic acid was achieved during run 1, barely than double observed for total VFAs. This is due to a lower inhibition effect of metabolism of lactic acid bacteria under strong acid conditions, as reported by Gottardo et al. (2017). This different behavior is probably due to the lower *pKa* (3.1) of lactic acid compared to the ones of VFAs. In this way, during the first experimental run lactic acid was still in undissociated form, thus it did not cross the plasma membrane, avoiding inhibition of its biosynthetic pathways.

During the second experimental run, all parameters were kept constant, while pH was adjusted toward a value of 5.5. Under these conditions, the reactor did not reach a steady state, and VFAs production rate followed a symmetric oscillation between 30 g/Ld and 5 g/Ld.

This phenomenon was already described by other authors for anaerobic digestion of food waste, and it was probably due to a detrimental effect of substrate overloading on system stability (Chen et al., 2012; Nagao et al., 2012).

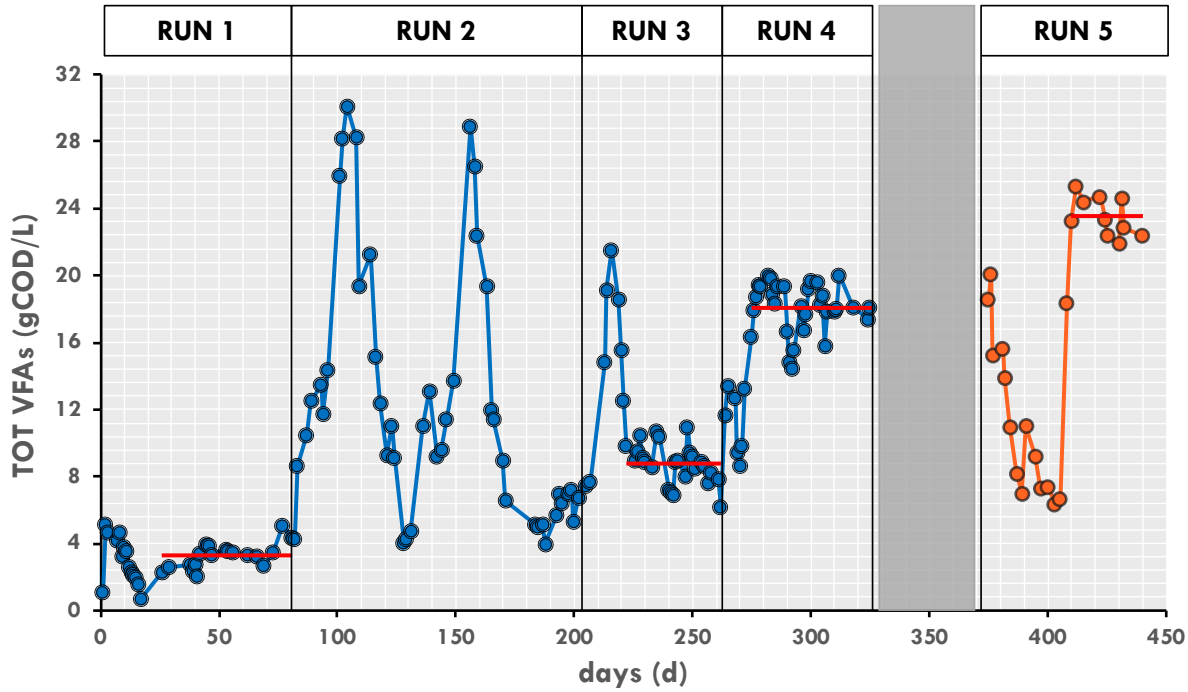

Figure 2. Total VFAs production trend. Blue curve is referred to mesophilic fermentation of HFW; orange curve is referred to thermophilic fermentation of HFW. Red lines show the average productivity at each steady-state; grey rectangle show the period of time during which the reactor was not fed to avoid washing-out phenomena

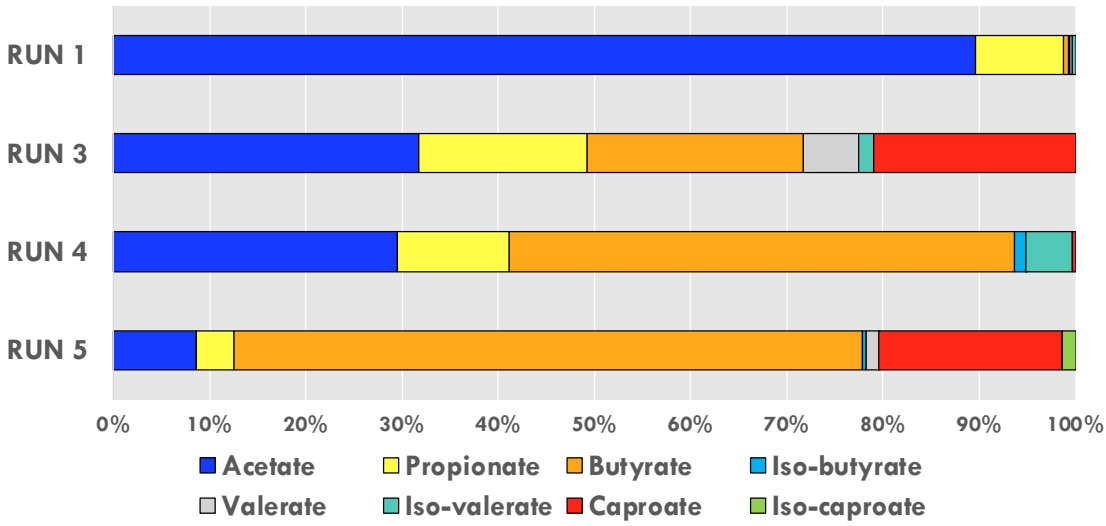

Figure 3. Average distribution pattern among VFAs for steady state at each experimental run. Run 2 is not included, due to the instability of the system

To recover the reactor from disturbance, in the third experimental run, the OLR was halved, from 22 to 11 gTS/Ld, while the pH was kept at 5.5. By this way, the reactor reached a steady state condition after around 15 days of operation, achieving a stable VFAs production of about 8.5 g/Ld, corresponding to a yield of 0.14 gVFA/gTVS fed. VFAs showed a wider distribution, as shown in Figure 3: acetic acid was still the main product, accounting for 32% of total, but also butyric, caproic, propionic, and valeric acids were present, accounting for the 23, 21,17, and 6 % of total VFAs, respectively. Finally, during the third run an average lactic acid production rate of around 7 g/Ld was achieved.

During the fourth experimental run, as consequence of a further increase of pH toward 7, the VFAs production rate reached a stable value of around 18 g/Ld, after 11 days of working, with a yield of 0.29 gVFA/gTVS fed. A similar yield, of 0.27 gVFA/gTS fed, was achieved by Zhang et al. (2005) from acidogenic fermentation of kitchen waste.

The improvement of acidogenic fermentation performance due to neutral pH conditions is confirmed by an increase of hydrolytic rate. At the third and fourth experimental runs, the effluent from reactor had an average sCOD of about 30g/L during run 3 and 50 g/L during run 4, which correspond to a solubilization rate of 0.27 and 0.59, respectively. This increase of the solubilization rate, is probably due to a higher metabolic activity, which results in a greater hydrolysis. Nevertheless, when pH was adjusted to 7, a greater acidification efficiency was achieved, since 38% of sCOD was composed of VFAs during the fourth run, while only 30% under pH 5.5 at the previous run. These results are in line with the ones from other authors. For example, Jiang et al.

(2013) studied the influence of different pH values, comprised in slightly acidic range (5-7), on VFAs production from synthetic kitchen waste. They found out that a pH value comprised among 6 and 7 brought to an increase of around 20% of hydrolysis rate, achieving a sCOD of 82 g/L. At lower uncontrolled pH, the sCOD was 60 g/L. This increase of solubilisation allowed to double VFAs production and other fermentation products by tenfold compared to the uncontrolled pH, as consequence of a higher hydrolytic enzymatic activity and avoidance of inhibition due to acidification of medium.

Despite of this increase of total VFAs production, relative distribution remained almost stable with respect to acetate and propionate, while caproate and valerate disappeared in favour of butyrate, which become the main product, accounting for around 50% of total VFAs, as displayed in Figure 3. During run 4, the average lactic acid production rate dropped to a value of about 1.09 g/Ld. This result is line with the findings of Gottardo et al. (2017), which observed a drastic reduction of lactic acid production under neutral conditions in favour of VFAs production.

In order to test the influence of thermophilic conditions, working temperature was increased to 55°C during the fourth run, keeping constant all the other operational parameters. In order to avoid the washing out of biomass, the reactor was not fed for 45 days, until a certain daily volumetric biogas production was observed. Under these conditions a stable total VFAs production of around 23.5 g/L was achieved during the last run, after 30 days of working. Butyric acid was the main product, accounting for around 65% of total VFAs, while caproic, acetic, and propionic acids constituted only the 19, 9, and 4% of total VFAs. Furthermore, a yield of 0.38 gVFA/gTVS fed was achieved. This greater yield was probably due to a better acidogenic fermentation metabolism, rather than a greater hydrolytic activity, since a solubilization rate of 0.63 was achieved during run 5, comparable to one observed for run 4 (0.59). Furthermore, a greater amount of sCOD was composed by VFAs during the last experimental run: 45% for run 5 compares to 38% for run 4. Finally, an increase of lactic acid production rate was observed with respect to the previous run, from 1.09 g/Ld for run 4 to 8.37 g/Ld for run 5. Probably, this was due to a favourable effect of increased temperature on lactic acid bacteria populations in the reactor.

Regarding gaseous products, CH_4 yield was approximately null for all experimental runs. This result is in line with what stated by Lee at al. (2014). According to these authors, CH_4 production easily occurs under alkaline conditions, since methanogenic archeabacteria have an optimum pH range growth of 8-10. Finally, H_2 occurred as fermentation by-product during all experimental runs, even though an appreciable volumetric yield, of about 67 mL/gTVS, was achieved only during the last run.

Figure 4 shows the mass balance of the system. The detrimental effect of acidification during run 1caused a very low percentage of bioconversion. Nonetheless, total VFAs production and lactic acid are capable to explain the 63% of COD conversion.

Halving OLR at run 3, along with an increase of pH to 5.5, allow to doubling TS, TVS and COD bioconversion. Moreover, in this case only 50% of COD was converted into VFAs and lactic acid, but, in this experimental run, a two times higher amount of CO_2 was produced (2.97 g vs 1.45 g) with respect to run 1, contributing to COD convertion.

During run 4, a 16% lower COD bioconversion took place with respect to run 3. Despite this, about 84% of COD bioconversion is related to VFAs production. Furthermore, the rest of COD bioconversion can be explained by the production of 5.12 g of CO_2.

During the last experimental run, a greater percentage of TS, TVS and COD bioconversion took place. Furthermore, in this case VFAs and lactic acid production is capable to explain the 95.67% of COD bioconversion. In addition, 5.21 g of CO_2 were produced during run 5. Thus, the mass balance for this run implies that the tested operational parameters setup had positive effect on promotion of acidogenic fermentation towards VFAs, rather than others fermentation products.

RUN 1 (HRT: 6 d - OLR: 22 gTS/Ld – pH: uncontrolled - °T: 37 °C)

INFLUENT		EFFLUENT		% BIOCONVERSION		PRODUCTS	
TS	90.04 g/d	TS	85.84 g/d	TS	4.66	CO_2	1.43 g/d
TVS	87.81 g/d	TVS	75.19 g/d	TVS	14.37	VFAs	2.12 g/d
COD	102.35 g/d	COD	88.32 g/d	COD	13.71		

RUN 3 (HRT: 6 d - OLR: 11 gTS/Ld – pH: 5.5 - °T: 37 °C)

INFLUENT		EFFLUENT		% BIOCONVERSION		PRODUCTS	
TS	45.02 g/d	TS	36.10 g/d	TS	19.81	CO_2	2.97 g/d
TVS	43.90 g/d	TVS	31.10 g/d	TVS	29.16	VFAs	6.15 g/d
COD	51.17 g/d	COD	28.28 g/d	COD	44.73		

RUN 4 (HRT: 6 d - OLR: 11 gTS/Ld – pH: 7 - °T: 37 °C)

INFLUENT		EFFLUENT		% BIOCONVERSION		PRODUCTS	
TS	45.02 g/d	TS	39.30 g/d	TS	12.70	CO_2	5.12 g/d
TVS	43.90 g/d	TVS	29.08 g/d	TVS	33.76	VFAs	12.67 g/d
COD	51.17 g/d	COD	36.08 g/d	COD	28.08		

RUN 5 (HRT: 6 d - OLR: 11 gTS/Ld – pH: 7 - °T: 55 °C)

INFLUENT		EFFLUENT		% BIOCONVERSION		PRODUCTS	
TS	45.02 g/d	TS	31.73 g/d	TS	29.52	CO_2	4.93 g/d
TVS	43.90 g/d	TVS	23.79 g/d	TVS	45.81	VFAs	16.47 g/d
COD	51.17 g/d	COD	27.83 g/d	COD	45.61		

Figure 4. Mass balance

2.3.3 Microbial community structure

The bacterial microbiome structure was estimated by high-throughput 16 S rRNA gene sequencing for all the experimental runs. The analysis was performed on samples taken

at the beginning and at the end of steady state for run1, 3, 4, and 5, reported as "A" and "B", respectively. For run 2, since the reactor did not reach a stable production, the analysis was conducted on a sample taken at time of maximum (run 2max) and minimum (run 2min) of total VFAs production.

Figure 5 summarizes the main bacterial genera found in the reactor for each sample analyzed, described as relative abundance of identified reads.

Despite the complex composition of the food waste, only lactobacilli populations were enriched during run 1. The analysis produced an amount of 36383 reads for run 1A, 99% of which were assigned to *Lactobacillus* genus. A similar result was obtained from run 1B. In this case 20235 reads were obtained, and, also for this sample, 99% of them belong to *Lactobacillus* genus. These results are not surprising, since an average yield of 0.07g per gram of TS fed of lactic acid was obtained during run 1, twice higher than total VFAs. The greater lactic acid yield can be explained by a certain grande of acid tolerance of lactobacilli, ensured by the presence of a constant gradient between extracellular and cytoplasmic pH. When the intracellular pH reaches a threshold value, a particular ATPase in plasma membrane of lactobacilli becomes active, generating a proton expulsion, increasing intracellular pH (Corcoran et al., 2005). Nonetheless, a certain production of VFAs is ensured by the presence of heterofermentative *Lactobacillus* spp. in the reactor. The analysis, in fact, allowed to reveal the presence of the following heterofermentative species of *Lactobacillus* in the reactor for run 1A and run 1B: *L. pontis*, *L. vaginalis, L. buchneri*, *and L. farraginis* (Vogel et al., 1994; Filya, 2003; Endo & Okada, 2007; Ibrahim, 2016).

The sequence analysis carried out for run 2 max generated 40688 reads. These were attributed to the genera *Lactobacillus*, *Caproiciproducens*, and *Clostridium sensu stricto* 12, representing the 68.75, 29.46, and 1.75% of total reads, respectively. The analysis conducted for run 2 min generated 39920 reads, and almost the totality of them belong to *Lactobacillus* genus. These results, along with the oscillation behavior of total VFAs production described above, underline that a certain phenomenon occurred.

Bacteria belonging to the genus *Caproiciproducens* are strictly anaerobic, isolated for the first time from an activated sludge sample, which was collected from a municipal wastewater treatment facility in Seoul, Republic of Korea. Their name comes from the capability of caproic acid production, along with acetic and butyric acids (Kim et al., 2015; Bengelsdorf et al., 2019). Moreover, *Caprociproduces* spp. are capable to make a lactate-based chain elongation. The pyruvate produced during lactate oxidation is further oxidized to acetyl-CoA and CO_2 with electrons released in form of reduced ferredoxin. The derived acetyl-CoA is used for butyrate and caproate formation through a reverse β-oxidation pathway.

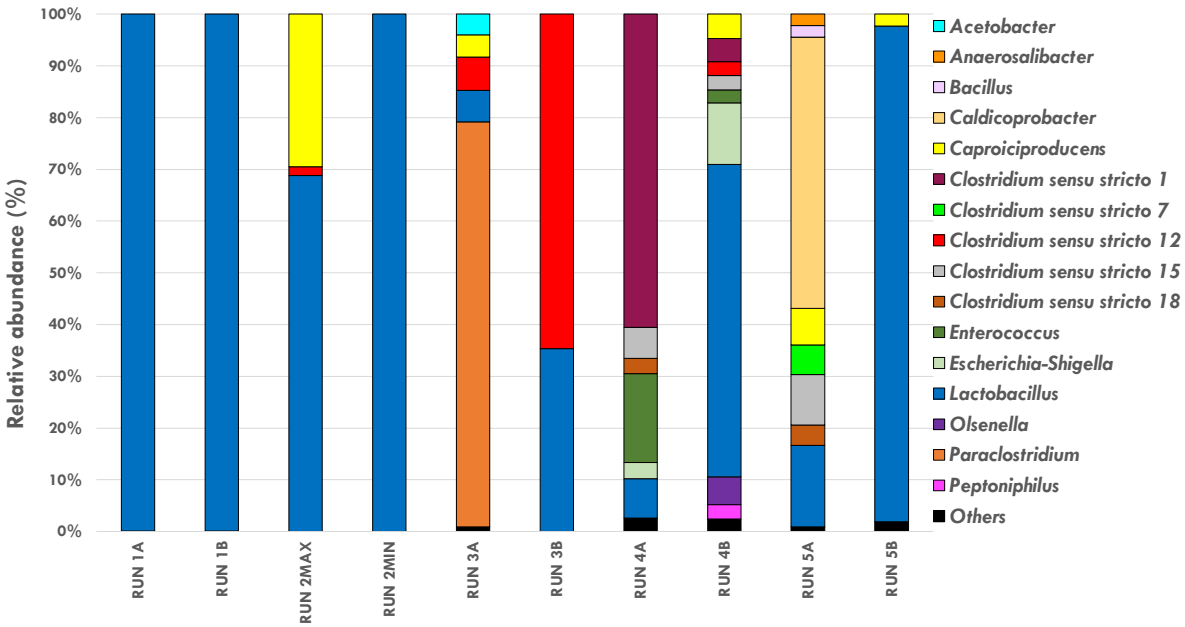

Figure 5. Main bacterial genera estimated by high-throughput 16 S rRNA gene sequencing in samples taken at different sampling times. Genera with a relative abundance lower than 1.5% are mentioned as other genera.

This pathway allows to elongate available acetate (C2) to butyrate (C4) by the condensation of two acetyl-CoA, and, if other molecules of acetyl-CoA are available, to elongate it to caproic acid (C6) (Kucek et al., 2016). Figure 7 shows the punctual distribution among VFAs produced at time of sampling during run 2. As can be noticed, at run 2max, when *Caproiciproducens* genus was present in the reactor along with lactobacilli, a greater amount of butyric and capric acids and a lower amount of acetic acid were produced, probably as consequence of a chain elongation process mediated by *Caproiciproducens*, using lactic acid produced by lactobacilli as electrons donor. During run 2 min, apart from the extremely lower VFAs production rate, acetic acid was the main fermentation product. Probably, this was due to the absence of *Caproiciproducens* in this phase. The correlation among lactobacilli and *Caproiciproducens* spp. was observed by Contreras-Dávila et al. (2019) during an experiment on caproic acid production by reverse β-oxidation pathway using food waste as feedstock. They observed that the process took place by two separate phases: an acidification phase towards lactate as main product, followed by a chain elongation phase. The oscillation between these two phases required an increase of OLR, as in the case of the second run.

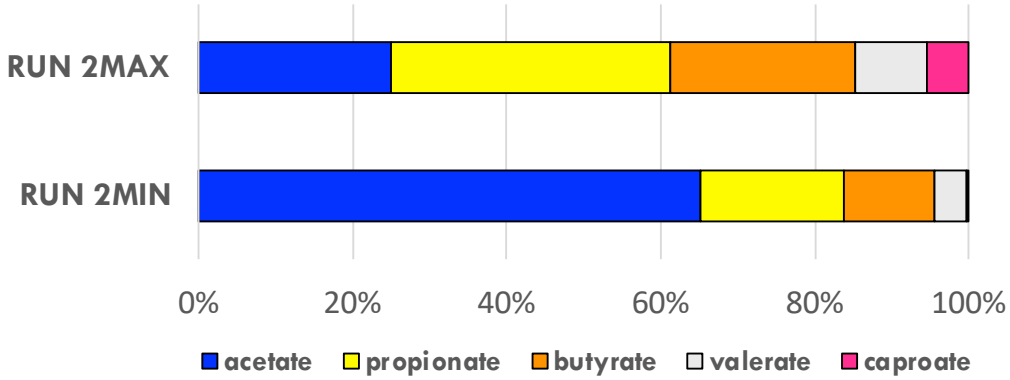

Figure 7. Pattern of distribution at time of sampling during the maximum and minimum of total VFAs production

For run 3A *Paraclostridium*, *Clostridium sensu stricto* 12, *Lactobacillus*, *Caproiciproducens*, *Acetobacter* were the main genera detected in the sample, accounting for the 78.16, 6.45, 6.04, 4.26, 4.02% of the total 47848 sequenced. Bacteria belonging to the genus *Paraclostridium* are Gram positive, rod-shaped cells capable to produce endospores. They are obligate anaerobes, that grow on a number of organic substrates, especially amino acids (Jyothsna et al., 2016). For run 3B and enrichment of two main genera took place: of 87226 sequenced reads, about 63% and 34% were attributed to *Clostridium sensu stricto 12* and *Lactobacillus*, respectively. Probably, a stable production of VFAs was detrimental for *Paraclostridium* population. The taxa presently placed into the *Clostridium sensu stricto* genera are generally obligately anaerobic rods, usually Gram positive. Nearly all members of the taxon *Clostridium sensu stricto* form butyrate as main fermentation product, but also other compunds, including various organic acids and alcohols, are produced. Thanks to various pathways, such as mixed acids, solventogenesis, and homoacetogenic fermentation, *Clostridium sensu stricto* show a wide physiological versatility, which can explain the greater distribution among VFAs achieved during this experimental run. Finally, the presence of lactobacilli in this run is reflected on lactic acid production rate, which reached a stable value of 7.43 g/Ld.

For run 4A a total of 27860 reads were sequenced. *Clostridium sensu stricto 1*, *Enterococcus*, *Lactobacillus*, *Clostridium sensu stricto 15*, *Escherichia-Shigella*, and *Clostridium sensu stricto 18* were the main genera, corresponding about to 60, 17, 8, 6, 3, 2.9% of total sequenced reads, respectively. This implies that the increase of pH toward neutrality led to an initial enrichment of bacteria belonging to the class *Clostridia*. The more representative specie belonging to *Clostridium sensu stricto 1* is C. *butyricum*, a strictly anaerobic, Gram-positive, spore-forming bacillus, so named for its capacity to produce high amounts of butyric acid (Wiegel, 2015). An interesting aspect

of the results obtained from this sample is related to lactic acid production. At the time of sampling, the lactic acid production rate was about 1.79 g/Ld, one of the lowest measured during the whole experimental campaign. This low production can be partially explained by the relative low abundance of reads attributable to *Lactobacillus* spp., but also by a possible mechanism of cross-feeding of lactate. It was found out that some bacteria, such as C. *butyricum*, are able to convert lactate and acetate to butyrate, thanks to a unique cellular complex, called Etf complex (Detman et al., 2019). For run 4B a total amount of 27802 reads were sequenced. The main genus was *Lactobacillus*, accounting for the 60.39% of total reads.

Escherichia-Shigella, *Olsenella*, *Caproiciproducens*, accounted for about the 5% of total reads, while *Clostridium sensu stricto 1*, *Peptoniphilus*, *Clostridium sensu stricto 15*, *Clostridium sensu stricto 12*, *Enterococcus* accounted for about 2.5%. This result can explain the distribution among fermentation products obtained during this run, which was however characterized by butyric acid as main fermentation product. Even in this case, some pathways of conversion of lactic acid to other production, such as caproic acid, probably took place in the system, as revealed by the presence of *Caproiciproduces* spp.. This could explain the low lactate production rate.

For run 5A an amount of 7844 were sequenced, which allow to defined the following genera: *Caldicoprobacter* (52.43%), *Lactobacillus* (15.72%), *Clostridium sensu stricto 15* (9.74%), *Caproiciproducens* (6.70%), *Clostridium sensu stricto 7* (5.79%), *Clostridium sensu stricto 18* (3.89%), *Bacillus* (2.28%), and *Anaerosalibacter* (2.19%). It is not surprising that the main genus is *Caldicoprobacter*, since the operating temperature was increased toward 55°C. Bacteria belonging to the genus *Caldicoprobacter*, in fact, are thermophilic microorganisms, Gram positive, spore-forming, nonmotile, straight to curved rods. Furthermore, they are neutrophilic, with a, optimum pH range for growth of approx5–9 (which is the operating pH during this run). The peculiarity of species belonging to this genus is the capability of growing on xylan, and they can degrade hemicellulose (Yokoyama et al., 2010). However, the system stability allowed to enrich *Lactobacillus* spp. during run 5. In fact, of total 5823 reads as many as 95% belonged to the genus *Lactobacillus*. This result is line with the description of a variety of thermophilic lactobacilli (Giraffa et al., 1998; Jensen et al., 2009; Slattery et al., 2010; Tian et al., 2019), even though the analysis was not able to identify bacteria at specie level in this case.

2.3.4 Statistical analysis

To understand the synergetic effect of the three operational parameters (pH, OLR, temperature) tested on total VFAs production, principal components analysis (PCA) was performed.

Figure 6 shows the results obtained. As shown in the loading plot, the component 1 (CP1) and component 2 (CP2) describe the 96% of covariance. Total VFAs production is described mainly by CP1, since the direction of autovector reported for total VFAs is not much divergent from CP1. A similar behavior, although with a lesser extent, is recognizable for pH. Furthermore, pH and OLR seem to have an opposite effect on the system, in fact they represented by two autovector with a nearly parallel direction, but an opposite versus. This result is confirmed by instability of system during the second run. Furthermore, to recover the system from disturbance, it was necessary to halve OLR. Finally, CP2 takes in to account the combined effect of OLR and temperature, since CP2 seems to be the vectoral sum of the autovector °T and OLR. The scoring plot confirms that CP1 can be assimilated to total VFAs production, since to a greater CP1 corresponds a greater point the scoring plot. Furthermore, points are spread down on CP1, since it describes total VFA production with a greater extent, along with a pH increase, as seen in the loading plot. It is important to notice that all the runs in the scoring plot have a similar distribution on CP2. This further confirms that CP2 is strongly dependent on OLR and temperature, in fact run 5 and run 1 are near down on CP2, since even if temperature was increased, the OLR was halved in run 5 with respect to run 1.

When the study of CPs was extended to the whole spectrum of variables measured for each experimental run, including bacteria populations reads, (except for run 2, due to instability of the system), none component was able to describe the majority of variance in the system. Rather, the variance of system resulted to be distributed more or less uniformly on six different CPs, as shown in Figure 7.

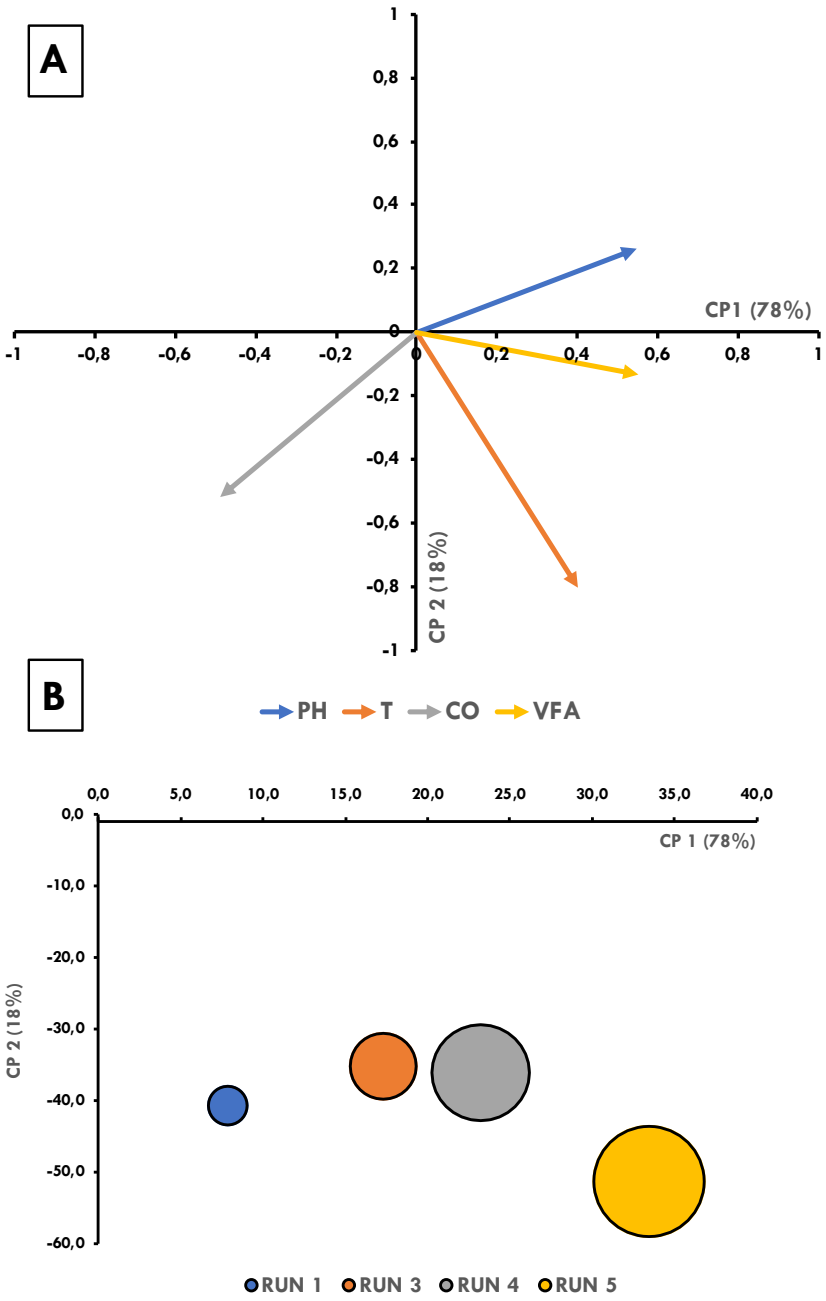

Figure 6. Loading (a) and scoring plot (b)

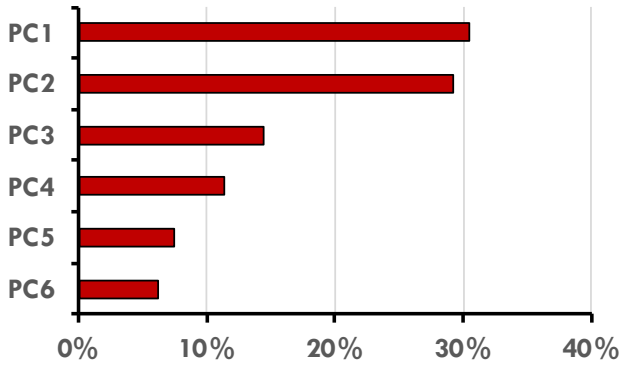

Figure 7. CPs describing the variance of the whole system

For this reason, a matrix of correlation was arranged, which is presented as heat map in Figure 8. The matrix obtained combining both variable of process, bacterial populations and fermentation products. It was properly rearranged in order to underline the blocks nature of matrix itself.

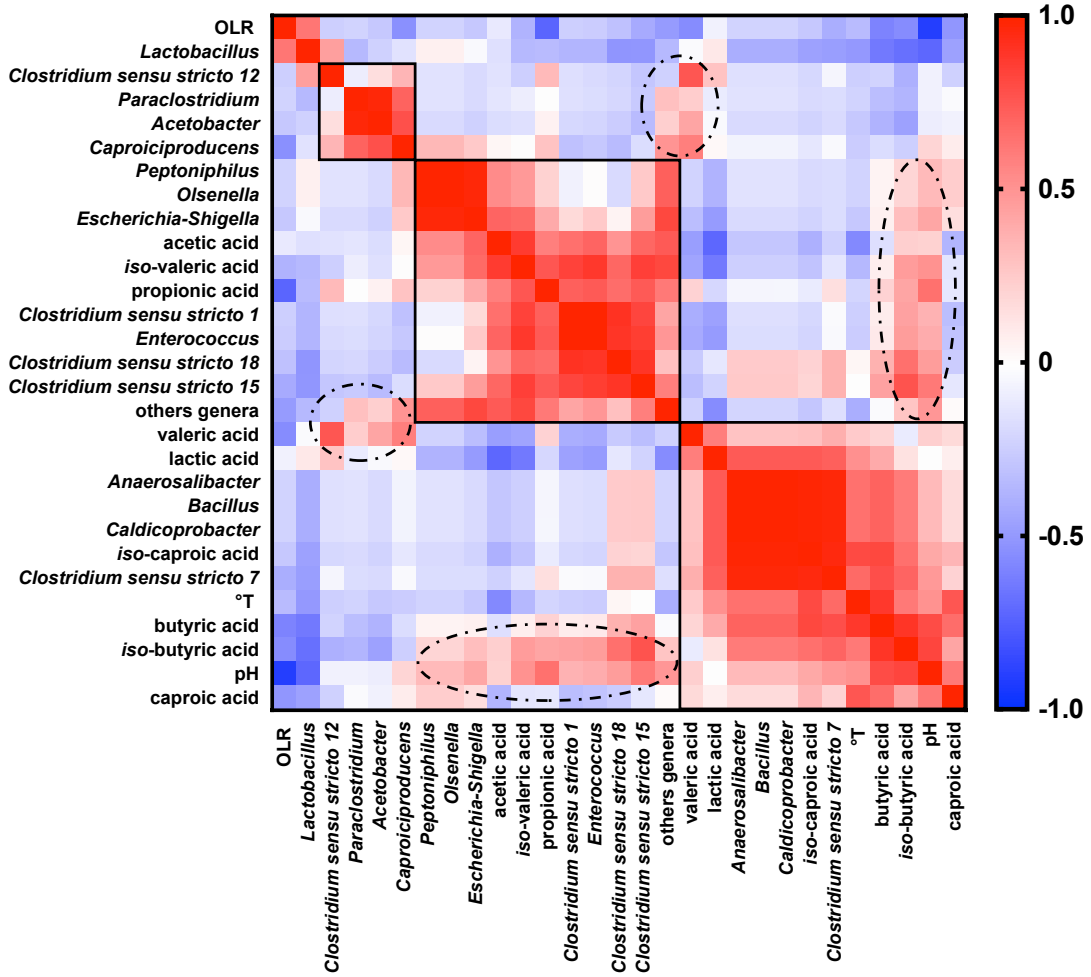

Figure 8. Heat map of correlation matrix among all the variables describing the reactor system in all the stable experimental runs

In this way, at least three groups of positively correlated variables were revealed, along with two subgroups of variables that seem to connect these three groups. Among fermentation products, propionic acid seems to be strongly positively correlated with *iso*-valeric acid and acetic acid. Furthermore, OLR negatively shows correlation with propionic and valeric acid. Nonetheless, valeric acid has a positively correlation with the genera *Clostridium sensu stricto 12*, *Paraclostridium*, *Acetobacter*, and *Caproiciproducens*. Similarly, butyrate, iso-butyrate, caproate and pH, which are part of the group in the lowest group have a positive correlation with the member of the

group in the center of the matrix. This result is not unexpected, since the central group comprise almost the total population of *Clostridia* found out in the system. Finally, capric acid is negatively correlated with *Lactobacillus*, which, evidently, are not directly involved in caproate synthesis.

2.4 CONCLUSIONS

The operational parameters set up strongly affected VFAs production, both in quantitative and qualitative terms. Even if a synergetic action on the system is recognizable for the three parameters (pH, OLR, temperature) tested, a direct effect on VFAs production is clear for each of them. pH had a crucial role on total VFAs production. A strong acid pH was detrimental on bacteria growth, leading to a very low VFAs production. Bacterial population required a neutral pH for maximizing growth rate and consequently VFAs production. OLR had a detrimental effect on process stability, and it was necessary to halved it for recover the system from disturbance. Temperature had a lower effect on VFAs production with respect to the increase of pH toward a value of 7. When pH was adjusted to 7 during run 4, a doubling of VFAs yield was observed with respect to the previous run, from 0.14 to 0.28 gVFAs/gTS fed. Whereas, an increase of temperature from mesophilic to thermophilic conditions during the last two runs, allowed a lower increment of VFAs yield, from 0.28 to 0.37 gVFAs/gTS fed. Finally, it is interesting to notice that, even if MMCs were used as intrinsic inoculum, operational parameters tend to select specific bacterial populations, leading to a different VFAs distribution pattern.

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CHAPTER 3: EFFECT OF C-SOURCE CHEMICAL NATURE AVAILABLE TO MICROORGANISMS ON ACIDOGENIC FERMENTATION

3.1 INTRODUCTION

When food waste is used as feedstock for acidogenic fermentation, its composition has a strong effect on VFAs biosynthesis, being able to influence both their quantity and their distribution pattern. The relative distribution among VFAs is a critical aspect for the subsequent utilization. For example, when VFAs are applied for nutrients removal in wastewater treatment plants, the low molecular weight VFAs (i.e. acetate and propionate) are considered as the high-quality carbon source. In PHAs production, even-carbon VFAs (acetic and butryric acids) favor the synthesis of 3-hydroxybutyrate, while the odd-carbon VFAs (propionate or valerate) promote the production of 3-hydroxyvalerate (Huang et al., 2019).

The VFAs quality, beside their quantity, is therefore of fundamental importance and should be carefully checked in a biorefinery approach.

Lipids, carbohydrates and proteins all give different results, as feedstock, in terms of fermentative products.

Generally, lipids in food waste are less suitable for fermentative metabolism than carbohydrates and proteins. Even if they give contribution to high COD levels into substrates, lipids have slower biodegradation kinetics (Alibardi & Cossu, 2016). Furthermore, hydrolysis of lipids produces glycerol and long-chain fatty acids (LCFAs). Although glycerol can be used as a fermentation substrate, LCFAs are able to adhere to cellular wall, affecting the transport of nutrients, and, consequentially, inhibiting the metabolism of anaerobic bacteria (Alibardi & Cossu, 2016; Shen et al., 2017).

Carbohydrates are easily converted by microbial metabolism into glucose, which is immediately available for glycolysis and thus for fermentation into VFAs (Shen et al., 2017). Moreover, it was reported that fermentation of pure carbohydrates, such as glucose, leads mainly to production of butyric, propionic, and acetic acid, in this exact order. Yin et al. (2016) investigated the pure glucose fermentation in batch reactor, at mesophilic range, under pH 6, using granular activated sludge as seed. They obtained a maximal VFAs production of 38.2 gCOD/L, with butyric, propionic, acetic, and valeric acid accounting for 50, 30, 17, and 3% respectively. Authors assumed that, despite a

higher theoretical high conversion efficiency of glucose into acetic acid, it was not accumulated into reactor as consequence of its consumption for the production of H_2 or for microbial growth. However, another study on fermentation of starch coming from fresh potatoes in a packed-bed reactor operating at mesophilic range and uncontrolled pH, led to a total VFAs production of around 18 g/L, with acetate, butyrate and propionate representing about the 45, 28, and 14% of total, respectively. Nevertheless, total VFAs and their profiling changed doubling the loading rate. VFAs production reached a slightly lower value, of around 17 g/L, with acetate representing about 70% of total (Parawira et al., 2004). Alibardi and Cossu (2016) found the fermentation of synthetic substrates, composed by fresh food rich in carbohydrates, was achieved a concentration of butyric acid close to that of acetic acid, with a butyrate/acetate ratio of around 0.8. These studies carried out using real substrates confirmed butyric, propionic and acetic acid as main carbohydrates fermentation products. The differences in terms of single percentage in VFAs depend on the complexity and heterogeneity of the substrates and of the different operative parameters, which can activate or inactivate a specific metabolic pathway (Feng et al., 2009).

Proteins are generally characterized by a lower biodegradability with respect to carbohydrates, due to their tertiary and quaternary structure, which make them less susceptible to protease action (Battista & Bolzonella, 2018). The result is that the efficiency of carbohydrates hydrolysis from food waste is up to 80%, while from protein is in the range 40–70%. For this reason, food waste proteins hydrolysis is considered as a rate-limiting step during acidogenic fermentation (Shen et al., 2017; Yin et al., 2016). Although fermentation of proteins leads to production of the same VFAs obtained from sugar metabolism (acetate, propionate, and butyrate), the relative ratio among them is quite different. Acetic acid is the main VFA obtained from fermentation of peptone, as reported by Yin et al. (2016), accounting for the 70% of total VFAs produced, while butyrate and propionate representing around the 10 and 15%. Furthermore, valeric acid production is mainly associated with proteins fermentation, as a result of redox Stickland reaction between couple of amino acids and of reductive deamination of sole amino acids (Parawira et al., 2004) but its concentration was only the 5% of total VFAs produced (Yin et al., 2016). As for carbohydrates, VFAs production seems also to be affected by origin of proteins. Lately, it was demonstrated that fermentation of animal or vegetal proteins lead to a different VFAs profiling. Shen et al. (2017) carried out a fermentation of two different proteinaceous substrates (i.e. proteins from tofu and eggs), finding out that metabolism of vegetal proteins leads to production acetic, propionic, butyric, and valeric acid with

a relative ratio of 56.3:15.7:10.4:17.6, while fermentation of animal proteins leads to an equal production of these acids. Likely, this different VFAs profiling can be related to a different amino acids composition of animal and plant proteins. The former, for example, usually shows a consistently lower concentration in lysine, sulphur-containing amino acids with respect to animal derived protein, and a similar difference can be found also in terms of threonine in cereal derived proteins (Sosulski & Imafidon, 1990; Young & Pellett, 1994).

Therefore, it is clear that acidogenic fermentation of a heterogeneous substrate such as food waste is the result of several contribution from different metabolic pathways.

In this scenario, the aim of this work is to investigate the effect of chemical nature of the main C-source available for MMCs on VFAs production, both in qualitative and quantitative terms. For this purpose, the synthetic HFW, formulated as reported in the previous Chapter, was divided into five fractions, which were used as fermentation substrate, under three different pH (uncontrolled, 5.5, and 7), and mesophilic conditions (37°C). In this way, in the context of a hypothetical multi-feedstock treating facility, such as an urban biorefinery, the results obtained from this work could be useful for the determination of the optimal ratio to mix together different fermentation substrates.

3.2 MATERIALS AND METHODS

3.2.1 Substrates

The synthetic HFW formulated for the semi-continuous experiment, reported in Chapter 2, was divided into five fractions, on the basis of their theorical content in terms of more or less complex carbohydrates, proteins, and lipids. For this purpose, the database on nutrients composition of food, provided by United States Department of Agriculture, USDA, was used (USDA, 2016). In this way, the following fractions were obtained:

- *proteins-rich*, made of parmesan cheese, tuna in brine, and canned beef meat;
- *lipids-rich*, made of olive oil;
- *starch-rich fraction*, made of bread and pasta;
- *cellulose-rich*, made of pure cellulose paper;
- *fibers and sugars-rich*, made of fruit and vegetables.

Every food in each fraction was added maintaining the same ratio stated for the whole HFW mixture.

Before starting the batch trials, chemical-physical characteristics of every fraction, as well as HFW mixture, were measured.

3.2.2 Experimental setup and analytical methods

The fractions so obtained were finely milled and the resulting substrates were fermented in 1 L batch reactors, with a working volume of 500 mL. As inoculum was employed the effluent from a pilot-scale fermenter treating agrowastes, located in Isola della Scala (Verona), Italy.

A F/M ratio of 7 was used, in terms of COD. For this purpose, an amount of 12 g of inoculum was added to each reactor, consequently changing the amount of substrate (26.0 g proteins-rich; 2.4 g lipids-rich; 37.7 g sugars and fiber-rich; 6.1 g cellulose-rich; 10.8 g starch-rich; 17.9 g of whole HFW mixture). Once a proper amount of substrate and inoculum were transferred in the reactors, the final volume was adjusted to 500mL with saline solution. After sampling, pH was properly adjusted, according to the three different pH tested, i.e. uncontrolled, 5.5, and 7, using a NaOH (30% w/v) or a H_2SO_4 (2.5 M) solution. The reactors were hermetically sealed with butyl rubber stoppers and the fermentation broth was manually shacked to ensure mixing of inoculum and substrate. The reactors were transferred into a laboratory stove, setting the working temperature to 37 ± 2°C.

In order to monitor the biogas production, the pressure into reactors was measured at time of sampling, by mean of a digital manometer (HD 2104.2, DeltaOHM, Italy). Thus, the pression values recorded were used to estimate the volume of biogas using the ideal gas law and molar volume. Finally, the cumulative volume of biogas was used to determine the specific gas production (SGP), as the ratio of milliliters of biogas and grams of COD inoculated in each reactor.

After pressure measuring, an aliquot of 7 mL was sampled and stored at -20 ± 1°C for evaluating the fermentation performances. Furthermore, at time of sampling, pH was measured and manually adjusted.

The whole experiment lasted 180 h. The first sample was taken after 12 h of incubation, while the following ones were taken every 24 h.

In addition to the five fractions, fermentation performance was evaluated for the whole mixture representing the standardized HFW. To evaluate the contribution of residual organic matter that was present in the inoculum, a blank reactor was prepared as control, adding the same amount of inoculum used for the reactors and tap water until the same working volume of 500mL. Therefore, the total VFAs production from substrates was given by deduction of average VFAs production in blank reactors. Finally, two series of batch trials were set up for the three pH conditions tested and for blank reactor. Alongside these reactors, set up for quantitative monitoring of fermentation performance, another set of reactors for each fractions and pH tested were initiated. In this case, pH was adjusted adding the same volume of NaOH or

H_2SO_4 solutions used for the respective quantitative reactor by mean of a needle. In this way, a proper amount of biogas accumulated in the headspace, allowing the analysis of its composition at the end of experiment.

Regarding the analytical methods, VFAs, TS, COD, sCOD, TKN, ammonia, biogas composition, were measured by mean of methods described in paragraph 2.2.2.

3.2.3 Microbial community structure: high-throughput 16 s rRNA gene sequencing

The microbial community structure was studied with high-throughput 16 s rRNA gene sequencing. The analysis was carried out as reported in paragraph 2.2.5, except for database used. In this case, in fact, NCBI database was used.

3.2.4 Statistical analyses

To emphasize the nature of correlation between individual bacterial populations identified in the reactors, operating pH, and relative amount of proteins, lipids, and carbohydrates fed, a correlation matrix was constructed on excel, then this was used to draw an heatmap using the software GraphPad Prism 8. For this purpose, theoretical contribution in terms of proteins, lipids, simple sugars, carbohydrates, and fiber given by each fraction was estimated on the basis of data on provided by United States Department of Agriculture, was used (USDA, 2016).

3.3 RESULTS AND DISCUSSION

3.3.1 Substrates and inoculum characterization

The chemical physical-characteristics of substrates and inoculum, in terms of TS, COD, and TKN are listed in Table 1.

It is interesting to notice the large amount of nitrogen in form of TKN of proteins-rich fraction, which could explain the capability of system buffering of this fraction itself. This aspect, along with a discussion on VFAs production, is accurately described in the follow paragraph.

Table 1. Chemical-physical characteristics of substrates and inoculum

Fraction	TS (g/kg)	TVS (g/kg)	COD (g/kg)	TKN (g/kg)
proteins	417.86 ± 27.18	348.35 ± 0.67	242.65 ± 11.34	31.08 ± 2.78
starch	634.93 ± 26.02	555.62 ± 0.78	581.83 ± 24.56	10.49 ± 2.10
sugars and fiber	134.03 ± 1.25	123.10 ± 0.54	167.29 ± 27.78	1.32 ± 0.08
cellulose	941.25 ± 1.76	938.87 ± 1.52	962.30 ±19.11	1.69 ± 0.15
lipids	n.d.*	n.d.*	2678.59 ± 84.76	2.86 ± 0.58
HFW	290.51 ± 19.50	253.78 ± 15.12	352.72 ± 8.86	9.90 ± 0.32
inoculum	48.58 ± 3.55	43.23 ± 2.77	75.10 ± 12.45	3.14 ± 0.44

*not determined

3.3.2 Effect of pH and C-source chemical nature on VFAs production

Among operational parameters, pH shows a key role on VFAs production via acidogenic fermentation carried out by MMCs (Strazzera et al., 2018). For this reason, to emphasize the combined effect of pH adjustment and C-source chemical nature on overall VFAs production, the experiment was carried out under three different pH, uncontrolled, 5.5, and 7, for each tested substrate.

Figure 1 shows the fermentation performances achieved from each fraction, identifying the amount of total VFAs measured at each time of sampling, and the relative distribution among fermentation products. Furthermore, the pH value measured in each reactor, before adjustment at time of sampling, is also reported.

The VFAs obtained from protein-rich fraction (Figure 1A) shows that the highest production was achieved when pH was adjusted to 7, reaching a total amount of 13.77 ± 0.89 gCOD/L after 132 h of incubation. The second highest production, of about 12 gCOD/L, was reached under pH 5.5, after just 108 h. The reactor set under uncontrolled pH showed the lowest production, of about 10 gCOD/L of total VFAs after 132 h of incubation. Moreover, a different pattern of distribution among fermentation products was observed. When pH was adjusted to 7, butyrate was the main fermentation product, accounting for about 53% of total VFAs, while *iso*-valeric, acetic, *iso*-butyric, propionic, *iso*-caproic, caproic, and valeric acids accounted for 14.97, 13.17, 6.95, 6.51, 2.03, 1.35, 1.31% of total VFAs, respectively. Setting up pH to a value of 5.5, a halved amount of butyrate (25.61%) was obtained with respect to pH

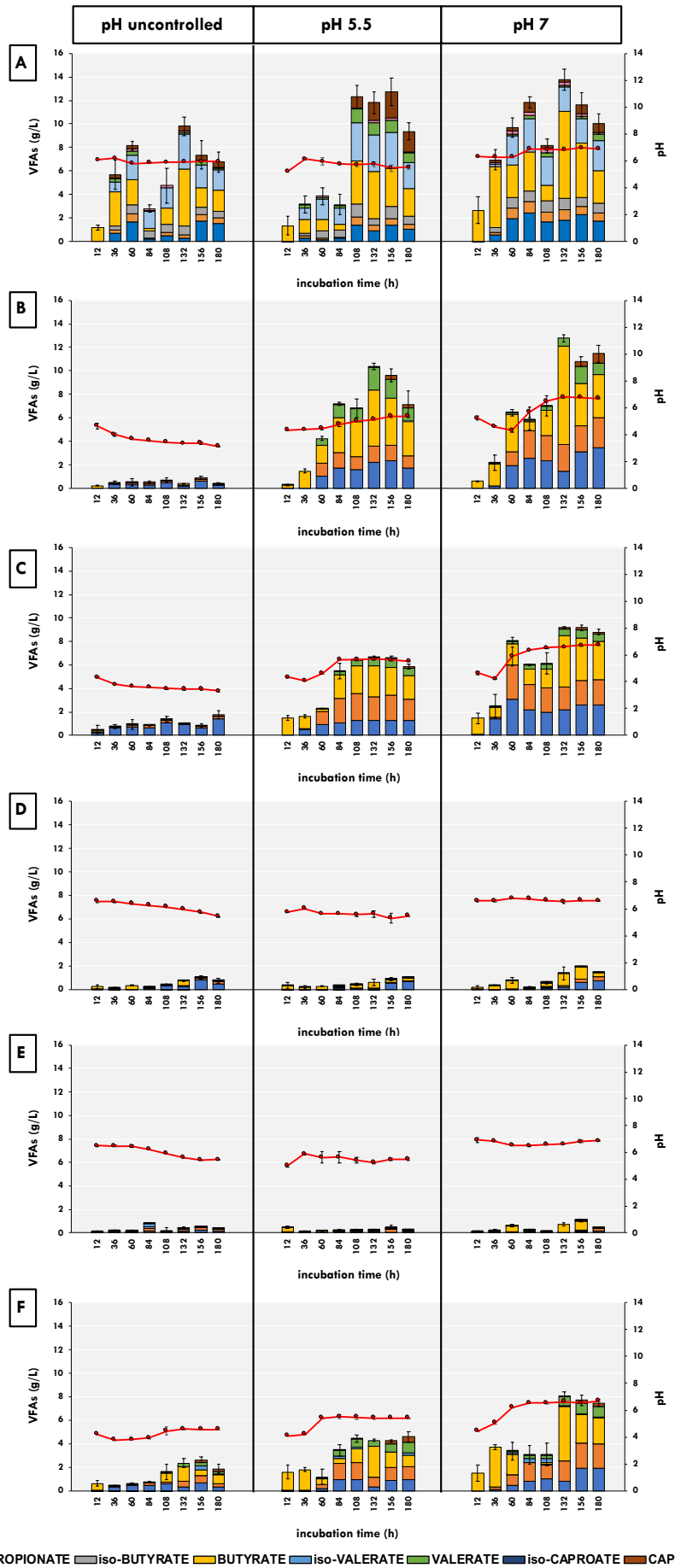

Figure 1. Fermentation performances from each substrate. a) protein-rich; b) starch-rich; c) fibers and sugars; d) cellulose-rich; e) lipid-rich fraction; f) HFW mix. Red curves are referred to the average pH value in the reactors at time of sampling

7. In addition, for caproate, *iso*-valerate, and valerate a greater percentage was reached, since they represented the 17.10, 23.74, and 8.57% of total VFAs obtained. These results are in line with those reported by other researchers, which observed a beneficial effect of a pH value around 5 on valeric acid production from proteinaceous substrate (Liu et al., 2012). Finally, under uncontrolled pH the main fermentation products were butyric and *iso*-valeric acids, accounting for the 49.06 and 30.46% of total VFAs. Although some differences were observed regarding distribution among fermentation products, it is important to stress that a comparable total VFAs production was achieved under the three tested pH values. Probably, this result can be explainable by pH behavior in the reactors. The system itself was able to buffer pH, maintaining a value close to 5.5, even without external adjustment, and this was due to the presence of abundant nitrogen compounds derived from proteinaceous substrate. It is important to notice that a greater valeric acid production was achieved under pH 5.5. Finally, it is important to notice that proteins-rich fraction was the only substrate capable to lead a production of branched VFAs, in particular *iso*-valeric acid. This result was not surprising, since when branched-chain amino acids, such as valine and leucine, undergo to oxidative deamination, they are converted to the branched-chain VFAs *iso*-butyric and *iso*-valeric acid (Apajalahti et al., 2019).

As for carbs, the best fermentation performance from substrates rich in more or less complex carbohydrates forms was obtained from starch-rich fraction (Figure 1B). A highest production was obtained adjusting pH toward neutrality, with a maximum VFAs production of 12.77 gCOD/L, after 132 h of incubation. The butyric acid was the main fermentation product, representing the 65.85% of total VFAs, while propionic, acetic, and valeric acid accounted for the 11.77, 17.37, 5.01% of total VFAs produced. When pH was adjusted to 5.5, the total VFAs decreased, reaching a maximum production of 10.38 gCOD/L, after 132 h. Even in this case, butyric acid was the main product, even if with a minor extent, representing the 45.48% of total VFAs. At the same time, the amount of acetate and valerate increased, representing the 21.53 and 18.57% of total VFAs, respectively, while the percentage of propionic acid remained almost the same. When the pH was not adjusted, it quickly dropped under 4, reaching a value of 3.14 at the end of incubation time. Under these conditions, total VFAs production was negatively affected, in fact, VFAs amount did not exceed 1 gCOD/L over the entire time of incubation. These results emphasize the key role of pH on acidogenic fermentation. When a strong acidification took place, VFAs are under undissociated form (H-Acid), becoming more liposoluble. Thus, it was easier for them to diffuse across the plasma membrane. Once they are in the cytosol, dissociation occurs, due to the neutral intracellular pH, decreasing the cytosolic pH, with a subsequent growth-

inhibiting effect on microorganisms (Palmqvist & Hahn-Hägerdal, 2000). Even under the two other conditions tested, pH had a crucial role on VFAs production, although different processes are probably involved in this case. Awasthi et al. (2018), in an experiment on microbial degradation of macromolecules found in food waste, observed that the activity of α-amylase, the crucial hydrolytic enzyme for starch solubilization, is strongly affected by pH. They studied the degradation process under a pH ranging from a value of 4 to 10, observing that a neutral pH had a favorable effect both on proliferation of microbial strains producers of α-amylase and on the hydrolytic activity of the enzyme itself. On the contrary, a gradual adjustment of pH both toward acid and alkaline conditions had a negative effect on α-amylase. This finding can explain the different VFAs production observed during the experiment. In fact, as pH increased, an increment of sCOD in liquid phase was observed.

When pH was adjusted to 5.5 and 7, a sCOD of 11.01 ± 0.09 g/L and 13.84 ± 0.36 g/L, respectively, were measured at time of maximum VFAs production. This means that about 76 and 95% of total COD was converted into sCOD under pH 5 and 7, respectively. Moreover, in both cases more than 90% of sCOD was composed by VFAs. These results could indicate that hydrolysis was not a limiting step for acidogenic fermentation when pH was adjusted (especially to 7). Whereas, under uncontrolled pH, an average sCOD of 4.73 ± 0.31 g/L was present in the reactor after 156 h of incubation, i.e. at the maximum VFAs production, which corresponds to a conversion of about 32% of initial total COD. This low conversion was probably due to a low hydrolytic activity of α-amylase, which limited the acidogenic fermentation process.

Figure 1C shows the fermentation performance achieved by sugars and fiber-rich fraction. As for the starch rich fraction, when the pH was not controlled, it quickly dropped to a value of about 3.50. Even in this case, the acidification of medium negatively affected VFAs production, that reached a maximum value of 1.74 ± 0.34 g/L at the end of incubation time, with acetic acid as main fermentation product, accounting for more than 80% of total VFAs. An increase of pH toward 5.5 allowed to increase VFAs production until a value of 6.51 ± 0.14 gCOD/L, just after 108 h of incubation, remaining more or less constant for the subsequent incubation period. This result is probably due to a greater bioavailability of simple sugars contained in fruits biomass, since VFAs represented more than 80% of sCOD after 108 h of incubation. Moreover, under these conditions, butyric acid was the main fermentation product, accounting for the 39.82% of total VFAs, while propionic, acetic, and valeric acid represent the 30.56, 18.97, and 10.57% of total VFAs produced, respectively. An adjustment of pH toward neutrality allowed to increase total VFAs production by 30% with respect to pH 5.5, reaching a maximum production of about 9 gCOD/L after 132

h of incubation. Finally, a similar relative distribution pattern among VFAs was observed with respect to pH 5. In fact, butyric, acetic, propionic and valeric acid accounted for 47.50, 23.81, 21.36, and 6.32% of total VFAs produced. This distribution is in line with typical distributions among fermentation products achieved by acidogenic fermentation process carried out by MMCs on simple sugars, such as glucose (Yin et al., 2016).

Figure 1D and 1E describe the VFAs productions observed starting from cellulose-rich and lipids-rich fractions. In both cases a very low VFAs production was observed under every pH value tested. Starting from cellulose-rich fraction, hydrolysis was probably the limiting step of bioconversion process, as shown by the low sCOD concentrations measured. While, regarding lipids-rich fraction, it is well known that LCFA have a detrimental effect on fermenting microorganisms, due to damage to cell membranes, which reduce nutrients transport and decrease cell permeability (Amha et al., 2017). The absence of acidogenic fermentation processes is further confirmed by the behavior of pH into reactor in both cases, which remain more or less constant at the initial value during the whole incubation process, without any other contribution of adjustment.

Finally, Figure 1F describes the fermentation performances obtained from the whole mixture representative of standardized HFW. The highest VFAs production was achieved under neutral pH, with a maximum of 8.02 $\pm$ 0.42 after 132 h of incubation. The main product was butyric acid, which accounted for about 58% of total VFAs. Afterwards, propionic, acetic, and valeric acid accounted for 21.18, 10.37, and 9.87% of fermentation products, respectively. Other VFAs were produced in traces. When pH was controlled to 5.5, a maximum VFAs production of about 4.5 gCOD/L was achieved, just after 108 h of incubation, and it was almost stable for the rest of incubation time. At the end of experiment, a wider distribution of fermentation products was observed with respect to pH 7. Acetate, propionate, and butyrate, in fact, accounted each for about 20%, while caproate and *iso*-valerate represented the 10.20 and 4.54% of total VFAs produced. Finally, under uncontrolled pH a maximum VFAs production of 2.64 $\pm$ 0.25 gCOD/L was reached after 156 h of incubation, with a distribution among fermentation products very similar to the one observed under pH 5.5. Even in this case, pH showed its crucial role in determining the fermentation performance. Without pH control, the system was partially able to buffer it, contrary to what happened with sugars rich fractions, as consequence of a relative abundance of nitrogen compounds, namely proteins, in the whole mixture. Even though an initial decrease until a value of 3.76 took place within the first 36 h of incubation, pH was restored above a value of 4.4 after 108 h, probably as consequence of proteins degradation, that released ammonia in the growth medium. This conclusion is supported by values of $N\text{-}NH_4^+$ measured in each reactor at the end of experiment. As expected, the highest amount

of $N\text{-}NH_4^+$ was found in the reactor inoculated with protein-rich fraction. In this case, a value of about 800 mg /L of $N\text{-}NH_4^+$ was measured at the end of experiment in all the reactors set up. For all the reactors inoculated with substrates rich in more or less complex carbohydrates, extremely lower values of ammonia, namely under 10 mg/L, were measured, which reflect the poor capability of buffering of those systems and, thus, the observed pH behavior. Finally, only the reactors inoculated with the whole HFW mixture, which had a sufficient amount of proteins, showed a concentration of about 50 mg/L of $N\text{-}NH_4^+$ at the end of experiment under uncontrolled pH, that can explain a certain degree of buffering capability, which prevented a strong acidification of the medium.

3.3.3 Biogas production

Figure 2 shows the biogas production performances from the four most productive substrates, namely proteins-rich, starch-rich, sugars and fiber-rich, and the mixture representative of the whole standardized HFW. Because of the poor metabolic activity observed from cellulose and lipids-rich fractions, almost none biogas was produced. For this reason, biogas production achieved by these substrates is not discussed below. First of all, it is important to underline that CH_4 production did not take place in any reactor, at least in trace from protein-rich fraction (~ 0.4%). Presumably, the absence of CH_4 production was due to accumulation of VFAs, that, along with an acid or just neutral environment, had a detrimental effect on methanogenic archeabacteria (Lee et al., 2014). Moreover, as expected, in all the reactors the two main gaseous products were CO_2 and H_2, typical by-products of acidogenic fermentation. Furthermore, a plateau for biogas production was observed after 132 h of incubation times in all the reactors tested.

The highest biogas production was achieved by starch-rich and sugars and fiber-rich fractions, when pH was adjusted to 7, as shown in Figure 2c. Under these conditions, in fact, a SGP of about 80 and 60 mL/g of fed COD were reached by the starch-rich and sugars and fiber-rich reactors, respectively. At the same time, analysis of biogas composition at the end of incubation time revealed that H_2 represented the 48.5 and 52.0%, respectively for these reactors. This result theoretically means that about 40 mL of H_2 can be produced from these substrates for each gram of COD fed. Nevertheless, biogas production from these two substrates decreased as pH was under more or less strong acid conditions, reaching a value of about 40 mL/g COD at pH 5.5 and below 20 mL/g COD under uncontrolled pH, as shown in Figure 2b and 2a. pH has a crucial effect on H_2 production, as reported from other authors.

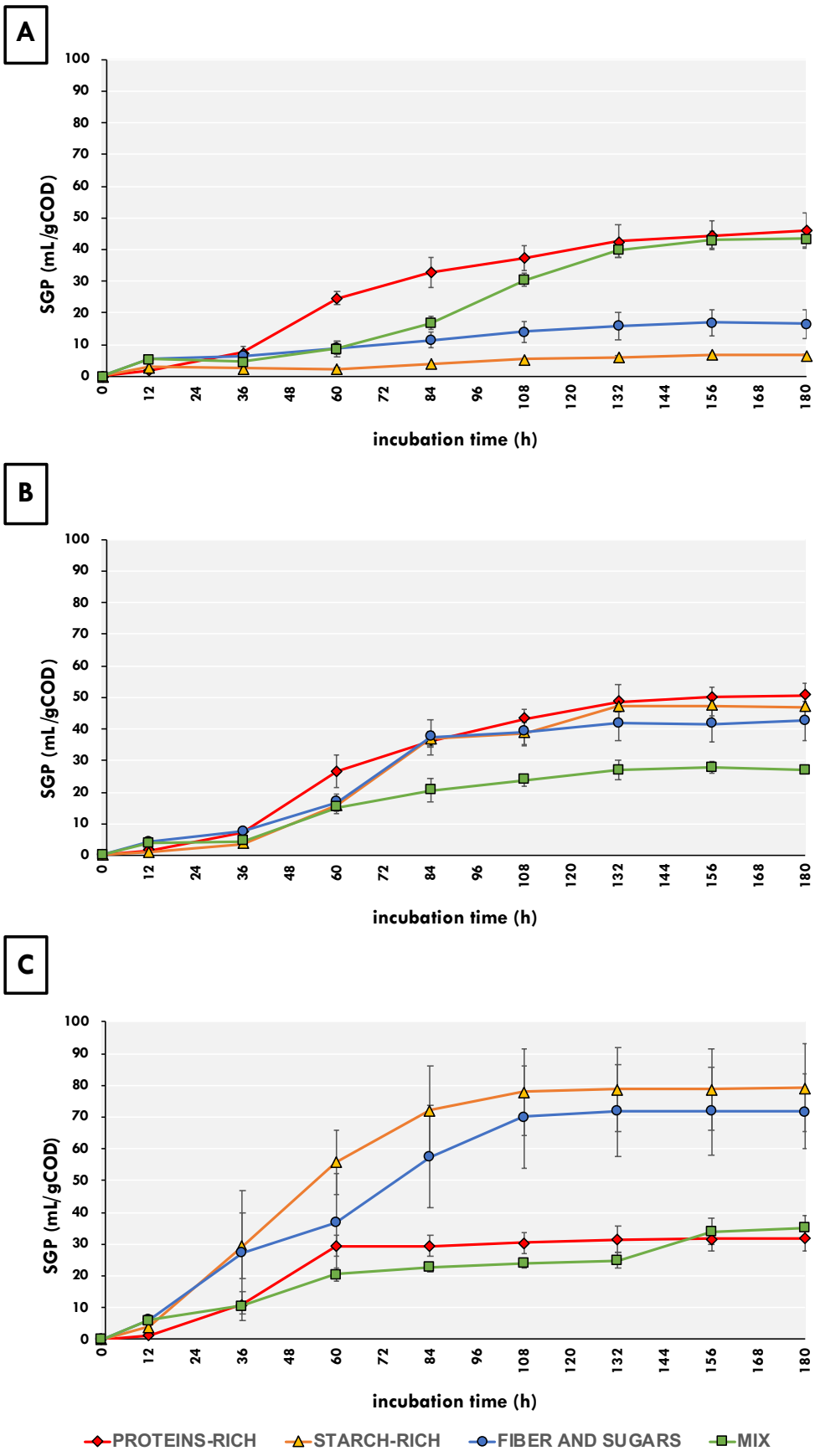

Figure 2. Specific biogas production: a) pH uncontrolled, b) pH 5.5, c) pH7

Generally, the optimal pH in terms of H_2 production is within a range of 5-7, probably as consequence of a positive effect both on activity of hydrogenases and on growth of fermentative microorganisms (Guo et al., 2010; Chinellato et al., 2013; Jarunglumlert et al., 2018). The greater H_2 production observed from more or less complex carbohydrates rich fractions, with respect to proteinaceous substrate, is line with reported productions for food waste rich in sugars. This is probably due to consumption of H_2 during oxidative deamination that took place for biodegradation of proteins (Kiran et al., 2014). For protein-rich and mixture representative of HFW, in fact, a lower SGP and H_2 were observed. However, pH adjustment had a lower effect on regulation of biogas and H_2 production from these substrates, since not a great different in terms of SGP and percentage of final volume of H_2 were observed. This was probably related to the intrinsic capability of buffering of these systems, compared to reactors fed with fraction rich in more or less complex carbohydrates.

Moreover, SGP showed an opposite behavior with respect to carbohydrates-rich fraction. In this case, in fact, greater SGPs were observed under acid conditions. From proteins-rich fraction a SGP of about 50 mL/g COD took place under acid pH values, i.e. uncontrolled and 5.5, while a SGP of about 30 mL/g COD was observed under neutral pH. Furthermore, at the end of incubation time 61.2, 52.4, and 53.7 % of biogas produced was composed by H_2 under pH 7, 5.5, and uncontrolled pH, respectively, which correspond to a specific H_2 production of about 18 mL/g COD for pH 7 and 24 mL/g COD for acid pH. This small difference in terms of H_2 production is probably due to a similar consumption of H_2 for oxidative deamination of proteins under the three pH tested, as proven by the fact that similar concentration of ammonia (~ 800 mgN-NH_4^+/L) observed in all the reactors inoculated with proteins-rich fraction at the end of the experiment. From the whole mixture representative of HFW a SGP of about 40 mL/g COD took place under acid uncontrolled pH, while a SGP of about 30 mL/g COD was observed under pH values of 5.5 and 7. In addition, the volume of biogas produced was composed by about 60% of H_2 at the end of incubation time in all the reactors inoculated with HFW, which correspond to a specific H_2 production of around 24 mL/g COD for under uncontrolled pH, and 18 mL/g COD under pH 5.5 and 7. Probably, two different processes can explain the lower biogas and H_2 production observed from HFW: (*i*) oxidative deamination of amino acids, that consumed H_2 in all the HFW reactors, and (*ii*) butyrate-acetate type fermentation, which is coupled with H_2 production. Indeed, acetate and butyrate formation during acidogenic fermentation is accompanied by H_2 production (Kim at al., 2010). Actually, under uncontrolled pH, butyrate represented around 40% of total VFAs produced from HFW at the end of experiment, twice what observed under pH 5.5 and 7. This result could mean that under

uncontrolled pH butyrate, and thus H_2 production, was favored, with respect to other metabolic pathways. Moreover, this match with a lower degree of consumption of H_2 for deamination of amino acids. In fact, under uncontrolled pH an amount of ammonia of about 40 mg $N\text{-}NH_4^+/L$ was measured, while 65 and 113 mg $N\text{-}NH_4^+/L$ were observed under pH 5.5 and 7, respectively, at the end of experiment.

3.3.4 Microbial community structure

The bacterial microbiome structure was estimated by high-throughput 16 S rRNA gene sequencing in both the inoculum utilized for the reactors start-up and in samples taken from each reactor at the end of incubation time. Data in Figure 3 describe the main genera found out in each reactor expressed as percentage of relative abundance of total reads classified at genus level on total reads. In order to highlight the main genera in each sample, genera with a relative abundance lower than 5% were merged in the group "others".

The analysis carried out on reactor inoculated with proteins-rich fraction produced several reads, ranging between 16188 and18873. The main genera found in the reactor inoculated with proteins-rich fraction under uncontrolled pH were *Klebsiella*, *Lactobacillus*, *Anaerolactibacter*, *Olsenella*, and *Enterobacter*, which account for about 30, 18, 7, 6, and 5% of total reads.

Klebsiella is a genus of facultative anaerobic bacteria, Gram negative, belonging to the family *Enterobacteriaceae*, which can be found in a variety of environmental sources such as soil, vegetation and water. (Brisse et al., 2006; Lin et al., 2019).

Klebsiella spp. are characterized by a rapid growth rate, requiring simple growth condition, with an optimal pH ranging between 5-7. This optimal pH matches with the average pH values measured in all the reactors inoculated with proteins-rich fraction, even the ones without pH control. It follows that *Klebsiella* spp. growth was favored in all these reactors. They are capable of producing a variety of fermentation byproducts, such as H_2. For this reason, some species such as *K. pneumoniae* were used for H_2 production, achieving good yields. Moreover, along with H_2, butyrate was the main fermentation product. This finding could explain the great amount of butyric acid under uncontrolled pH, despite the absence of other butyrate producers like *Clostridia* (Niu et al., 2010; Estevam et al., 2018).

Even the genus *Enterobacter* belongs to the family *Enterobacteriaceae*, and comprises motile, Gram negative, facultatively anaerobic bacteria, which are able to ferment

glucose to biogas under mesophilic conditions. In particular, the main fermentation products are CO_2 and H_2, in a 2:1 ratio (Brenner et al., 2005).

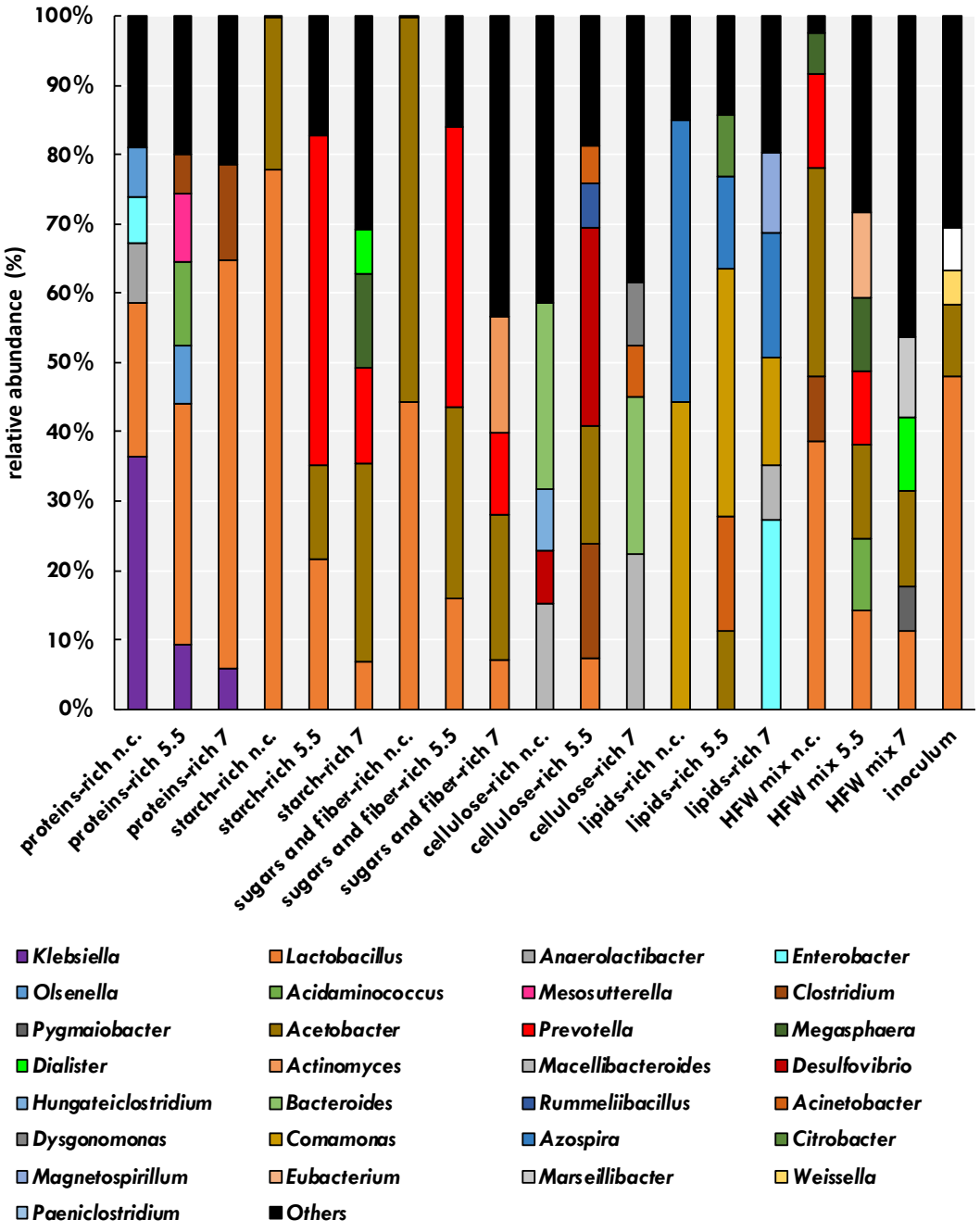

Figure 3. Main genera found at the end of incubation time in each reactor

Among lactobacilli, *L. mucosae* was the main specie found out in the reactor. Bacteria belonging to this species are heterofermentative facultative anaerobic lactobacilli. It was reported that the addiction of *L. mucosae* during fermentation of brewers grain, carried out by rumen bacteria, enhanced total VFAs production (Soriano et al., 2014).

Olsenella genus belongs to the class *Actinobacteria*, and, like the other members of this class, it includes Gram positive, non-motile, microaerotolerant, obligately heterofermentative bacteria that ferment carbohydrates mainly to lactic, but also formic, and acetic acids via anaerobic pyruvate-formate lyase system, contributing to maximize VFAs production (Kraatz et al., 2011; Goodfellow et al., 2012; Zhang et al., 2017). When pH was adjusted to 5.5, the main genera found in the reactor were *Lactobacillus*, *Acidaminococcus*, *Mesosutterella*, *Klebsiella*, *Olsenella*, and *Clostridium*, which account for about 32, 11, 9, 8, 7, and 5% of total reads. The relative abundance of heterofermentative bacteria belonging to *Lactobacillus* genus, such as *L. mucosae*, can probably explain the increase of acetic acid production when pH was rose toward a value of 5.5. Actually, under uncontrolled pH, when the main genus was *Klebsiella*, acetate was only the 3% of total VFAs, while it triplicated under pH 5.5, representing around 10% of total VFAs. An interesting effect of coexistence of these two groups, *Lactobacillus* and *Klebsiella*, on fermentation of proteinaceous substrate was found by Rosales-Bravo et al. (2017) using a combination of *Lactobacillus* strains and *K. variicola*. They found out that these two groups had a synergetic effect on milk fermentation, leading to an increment of overall proteolysis, allowing a 5-6 folds higher concentration of free amino acids in fermentation broth. In particular, the concentration of branched ones, leucine and iso-leucine, increased, which are recognized as precursors in fermentative production of branched VFAs (Zhang et al., 2013). Probably, a similar interaction took place in these reactors, leading to the production of branched VFAs, as discussed above. Moreover, under pH 5.5 a remarkable increased of caproic acid was noticed, representing around the 17% of total VFAs. This is line with results reported by Rosales-Bravo et al. (2017), which observed an increase of acetic and caproic acid when *K. variicola*, the main species found in our reactors, was added to a consortium of *Lactbacillus* strains. Furthermore, maintenance of *Klebsiella* spp. during acidogenic fermentation required a pH of about 5.5 (Maintinguer et al., 2011). In this scenario it is possible to assume that some synergetic effect on acidogenic fermentation among this two microbial groups took place also in our reactors.

Acidaminococcus is a genus in the class *Negativicutes*, whose members, like other members of the class, are Gram negative, despite being part of phylum *Firmicutes*, which normally includes Gram positive bacteria. These bacteria are anaerobic diplococci able to use amino acids, of which glutamic acid is the most important, as the sole energy source for growth. The main fermentation products are acetic and butyric acids and CO_2 (generally with a ratio 1:2 of butyrate/acetate). Actually, these bacteria could give a contribution to acetic acid production, since about a five folds greater amount of acetate was produced under pH 5.5 with respect to uncontrolled

pH, accounting for 10% of total VFAs. Furthermore, some reports indicate that some species, such as *Acidaminococcus fermentans*, convert glutamate to produce H_2, NH_4^+ and acetate. *Acidaminococcus* are mesophilic bacteria, with an optimum pH value below 6.5 for their growth. This optimal pH could explain why they disappeared when pH was adjusted to 7 (Rogosa, 1969; Chang et al., 2008; Lay et al., 2010).

Mesosutterella genus comprises bacteria asaccharolytic, since it not surprisingly to find them on a proteins-rich substrate, like observed in these reactors (Sakamoto et al., 2018).

Despite the low butyric acid production under pH 5.5 with respect to uncontrolled pH, one of the main genera is represented by *Clostridium*, even if with minor extent. At the same time, the presence of these bacteria could explain the increment of odd number VFAs, in particular valeric acid, which production increased from about 2 to 9%. It was found out that the same factors have a contribution in maintenance of *Clostridium* and *Klebsiella* spp. in the anaerobic conditions, such as high concentration of proteinaceous substrate and controlling of working pH at 5.5. These operational conditions match with the ones maintained during this experiment, so the coexistence of this two groups is not surprisingly (Maintinguer et al., 2011).

When pH was set up to a value of 7, the main genera found out in the reactor inoculated with proteins-rich fraction were *Lactobacillus*, *Clostridium*, and *Klebsiella*, which represented the 56, 13, 6% of total reads analyzed, respectively.

The increase of pH toward neutrality had a favorable effect on populations of *Lactobacillus*, which become predominant over the other populations in the reactor, especially on *Klebsiella*. Probably this decreased of viability of *Klebsiella* populations during the fermentation process was due to stronger presence of *Lactobacillus* in the mixed culture, as previously reported by other authors (Rosales-Bravo et al., 2017).

The increase of lactobacilli, along with consequent decrease of *Klebsiella* populations, can probably explain part of the difference observed for biogas production, in particular H_2, from proteins-rich fraction under the three tested pH. Despite the behavior of biogas and H_2 production observed in the reactors fed with more or less complex carbohydrates, a lower SGP was observed under pH 7. The lack in population of *Klebsiella* resulting in a lower H_2 production, even thought it was partially offset by an increase of *Clostridium* spp.. pH value close to neutrality has a beneficial effect both on cell growth and acetate and butyrate production, besides on H_2, by bacteria belonging to *Clostridium*, which can explain in part the results obtained from this reactor (Charalambous et al., 2020).

The sequencing analysis carried out on reactor inoculated with starch-rich fraction produced a number of reads ranging between 10654 and18104. The main genus

found in reactor working under uncontrolled pH was *Lactobacillus*, representing about 80% of total reads. This is probably due to the ability of these bacteria to resist under strong acidification of growth medium. When the intracellular pH decreases, as consequence of diffusion into cells of undissociated acids, a particular ATPase in plasma membrane of lactobacilli becomes active, generating a proton expulsion, increasing intracellular pH (Corcoran et al., 2005).

When pH was adjusted to 5.5 the main genera in the reactors were *Prevotella*, *Lactobacillus*, and *Acetobacter*, which accounted for 47, 21, and 13% of total reads.

Bacteria belonging to *Prevotella* spp. are strictly anaerobes, belonging to *Bacteroidia* class. Like other members of the class, *Prevotella* are characterized by the ability to grow on a variety of carbohydrates. In particular, they are able to degrade starch, thanks to α-amylase activity, producing acetic, succinic, *iso*-butyric, and *iso*-valeric acids (Janeček & Blesák, 2011; Dos Reis et al., 2015 Charalambous et al., 2020). The main species was identified as *P. cerevisiae*. These bacteria are able to ferment several sugars mainly into acetic and succinic acids. Moreover, they exhibit an optimum pH growth ranging between 5 and 7, which can explain because they were not detected under strong acid uncontrolled pH (Nakata et al., 2019). Moreover, *Prevotella* spp. are reported as H_2 producers, thus probably they had a contribution on the overall H_2 production observed in these reactors. Interestingly, one of the main genera found under pH 5.5 was *Acetobacter*. This genus is part of acetic acid bacteria group. Although these bacteria are thought to be strict aerobes, unable to grow or survive in the absence of oxygen, they could remain viable during fermentation processes under anaerobic conditions, like during wine production. This is due to use electrons acceptors other than molecular oxygen, which allows them to have a limited metabolic activity under anaerobic conditions (Jackson, 2008). Probably, *Acetobacter* used ethanol produced by heterofermentative lactobacilli, such as *L. mucosae*, to sustains their growth in the reactors. Actually, they are able to oxidize ethanol to acetic acid or other alcohols to their corresponding acids, and this represents one possible explanation of distribution among VFAs observed in these reactors (Rich et al., 2015; Arai et al., 2016).

When pH was adjusted to a value of 7, the main genera found in the reactors inoculated with starch-rich fraction were *Acetobacter*, *Prevotella*, *Megasphaera*, *Lactobacillus*, and *Dialister*. The former three genera accounted for 27, 13, and 12%, respectively, of the total sequences, while *Lactobacillus* and *Dialister* accounted for the 6% each one. Among them, the most interesting genus, in the light of results obtained under pH 7, is *Megasphaera*, a genus belonging to the family *Veillonellaceae*, part of the class *Negativicutes*. They are anaerobic, Gram negative, non-motile cocci. The most abundant species found in our reactors was *M. elsdenii*, which requires a pH raging

from 5 to 8 for an optimal growth (Nelson et al., 2017). Bacteria belonging to this species have a fermentative type of metabolism and can use both carbohydrates and organic acids, involving different metabolic pathways. For this reason, the composition of their fermentation end products is variable. For example, products from lactate fermentation are acetate, propionate, C4 straight- and branched-chain fatty acids, valerate, little or no caproate and formate. Furthermore, several studies showed that butyrate was the main fermentation product starting from glucose. Nonetheless, products from glucose differed from those from lactate by formate and caproate production, a lesser production of valerate, little or no production of acetate. This is line with distribution among VFAs observed for fermentation of starch-rich fraction under pH 7, where butyric acid accounted for more than 60% of total VFAs, while acetic and propionic acid accounted for 12 and 17% of total VFAs respectively (Vos et al., 2011; Ohnishi, 2015; Nelson et a., 2017). Moreover, the presence of bacteria belonging to *Megasphaera* genus could make a contribution to H_2 production, utilizing lactate produced by *Lactobacillus* that were present in the system (Dos Reis et al., 2015).

Bacteria belonging to the genus *Dialaster* are also part of family *Veillonellaceae*, like *Megasphaera*. They are obligately anaerobic or microaerophilic, nonmotile, small Gram negative, whose metabolic end products are variable amounts of acetate, lactate, and propionate. Moreover, *Dialister* spp. is not a hydrogen-producing organism, even if they are found in hydrogen-producing system (Lin et al., 2008; Vos et al., 2011).

Sequencing analysis carried out on samples taken from reactors inoculated with sugars and fiber-rich fraction allowed to produce from 19128 to 15870 reads. The results obtained from this fraction were very similar than those observed from starch-rich fraction, probably as consequence of similar selection effect, exerted by sugars abundance in the medium. Under strong acid uncontrolled pH, the main genera found were essentially two, *Acetobacter* and *Lactobacillus*, which accounted for 51 and 40% of total sequences, similarly to what observed for starch-rich fraction. Even under pH 5.5 the main genera found in these samples are similar to the ones observed from starch-rich fraction under the same conditions, namely *Prevotella* (38%), *Acetobacter* (26%), and *Lactobacillus* (15%). When pH was adjusted to 7, the main genera found in the reactors are the same reported in starch-rich reactors working at the same pH value. Nevertheless, starting from sugars and fiber-rich fraction variability among genera increased, since the group that includes genera representing less than 5% of total sequences showed a remarkably increment, representing about 24%, indicating that VFAs production is the result of coexistence of great variety of populations.

Total sequences obtained from reactors inoculated with cellulose-rich fraction ranging between 11518 and 15432 reads. Whereas a very low VFAs and H_2 production took

place, probably none of the fermentative populations present in these reactors were in optimal conditions for growth and, subsequently, for metabolism of cellulose, even though some genera capable of cellulolytic activity were detected. For example, some bacteria belonging to *Bacteroides*, the main genus found both under uncontrolled and neutral pH, are able to degrade more or less complex carbohydrates. The *Bacteroides* genus was discovered in 1919 and defined as the type genus of the *Bacteroidaceae* family. This genus currently comprises 52 validated species of nonsporulating, anaerobic, Gram negative and rod-shaped bacteria (Andrieu et al., 2018). In particular, the highest number of reads belong to the specie *B. graminisolvens*. These bacteria are mesophilic strictly anaerobes, isolated from a methanogenic reactor treating cattle waste. *B. graminisolvens* utilizes xylan, as well as various sugars including arabinose, xylose, glucose, mannose, cellobiose, raffinose, starch and pectin, producing acetate, propionate, and succinate. The optimum pH for growth was approximately 7.5, which is above the working pH in the experiment (Nishiyama et al, 2009). Presumably, this aspect, along with the absence of certain specific nutrients, caused a failure of metabolism of *B. graminisolvens*.

Another main genus in reactors inoculated with cellulose-rich fraction, under uncontrolled and pH 7, was *Macellibacteroides*, a genus belonging to the family *Ruminococcaceae*. *Macellibacteroides* are known to ferment various sugars to produce organic acids such as acetic, butyric, and lactic acids (Baek et al., 2016). The main species was *M. fermentans*, which includes obligately anaerobic, non-spore-forming, rod-shaped mesophilic bacteria, which stained Gram positive even if they have the typical cell wall structure of Gram negative (Jabari et al., 2012). The analysis of whole genome of *M. fermentans* also revealed a number of calcium dependent carbohydrate binding module families, that are implicated in the degradation of a number of cellulosic structures (Rout et al., 2017). Probably, the lack of nutrients, along with an optimum pH above 7, inhibited their metabolism in this system.

Finally, another interesting genus identified in these reactors were *Hungateiclostridium*. These bacteria belong to the family *Hungateiclostridiaceae*, in the order of *Clostridiales*. Some species of this genus are reported as degraders of complex carbohydrates. For example, *Hungateiclostridium thermocellum*, previously known to be *Ruminiclostridium thermocellum* or *Clostridium thermocellum*, is a species of Gram positive anaerobes, capable to hydrolyze plant cell polysaccharides converting them into valued biochemical products such as biofuels, oligosaccharides, acetic acid and bio-hydrogen (Sharma et al., 2019).

H. mesophilum is a species of mesophilic, cellulolytic and spore-forming bacterium, first isolated from a biogas fermenter fed with maize silage. These bacteria show a

cellulolytic activity and optimal growth were observed at 45 °C and neutral pH (optimum, pH 7.5) (Rettenmaier et al., 2019).

Reads identified from reactors treating lipidis-rich fraction ranging between 22190 and 15624. The absence of fermentative metabolism these reactors, in addition to very low biogas and VFAs production, is also proven by the presence of obligate anaerobic genera, such as *Comamonas* and *Azospira* (Brenner et al., 2005).

For samples taken from reactors inoculated with the mixture representative of HFW a total number of reads ranging between 10294 and 15278 were obtained. When the pH was uncontrolled, the main genera found were *Lactobacillus* (38%), *Acetobacter* (30%), *Prevotella* (13%), *Clostridium* (9%), and *Megasphaera* (6%). *Lactobacillus* and *Acetobacter* spp. explain the largest part of distribution among sequences. This result is in line with that observed under uncontrolled pH for more or less complex carbohydrates-rich fractions, even thought acidification of medium occurred with a lower extent in these reactors, allowing survival of bacteria belonging to other genera. This can explain the greater VFAs production observed under uncontrolled pH in these reactors with respect to reactors treating carbohydrates-rich substrates. An increase of pH first toward 5.5, and later to 7, allowed to promote the coexistence of a wide number of genera, none of which became strongly predominant on the others. This result can explain, as well as the good yield, the wide distribution among fermentation products obtained in these reactors.

Finally, Figure 4 shows the correlation matrix obtained combining the number of reads found for each bacterial population with pH and contribution of substrates in terms of proteins, carbohydrates, simple sugars, lipids. The contribution in terms of macromolecules from each substrate was estimated using USDA database (USDA, 2016), as shown in Table 2. To highlight the blocks nature of the matrix, it was properly rearranged. In this way, it is easily possible to recognize groups of highly correlated species and relate them to the different carbon sources.

On the lower right of the matrix there is a group of bacterial populations with a positive correlation with simple sugars, i.e. *Actinomyces*, *Acetobacter*, and *Prevotella*.

Going up towards the center of the matrix, it is possible to observe a second group of bacteria populations, that, in this case, show a positive correlation with pH. They are essentially *Marseillibacter*, *Pygmaiobacterm*, and *Dialister*.

Above this, there are another two groups of microorganisms, one includes *Bacteroides*, *Macellibacteroides*, *Dysgonomonas*, *Hungateiclostridium*, while the second comprises *Clostridium*, *Desulfovibrio*, *Rummeliibacillus*.

Table 2. Theorical macromolecular contribution of each fraction put in the different reactors, estimated using USDA database (USDA, 2016)

Fraction	Water (g)	Proteins (g)	Fat (g)	Carbohydrates (g)	Fiber (g)	Sugars (g)
protein	15.42	4.99	3.06	1.45	0.06	0.03
starch	5.02	0.81	0.25	4.48	0.25	0.08
sugars and fiber	31.16	0.24	0.09	6.04	0.90	3.85
cellulose	0	0	0	0	12.20	0
lipid	0	0	2.40	0	0	0
HFW	12.75	0.98	0.72	2.74	0.77	1.20

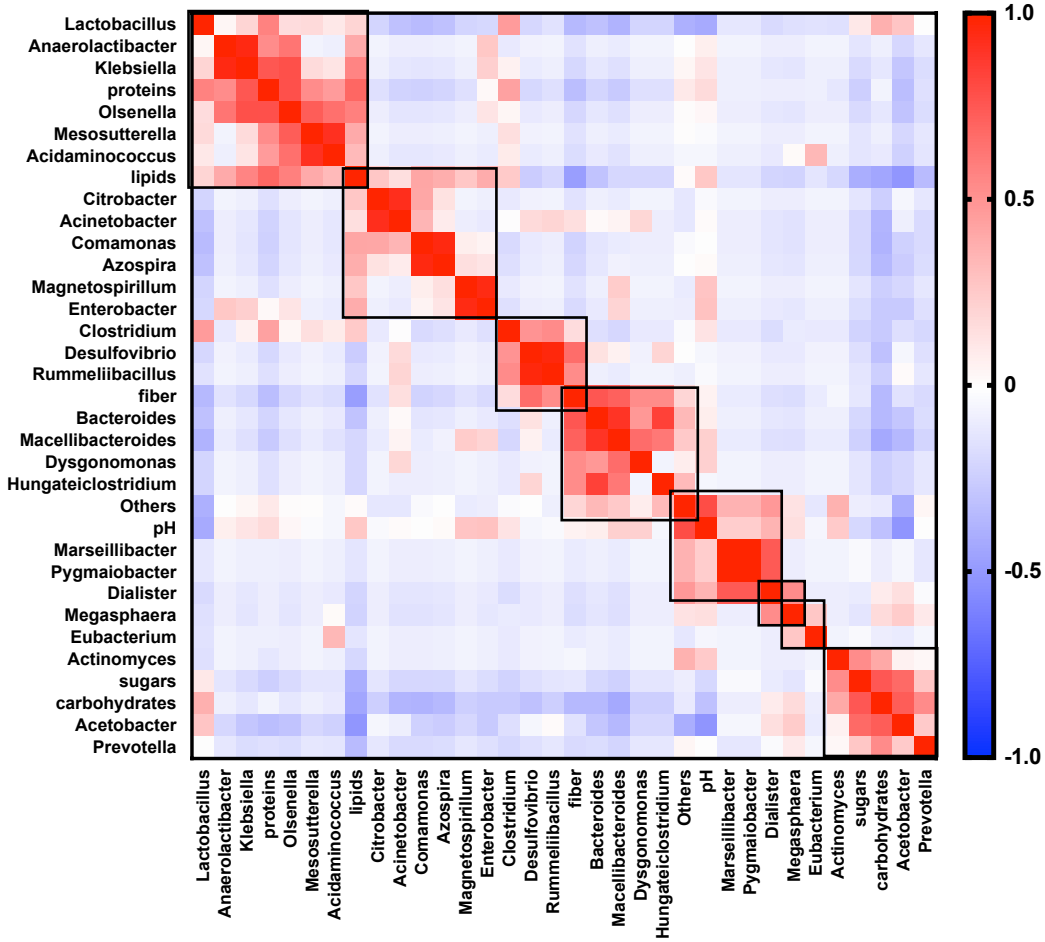

Figure 4. Correlation matrix among bacterial populations and theoretical content in terms of proteins, lipids, carbohydrates, simple sugars, and fiber, coming from each fraction

Both these groups show a positive correlation with fiber, but mutually independent. Finally, up on the left of matrix there are populations with a positive correlation with proteins and lipids, i.e. *Lactobacillus*, *Anaerolactibacter*, *Klebsiella*, *Olsenella*, *Mesosutterella*, and *Acidaminococcus*. Even *Clostridium* shows a positive correlation with

this group. It is interesting to notice the behavior of *Lactobacillus*. This population, in fact, has a positive correlation with the groups of populations positively correlated with proteins and lipids, but also with the one characterized by a positive correlation with simple sugars and carbohydrates. At the same time, *Lactobacillus* has a negative correlation with population positively correlated with fibers and less acid environment.

3.4 CONCLUSION

This work confirmed the crucial role of pH on acidogenic fermentation. The absence of intrinsic buffering capability of systems, in fact, led to a strong acidification in presence of readily biodegradable sugars, as consequence of VFAs accumulation. At the same time, when a control of pH was established, the chemical nature of the main carbon source available to microorganisms, led to a selection of certain populations at the expense of other, which had a fundamental effect on final VFAs profiling. For this reason, the composition of residual biomass streams should be well addressed in the perspective of an integrate treatment of wastes.

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CHAPTER 4: ACIDOGENIC CO-FERMENTATION OF HOUSEHOLD FOOD WASTE, SEWAGE SLUDGE, AND DISPOSABLE DIAPERS FOR VOLATILE FATTY ACIDS PRODUCTION

4.1 INTRODUCTION

The worldwide production of solid waste has reached the alarming level of 17 billion tons and it has been estimated it will be around of 27 billion tons within 2050 as consequence of the human population growth (Laurent et al., 2014). Thus, waste management is one of the main areas where further improvements are needed to reach higher levels of waste prevention, reuse and recycling. European Commission has adopted a new formula, more general and not limited to the waste recycling topic, named "action plan for a circular economy" in which waste is only a part of the product's life-cycle. In particular, Europe promoted the so called "waste hierarchy" which ranks different waste management options: disposal in landfills or incineration with little or no energy recovery, are the least favorable options in the light of the reduction of greenhouse gas emissions. Waste prevention, reuse and recycling have instead the highest potential to reduce those emissions (European Commision, 2008).

With an annual amount production of approximately 2 billion metric tons and 10 million tons on a dry matter based, HFW and sewage sludge are the most abundant organic wastes (substrates) produced in urban environment. For their huge abundance and their chemical composition, they are potentially exploitable as renewable carbon sources within a multi-feedstock and multi-purpose biorefinery approach, based on the waste hierarchy (Battista et al., 2019).

Beside HFW and sewage sludge, another important waste stream in urban context is represented by disposal diapers. They account for 2-15% of municipal solid waste and their management is essentially based on landfill or incineration, due to collection of unsorted municipal solid wastes (Karimi et al., 2020; Tsigkou et al., 2020). When diapers are landfilled, their natural decomposition requires a very long period of time, due to both low biological activity in landfills and the fact that consumers tend to dispose diapers by enfolding them in plastic (Karimi et al., 2020).

Nonetheless, diapers are mostly composed by cellulose and synthetic fibers, that can undergo to biological processes. Typically, baby diapers are composed by 36.6%

cellulose pulp, 30.7% sodium polyacrylate (SAP), 16% polypropylene, 6.2% low-density polyethylene, and 10.5% of elastic and adhesive tapes. Moreover, cellulose fibers are not the biodegradable material within disposable nappy, since the percentage of organic content can reach almost 87% if urine and excreta are also added (Tsigkou et al., 2019).

Acidogenic fermentation is a very conventional biological stage in biorefinery having the aim to degrade FWs and SS in valuable simple molecules, the Volatile Fatty Acids (VFAs). They are short-chain aliphatic mono-carboxylate compounds with two (acetic acid) to six (caproic acid). The optimization of VFAs production is attracting more and more interest, as they are considered biological precursors for different biofuels and high added value compounds, in particular bioplastics (Bhatia & Yang, 2017).

The food wastes include a very heterogeneous types of wastes differing concentration and composition of organic matter, which influences the choice of best operating parameters for the VFAs production. In general, the best operating conditions for increasing the amounts of VFAs are as follows: *i*) a slightly controlled pH (6.0-7.0), *ii*) short HRT (1-7 days depending on the substrates complexity), *iii*) mesophilic or thermophilic temperatures and *iv*) relatively high OLR (approximately 10 gTS/Ld) (Strazzera et al., 2018).

With specific reference to the VFAs profile this is also depending on the food waste chemical nature beside the process operational conditions.

Generally, the lipids in FWs are less prone to fermentation than the carbohydrates and proteins because they have low solubility and slower biodegradation kinetics (Li et al., 2017). Furthermore, the hydrolysis of lipids produces glycerol and long chain fatty acids (LCFAs) (Alibardi & Cossu, 2016; Shen et al., 2017). On the contrary, Carbohydrates are immediately available for glycolysis and fermentation into VFAs (Shen et al., 2017). In particular, glucose conversion privileges the formation of acetic acid, which is immediately used for the production of H_2 or for microbial growth by microorganisms, resulting in a low final concentration. Instead, more complex carbohydrates, led to a total VFA production of approximately 18 g/L, with acetic acid, butyric and propionic acids as most abundant VFAs (Yin et al., 2016). Proteins are generally characterized by lower kinetics due to their tertiary and quaternary structures. Anyway, also in this case acetic, propionic and butyric acids are the most relevant products of protein fermentation, with a dominant presence of HAc, which usually accounts for 70% of the total VFAs produced (Yin et al., 2016; Strazzera et al., 2018).

Regarding the VFA production from SS alone, this is not advantageous, being only partially and slowly biodegradable. In addition, some recalcitrant inhibiting compounds

(humic acid-like compounds, melanoidins, nitrogen heterocycles, and phenols) were also found in SS. Thus, SS co-digestion with FWs is highly recommended (Battista et al., 2019).

Li et al. (2017) investigated the effect of FW and SS co-digestion on hydrogen and VFA production. They observed an increase in the hydrogen content that was approximately 30% higher than that obtained by FW fermentation alone. In addition, VFA production reached 281.84 mg/gVS, with an improvement of approximately 10%. This improvement was explained by the multi-substrate characteristics, which were obtained by supplying a higher soluble chemical oxygen demand (25–35 g/L) and a suitable pH (6.0–7.0), which decreased the total ammonia nitrogen by 18.67% and ensured a proper carbon/nitrogen ratio (15–25).

Up to date, few studies were conducted on biological treatments of disposable diapers. These processes include dark fermentation (DF), AD, composting, and fungal treatment (Sotelo-Navarro et al., 2019). In a work carried out by Tsigkou et al. (2019), batch DF of fruit and vegetables wastes and nappies hydrolysate (2:3 v/v) was conduct under mesophilic conditions, testing a pH range from 4.5 to 7.5. They found that a maximum total VFAs production of about 18 g/L occurred under pH 7.5, with butyric and acetic acids accounting for 40% of total fermentation products each one. Moreover, 1.5 mol H_2/mol glucose was obtained. In another study, batch fermentation of diapers was carried out to test the effects of working temperature (35, 55 °C) mainly on H_2 production, demonstrating that a higher production of the gas, along with byproducts acetate and butyrate, was achieved under thermophilic conditions, probably owing to a more rapid microbial metabolism in the thermophilic regime, that could face with complex structure of cellulose (Sotelo-Navarro et al., 2017). A study on co-digestion in semi-continuous of the cellulose from diapers with waste activated sludge demonstrated that a moderate biomethane production of about 280 ml CH_4/g VS_{fed}, with a TVS removal efficiency of 52% (Torrijos et al., 2014).

The aim of this work was to assess the possibility of a simultaneous fermentation of the main waste streams produced in urban areas, i.e. HFW, sewage sludge, and cellulose from diapers, for VFAs production, in the perspective of exploitation of acidogenic fermentation process into an integrate urban biorefinery platform. Actually, two different fermentation schemes were employed. First of all, VFAs production potential of the two readily biodegradable substrates, i.e. HFW and SS, was assessed. Moreover, acidogenic fermentation performances were evaluated adding cotton wool (CW) to the other two substrates, to figure out the effect of cellulose fibers contribution on overall VFAs production. Due to the recalcitrant nature of cellulose toward hydrolysis, acidogenic fermentation processes were carried both under mesophilic and

thermophilic conditions, to asses a possible benefit of increased temperature on hydrolysis.

4.2 MATERIALS AND METHOD

4.2.1 Substrates

A standardized HFW was used for co-fermentation trials. It was prepared as reported in Chapter 2, opportunely milled, and stored at -20 ± 2°C until use.

SS was collected from a secondary settler of a municipal wastewater treatment plant (Verona, Italy). It was stored at 4 ± 2°C until use.

To mime the contribution of cellulose fibers derived from diapers, CW made of pure cellulose was employed, storing it in a vacuum desiccator until use. This methodological choice is the result of the need to exclude SAP from fermentation substrate, as well as plastic residues. SAP is the sodium salt of polyacrylic acid, known to be efficient water absorbent and it is used widely for this purpose in industry (Tsigkou et al., 2019). The presence of SAP in fermentation broth would cause two issues: *i*) limiting volume of free water available for microorganisms; *ii*) process failure of anaerobic system fed with SAP, since few fermentative microorganisms can face with degradation of polyacrylates (Tsigkou et al., 2019). Moreover, techniques reported for separation of cellulose fibers from diapers at lab-scale are extremely complicated, mainly employing the patent filed by Conway et al. (1996). Briefly, it requires the following steps: *i*) shredding diapers; *ii*) pulping of the resulted material, adding a chemical agent to prevent swelling of SAP; *iii*) separation of the components using different filtration systems (Tsigkou et al., 2014).

Before employing, the three substrates were characterized for their chemical-physical characteristics.

4.2.2 Experimental setup and analytical methods

Co-fermentation trials were carried out in semi-continuous fed batch systems. Two different schemes of co-fermentation were adopted. One reactor (RA) was fed only with HFW and SS, while a second reactor (RB) was fed with HFW, SS, and CW. Table 1 summarizes the operation setup used at each experimental run and the different ratio among substrates used for the two reactors.

All the experimental runs were carried under the same HRT (6 d), OLR (11 gTS/Ld), pH (7), since these operational parameters set up was found to be the best for maximizing VFAs production. This conclusion is corroborated by both the findings from the experiments described in the previous chapters and results reported by other authors (Strazzera et al., 2018; Moretto et al., 2019).

Table 1. Operational parameters setup for co-fermentation

Reactors	Substrates ratio*	°T (°C)	HRT (d)	OLR (gTS/Ld)	pH
RA	HFW+SS 6:4	37	6	11	7
RB	HFW+SS + COTTON WOOL 5:2.5:2.5	37	6	11	7
RA	HFW+SS 6:4	55	6	11	7
RB	HFW+SS + COTTON WOOL 5:2.5:2.5	55	6	11	7

* in terms of TS

Continuous stirred tank reactor systems could not be used for this work, due to issues related to rheologic properties of CW. Although CW were finely shredded, a preliminary test demonstrated that CW tend to wrap up the propellers, causing an exertion of engine, and consequent failure of agitation process. Moreover, an efficient continuous feeding was very difficult to carry out. For these reasons, two equal 2.5 L glass batch reactors were employed, emetically sealed with screw caps. The caps were opportunely modified by adding valves, in order to monitoring both volumetric biogas production and composition. To keep a constant temperature, reactors were transferred in a thermostatically controlled water bath (FA90, Falc, Italy). After addition of a proper amounts of substrates, the working volume was adjusted to 2.1 L, by mean of tap water. No inoculum was used, and acidogenic fermentation was carried out by microorganism that were still present in the substrates.

Reactors were daily fed, expect during weekends, following the ratio listed in Table 2 in terms of TS. During feeding a sample was taken from each reactor effluent, and they were stored at -20 ± 2°C for subsequent evaluation of fermentation performances. At time of sampling, pH was measured and adjusted with a proper volume of a NaOH (30% w/v) solution. Each experimental run lasted 60 days, corresponding to 10 cycles

of feeding. As temperature was risen from 37 to 55°C, reactors were not fed for a period of time of 16 days, in order to avoid washing out phenomena.
Regarding the analytical methods, VFAs, lactic acid, TS, TVS, COD, sCOD, TKN, pH, ammonia, biogas production and composition, were measured by mean of methods described in paragraph 2.2.2.

3.2.3 Microbial community structure: high-throughput 16 s rrna gene sequencing

The microbial community structure was studied with high-throughput 16 s rRNA gene sequencing. The analysis was carried out as reported in paragraph 2.2.5, except for database used. In this case, in fact, NCBI database was used.

4.3 RESULTS AND DISCUSSION

4.3.1 Substrates characterization

Table 1 summaries the characteristics of the three substrates used during the experimental work.

Table 2. Characteristics of substrates

Substrate	HFW	SS	CW
pH	5.83 ± 0.12	7.57 ± 0.28	nd*
TS (g/kg)	245.17 ± 2.56	40.96 ± 8.21	956.50 ± 1.89
TVS (g/kg)	233.76 ± 0.29	31.40 ± 7.62	934. 32 ± 2.33
COD (g/kg)	219.76 ± 9.86	32.24 ± 8.94	967.15 ± 50.17
TKN (g/kg)	8.59 ± 0.31	2.48 ± 0.69	nd*
Ammonia (mg N-NH_4^+/L)	664.95 ± 0.66	1071.15 ± 35.55	nd*

not determined*

It is clear how the low amount of COD present in SS was compensated by the addition of HFW and/or CW.

4.3.2 Acidogenic fermentation performances

Figure 1 shows the total VFAs production achieved during the di- and tri-fermentation experiments.

Under mesophilic conditions, the reactor fed with HFW and SS (RA) reached a stable total VFAs production of about 26 gCOD/L, after 13 days of operation, which corresponds to a yield of 0.40 gVFAs/gTS fed. A wide distribution among fermentation products was achieved, as shown in Figure 2a. In fact, caproate, butyrate, *iso*-valerate, valerate, acetate, propionate, and *iso*-butyrate accounted for 26.69, 24.73, 11.50, 10.95, 10.22, 8.88, and 6.72% of total VFAs produced, respectively, while *iso*-caproic acid was observed only in traces.

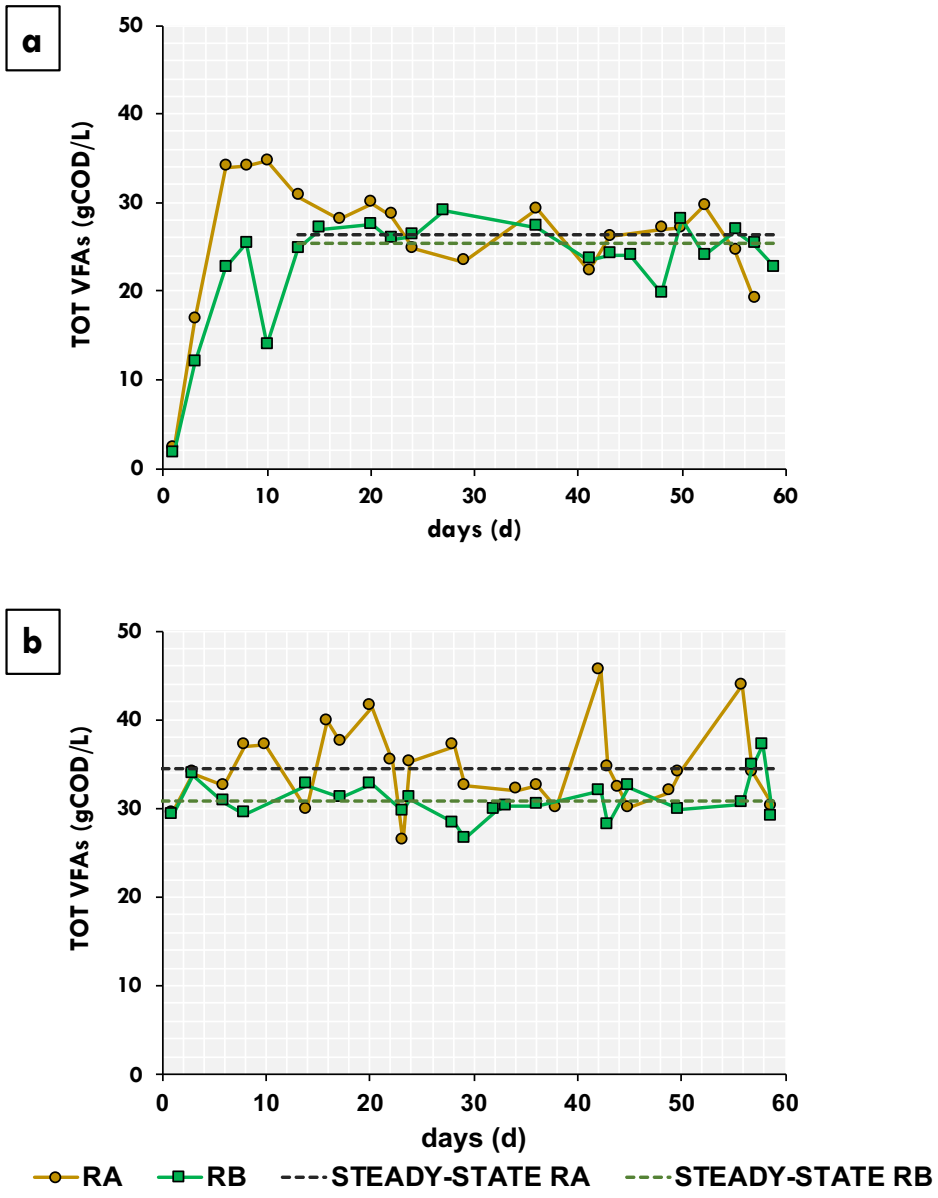

Figure 1. Total VFAs production: a) mesophilic, b) thermophilic conditions

These results emphasize that good acidogenic fermentation performance took place, with respect to results from similar works found in literature. For example, in a study on effect of food waste and sewage sludge mix ratio on VFAs production in batch reactors, it was found out that a ratio of food waste/SS of 3:2, under pH 6 and mesophilic conditions, allowed to obtain a VFAs production of only 9.37 g/L, with a yield of about 0.16 gVFAs/gTS fed. Moreover, butyric and acetic acids represented the main fermentation products, accounting for around 50 and 46% of total VFAs (Cheng et al., 2016).

Results comparable with ones obtained from this experiment were achieved in a work carried out by Zhang et al. (2016), even if a pretreatment of substrates by mean of microwave was required. Under mesophilic conditions and neutral pH, a total VFAs production of 22 g/L, corresponding to a yield of about 0.31 gVFAs/gTS fed, was achieved by co-fermentation of pretreated food waste and SS (3:2 in terms of TS). A similar yield, of around 0.34 gVFAs/gTS fed, was reached, under the same conditions, using food waste and pretreated SS. In both cases, acetic acid was the main product, accounting for 50% of total VFAs.

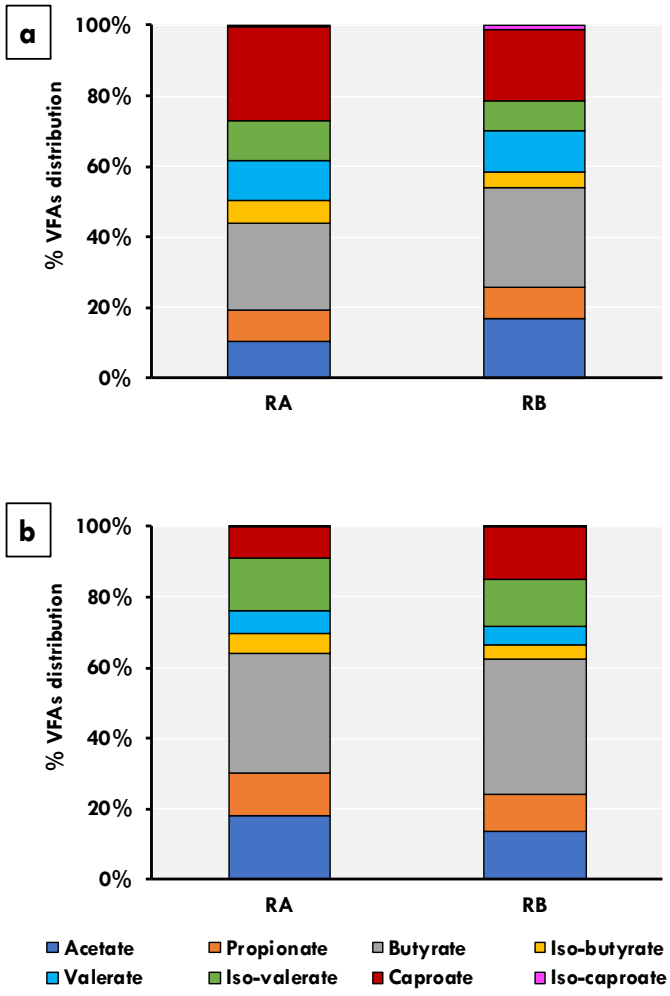

Figure 2. VFAs distribution patter: a) mesophilic, b) thermophilic conditions

The reactor fed with the three substrates (RB) reached a steady state after 13 days of operation, achieving an average total VFAs production of 25 gCOD/L and a yield of 0.30 gVFAs/gTS fed, under mesophilic conditions. As well as RA, a great distribution among fermentation products was observed in RB, even thought, the main fermentation product in this case was butyric acid, which accounted for 28.50% of total VFAs. However good percentages were observed also for caproate, acetate, valerate, propionate, *iso*-valerate, *iso*-butyrate, and *iso*-caproate, which accounted for the 20.20, 17.00, 11.70, 8.66, 8.32, and 4.29%, respectively. Moreover, a little increase of *iso*-caproate was observed with respect to RA, representing the 1.33% of total VFAs. In a work on mesophilic co-digestion of cellulose hydrolysate from diapers with fruits and vegetables wastes, Tsigkou et al. (2020) obtained a total VFAs production of about 11.8 g/L, under a pH value of 6, HRT 2 d, and an OLR of 21 gCOD/Ld, corresponding to a TS concentration into reactor of about 10 g/L. Even in this work, a large amount of caproic acid was obtained, which accounted for about 50% of total VFAs, while butyric and acetic acids represented the 30% each one. Moreover, Tsigkou et al. (2019), in a previous study carried out using the same substrates under mesophilic conditions, observed that caproic acid was produced only under pH value of 7, and disappeared at pH 6.5 and 7.5. However, under all the three tested pH values acetic acid was the main fermentation product, accounting for no less than 50% of total VFAs. Figure 1b shows that both RA and RB were readily able to restore a stable production, as reactors feeding restarted. Under these conditions, an average total VFAs production rate of 34.45 gCOD/L was achieved in RA, while in RB a lower production of 30.86 gCOD/L was observed. These values correspond to a yield of 0.55 and 0.48 gVFAs/gTS fed for RA and RB, respectively. Despite this little difference in terms of yield, a similar distribution among fermentation products was achieved in both reactors, as reported in Figure 2b. In RA butyrate, acetate, *iso*-valerate, propionate, caproate, valerate, and *iso*-butyrate accounted for 34.07, 18.01, 14.70, 12.11, 8.93, 6.37, and 5.72% of total VFAs, respectively. A very similar distribution was observed in RB, with the only difference that a little smaller amount of acetic, propionic, *iso*-butyric, and *iso*-valeric acids were produced, in favor of caproic acid. Indeed, butyrate, caproate, acetate, *iso*-valerate, propionate, valerate, and *iso*-butyrate accounted for 38.12, 14.89, 13.75, 13.21, 10.42, 5.58, and 4.01%, respectively. No *iso*-caproic production was observed in both reactors.

Although different total VFAs production was achieved in RA and RB, the increment of working temperature toward thermophilic conditions had a favorable effect on fermentation performances in both reactors, since total VFAs increased as consequence of increment of temperature. Probably, the greater production can be due to a higher

hydrolysis rate, since sCOD showed an increment in both the reactors. In fact, an increase of 32% of sCOD took place in RA, from an average value of 30.06 g/L, when reactor was incubated at 37°C, to 44.56 g/L, rising temperature at 55°C. The increase of hydrolysis is also proven by a greater amount of ammonia in the reactor, that is indicative of a greater hydrolysis of proteins. In fact, a value of 1.89 gN-NH_4^+/L was measured under mesophilic conditions, while it increased to 2.21 gN-NH_4^+/L under thermophilic conditions. A similar behavior was observed in RB, even if with minor extent, probably as consequence of the recalcitrant nature of cellulose fibers toward hydrolysis. An increment of about 28% of sCOD took place in the reactor under thermophilic conditions, from 27.38 g/L measured under mesophilic conditions to a value of 38.18 g/L. Even an increment of ammonia was observed, since it increased from 1.13 gN-NH_4^+/L to a value 1.58 gN-NH_4^+/L.

Table 3 shows biogas production in RA and RB under the two tested temperature.

Table 3. Average CO_2, CH_4 and H_2 yields

MESOPHILIC CONDITIONS			
	CO_2 yield (mL/gTVSd)	CH_4 yield (mL/gTVSd)	H_2 yield (mL/gTVSd)
RA	128.21	73.64	31.18
RB	56.43	21.53	13.23
THERMOPHILIC CONDITIONS			
	CO_2 yield (mL/gTVSd)	CH_4 yield (mL/gTVSd)	H_2 yield (mL/gTVSd)
RA	84.04	27.24	14.43
RB	63.52	18.74	21.90

During di-fermentation of HFW and SS an average volume of 4.78 L/d of biogas was production under mesophilic conditions. CH_4 represented the 30.72% of total biogas, corresponding to a yield of 73.64 ml/gTVSd. Whereas, H_2 was the about 13% of total biogas volume, with a yield of 31.18 mL/gTVSd. Increasing temperature, a lower biogas production of 2.69 L/d was observed in RA. Moreover, both CH_4 and H_2 decreased, in favor of CO_2, accounting for 21.24 and 11.25% of total volumetric production, corresponding to yields of 27.24 and 14.43 mL/gTVSd, respectively.

During tri-fermentation of HFW, SS, and CW a lower biogas production was achieved with respect to di-fermentation. Under mesophilic conditions, in fact, an average biogas volume of 1.98 L/d was measured in RB, about 60% less compared to RA. It was composed of 22.82% CH_4 and 11.25% H_2, with a yield of 21.53 and 13.23 mL/gTVSd

for CH_4 and H_2, respectively. Biogas production in RB was even lower under thermophilic conditions, with respect to RA. In this case a biogas production of 2.30 g/L was achieved, with a CH_4 and H_2 yields of 18.74 and 21.90, respectively.

4.3.3 Mass balance

Table 4 shows the mass balance for the two systems. A COD bioconversion of about 60% took place in RA under mesophilic conditions. Most of this COD was converted into VFAs, since they account for about 78% of converted COD. Moreover, CO_2, which was the main gaseous fermentation byproduct, could explain another remarkable part of COD conversion, since an average amount of 5 gCO_2/d was obtained in RA. When the temperature was increased toward thermophilic conditions, a greater COD conversion of about 71% took place in RA. In this case, VFAs production can explain well 90% of COD bioconversion. In addition, an average amount of 3.46 gCO_2/d was obtained from RA under thermophilic conditions.

Table 4. Reactors mass balance

Mesophilic conditions						
	RA			RB		
Parameter	Influent	Effluent	% Bioconversion	Influent	Effluent	% Bioconversion
gTS/d	22.90	12.41	45.81	22.97	13.36	41.85
gTVS/d	19.96	8.80	55.87	20.97	13.01	37.96
gCOD/d	19.93	8.07	59.52	20.88	11.25	46.12
Thermophilic conditions						
	RA			RB		
Parameter	Influent	Effluent	% Bioconversion	Influent	Effluent	% Bioconversion
gTS/d	21.88	9.48	48.01	22.34	11.71	47.56
gTVS/d	20.98	5.42	70.30	21.60	10.85	49.78
gCOD/d	18.24	5.23	71.00	19.83	8.32	58.04

A total COD conversion of about 46% was observed in RB under mesophilic conditions, 92% of which can be explained by VFAs production. Furthermore, an average production of 1.85 gCO_2/g was measured in RB under mesophilic conditions. An increment of temperature toward 55°C allowed to increase COD conversion in RB, until a value of 58.04%. In this case about 93 % of COD converted can be explained by

total VFAs production. Moreover, an average CO_2 production of 2.70 gCO_2/d was reached in RB under thermophilic conditions.

4.3.4 Microbiome structure

Figure 3 shows the class of bacteria found in RA and RB at the end of both mesophilic and thermophilic experimental runs.

In RA the sequencing analysis carried out at the end of mesophilic run allowed to obtain 11888 reads, most of which were classified under *Clostridia*, *Erysipelotrichia*, *Planctomycetia*, class (Figure 3a). These class, in fact, accounted for about 39, 14, and 11% of total reads. Among *Clostridia*, the main genera identified were *Saccharofermentans*, *Mobilibacterium*, *Anaerolactibacter*, and *Clostridium*, that represented the 19, 6, 4, 3% of total reads, respectively (Figure 4).

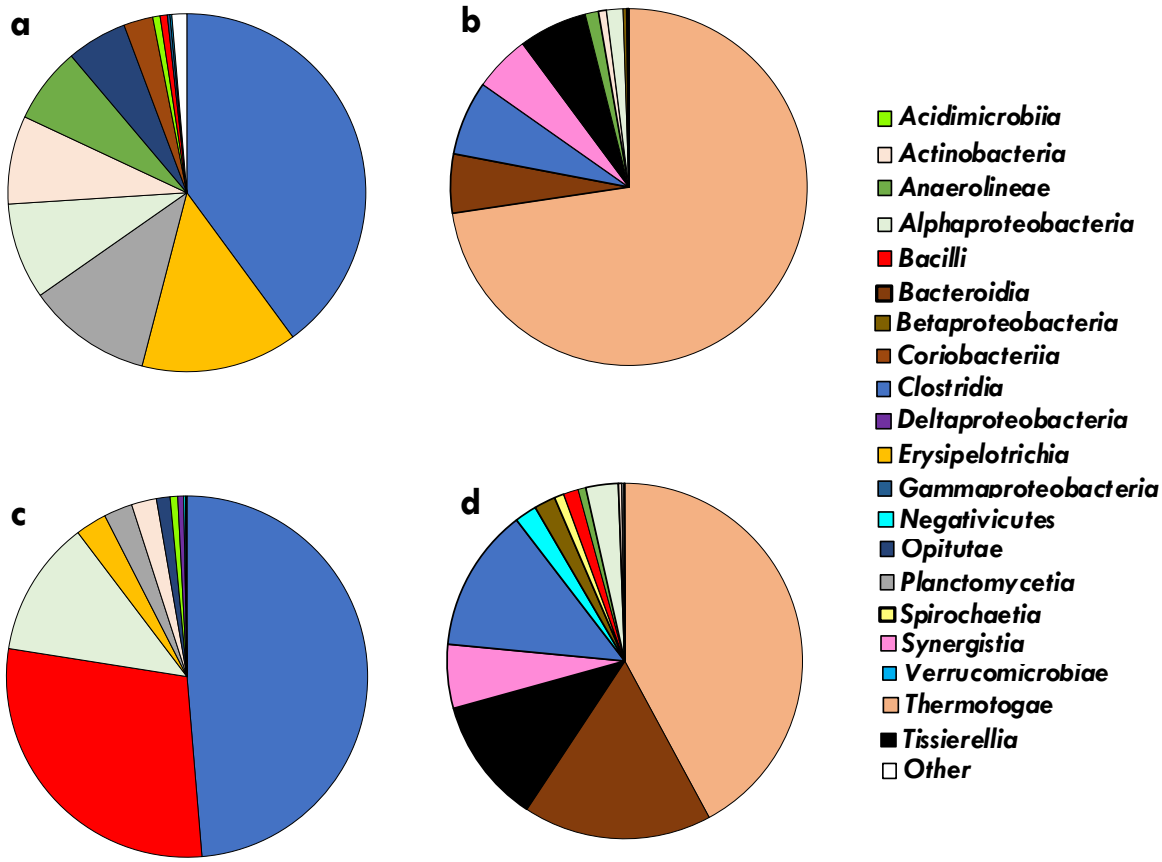

Figure 3. Class of bacteria found in the reactors a) mesophilic RA, b) thermophilic RA, c) mesophilic RB, d) thermophilic RB

With regard to the genus *Saccharofermentans*, all the sequences were attributed to the specie *S. acetigenes*. Bacteria belonging to this species are Gram negative, non-motile, mesophilic, obligately anaerobes, whit optimal growth under neutral pH. These bacteria are able to ferment several sugars, such as glucose, D-fructose, sucrose, and starch, producing mainly acetate, lactate, fumarate, and traces of CO_2 and H_2. However, *S. acetigenes* is not able to degrade cellulose (Chen et al., 2010).

Sequences found in RA for *Anaerolactibacter* genus were all attributed to the specie *A. massiliensis*. Bacteria of this species are Gram negative, non-motile, non-spore-forming, strictly anaerobes. These strains were able to grow at pH levels ranging from 6.5 to 8, but the optimum was observed at pH 7.5. They are able to ferment carbohydrates such as cellobiose, maltose, sucrose and trehalose (Togo et al., 2019).

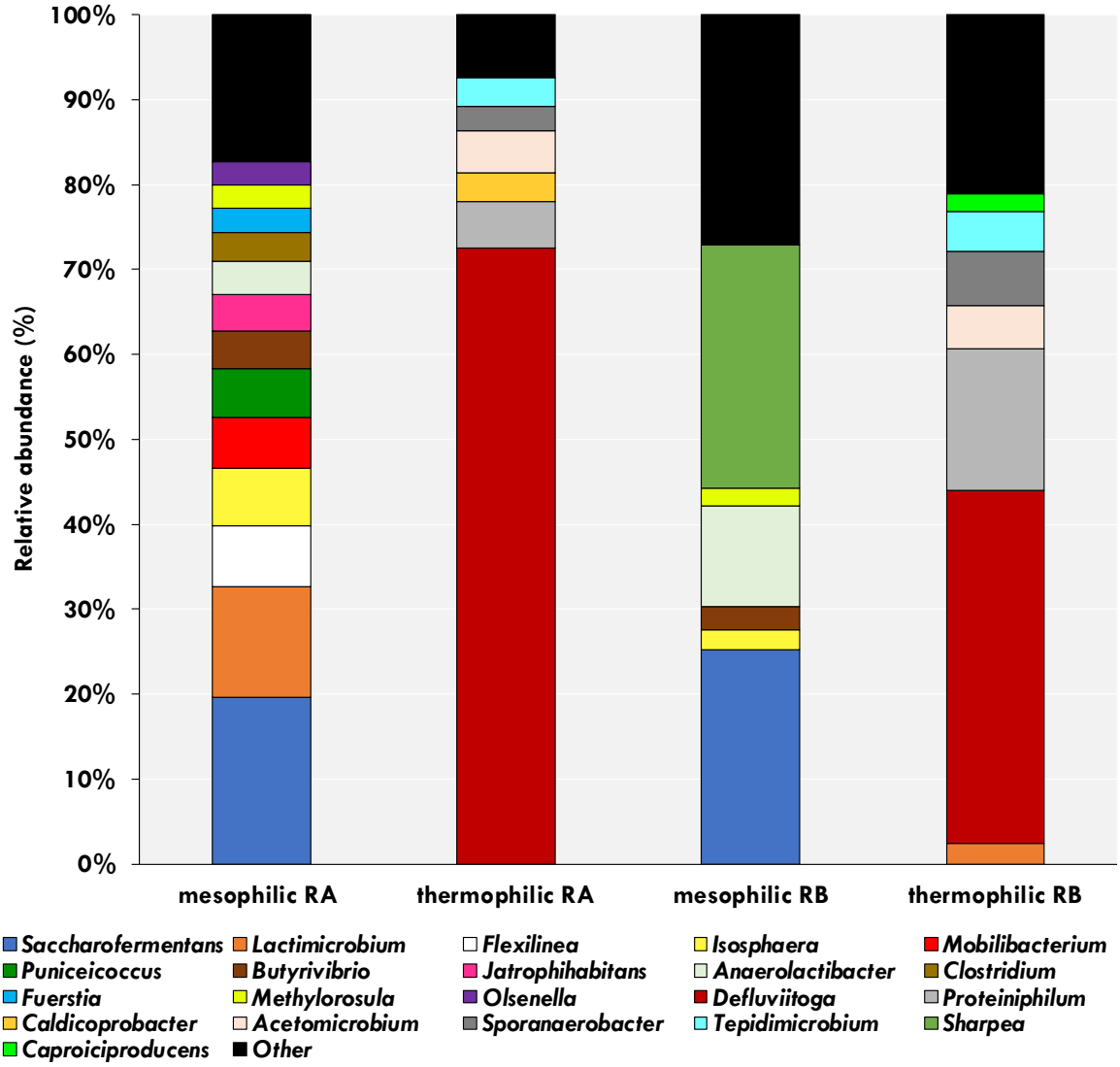

Figure 4. Relative abundance of genera in RA and RB, with respect to total identified reads. Genera which accounted for less than 2% of total reads were categorized under "other" group

Finally, the presence of bacteria belonging to the genus *Clostridium* in RA could explain the good butyric acid production observed in this reactor under mesophilic conditions, since these organisms are recognized as butyrate producers (Wang et al., 2018).

Within the class *Erysipelotrichia*, the main genus was *Lactimicrobium*, in particular the species *L. massiliense*, which was recently isolated and described, showing similar metabolic characteristics of *A. massiliense* (Togo et al., 2019).

The class of *Planctomycetia* belongs to the phylum *Planctomycetes*, that includes ubiquitous, environmentally important bacteria, which have a crucial role in global carbon and nitrogen cycles. These microorganisms, in fact, are able to carry out an anaerobic oxidation of ammonium. For this reason, *Planctomycetes* are useful bacteria for wastewater treatment (Kohn et al., 2016).

The analysis carried out from sample taken from RA at the end thermophilic run allowed to obtain a total of 13979 reads. In this case, the main class found in the reactor was *Thermotogae*, which account for 72% of total reads (Figure 2b). This is a clear indication that thermophilic conditions had a serious impact on microbiome, tending to select a lower number of bacteria populations. From a biotechnological point of view, *Thermotogae* species are of importance because they encode thermo-stable proteins that potentially are applicable in biomass conversion processes (Maus et al., 2016). Within *Thermotogae* class, the only genus found in RA was *Defluviitoga*, in particular the species *D. tunisiensis* (Figure 3). This species includes Gram negative thermophilic, anaerobic and slightly halophilic, with an optimum growth under pH 7 (Hania et al., 2012; Klippel et al., 2019). They are able to metabolize a large variaty of complex carbohydrates, including cellobiose and xylan, for the production of acetate, H_2, and CO_2 (Cappelletti et al., 2012; Maus et al., 2015).

A total number of 8782 reads were obtained starting from sample taken from RB at the end of mesophilic run. The most abundant class found were *Clostridia* (49%), *Bacilli* (29%), and *Alphaproteobacteria* (12%) (Figure 2c). Among *Clostridia*, the main genera were *Saccharofermentans*, *Anaerolactibacter*, and *Butyrivibrio*. Unlike *Butyrivibrio*, the first two genera were also abundant in RA the same conditions. Bacteria belonging to *Butyrivibrio* are Gram negative, but structurally Gram positive, non-spore forming, mesophilic, strictly anaerobes. They exhibit a fermentative metabolism on more or less carbohydrates as substrates, such as starch and cellulose, with butyrate, as well as lactate and acetate as end products. Bacterial species belonging to the genus *Butyrivibrio*, in fact, are important degraders and utilizers of lignocellulosic plant material, since they degrade polysaccharides and ferment the released monosaccharides to yield VFAs (Palevich et al., 2019). Probably, they had a crucial role in degradation of cellulose fibers.

With regard to *Bacilli* class, the main genus found in RB was *Sharpea*, which includes lactobacilli typically of rumen, whit a homofermentative metabolism (Kumar et al., 2018).

Finally, a total amount of 10261 reads was obtained from sample taken from RB at the end of thermophilic run. As shown in Figure 2d, in this case the main class were *Thermotogae*, *Bacteroidia*, *Clostridia*, and *Tissierellia*, which accounted for 42, 17, 13, and 11%, respectively. This result indicates that microbiome in RB was shaped by selective pressure of temperature increase with a less extent with respect to RA. *Bacteroidia* belongs to the phylum *Bacteroidetes*, the second most abundant group of the typical microbial communities found in biogas reactors. This phylum is a metabolically heterogeneous group, that includes species with a broad range of capabilities, such as hydrolysis of polysaccharides and proteins, fermentation of sugars and production of organic acids such as acetic, propionic, succinic and butyric acids (Hahnke et al., 2016). The main genus found in the reactor within *Bacteroidia* class was *Proteiniphilum*. Bacteria belonging to this genus are Gram negative, obligately anaerobic, with an optimum temperature for growth ranging between 20 and 45°C. Moreover, there are not saccharolytic, being not able to use carbohydrates. Their name, in fact, comes from the capability of proteins degradation, which leads to generation of acetic and propionic acids as end products of fermentation (Chen & Dong, 2005).

Members of the class *Tissierellia* have a Gram positive cell wall structure and their reaction to Gram stain is positive or variable. The class includes obligatory anaerobic organisms, with a versatile metabolism. Although they use amino acids as main sources of energy, some species are able to ferment carbohydrates. Futhermore, a few members show the ability to reduce Fe(III) (i.e. *Tepidimicrobium*), to use inorganic sulfur-containing compounds like thiosulfate and/or elemental sulfur as electron acceptors (i.e. *Soehngenia*, *Sporanaerobacter* and *Tepidimicrobium*) (Alauzet et al., 2014)..

Finally, *Clostridia* class showed an enrichment as consequence of temperature increase, since different genera found in the reactors belongs to this class, even if they individually showed a low relative abundance.

4.4 CONCLUSION

This work asses the possibility of simultaneous fermentation of three abundant organic waste streams of urban areas, HFW, SS, and cellulose from disposable diapers. Although good yields were achieved, in terms of VFAs production, some issues needed to be overcome for a scale up of this process, main of which is optimization of organic content isolation from diapers. Finally, temperature had a crucial role in promoting of

hydrolysis and consequently VFAs production, but a detail cost-benefit analysis must be carried out for scaling up of the proposed process.

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