Enseñar la explotación de la tierra, no la del hombre

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DOCTORADO EN CIENCIAS AGROALIMENTARIAS

DINAMICA MICROBIANA DE BIODIGESTORES ENRIQUECIDOS CON PROPIONATO

THESIS

In partial fulfillment of the
requirements for the degree of
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By
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Under supervision of
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ABBREVIATIONS

AD	Anaerobic digestion
AcoD	Anaerobic co-digestion
Al	Aluminum
BMP	Biochemical methane potential
C	Carbon
Ca	Calcium
CH_4	Methane
CL	Chicken litter
Co	Cobalt
CO	Carbon monoxide
CO_2	Carbon dioxide
Cr	Chromium
Cu	Copper
C/N	Carbon/nitrogen ratio
DIET	Direct interspecies electron transfer
DNA	Deoxyribonucleic acid
FA	Free ammonia
GHG	Greenhouse gas
H	Hydrogen

HRT	Hydraulic retention time
H_2	Molecular hydrogen
H_2S	Hydrogen sulfide
ISR	Inoculum/substrate ratio
K	Potassium
M	Molar concentration
Mg	Magnesium
MMC	Methylmalonyl-CoA
MPB	Methane producing bacteria
N	Nitrogen
Na	Sodium
NH_3	Ammonia
NH_4^+	Ammonium
Ni	Nickel
N_2	Molecular nitrogen
O	Oxygen
OLR	Organic loading rate
pH	Potential of hydrogen
p_{H2}	Partial pressure of molecular hydrogen
p_{CH4}	Partial pressure of methane

Pr	Propionate
rRNA	Ribosomal ribonucleic acid
SPOB	Syntrophic propionate-oxidizing bacteria
SRB	Sulfate reducing bacteria
SRT	Solid retention time
T	Temperature
TAN	Total ammonia
TS	Total solid
VFA	Volatile fatty acid
VS	Volatile solid
Zn	Zinc
ΔG°’	Standard Gibbs free energy change
μm	Methane production rate per day
λ	Lag phase time

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With love,

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From 2009 to 2010 I collaborated as laboratory analyst in the project "Validación de la producción de miel de maguey por el grupo Productoras de Totolapa SPR de RI" In the period from 2013 to 2016 I was manager of WWTP (Wastewater Treatment Plant) and Member of the Board of Potable Water and Sewerage Silao (SAPAS).

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RESUMEN GENERAL

Dinámica microbiana de digestores anaeróbicos enriquecidos con propionato

El objetivo de este trabajo fue evaluar la influencia de un consorcio microbiano obtenido bajo presión de selección con altos niveles de propionato, en la producción de metano de un sistema de codigestión alimentado con pollinaza (CL) y propionato (Pr), en condiciones mesofílicas. Para lograr este objetivo, el proyecto se dividió en dos secciones: 1) a través de una prueba de PBM en microcosmos (250 ml de volumen de trabajo) se llevó a cabo una codigestión anaeróbica de CL (3% TS) con Pr (4895 ppm). El proceso se detuvo después de 280 días; se observó un comportamiento triple sigmoidal con un PBM de 364.52 mL de CH4 g_{SVfed}^{-1}. 2) El experimento se realizó por triplicado. El sustrato utilizado fue pollinaza (3% de TS) que se degradó usando digestores con un volumen de trabajo de 10 L, durante 191 días. De acuerdo con el modelo modificado de Gompertz, los valores de PBM, μm y λ fueron 183.66 mL CH_4 $g_{vsfed}^{-1}d^{-1}$ ± 23.36, 3.70 mLCH_4 g_{vsfed}-$^{1}d^{-1}$ ± 0.52 y 14.86 d ± 12.18, respectivamente. Con el fin de dilucidar el papel del microbioma en la producción de metano, se llevó a cabo la secuenciación y el análisis bioinformático de 18 muestras de ADN, las muestras se tomaron en puntos estratégicos de la curva de rendimiento de metano acumulado. La información obtenida, utilizando diferentes herramientas bioinformáticas, reveló que el filo dominante es *Proteobacteria* y el principal microorganismo responsable de producir metano es *Methanoculleus marisnigri* a través de la vía de metanogénesis hidrogenotrófica; mientras que el metano producido por la vía acetoclástica se atribuyó principalmente a *Methanosarcina mazei*. Por otro lado, la degradación del propionato se llevó a cabo principalmente por *Syntrophomonas wolfei* y *Pelotomaculum thermopropionicum*, aumentando su población durante el tiempo del experimento. Además, la presencia de bacterias reductoras de sulfato (SRB) podría indicar la existencia de una relación sintrófica entre este grupo de microorganismos, bacterias sintróficas y metanógenos.

Palabras clave: Análisis de metagenomas, análisis de redes, metanógenos, PBM, pollinaza

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GENERAL ABSTRACT

Microbial dynamics of anaerobic digesters enriched with propionate

The objective of this work was to evaluate the influence of a microbial consortium, obtained under selection pressure with high levels of propionate, on methane production of a co-digestion system fed with chicken litter (CL) and propionate (Pr), under mesophilic conditions. To achieve this objective the project was divided in two sections: 1) through a BMP test in microcosms (250 mL working volume) an anaerobic co-digestion of CL (3 % TS) with Pr (4895 ppm) was carried out. The process stopped after 280 days; a triple sigmoidal behavior was observed. The BMP obtained was 364.52 mL CH4 g_{SVfed}^{-1}. 2) The experiment was carried out in triplicate. Chicken litter (3 % TS) was used as substrate, it was degraded using digesters with a working volume of 10 L, for 191 days. According to the modified Gompertz model, the value of BMP, μm and λ were, 183.66 mL CH_4 $g_{vsfed}^{-1}d^{-1}$ ± 23.36, 3.70 mLCH_4 $g_{vsfed}^{-1}d^{-1}$ ± 0.52 and 14.86 d ± 12.18, respectively. In order to elucidate the role of the microbiome on the methane production, sequencing and bioinformatic analysis of 18 DNA samples were carried out, the samples were taken at strategic points of the accumulated methane yield curve. The information obtained, using different bioinformatic tools, revealed that the dominant phylum is *Proteobacteria* and the main microorganism responsible to produce methane is *Methanoculleus marisnigri* through the hydrogenotrophic methanogenesis pathway. While the methane produced by acetoclastic pathway was mainly attributed to *Methanosarcina mazei*. On the other hand, the degradation of propionate was carried out primarily by *Syntrophomonas wolfei* and *Pelotomaculum thermopropionicum*, increasing its population through the time of the experiment. In addition, the presence of reducing sulfate bacteria (SRB) may indicate the existence of a syntrophic relationship among this group of microorganisms, syntrophic bacteria and methanogens.

Keywords: BMP, chicken litter, metagenome analysis, methanogens, network analysis.

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1. GENERAL INTRODUCTION

Along with increase d energy efficiency, substitution of fossil fuel-derived energy with renewable sources is crucial in achieving the goal of reduced emissions of anthropogenic GHG. Biogas produced through anaerobic degradation of organic residues has good potential in climate change mitigation and involves indirect environmental benefits such as reduced emissions of air pollutants and ammonia. Moreover, biogas provide a renewable source of energy in the form of methane (2004; Divya, Gopinath, & Merlin Christy, 2015).

AD process consists in the biodegradation of organic compounds contained in a biomass (Nzila, 2017) that can be originated from plants, animals or microorganisms (Prabir, 2013). In particular, the use of animal manure as biomass has been proposed with a dual purpose: a) renewable energy source and, b) constitutes a safe and economical disposal of an abundant and dangerous waste since, it contaminates the groundwater, it is a source of propagation of pathogens and contributes to the emission of GHG (Santos Dalólio et al., 2017; Y. Zhang et al., 2011). In this sense, chicken litter constitutes a source of biomass, since it is an organic waste material, rich in nutrients, such as nitrogen, phosphorus and potassium, besides being abundant and of low cost, it also has a content of relatively high energy compared to cow and pig manure, making it highly suitable for AD process (Bayrakdar, Molaey, Sürmeli, Sahinkaya, & Çalli, 2017). However, if not treated properly it causes serious damage to the environment, contaminating water and air, with GHG emissions of 6.93, 145.5 and 0.570 gigagrams of CH_4, CO_2eq and N_2O per head, respectively (FAOSTAT, 2016). The main problem with the use of a poultry manure as biomass for the production of methane is the effect of the accumulation of NH_3 and VFA (Abouelenien, Namba, Kosseva, Nishio, & Nakashimada, 2014; Bayrakdar, Sürmeli, & Çalli, 2017; Molaey, Bayrakdar, Sürmeli, & Çalli, 2018).

The presence of high levels of VFA decreases the yield in the production of biogas (Franke-Whittle, Walter, Ebner, & Insam, 2014); specifically, propionate, which is considered one of the precursors of methanogenesis since it may contributes 35%

of the total methane production (Koch, Dolfing, Wuhrmann, & Zehnder, 1983), has a detrimental impact on methanogenic microorganisms (Rétfalvi, Tukacs-Hájos, Albert, & Marosvölgyi, 2011) at concentrations of 37 - 100 mM (Pullammanappallil, Chynoweth, Lyberatos, & Svoronos, 2001), modifying the abundance of some microbial groups, because it causes inhibition in the growth of various microbial species (W. Kim, Shin, Han, Cho, & Hwang, 2015; Tian, Zhang, Li, Chi, & Yang, 2015). Therefore, the degradation of propionate and limitation of its accumulation are important parameters that must be considered to improve the process of anaerobic digestion in a reactor (Ahlert, Zimmermann, Ebling, & König, 2016). A solution to this problem is the addition of microbial cultures that use propionate or are VFA degraders, in such a way that they reduce the accumulation of this metabolite and improve the process (Ying Li et al., 2017).

However, despite its widespread use worldwide, the biological mechanisms of AD are not yet fully understood, mostly due to the complexity of the microbial communities involved (Nelson, Morrison, & Yu, 2011). In addition to this, one of the challenges in AD, is the control of the propionate concentration by investigating microbial propionate degradation. Up to now, studies concentrating on propionate degradation in chicken litter anaerobic degradation have been underrepresented. Further there is a lack of knowledge linking microbial community content, dynamics, and activity with reactor performance. Thus, detailed studies of the composition of AD microbial consortia and their metabolic functions are required. Insight in the microbial mutualism that underpins AD and functions close to the thermodynamic limits, is the key to elucidate the black box and upgrade this process with a new renaissance (Tan, Lee, & Shih, 2016).

The main objective of this study was to evaluate the influence of a consortium, obtained under selection pressure with high levels of propionate, on methane production, in a co-digestion system fed with chicken litter and high concentrations of propionate likewise, to determine the structural changes in microbial community on an AD of chicken litter. To achieve these objectives, it was necessary to perform a biochemical methane potential test (BMP) in

mesophilic conditions and performing a metagenome analysis using Illumina technology and bioinformatics tools in order to know microbial communities associated with the functional diversity, and to evaluate the resilience of the process under a wide range of propionate concentrations. To achieve the above, 3 stages were developed, which are described from Chapter 3 of this thesis.

Chapter 3. This is a review article with the most relevant aspects of anaerobic digestion, and the challenges of the chicken litter degradation represents for this system.

Chapter 4. A batch experiment in digesters with a working volume of 10 L, for 191 days was carried out. The main objective was to determinate the microbial structure changes through the whole methane production process, when the system is disturbed with large fed volume. To achieve this goal, a BMP test was carried out and the metagenome was analyzed. DNA was sequenced by Illumina HiSeq, the results were analyzed using bioinformatic tools for metagenome analysis (MG-Rast, Network analysis, etc).

Chapter 5. BMP test in 330 mL serological bottles, in which the AD of CL added with propionate (4895 ppm), was carried out. This was done to know if the microbial consortium that had been adapted to high concentrations of propionate (approximately 3500 ppm at that time) was able to degrade higher concentration of this metabolite, resulting in a high yield in the production of methane compared with previous research.

2. GENERAL LITERATURE REVIEW

2.1. Climate changes

Some of the greatest challenges human beings face in the 21st century are the environmental pollution and energy insecurity. There is a general consensus that global warming is due to the large-scale anthropogenic emission of greenhouse gases, which are mainly caused by the generation of heat and power (Appels, Baeyens, Degrève, & Dewil, 2008), which causes an increase in global temperature that mainly affects ecosystems, wildlife, food chains and eventually human life.

The use of renewable energy as an alternative to fossil fuels will play a crucial role to help mitigate CO_2 emission (Hagos, Zong, Li, Liu, & Lu, 2017), helping to improve the living standard of developing countries, reducing cost effective energy and utilizing bioenergy efficiency (Ebner et al., 2015). In this regard, the energy source of biomass has become one of the most promising futures of renewable energy sources. Waste materials like sewage sludge, manure and crop waste are of specific importance since these sources do not compete with food crops in agricultural land usage. In this sense, forestry resources, agricultural resources, sewage and industrial organic wastewater, municipal solid wastes, livestock and poultry dung and biogas are major categories for use (Scarlat, Dallemand, & Fahl, 2018).

In accordance with Appels, Lauwers, et al. (2011), there are various technologies available for power generation from biomass and waste (known as bioenergy) and can be subdivided into thermochemical, biochemical and physicochemical conversion processes. A schematic overview is presented in Figure 1.

2.2. Bioenergy and biogas

Bioenergy can play a central role in promoting renewable alternatives, is a nearly GHG-neutral replacement for fossil fuels (Haberl et al., 2012) due to its renewable and widely applicable characteristics. Biogas, which is generally referred to as a type of bioenergy, is a gas obtained from the degradation of organic matter carried

out by microorganisms under anaerobic conditions. Biogas is a promising means of addressing global energy needs and providing multiple environmental benefits, as shown in Table 1 (Mao, Feng, Wang, & Ren, 2015; Scarlat et al., 2018). Raw biogas typically consists of CH_4 (50–75%), CO_2 (25–50%), and smaller amounts of N_2 (2–8%). Trace levels of H_2 sulfide, ammonia, and various volatile organic compounds are also present depending on the feedstock (Yin Li et al., 2019).

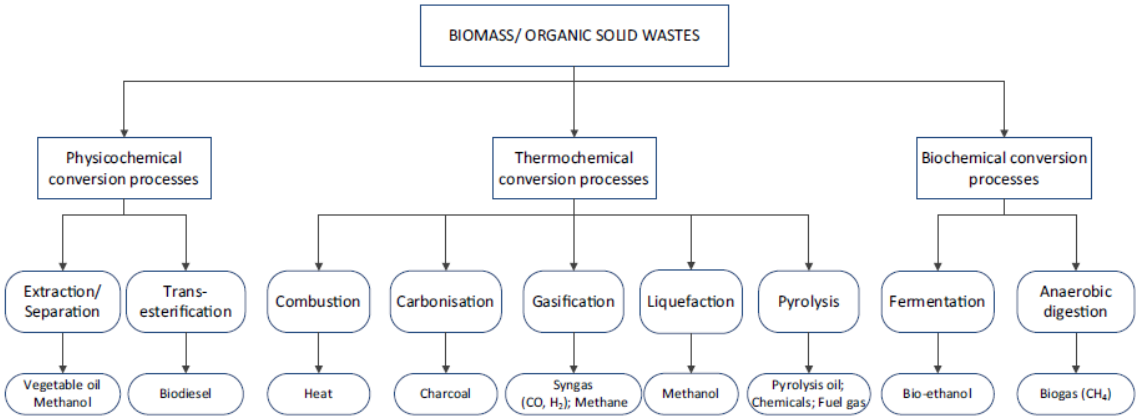

Figure 1. Biomass and waste conversion technologies (Appels, Lauwers, et al., 2011).

Most biogas production occurs in the United States and Europe, although other regions increasingly are deploying the technology as well. Some examples of the successful use of biogas are: Italy, 3405 GW h of electricity was produced from biogas in 2011 (Bacenetti, Negri, Fiala, & González-García, 2013); in the EU policy estimates that at least 25% of all bioenergy can be derived from biogas (Holm-Nielsen, Al Seadi, & Oleskowicz-Popiel, 2009); in Germany, approximately 4000 agricultural biogas production units were operated on German farms at the end of 2008, which is beneficial for farmer living-environment (Weiland, 2010); in China about 248 billion m^3 of biogas were produced in 2010 (Yanfei Deng, Xu, Liu, & Mancl, 2014). Furthermore, from a socio-economic point of view, biogas not only significantly reduces the costs of treating waste but also has a relatively low feedstock cost. In addition, biogas has a lower sale price compared with diesel and petrol.

Table 1. Biogas applications (Scarlat et al., 2018).

Advantages	Applications
Green energy production	Electricity
	Heat
	Vehicle fuel
Organic waste disposal	Agricultural residues
	Industrial wastes
	Municipal solid wastes
	Household wastes
	Organic waste mixtures
Environmental protection	Pathogen reduction through sanitation
	Less nuisance from insect flies
	Air & water pollution reduction
	Eutrophication and acidification reduction
	Forest vegetation conservation
	Replacing inorganic fertilizer
Biogas-linked agrosystem	Livestock-biogas-fruit system
	Pig-biogas-vegetable greenhouse system
	Biogas-livestock and poultryfarms system
GHG emission reduction	Substituting conventional energy sources

Biogas upgrading to higher quality biomethane is also increasing for use as a vehicle fuel or for injection into the natural gas grid. In developing countries, biogas is mainly produced in small, domestic-scale digesters to provide fuel for cooking or even lighting, in comparison to developed countries, where biogas developments focused on larger scale, farm based and commercial, electricity and heat biogas plants.

Different biogas support programs have been carried out to develop household biogas systems to provide people with biogas for cooking, as an alternative energy source, to reduce firewood consumption and avoid deforestation, decrease indoor air pollution and improve soil fertility (Vijay, Kapoor, Trivedi, & Narale, 2015; Vögeli, C. Lohri, Gallardo, Diener, & Zurbrugg, 2014). In recent years, one of the most attractive renewable energy pathways to generate this kind of bioenergy is AD of waste and residues from agriculture and industry, municipal organic waste, sewage sludge, etc.

2.3. Anaerobic digestion (AD)

Anaerobic digestion, classified within the biochemical conversion processes, is a robust process widely applied and it is also predicted to play a vital role in the future of renewable energy production (Surendra, Takara, Hashimoto, & Khanal, 2014). AD can reduce organic pollution from the liquid outputs of homes, industry and agriculture, while potentially offsetting the use of fossil fuels at the same time. In addition, it offers numerous other significant advantages, such as lower energy requirements, and less sludge production compared with traditional aerobic treatment (Y. Chen, Cheng, & Creamer, 2008; Franke-Whittle et al., 2014). Different studies on anaerobic digestion have been carried out in order to minimize process instability and increase methane yield (Table 2), focusing mainly on the type of technology, mechanism, factors that affect efficiency, substrate, etc.

Table 2. Recent studies on AD process.

Relevant aspect	**References**
Operational factors	Zahan and Othman (2019)
Inhibition factors	W. Zhang, Zhang, Li, and Jianxiong Zeng (2019)
Pretreatment techniques	Ma and You (2019)
Co-digestion	Meneses-Reyes, Hernández-Eugenio, Huber, Balagurusamy, and Espinosa-Solares (2018)
Environmental conditions	Bi et al. (2019)
Microbial community	Song, Song, Li, Liu, and Niu (2019)
Metagenomic and metabolic analysis	Zhu, Campanaro, Treu, Kougias, and Angelidaki (2019)

AD consists of an integrated system of a physiological process of microbial and energy metabolism that convert organic matter to methane and carbon dioxide, and can take place under psychrophilic (below 20 °C), mesophilic (25–40 °C) or thermophilic (50–65 °C) conditions, although biodegradation under mesophilic conditions is most common (H. H. Chen & Lee, 2014). Nevertheless, the efficient conversion of organic matter to methane in an anaerobic digester is dependent on the mutual and syntrophic interactions of functionally distinct microorganisms

(Akuzawa et al., 2011). Therefore, we must consider that this microbial community is sensitive to variations in the operating conditions applied and thus, the AD process, if improperly managed, would become unstable and result in reduced biogas production.

2.3.1. Substrate

There is a high range of organic material that can be used as feedstock for AD, and the feedstock choice depends mostly on regional availability. The most common feedstocks used for AD are listed in Table 3 (Vasco-Correa, Khanal, Manandhar, & Shah, 2018).

The choice of feedstock has high influence in different aspects of the AD process, including pre-processing (separation, reduction size, mixing); pretreatment, which is performed to enhance the digestibility of some feedstocks and could contribute significantly to the cost of the processing (Carlsson, Naroznova, Møller, Scheutz, & Lagerkvist, 2015); retention time, which is dependent on the digestibility of the material; biogas yield and overall economics of the process; and composition of the digestate (Weiland, 2010). Most feedstocks used for AD have an intrinsic variability that could significantly affect the AD process. The total solid (TS) content of the most common feedstocks varies from 2 % in manure and other liquid wastes to 80 % in agricultural residues.

A huge amount of nitrogen-rich waste from industrial and agricultural production (such as animal manure, slaughterhouse waste, and waste active sludge) is produced annually. This nitrogen-rich waste contaminates the environment (Lu, Kitamura, Takisawa, & Jiang, 2015), and creates difficult challenges for the environmental health and sustainability of the poultry industry (Wandera et al., 2018). In this sense, manure is a frequently used feedstock for anaerobic digesters because it is readily available and very suitable for the development of anaerobic microorganisms because of its high nitrogen content (Appels, Lauwers, et al., 2011).

Table 3. Common feedstock used for anaerobic digestion with their main features (Vasco-Correa et al., 2018).

Feedstock	Main features	Biogas yield (m^3 kg^{-1} VS^{-1})	TS (%)	C/N ratio
Animal manure	Usually codigested with bedding material or other biomass high in carbon High buffer capacity Complete source of nutrients and trace elements	0.1 – 0.6	2 – 20	3 – 15
Organic fraction of municipal solid waste	Needs separation at the source, or high pre-processing Requires size reduction High variability in composition	0.3 – 0.6	20 – 50	35
Food waste	Produced by hotels, restaurants, markets, and food processing companies Requires size reduction High variability in composition Easily digestible, could generate inhibition by acidification	0.3 – 0.8	5 – 30	15 – 30
Agricultural residues and energy crops	Abundant in availability Composed mostly of cellulose, hemicellulose and lignin, and/or starch. Residues include leaves, stover, straws, etc. Energy crops can be ensiled for storage. Highly recalcitrant needs pretreatment to enhance digestibility	0.2 – 0.5	20 – 35	40 – 150
Sewage sludge	Byproduct of wastewater treatment. High in solids, pathogens and nutrients. Highly contaminant, needs stabilization. Low digestibility: pretreatment or co-digestion with another feedstock could improve it.	0.8 – 1.2	20 – 35	40 – 70
Algae	Macroalgae or microalgae. Low digestibility, recalcitrant structure, especially if cell wall has not been disrupted. Could improve with pretreatment	0.5 – 0.8	2 – 30	5 – 25

Chicken litter

The global poultry production was 111,000 thousand metric tons in 2015. World poultry production is projected to increase by 24 % over the next decades, reaching 131,255 thousand metric tons in 2025. Moreover, global production of chicken meat is forecast 4 percent higher in 2020 to a record 103.5 million tons, surpassing pork production (USDA, 2019). This intensive chicken industries produce a huge amount of manure that needs to be properly treated to prevent environmental pollution. Besides that, CL is an interesting substrate for biogas production due to the potentially high energy yield that can be gained (Wandera et al., 2018) and also, that a biologically stable material, i.e. digestate, can be produced and used as a fertilizer (Riva et al., 2016). CL consists of the animal waste plus the bedding material which contains wood chips and feed residue (Sharma, Espinosa-Solares, & Huber, 2013). It must be remembered that CL has high nitrogen content due to uric acid and protein, both of which are degraded to ammonia. The total ammonia (TAN) contents are NH_4^+ and NH_3 often referred as free ammonia (FA). The FA has been shown to be pH, temperature and TAN dependent and is known to be a significant cause of inhibition due to enzyme inactivation. In addition, affects the cellular diffusion process causing disturbances in AD processes (Bi et al., 2019; Kadam & Boone, 1996).

2.3.2. Biochemistry of Anaerobic Digestion

AD is a series of complex biological process that can be divided into three major microbial steps, i.e. hydrolysis, acidogenesis/acetogenesis, and methanogenesis represented in Figure 2, mainly occurs in absence of oxygen (Mehariya, Patel, Obulisamy, Punniyakotti, & Wong, 2018; Monge A, Certucha B, & Almendariz T, 2013). During **hydrolysis**, a consortium of bacteria breaks down complex organics (e.g. proteins, carbohydrates, and lipids) from the influent into soluble monomers such as amino acids, simple sugars, glycerols, and fatty acids (J. L. Chen, Ortiz, Steele, & Stuckey, 2014; Nielsen, Uellendahl, & Ahring, 2007). Hydrolysis of these complex polymers, some of which are insoluble, is catalyzed by extracellular enzymes such as cellulases, proteases, and lipases. Hydrolysis

of insoluble polymers is generally considered as rate limiting step in the AD of solid organic wastes, because the hydrolytic enzyme should be primarily adsorbed on the surface of solid substrates, although with soluble feeds methanogenesis is regarded as the key step in anaerobic digestion (Appels et al., 2008; Coelho, Droste, & Kennedy, 2011).

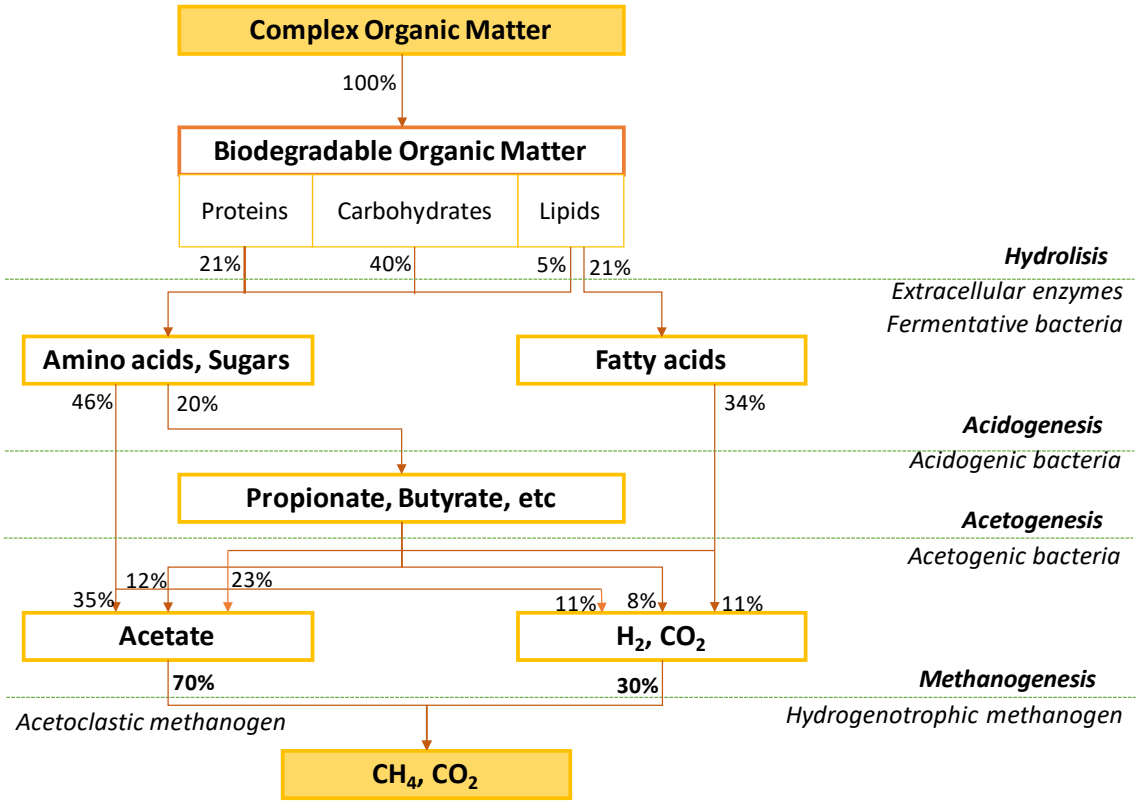

Figure 2. Schematic representation of the main conversion processes in anaerobic digestion (modified from Gujer & Zehnder, 1983).

The second stage of the process is **acidogenesis**, which includes fermentation and anaerobic oxidation (β-oxidation), processes that are carried out by fermentative acidogenic and acetogenic bacteria, respectively (Batstone & Jensen, 2011). This step provides intermediates from substrate metabolism as electron acceptor (Yue Li, Chen, & Wu, 2019). Fermentative acidogenic bacteria convert sugars, amino acids, and fatty acids to organic acids (e.g. acetic, propionic, formic, lactic, butyric, or succinic acids), alcohols and ketones (e.g. ethanol, methanol, glycerol, and acetone), acetate, CO_2, and H_2 (Franke-Whittle et al., 2014; C. Zhang, Su, Baeyens, & Tan, 2014). Acetate is the major product

of carbohydrate fermentation. Acetogenic bacteria convert fatty acids (e.g. long chain fatty acids) and alcohols into acetate, H_2, and CO_2, which are used by the methanogens to produce biogas.

In the methanogenesis process, generally, the substrates which could be utilized by methanogens include three types:(1) VFA (C1–C6); (2) n- or i-alcohols; (3) gases (CO, CO_2 and H_2) (Appels, Assche, et al., 2011).There are four main pathways for methane (CH_4) production: (i) acetoclastic methanogens utilize acetate to directly produce CH_4 and CO_2; ($CH_3COOH \rightarrow CH_4+CO_2$, $\Delta G°' = -36$ kJ mol^{-1}), (ii) hydrogenotrophic methanogens use H_2 or formate to reduce CO_2 to CH_4; ($CO_2+4H_2 \rightarrow CH_4+2H_2O$, $\Delta G°' = -131$ kJ mol^{-1}), (iii) methylotrophic methanogens metabolize methyl compounds to produce a small amount of CH_4 as shown in Figure 3; and (iv) syntrophic partnerships of acetate-oxidizing bacteria and hydrogenotrophic methanogens convert acetate to CH_4 via the intermediates H_2 and CO_2 (de Bok, Plugge, & Stams, 2004; Leng et al., 2018).

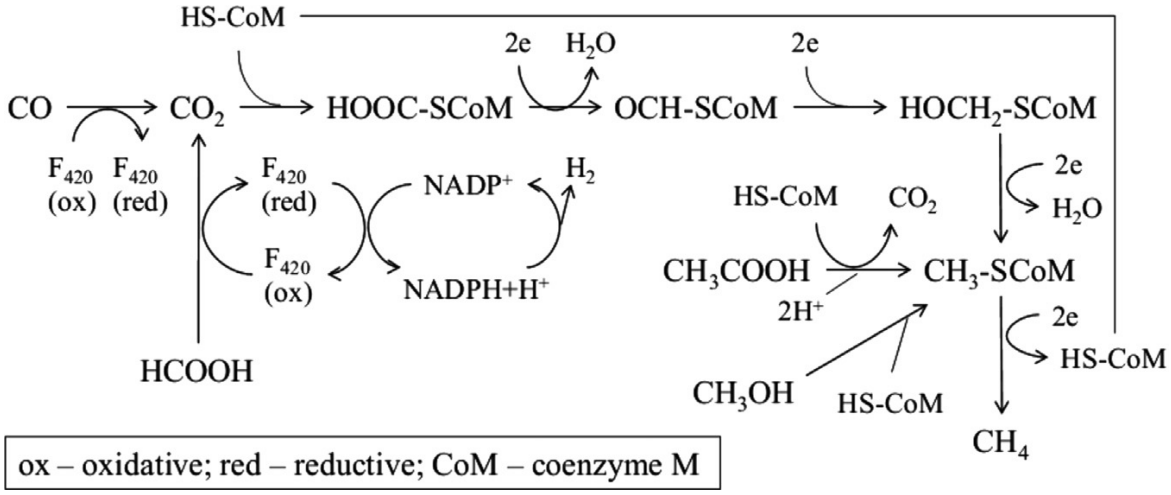

Figure 3. Metabolic process from CO, CO_2, formate, carbinol and acetate to methane (de Bok et al., 2004).

During the methane formation process, the co-enzymes M and F_{420} play a significant role in formate and CO transformation. which are firstly transformed into CO_2 by F_{420}, and then CO_2 is reduced into CH_4 along with the action of co-enzyme M. Moreover, the co-enzyme M also plays an important role in acetate and carbinol transformation. Since VFA is the main product during anaerobic

digestion, most of the methane is produced through the acetate route (Appels, Assche, et al., 2011; Molino, Nanna, Ding, Bikson, & Braccio, 2013). According to Cai et al. (2016), in thermodynamics terms, the overall energy generated via acetoclastic methanogenesis (Pathway i) is the same as the energy generated via acetate oxidation and hydrogenotrophic methanogenesis based on anaerobic syntrophy (Pathway iv) (Figure 4).

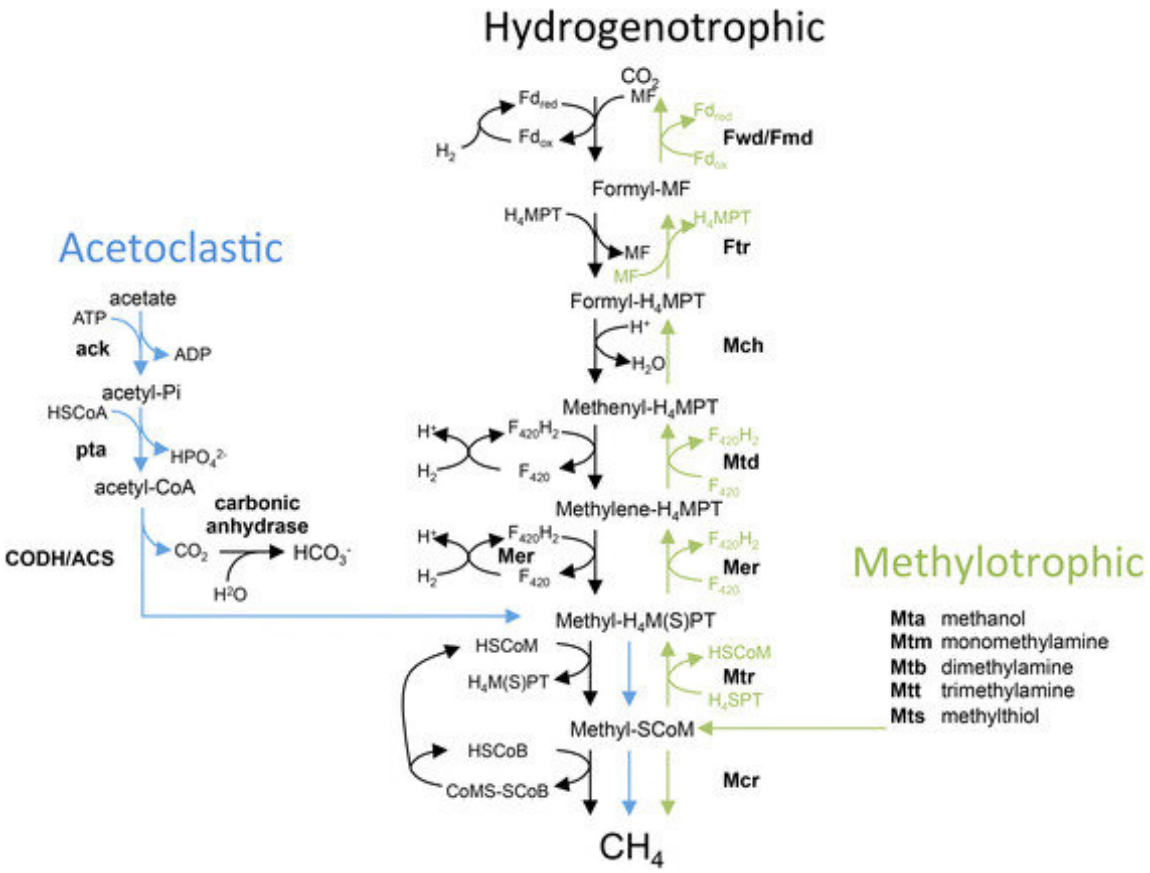

Figure 4. The three methanogenesis pathways (Lambie et al., 2015).

The difference is that in acetoclastic methanogenesis all energy goes to one type of microorganisms. In syntrophic acetate oxidation, the energy is shared by two different species. Based on energetics alone, acetoclastic methanogens should outcompete syntrophic acetate-oxidizing bacteria. Hydrogenotrophic methanogens are crucial for the electron flow in the AD process because of their ability to scavenge H_2/formate at low levels and promote syntrophic acetogenesis.

Because the degradation phases are all closely connected with each other, an imbalance between the bacterial and archaeal communities can cause a deterioration in reactor performance, and thus changes in the amount of methane produced (Akuzawa et al., 2011; Rastogi, Ranade, Yeole, Patole, & Shouche, 2008).

2.3.3. Microbiome

The degradation process is complex and depends on a balanced action of several microbial groups (Nielsen et al., 2007).However, the small amount of energy generation in AD forces the microorganisms into a very close and efficient cooperation. Syntrophy is a particularly mutualistic partnership in AD, defined as a thermodynamically interdependent life style where neither partner can operate without the other (Morris, Henneberger, Huber, & Moissl-Eichinger, 2013).The efficient and stable operation of methane fermentation relies on syntrophic relationships among a community of microbes, including fermenting bacteria, specialized acidogenic and acetogenic syntrophs, and methanogenic archaea with diverse and parallel pathways for substrate metabolism (Briones & Raskin, 2003).

The AD process begins with the microbial hydrolysis of proteins, fats, carbohydrates, and some other biodegradable polymers, releasing amino acids, fatty acids, and sugars. Hydrolytic bacteria are phylogenetically diverse but mostly fall into two phyla, *Bacteroidetes* and *Firmicutes* (Venkiteshwaran, Bocher, Maki, & Zitomer, 2016). Acidogenic bacteria then convert amino acids, fatty acids, and sugars into ammonia, VFA, CO_2, H_2, and alcohols. Most species of acidogenic bacteria belong to the phyla *Firmicutes*, *Bacteroidetes*, *Proteobacteria*, *Chloroflexi*, and *Actinobacteria*. The genera under these phyla have been commonly identified as *Clostridium* and *Bacillus*, *Bacteroides* and *Proteiniphilum*, *Desulfovibrio* and *Geobacter*, *Chloroflexus*, and *Mycobacterium* (Cai et al., 2016). While acetate, formate, H_2/CO_2, and methyl compounds can be directly utilized by methanogens, other compounds resulting from acidogenesis such as butyrate, propionate, lactate, and ethanol are further biodegraded by a group of syntrophic

acetogens into acetate, formate, and H_2/CO_2. Syntrophic acetogens generally include *Syntrophobacter*, *Pelotomaculum*, *Smithella*, *Syntrophus*, *Syntrophomonas*, and *Syntrophothermus*. The first three genera are typically involved in propionate degradation, whereas the others are commonly responsible for the oxidation of butyrate and other fatty acids (Venkiteshwaran et al., 2016). Syntrophic acetogenesis is thermodynamically unfavorable under standard conditions. The syntrophic partnership with methanogens, which maintain a low H_2 partial pressure (p_{H2}) and low formate and acetate concentrations, allows this process to occur (Stams & Plugge, 2009).

In the methanogenesis step, acetate, hydrogen, and carbon dioxide are converted into methane by methanogenic microorganisms, which are also classified as archaea composed of both gram-positive and gram-negative bacteria with a wide variety of shapes, e.g., coccoid and bacilli (Michael & Constantinos, 2006). Differences in environmental conditions as well as reactor operating conditions (pH, temperature, HRT, input material) have been reported to affect the composition of these communities (Burak Demirel & Scherer, 2008; W. Kim et al., 2015).

The most abundant genus of methanogens found in mesophilic anaerobic digesters was *Methanosarcina* (Cai et al., 2016), which are facultative acetoclastic methanogens that can also utilize H_2/CO_2 and C-1 compounds for methane production (Yuchen Liu & Whitman, 2008). *Methanosaeta* are obligate acetoclastic methanogens that are known to use only acetate or acetate plus electrons obtained via DIET (Venkiteshwaran et al., 2016). DIET raises the intriguing possibility that the organism gets additional energy with electrons transferred via DIET (Shrestha et al., 2013). *Methanoculleus*, *Methanospirillum*, *Methanoregula*, *Methanosphaerula*, *Methanobacterium*, *Methanobrevibacter*, and *Methanothermobacter* are the most commonly observed hydrogenotrophic methanogens in anaerobic digesters (Cai et al., 2016). DIET proceeds via electrically conductive pili or c-type cytochromes from anaerobic syntrophs to methanogens resulting in methane production from ethanol, as observed for

associations between *Geobacter* as electron producer and *Methanosaeta* or *Methanosarcina* as electron consumer (Rotaru et al., 2014).

2.3.4. Perturbations and stress on AD process

The growth rates and the sensitivity toward environmental changes differ widely between the different microbial groups. As a consequence, an unrestrained reactor operation can lead to disturbances in the balance between the different microbial groups, which might lead to reactor failure (Nielsen et al., 2007).

The reactor conditions can explain the community structures in functionally stable (LaPara, Nakatsu, Pantea, & Alleman, 2002) and unstable (Goberna, Insam, & Franke-Whittle, 2009) bioreactors. According to Allison and Martiny (2008), resistance, resilience and redundancy are the three main factors shown in the population dynamics. With resistance, a population maintains an abundance over time, with resilience a population rebounds following a disturbance, and with redundancy a disturbed population is replaced by a new population whose function makes the original population redundant (Niu, Takemura, Kubota, & Li, 2015). Normally, during a process imbalance, intermediates such as H_2, VFA and alcohols will accumulate, accompanied by fluctuations in gas production. In theory, this provides several parameters that can be used as indicators of process instability. However, the complexity of the process has made it difficult to find a simple and suitable control parameter reflecting the metabolic state of the entire process (Nielsen et al., 2007).

Reactor acidification through reactor overload is one of the most common reasons for process deterioration in anaerobic digesters (Akuzawa et al., 2011). This occurs because of a build-up of volatile fatty acids (VFA) which are produced by acidogenic and acetogenic bacteria and reflects a kinetic uncoupling between the acid producers and consumers (Ahring, Sandberg, & Angelidaki, 1995). Y. Wang, Zhang, Wang, and Meng (2009) reported for mesophilic conditions, acetic acid and butyric acid concentrations of 2400 and 1800 mg L^{-1}, respectively, resulted in no significant inhibition of the activity of methanogens, while a propionic acid

concentration of 900 mg L^{-1} resulted in significant inhibition of the methanogens. Opinions vary regarding which VFA is the best indicator for impending reactor failure, with different authors suggesting i-butyric, i-valeric, propionic acid, or the ratio of propionic/acetic acid as the most appropriate indicator (Boe, Dolin, & Middleton, 2006). Nonetheless, it does not appear to be possible to define VFA levels to indicate the state of an anaerobic process, as different systems have their own levels of VFA that can be considered 'normal' for the reactor, and conditions that cause instability in one reactor do not cause problems in another reactor (I. Angelidaki, Ellegaard, & Ahring, 1993).

2.3.5. Key parameters of AD

AD process, including the way in which microorganism interact within the system is governed by different key parameters such as temperature, VFA, pH, ammonia, nutrients, trace elements balance, OLR, HRT and pretreatment (Xu et al., 2018). It is therefore extremely important to maintain the key parameters within the appropriate range for long term operation of AD (C. Zhang et al., 2014). These key parameters are discussed below.

Temperature

Temperature is one of the most significant parameters influencing AD, because it not only influences the activity of enzymes and co-enzymes, but also influences the methane yield and digestate (effluent) quality (Appels, Assche, et al., 2011; Sánchez, Borja, Weiland, Travieso, & Martín, 2001). Generally, anaerobic bacteria can grow at psychrophilic (10–30 °C), mesophilic (30–40 °C) and thermophilic (50–60 °C) conditions (Kwietniewska & Tys, 2014). The performance of AD however increases with an increasing temperature (El-Mashad, Zeeman, van Loon, Bot, & Lettinga, 2004), the advantages of the thermophilic operation with its higher metabolic rates, higher specific growth rates, higher rates of the destruction of pathogens along with higher biogas production (J. K. Kim, Oh, Chun, & Kim, 2006). Moreover, the rates of organic nitrogen degradation and phosphorus assimilation also increased with temperature (Sánchez et al., 2001). According to Appels, Assche, et al. (2011), thermodynamics show that a higher

temperature is a benefit to endergonic reactions (e.g., the breakdown of propionate into acetate, CO_2, H_2), but not favorable to exergonic reactions such as hydrogenotrophic reactions and methanogenesis.

Furthermore, temperature could also affect the passive separation of solids which is demonstrated to be better under thermophilic than psychrophilic conditions (Kaparaju & Angelidaki, 2008). Although several advantages were observed under thermophilic condition, some disadvantages are worth considering since the thermophilic process is more sensitive to environmental changes than the mesophilic process (Ahn & Forster, 2002; M. Kim, Ahn, & Speece, 2002). Appels, Lauwers, et al. (2011) reported that process failure can be obtained when the rate of temperature changes exceeds 1 °C day^{-1} so, to maintain a stable digestion the changes in temperature should be less than 0.6 °C day^{-1}.

In general, the mesophilic process often is more stable compared to the thermophilic process (Appels et al., 2008). Many mesophilic bacteria cannot survive in the thermophilic ranges of temperature, whereas thermophilic bacteria can survive in mesophilic ranges of temperature, but their growth rate is slow (Gou C. et al., 2014).

VFA and pH

Volatile Fatty Acids (VFA) which mainly include acetic acid, propionic acid, butyric acid, and valeric acid, are the main intermediate products during AD of organic wastes (Cysneiros, Banks, Heaven, & Karatzas, 2012). Generally, VFA produced in the anaerobic process could be ultimately transformed into CH_4 and CO_2 by syntrophic acetogens and methanogenic archaea. Short chain VFA are not toxic themselves, however, VFA can accumulating high organic loading, resulting in the decrease of pH and even the failure of AD (Pham, Nam, Jeon, & Yoon, 2012). Among the four acids, acetic and propionic acids play a dominant role in biogas production (C. Zhang, Su, & Tan, 2013), and their concentrations could be used as indicators of the performance of AD or as an indicator of digestion imbalance (Buyukkamaci & Filibeli, 2004; Marchaim & Krause, 1993).

According to Q. Zhou, Jiang, Li, and Jiang (2016), the methanogens will not be able to metabolize the acetate produced by the acetogenic organisms until the abundance methanogenic organisms has increased sufficiently. This is especially true of feedstocks which are rapidly hydrolyzed. Previous research demonstrated that a propionic acid to acetic acid ratio exceeding 1.4 or the concentration of acetic acid exceeding 0.8 g L^{-1} lead to AD failure (Pullammanappallil et al., 2001).

VFA affect the pH which is also one of the most important parameters affecting AD. Anaerobic bacteria need different pH ranges for their growth, e.g., a comprehensive pH range of 4.0 – 8.5 is required by fermentative bacteria while a limited range of 6.5 – 7.2 is favorable for methanogens' growth (Ramírez-Sáenz, Zarate-Segura, Guerrero-Barajas, & García-Peña, 2009; Yin, Liu, Zhai, & Li, 2019).

The pH value is an important factor because it influences the proportion of ionized and non-ionized forms (excessive hydrogen sulfide, fatty acids, and ammonia are toxic in their non-ionized forms) (Ward, Hobbs, Holliman, & Jones, 2008). Previous reports pointed out that VFA could be significantly affected by pH of anaerobic digester: at low pH the main VFA are acetic and butyric acids, while acetic and propionic acid played a dominant role when pH was 8.0 (Horiuchi, Shimizu, Kanno, & Kobayashi, 1999). Moreover, both types and abundance of acid-producing bacteria could be controlled by pH (Horiuchi et al., 1999).

Propionate

Propionate is a common fermentation product during the degradation and fermentation of biomass to biogas. The presence of propionic acid tends to upset anaerobic digestion processes due to its toxicity among the VFA (Musa, Idrus, Che Man, & Norsyahariati, 2018). However, operational mismanagement (e.g., overloading) or inadequate substrate compositions (inhibitor substances, growth factor deficiencies) can hamper the process, leading to propionate accumulation, causing a decrease in pH and microbial inhibition (Karlsson et al., 2012; Nielsen

et al., 2007). Affecting digester efficiency and stability. Therefore, propionate degradation is a limiting factor of anaerobic fermentation (Ahlert et al., 2016).

The accumulation of propionate is especially challenging, due to the thermodynamic constraints on its degradation (Table 4). Its degradation into acetate and H_2/CO_2 (and then to CH_4) accounts for approximately 6 – 35 % of the total methanogenesis (Smith & McCarty, 1989). Under standard conditions, propionate degradation is an endergonic process (J. Li, Q. Ban, L. Zhang, & A. K. Jha, 2012; Thauer, Jungermann, & Decker, 1977).

Table 4. Reactions involved in syntrophic propionate metabolism (Thauer et al., 1977)

Reaction	ΔG°' (kJ mol^{-1})
Proton reducing bacteria	
$Propionate^- + 2H_2O \rightarrow acetate^- + CO_2 + 3H_2$	+ 76.0
$Propionate^- + 2H_2O + 2CO_2 \rightarrow acetate^- + 3HCOO^- + 3H^+$	+ 65.3
Methanogens	
$4H_2 + CO_2 \rightarrow CH_4 + 2H_2O$	- 131.7
$4HCOO^- + 4H^+ \rightarrow CH_4 + 3CO_2 + 2H_2O$	- 144.5
$Acetate + H^+ \rightarrow CH_4 + CO_2$	- 36.0

Concentration of 1 M, pH 7.0 and T = 25 °C

The oxidation of propionate depends on stable hydrogen consumption (or respective electrons) by associated processes (Stams & Plugge, 2009) so, can be oxidized only if a syntrophic association occurs between propionate-oxidizing bacteria and H_2-consuming methanogens (McInerney, Sieber, & Gunsalus, 2009).

According to Bernhard Schink and Stams (2013) methanogenic environments depend on hydrogen-consuming microorganisms, which dispose of hydrogen in syntrophic cooperation. Hydrogenotrophic methanogens especially are often considered as optimal partner organisms for syntrophic propionate-degrading bacteria (SPOB). However, SPOB play an imperative role in the metabolic network (Dong, Plugge, & Stams, 1994; Leng et al., 2018).

The oxidation of propionate to acetate and methane by syntrophic hydrogenotrophic methanogens is an exergonic process, even under standard conditions ($\Delta G^{\circ\prime} = -25.2$ kJ per reaction, Equation 1) (Stams, 1994).

$$Propionate^{-} + 1.75H_2O \rightarrow Acetate^{-} + 1.75CH_4 + 1.25HCO_3^{-} + 0.25H^{+} \cdots\cdots\cdots \text{Equation 1}$$

There are essentially two known pathways that are responsible for propionate metabolism. Most of the syntrophic propionate oxidation is accomplished via the randomizing methylmalonyl-CoA (MMC) pathway, which is also referred to as the classical and common pathway (Kosaka et al., 2006). So far, at least ten species of SPOB have been identified, isolated and metabolically analyzed belong to the phyla of gram-positive *Firmicutes* (*Desulfotomaculum*, *Pelotomaculum*) and gram-negative *Deltaproteobacteria* (*Smithella*, *Syntrophobacter*). (J. Li et al., 2012).

Yitai Liu, Balkwill, Aldrich, Drake, and Boone (1999) identified a propionate-degrading syntroph of the genus *Smithella*. Methanogens that are syntrophycally related to *Smithella* produce less methane and more acetate than those that work in syntrophy with *Syntrophobacter* (uses MMC pathway). It differed greatly in the substrate range and catabolic products, forming small amounts of butyrate during propionate degradation. *Smithella* spp. utilize propionate in a non-randomizing pathway in which propionate is dismutated to acetate and butyrate via a six-carbon intermediate before being degraded via β-oxidation. This novel dismutation pathway is also known as the *Smithella* pathway (de Bok, Stams, Dijkema, & Boone, 2001).

In 2013, Dolfing concluded that the *Smithella* pathway is less sensitive to H_2 than MMC pathway, and under a significant range of conditions (Table 5) propionate oxidation via the *Smithella* pathway is exergonic whereas the MMC pathway is endergonic, Figure 5 shows the Gibbs free energy change over a wide range of p_{H2} for both processes. *Smithella* species have been detected in various anoxic environments, suggesting that this pathway is widespread (W. Liu et al., 2016).

Table 5. The window of opportunity corresponding to estimated range of H2 concentration.

Pathway	Hydrogen concentration at 25 °C (Pa)	Hydrogen concentration at 55 °C (Pa)
Smithella pathway	0.002 – 500.34[a]	1.8 – 580[b]
MMC pathway	0.002 – 1.82[a]	1.8 – 7.3[b]

Conditions: [acetate] = [propionate] = 1 mM; [bicarbonate] = 50 mM; p_{CH4} = 1 atm; pH = 7.
a Leng et al. (2018)
b Qiao et al. (2016)

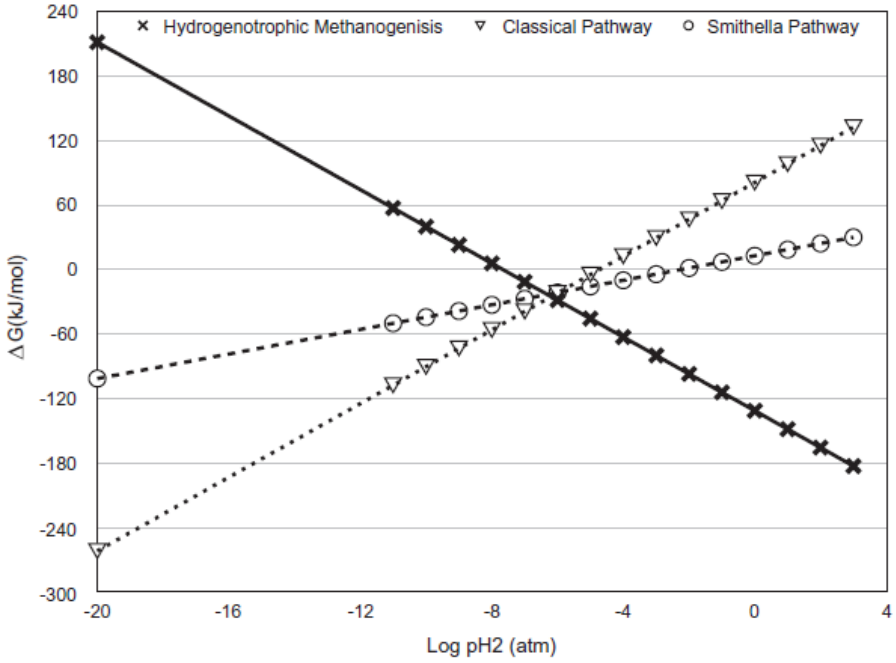

Figure 5. ΔG° values of hydrogenotrophic methanogenesis and propionate degradation, via classical or MMC pathway and via *Smithella* pathway at 25 °C.

C/N ratio

The performance of AD is significantly affected by C/N ratio (Zeshan K. O. P. & Visvanathan, 2012). An optimum C/N ratio is needed for AD because an appropriate nutrient balance is required by anaerobic bacteria for their growth as well as for maintaining a stable environment. According to Puyuelo, Ponsá, Gea, and Sánchez (2011) generally, a C/N ratio range of 20–30 is considered to be the optimum condition for AD. Anaerobic co-digestion (AcoD) of three organic substrates (dairy, chicken manure and wheat straw) was performed by Wang X.,

Yang G., Feng Y., Ren G., and X. (2012), explained that the maximum methane potential was achieved at the C/N ratio of 27.2, with stable pH and low concentrations of TAN and FA. However, recent studies pointed out that digestion proceeded well at low C/N ratios (15–20) (C. Zhang et al., 2014). Zhong W. et al. (2013) also found that the optimal C/N ratio for the co-digestion was 20. However, Sharma et al. (2013) reported a low C/N (7.39) for the co-digestion of poultry litter with thin stillage in thermophilic conditions. The above findings indicated that the optimum C/N ratio for AD depends on both feedstock and the inoculum. No matter what the C/N ratio is, an appropriate balance between C and N is required for effective digestion. An optimum carbon content had a positive effect on avoiding excessive ammonia inhibition (S. Park & Li, 2012).

Ammonia

Ammonia is formed during the biodegradation process of protein or other nitrogen-rich organic substrates, and mainly exists in the form of NH_4^+ and NH_3 (Yenigün & Demirel, 2013). It could be utilized as an essential nutrient for bacterial growth though it can also be toxic to microbes in the presence of high concentrations (Walker M., Iyer K., Heaven, & Banks, 2011). It is well known that ammonia plays an important role in balancing the C/N ratio which could affect the performance of AD significantly (Wang X. et al., 2012). Many previous papers have reported that ammonia could enhance the buffer capacity of AD, because VFA formed during digestion process could be neutralized by NH_3 (H. Wang, Fotidis, & Angelidaki, 2015; Q. Wang, Peng, & Su, 2013; Wang X. et al., 2012; C. Zhang et al., 2013).

VFA accumulation will be observed when the OLR increases, leading to a risk of digestion failure. The ammonia however could react with these VFA, avoiding the inhibition from VFA and allowing enough VFA for biogas production. Despite its buffer capacity, NH_3 was proven to be an inhibitor to many bacteria at high concentrations (Y. Chen et al., 2008; Fernandes T.V., Keesman K.J., Zeeman G., & Van Lier, 2012), since FA can diffuse through the cell membrane and further

hinder cell functioning through disrupting the potassium and proton balance inside the cell (Kayhanian, 1999).

Many previous reports pointed out that sensitivity to ammonia of acetoclastic methanogens, which convert acetate into CH_4 and CO_2, is much higher than the hydrogenotrophic methanogens (Banks, Zhang, Jiang, & Heaven, 2012; A. Schnürer & Nordberg, 2008), and thus more likely to cease methane production, due to the accumulation of acetate up to inhibitory levels, thus contributing further to a negative feedback mechanism that eventually leads to complete reactor failure (H. Wang et al., 2015). Under such high concentrations of ammonia and/or acetate, the so-called syntrophic acetate-oxidizing bacteria (SAOB) are able to reverse the homoacetogenic pathway and convert acetate to CO_2 and H_2 (Anna Schnürer, Zellner, & Svensson, 1999). This process is thermodynamically favorable through the concomitant consumption of H_2 by hydrogenotrophic archaea and, therefore, the syntrophic association between SAOB and methanogenic archaea prevents the inhibition of methanogenesis during the AD of nitrogen-rich substrates (Ruiz-Sánchez et al., 2019).

Among methanogens, *Methanosaeta concilii* and *Methanosarcina barkeri* showed higher sensitivity to increasing free ammonia concentrations (Sprott & Patel, 1986). The FA concentration increases with increasing temperature and pH value. For example, for the condition at pH 7 and 35 °C, less than 1% of the TAN is in the form of FA. However, at the same temperature, the FA increases to 10% at pH 8, as shown in Fig. 4 (Fernandes T.V. et al., 2012).

Yenigün and Demirel (2013) stated that digestion failure could be caused by the ammonia concentration of 1.7–1.8 g L^{-1} (free NH_4^+) and specifically illustrated that the inhibition was due to FA rather than the ammonium ions. Besides, J. L. Chen et al. (2014) summarized the critical concentrations and pointed out that a 50% reduction in the methane yield will occur as a result of the ammonia concentration of 1.7 – 14 g L^{-1}. The wide range of ammonia concentration for causing inhibition depends on the differences in feedstock, inoculums, environmental conditions (e.g., temperature and pH) (Yenigün & Demirel, 2013) and acclimatization periods

(Y. Chen et al., 2008). High concentration of ammonia could not only lead to lower biogas production and even digestion failure, but also result in ammonia emission from effluent (J. Park, Jin, Lim, Park, & Lee, 2010).

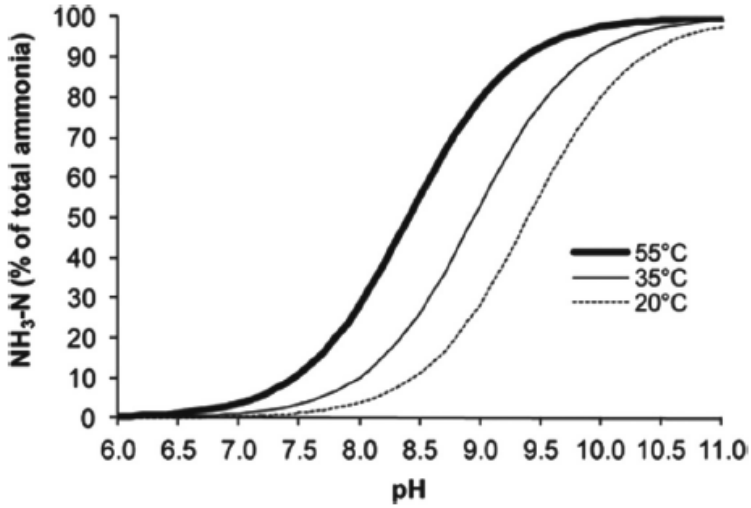

Figure 6. Free ammonia and ammonium percentages present in solution at 20, 35 and 55 °C (Fernandes T.V. et al., 2012).

Sulfur

Refers to a wide variety of compounds, the most common forms in sludge are as organic sulfur, sulfate and sulfide. H_2S can be derived from the reduction of sulfates, the hydrolysis of organic sulfur, and the direct conversion of sulfides during anaerobic digestion (Yan et al., 2018) as shown in Figure 7. Sulfur (in form of sulfate or sulfide) may become an inhibitor in the anaerobic digestion process and has been considered as a key factor affecting the competition between sulfate reducing bacteria (SRB) and methane producing bacteria (MPB).

On the other hand, H_2S produced in the biogas can cause many problems, such as inhibition of anaerobic digestion process because it is toxic as it diffuses into the cytoplasm through cell membranes and may form disulfide cross-links between polypeptide chains and denature the proteins (Kwietniewska & Tys, 2014; Yan et al., 2018), decrease of biogas production and methane content (Q. Zhou et al., 2016). Further, it was also observed that toxicity of sulfide increases with pH (Y. Chen et al., 2008).

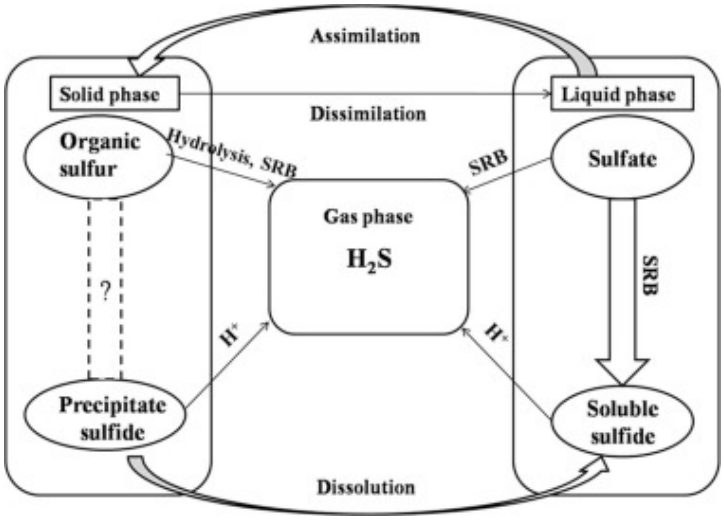

Figure 7.Conversion of sulfur in sludge during anaerobic digestion (Yan et al., 2018).

Metal elements

AD process also requires nutrient elements (C, H, O, N), metal elements including light metal ions (Na, K, Mg, Ca, Al) and heavy metal ions (Cr, Co, Cu, Zn, Ni, etc.) (Jin P., Bhattacharya S.K., Williama C.J., & H., 1998), because these cations play an important role in enzyme synthesis as well as maintaining enzyme activities (Schattauer A., Abdoun E., Weiland P., Plohl M., & M., 2011). However, inhibition could be caused by both of light and heavy metal elements when their concentrations are too high (Appels, Assche, et al., 2011). Y. Chen et al. (2008) reported that the optimum concentration of sodium for mesophilic hydrogenotrophic methanogens was 350 mg L^{-1}, and at the concentration of less 400 mg L^{-1} potassium could enhance the performance of both of thermophilic and mesophilic AD. Kugelman and McCarty (1965) reported a toxicity threshold concentration of 200 mg L^{-1} for calcium and a moderate inhibition occurred at the concentration of 2500–4000 mg L^{-1}, a strong inhibition occurred at a concentration of 8000 mg L^{-1}.

Heavy metal elements could cause inhibition to anaerobic organisms due to the disruption of the enzyme function and structure (J. L. Chen et al., 2014). Many previous findings have pointed out that the inhibition degree depends upon many

factors, such as the total metal concentration, chemical forms of the metals, pH, and redox potential (B. Demirel & Scherer, 2011; Lin & Chen, 1999; Zayed & Winter, 2000). On the other hand, it was reported that selenium is essential for both propionate oxidation and syntrophic hydrogenotrophic methanogenesis. Selenium supplementation allows digestion to proceed at substantially higher OLR (Banks et al., 2012).

Organic Load Rate (OLR)

OLR represents the amount of volatile solid (VS) fed into a digester per day under continuous feeding. With increasing OLR, the biogas yield increases to an extent, but the equilibrium and productivity of the digestion process can also be greatly disturbed (C. Zhang et al., 2014). Braz, Fernandez-Gonzalez, Lema, and Carballa (2018) report that adding a large volume of new material daily may result in changes in the digester's environment and temporarily inhibits bacterial activity during the early stages of fermentation. This bacterial inhibition occurs due to an extremely high OLR leading to higher hydrolysis/acidogenesis bacterial activity than methanogenesis bacterial activity and thus increases VFA production, which eventually leads to an irreversible acidification (Gou C. et al., 2014). Thereafter, the pH of the digester decreases, and the hydrolysis process is inhibited such that the restricted methanogenesis are not able to convert as much VFA to methane. Moreover, the structure of bacterial communities can vary with OLR.

For instance, Ferguson R.M.W., Coulon F., and R. (2016) in their study of the effects of sequential OLR shocks on sludge digesters observed that the microbiome became mainly enriched in *Firmicutes* after the increase in OLR. In contrast, Regueiro, Lema, and Carballa (2015), who used biodiesel waste to induce an OLR shock (from 2 to 10 g COD L^{-1} day^{-1}) in pig-manure digesters, observed that the disturbance caused an increase in *Bacteroidetes* and *Actinobacteria* phyla. Likewise, the archaeal community could be altered during organic overloading events. High concentrations of VFA activate the hydrogenotrophic methanogenesis pathway (Wirth R. et al., 2012) with the consequent community enrichment in hydrogenotrophic Archaea.

Retention time

The retention time is the time required to complete the degradation of organic matter. It is associated with the microbial growth rate and depends on the process temperature, OLR and substrate composition. Moreover, it is an important parameter because it determines the amount of organic matter and volatile solids than can be fed into the digester (Odey et al., 2016).Two significant types of retention time are herein discussed: solid retention time (SRT) or sludge age, which is defined as the length of of time a particulate material spends in a digester, and hydraulic retention time (HRT) which is defined as the length of the time the liquid and dissolve material remain in the reactor (Ekama & Wentzel, 2008). HRT is an important operational parameter for the anaerobic reactors which can affect the conversion of VS into biogas (Dareioti & Kornaros, 2014). Obtaining an effective HRT depends on the substrate composition and OLR; typically, an average retention time of 15 – 30 days is required to treat waste under mesophilic conditions (Mao et al., 2015). Decreasing the HRT usually leads to VFA accumulation, whereas, a longer than optimal HRT results in insufficient utilization of digester components. For algal biomass, an HRT below 10 days results in low methane productivity (Kwietniewska & Tys, 2014).

Biodegradability and Bioaccessibility

Biodegradation is the process of chemical decomposition performed by microorganisms; therefore, biodegradability is the property of a material to safely biologically degrade in the system by microorganisms in anaerobic conditions (Irini Angelidaki & Sanders, 2004). There are different factors (chemical, physical and physiological) in the environment that affect the biodegradation of organic compounds, such as concentration, pH, temperature, moisture, bioavailability and bioaccessibility (Hagos et al., 2017). Due to the complex structure of organic materials, bioavailability and bioaccessibility are mainly defined for the organic materials by their accessibility to degradation by microorganisms, depending on the digestion time, hydrolytic activity and the pre-treatment applied to the organic materials (Mottet et al., 2013). In AcoD systems, the disintegration and hydrolysis

steps of the biomass are the limiting and responsible factors for biodegradability, bioaccessibility, and degradation rates (Hagos et al., 2017). Traditionally, characterization of both factors can be obtained from the batch test using BMP experiments.

Inoculum/substrate ratio (ISR)

The ISR is defined as a ratio of active inoculum along with feedstock (Mehariya et al., 2018). It is a key parameter that affects the efficiency of anaerobic degradation and more importantly the accuracy of an AD experiment. Previous studies have shown that increasing ISR positively affected the ultimate practical methane yield (Dechrugsa, Kantachote, & Chaiprapat, 2013).

According to Zeng, Yuan, Shi, and Qiu (2010), there is a direct relationship between inoculum / substrate ratio and methane yield. If the inoculum / substrate ratio is low, the methane yield is low even when biogas production is high (Figure 8). The amount of substrate contributed substantially in increasing the percentaje of methane in the biogas (Raposo, Banks, Siegert, Heaven, & Borja, 2006).

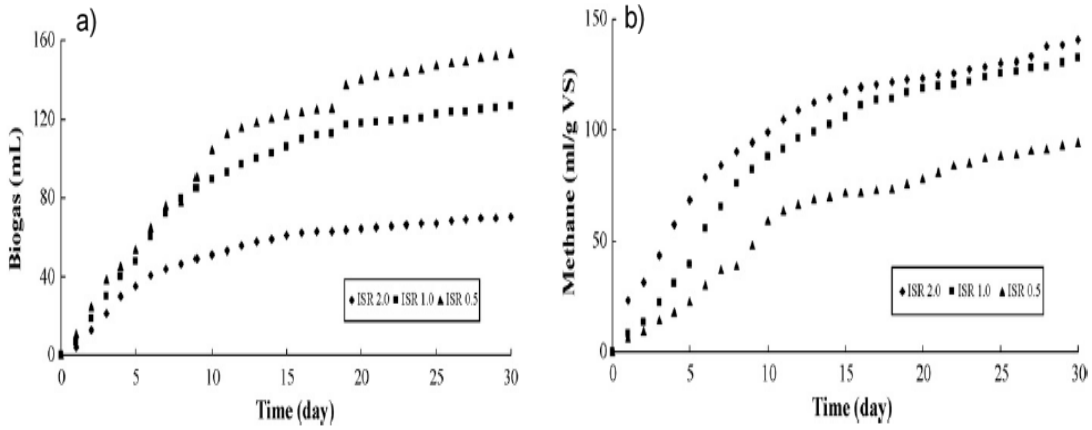

Figure 8. a) Variation of the cumulative biogas production and b) variation of the cumulative methane yield with digestion time at different ISRs (Zeng et al., 2010).

2.4. Anaerobic co-digestion (AcoD)

AcoD is an energy-efficient process that can improve fermentation performance by adding a secondary substrate that supplies nutrients that initial substrate is

lacking or with complementary characteristics (Hagos et al., 2017; S. Park & Li, 2012). AcoD can therefore lead to more stable digestion and enhanced gas yields. For example, Shah et al. (2015) found that AcoD can enhance biogas production from 25% to 400% over the mono-digestion of the same substrates, which improves economics due to higher methane production. In addition, AcoD can give the following important benefits: (i) improve the process stabilization, (ii) dilution of inhibitory and/or toxic compounds, (iii) alleviate imbalance of nutrients, (iv) improve of the required moisture contents in the digester feed, (v), reduce the emission of greenhouse gase, (vi) synergistic effects of microorganisms, (vii) adjustment of the carbon-to-nitrogen (C/N) ratio, (viii) preventing inhibition and, (ix) increasing the load of biodegradable organic matter (Shah et al., 2015; Sharma et al., 2013).

2.5. Analysis of AD system

Elucidating the relationship between microbial diversity and functions in microbial communities has been a challenge due to the different bioreactor designs, operational parameters and raw materials used in AD. To understand and improve the AD process, it has been necessary to combine different analyzes, some to understand the efficiency of AD (from an operational scheme), and others to know how the microbial structure and its interactions change during the process.

2.5.1. BMP

The BMP experiments have been broadly used to determine the anaerobic biodegradability, potential efficiency, and concentration of organics in different feedstocks that can be converted by anaerobic digestion to methane (Labatut, Angenent, & Scott, 2011). According to Raposo et al. (2011) BMP experiments can be used to determine: (i) the amount of organics in the substrates that can be converted to methane (biogas) in a given specific time, (ii) remaining organic material for further management, (iii) the amount of non-biodegradable remain after treatment and (iv) to assess the potential efficiency of the process for a given combination of substrates. It can also give essential information to researchers to

determine the most efficient way of mixing substrates for co-digestion process (Hagos et al., 2017). As the application and importance of AcoD process increase, it is necessary to find the mechanism to assess the biodegradability and bioavailability of substrates and the biogas production performance. In this case, BMP experiments can give relevant information to estimate the degradation related parameters and hydrolysis rates (P. Jensen, Ge, & Batstone, 2011; Mou, Scheutz, & Kjeldsen, 2014).

The BMP experiments have been performed to characterizing the physicochemical parameters at the mesophilic temperature in co-digestion with different mixing ratios, and in the single substrate systems as well (Cabbai, Ballico, Aneggi, & Goi, 2013). The information given by BMP tests is valuable for characterizing and evaluating the biodegradability and potential efficiency substrates, and for optimal design and performance of AD and AcoD process. In addition, BMP tests can provide a key database of AcoD process parameters to advance the modeling and utilizing different biomasses in AD and help to avoid the possible causes of synergism observed in co-digestion mixtures (Astals, Batstone, Mata-Alvarez, & Jensen, 2014). The BMP tests are useful method to determine the suitability of substrates for the AcoD process of biogas production (Cabbai et al., 2013; Lisboa & Lansing, 2013).

2.5.2. Metagenome analysis

Genome sequencing analysis can identify the keystone species and their interactions with other taxa. It helps to understand how synergistic biochemical reactions of AD-related microbiomes are affected by the different operational parameters. Nucleic acid–based molecular methods have revolutionized environmental biotechnology research by making it possible to study microbial communities without culturing (Kumaraswamy et al., 2014). DNA fingerprinting methods such as denaturing gradient gel electrophoresis (DGGE) have been employed widely to study AD systems. However, DNA fingerprinting and other early molecular methods suffer from limited coverage and depth. This limitation conflicts with the high diversity and importance of low abundance populations

within the AD microbiome, making it difficult to accurately monitor the activity of rare, but functionally important microorganisms (Amha et al., 2018). Thus, sequencing-based approaches using pyrosequencing, Illumina sequencing, or other high-throughput platforms are now widely used to study AD systems. Few years before, the microbial identification in AD systems had traditionally been accomplished by the construction of 16S rRNA gene clone libraries followed by Sanger sequencing (Rivière et al., 2009), which in recent years has been replaced by high-throughput next-generation sequencing of 16S rRNA gene amplicons (Werner et al., 2011). However, both these methods focus on taxonomic identification, and the metabolic pathways present can be determined only indirectly (Cai et al., 2016).

Recently, shotgun metagenomic sequencing, which directly sequences the extracted DNA, can provide more detailed information on the identity of the microbes and their metabolisms as well as other biological information including novel genes (Ferrer, Martínez-Abarca, & Golyshin, 2005). In addition to these advantages, metagenomic sequencing is gaining importance in the study of microbial communities because the decreasing cost and increasing sequencing depth have enabled high-resolution analysis of complex environmental samples, such as soils (Fierer et al., 2012), seawater (Tang, Liu, Jiao, Zhang, & Chen, 2013b), human gut (Qin et al., 2010), and freshwater (Iliev et al., 2017).

Metagenomics was first applied to AD in 2008 with the analysis of a German full-scale biogas plant treating farm waste (Schlüter et al., 2008). Several further studies have since examined AD metagenomes, with the focus mainly on taxonomy and gene-centric functional analyses (De Vrieze, Pinto, Sloan, & Ijaz, 2018; Wandera et al., 2019). Recently, metagenomic analysis has shifted toward reconstructing important metabolic pathways and genomes present in AD systems (Bedoya, Coltell, Cabarcas, & Alzate, 2019; Zhu et al., 2019).

In addition, using advanced molecular tools (for example, network analysis), we now know that syntrophic acetate oxidation (SAO), a thermodynamically unfavorable reaction (De Vrieze & Verstraete, 2016), is possible by more diverse

microbial populations than originally assumed (Lee et al., 2015; Treu, Kougias, Campanaro, Bassani, & Angelidaki, 2016; Werner et al., 2014). However, we continue to struggle with connecting community structure and function in AD systems (Carballa, Regueiro, & Lema, 2015) and have not yet unraveled the 'black-box' microbial ecology of AD (Nobu et al., 2015).

2.5.3. Network analysis

Microorganisms coexisting in nature forming complex ecological interactions webs, resulting in collaboration (positive impact), competition (negative impact) or neutral interactions between individual community members (Embree, Liu, Al-Bassam, & Zengler, 2015; Faust & Raes, 2012). Advances in microbial ecology have revealed high levels of species diversity and complexity in most communities (Gans, Wolinsky, & Dunbar, 2005; Khan et al., 2019; Wu et al., 2016). The possible combinations of positive, negative and neutral outcomes for two interaction partners allow the classification of various interaction types as is shown in Figure 9 (Faust & Raes, 2012). For example, for mutualism are certain cases of cross-feeding (also known as syntrophy), in which two species exchange metabolic products to the benefit of both (Woyke et al., 2006). Amensalism — in which one partner is harmed without any advantage to the other — occurs, for example, when metabolic by-products of a microbial species alter the environment to the detriment of other microorganisms (for example, lactobacilli lowering the pH of the surrounding environment). Detecting and investigating these various types of interactions in microbial ecosystems is far from straight-forward (Raes, Foerstner, & Bork, 2007).

Novel approaches towards the reconstruction of ecosystem-wide association networks can open the way towards global models of ecosystem dynamics. Ultimately, such models will be able to predict the outcome of community alterations and the effects of perturbations, and they could, in the long run, help with the engineering of complex microbial communities.

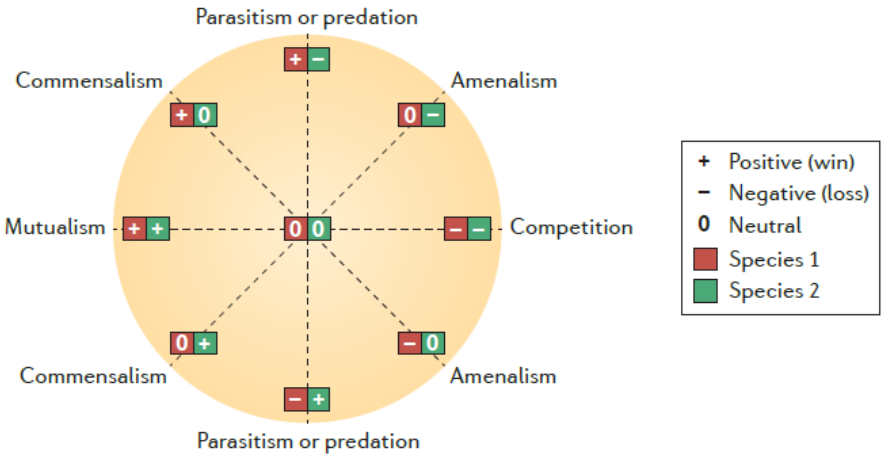

Figure 9. Summary of ecological interactions between members of different species (Faust & Raes, 2012).

Predicting relationships from this principle is straightforward: when two species (or any taxonomically relevant units) co-occur or show a similar abundance pattern over multiple samples, a positive relationship is assumed; when they show mutual exclusion (as in the checkerboard pattern) or anticorrelation, a negative one is predicted. However, interpretation of the ecological relevance of these relationships is far from easy. For instance, a positive relationship can be due to cross-feeding, co-aggregation in biofilms, co-colonization, niche overlap or other reasons, whereas a negative relationship may result from amensalism, a prey–predator relationship, competition, and so on. In addition, relationships can be time-lagged (for example, if one species increases its abundance at a certain moment, another species might only disappear at a later time) (Faust & Raes, 2012).

Network inference techniques are widely applied in genomics (Szklarczyk et al., 2010) and are starting to be adopted in ecology as well (Milns, Beale, & Smith, 2010). Here, we distinguish between two groups of network inference methods: those that can predict relationships between two species (namely, pairwise relationships) and those that can predict more complex ones.

Pairwise relationships: similarity-based network inference assesses the co-occurrence and/or mutual exclusion pattern of two species over multiple samples using a measure that quantifies the similarity of two species distributions. In a second step, the significance of the similarity score is assessed. After assessing all possible combinations of species in a given abundance data set, all significant pairwise relationships are then combined to construct a network (Figure 10) (Hecker, Lambeck, Toepfer, van Someren, & Guthke, 2009).

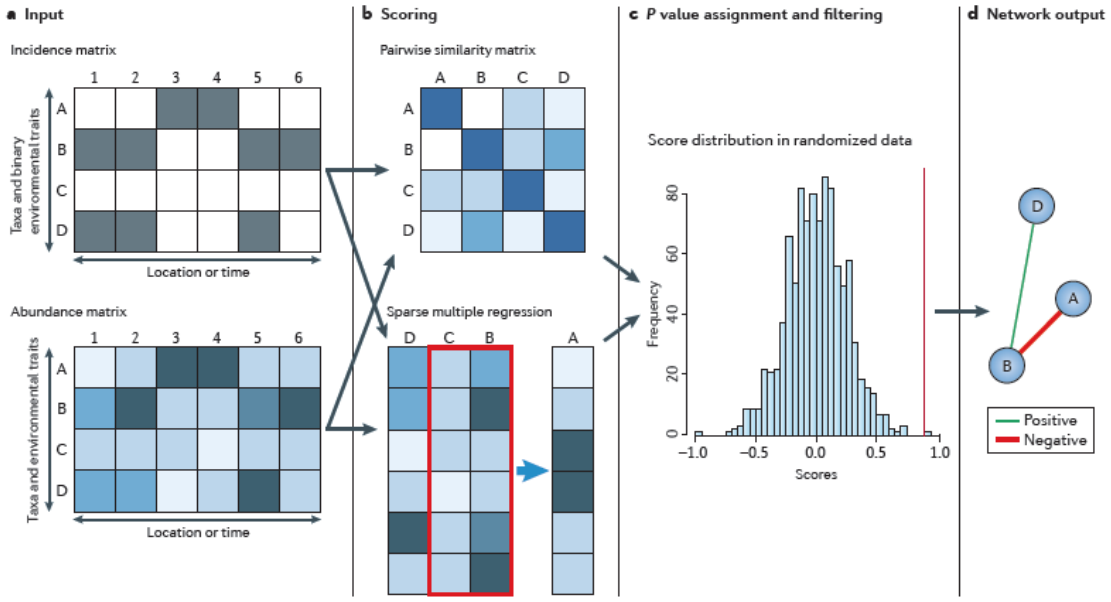

Figure 10. Principle of similarity- and regression-based network inference. The goal of network inference is to identify combinations of microorganisms that show significant co-presence or mutual exclusion patterns across samples and to combine them into a network. a) Network inference starts from an incidence or an abundance matrix, both of which store observations across different samples, locations or time points. b) Pairwise scores between taxa are then computed using a suitable similarity or distance measure (Faust & Raes, 2012).

Complex relationships: regression- and rule-based networks. Pairwise relationships do not capture more complex forms of ecological interactions, in which one species depends on (or is influenced by) multiple other species. A complex relationship that is inferred through multiple regression or association rule mining can be represented in the resulting network as an edge that connects more than two nodes in a directed way to point from the independent taxa to the dependent taxon (Chasman, Fotuhi Siahpirani, & Roy, 2016). Networks with such edges are formally known as directed hypergraphs. displays a network inferred

from a similarity-based approach, in which pairwise relationships are represented by edges connecting two nodes, whereas FIG. 3b gives an example of a directed hypergraph that results from association rule mining in a global microbial presence–absence data set and that visualizes complex relationships with hyper-edges connecting up to three nodes (Shaw, Liu, Weng, Chou, & Wang, 2017).

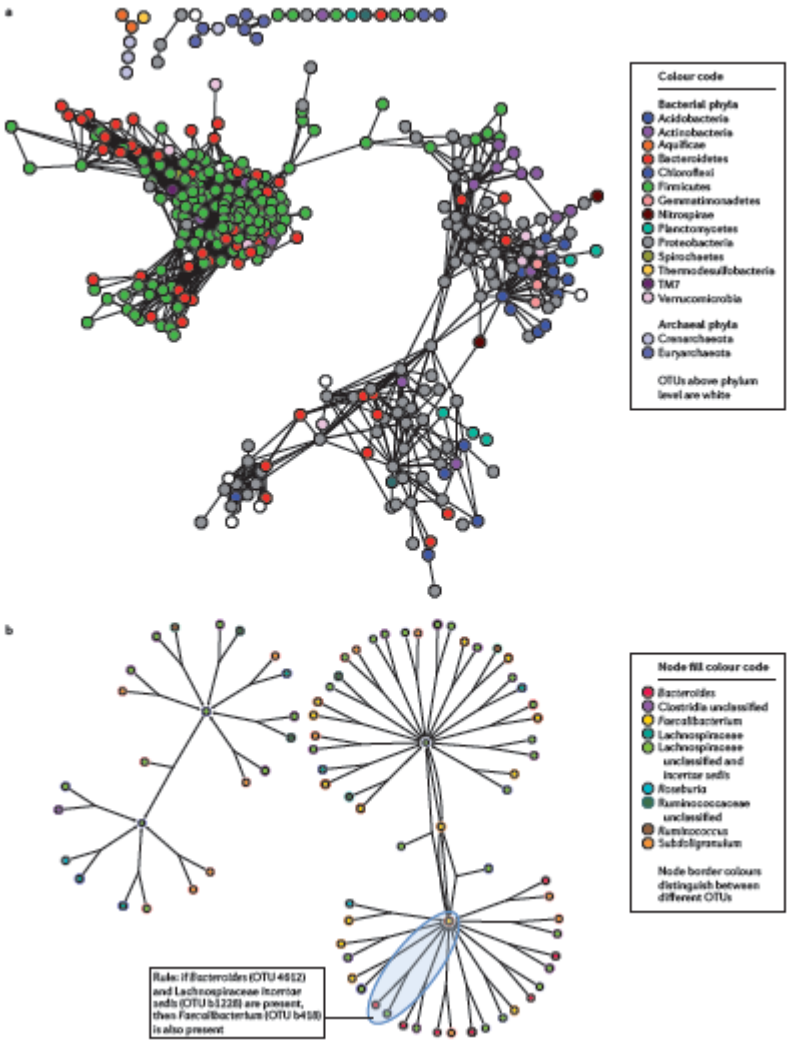

Figure 11. Examples for the prediction of pairwise versus complex relationships. Pairwise (a) and complex relationships (b) were inferred from a global microbial operational taxonomic unit (OTU) presence–absence data set (Shaw et al., 2017). a) Each node represents an OTU, and each edge represents a significant pairwise association between them. The edge thickness increases with significance. b) This network summarizes association rules mined with the a priori algorithm and filtered with the multiple testing correction (Faust & Raes, 2012)

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3. ANAEROBIC DIGESTION PROCESS OF CHICKEN LITTER: CONCEPTS, CHALLENGES AND NEW METHODS OF ANALYSIS

3.1. Abstract

An alternative to fossil fuel use is the production of methane through anaerobic digestion of chicken litter, which constitutes a source of biomass rich in nutrients, such as nitrogen, phosphorus and potassium. However, the main problem with its uses in methane production is the effect of the accumulation of ammonia and volatile fatty acids, which have a detrimental impact on microbial community specifically, on methanogenic microorganisms. In order to know in depth these changes in the microbial community and elucidating their possible effect on metabolism and inter- and intra- species relations, new technologies have been developed in recent years. The study of microbial communities has been revolutionized with the development of high-throughput sequencing technologies. The present review aims to present an overview of the process of anaerobic digestion of chicken litter and its challenges, as well as the new technologies and tools that are used to know the taxonomic and functional diversity of the system.

Keywords: Anaerobic digestion, chicken litter, high-throughput sequencing, Metagenomic analysis, propionate

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Thesis of Ph.D. in Ciencias Agroalimentarias, Doctorado en Ciencias Agroalimentarias, Departamento de Ingenieria Agroindustrial, Universidad Autonoma Chapingo
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3.2. Anaerobic digestion (AD) as an alternative for chicken litter treatment

Due to human population increase, chicken meat consumption increased around 38.5% (from 14.8 kg to 24.1 kg per capita) from 2000 to 2018. In the period of 2000 - 2014, global egg production increased by 36.5% or an average of 2.8% per year (Association of Poultry Processors and Poultry Trade in the EU countries, 2018). As a result, large volumes of chicken litter are accumulated in concentrated areas, causing greenhouse gas emissions of 6.93, 145.5 and 0.570 gigagrams of CH_4, CO_2eq and N_2O, per animal, respectively (FAOSTAT, 2016). The sustainable operation of such large production units is only achieved when chicken manure is adequately reused (Fuchs, Wang, Gabauer, Ortner, & Li, 2018). Since the organic matter in CL is highly biodegradable, an ecofriendly treatment alternative is anaerobic digestion (AD) (Fuchs et al., 2018), which in addition to minimizing waste, offers additional benefit to allow the recovery of the caloric value through biogas generation (Wandera et al., 2018). CL has high nitrogen content due to uric acid and protein, both of which are degraded to ammonia. The TAN (Total ammonia) contents includes ammonium (NH_4^+) and ammonia (NH_3) often referred as free ammonia (FA) (Yenigün & Demirel, 2013). The FA causes disturbances in AD due to its independence on pH, temperature and TAN. FA is known to be a significant cause of inhibition inactivating enzymes and affecting cellular transport of nutrients (Bi et al., 2020). In addition, FA can diffuse across the cell membrane and further hinder cell functions through disrupting the potassium and proton balance inside the cell (Kayhanian, 1999). Because of that the key to successful chicken manure AD and its main challenge, is to overcome ammonia inhibition.

Different studies on anaerobic digestion have been carried out in order to minimize process instability and increase methane yield, focusing mainly on the type of technology, operational factors that could affect efficiency, substrate, etc. Specifically, for the utilization of CL a huge variety of options, including dilution, change in process parameters (i.e., pH or temperature) (Niu, Qiao, Qiang, Hojo, & Li, 2013), biomass adaptation (Huber et al., 2018), bioaugmentation (Ioannis A.

Fotidis et al., 2014), co-digestion (Meneses-Reyes et al., 2018) and use of additives (e.g., zeolites or trace elements) (Kuttner, Weißböck, Leitner, & Jäger, 2015), have been under investigation. More recently, technical options for N removal have been under intensive investigation. In this context, significant technological progress has been achieved to overcome restrictions associated with the use of chicken manure as a substrate for biogas production (Fuchs et al., 2018).

AD is a robust microbial conversion process (Surendra et al., 2014), which can reduce organic pollution while potentially offsetting the use of fossil fuels. Biogas, which is generally referred to as a type of bioenergy, is a gas obtained from the degradation of organic matter carried out by microorganisms under anaerobic conditions (Mao et al., 2015; Scarlat et al., 2018). Raw biogas typically consists of CH_4 (50–75%), CO_2 (25–50%), and smaller amounts of N_2 (2–8%). Trace levels of hydrogen, sulfide, ammonia, and various volatile organic compounds are also present depending on the feedstock (Yin Li et al., 2019). AD consists of an integrated system of a physiological processes of energy metabolism that convert organic matter to methane and carbon dioxide (Figure 12). Usually is conceptually divided into three or four stages, hydrolysis acidogenesis and methanogenesis (Alvarado, Montañez-Hernández, Palacio-Molina, Oropeza-Navarro, et al., 2014). During hydrolysis, a consortium of bacteria breaks down complex organics (e.g. proteins, carbohydrates, and lipids) into soluble monomers (amino acids, simple sugars, glycerols, and fatty acids) (J. L. Chen et al., 2014). Hydrolytic bacteria are phylogenetically diverse but mostly fall into two phyla, *Bacteroidetes* and *Firmicutes* (Venkiteshwaran et al., 2016).

Acidogenesis includes fermentation and anaerobic oxidation (β-oxidation), processes that are carried out by fermentative acidogenic and acetogenic bacteria, respectively (Batstone & Jensen, 2011). They convert sugars, amino acids, and fatty acids to organic acids (e.g. acetic, propionic, formic, lactic, butyric, or succinic acids), alcohols and ketones (e.g. ethanol, methanol, glycerol, and acetone), acetate, CO_2, and H_2 (Franke-Whittle et al., 2014; C. Zhang et al.,

2014). Most species of acidogenic bacteria belong to the phyla *Firmicutes*, *Bacteroidetes*, *Proteobacteria*, *Chloroflexi*, and *Actinobacteria* (Cai et al., 2016). In addition, other compounds resulting from acidogenesis such as butyrate, propionate, lactate, and ethanol are further biodegraded by a group of syntrophic acetogens into acetate, formate, and H_2/CO_2. Syntrophic acetogens generally include *Syntrophobacter*, *Pelotomaculum*, *Smithella*, *Syntrophus*, *Syntrophomonas*, and *Syntrophothermus*. The first three genera are typically involved in propionate degradation, whereas the others are commonly responsible for the oxidation of butyrate and other fatty acids (Venkiteshwaran et al., 2016).

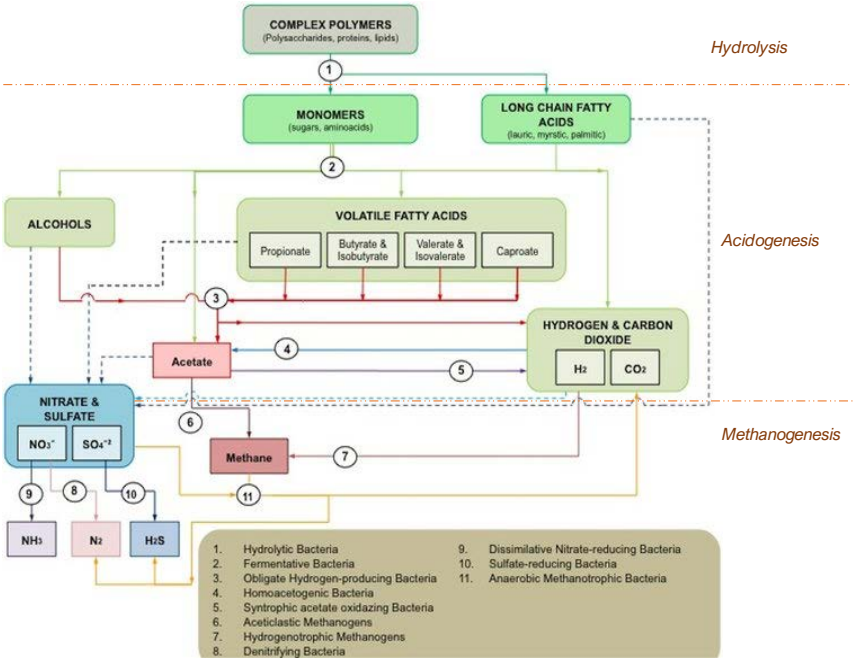

Figure 12. Anaerobic digestion process and microbial interactions. Solid lines represent the usual pathways in anaerobic digestion. Dash lines indicate alternative pathways in which the final electron acceptors are either sulfates or nitrates. Modified from Alvarado, Montañez-Hernández, Palacio-Molina, Oropeza, et al. (2014).

In the methanogenesis phase, generally, the substrates which could be utilized by methanogens include three types: short chain VFA, n or i-alcohols and gases (CO, CO_2 and H_2) (Appels, Assche, et al., 2011).There are four main pathways for methane (CH_4) production: (i) acetoclastic methanogens utilize acetate to directly produce CH_4 and CO_2, (ii) hydrogenotrophic methanogens use H_2 or

formate to reduce CO_2 to CH_4, (iii) methylotrophic methanogens metabolize methyl compounds to produce a small amount of CH_4 (Figure 13); and (iv) syntrophic partnerships of acetate-oxidizing bacteria and hydrogenotrophic methanogens convert acetate to CH_4 via the intermediates H_2 and CO_2 (de Bok et al., 2004; Leng et al., 2018).

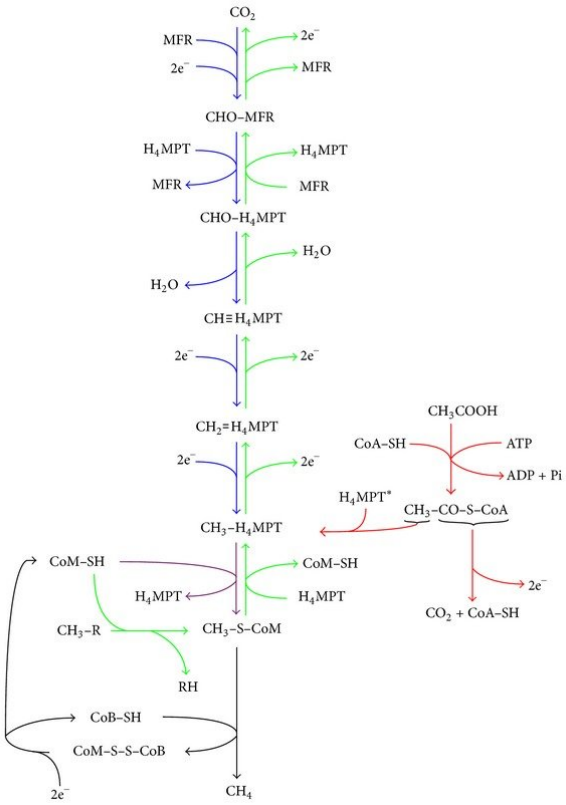

Figure 13. Overview of the three major known methanogenic pathways in Archaea. Color coding indicates the steps common to all three types (black), unique to the methylotrophic pathway (green), unique to the hydrogenotrophic (or CO2 reducing) pathway (blue), unique to the aceticlastic pathway (red), and shared between hydrogenotrophic and aceticlastic methanogenesis (purple). 2e− represents reducing equivalents, produced or consumed during each reaction. MFR: methanofuran; H4MPT: tetrahydromethanopterin; CoM-SH: coenzyme M; CoB-SH: coenzyme B; CoA-SH: coenzyme A; CoM-S-S-CoB: heterodisulfide of coenzyme M and coenzyme B; ATP: adenosine triphosphate; R: ligand bound to methylated compound that serves as substrate for methylotrophic methanogenesis. *Tetrahydrosarcinapterin is a functional analogue of H4MPT found in the Methanosarcinales order of methanogens (Browne & Cadillo-Quiroz, 2013).

The most abundant genus of methanogens found in mesophilic anaerobic digesters was *Methanosarcina* (Cai et al., 2016), which are facultative acetoclastic methanogens that can also utilize H_2/CO_2 and C-1 compounds for methane

production (Yuchen Liu & Whitman, 2008). *Methanosaeta* are obligate acetoclastic methanogens that are known to use only acetate or acetate plus electrons obtained via direct interspecies electron transfer (DIET) (Venkiteshwaran et al., 2016). *Methanoculleus*, *Methanospirillum*, *Methanoregula*, *Methanosphaerula*, *Methanobacterium*, *Methanobrevibacter*, and *Methanothermobacter* are the most commonly observed hydrogenotrophic methanogens in anaerobic digesters (Cai et al., 2016). Nevertheless, the efficient conversion of organic matter to methane in an anaerobic digester is dependent on the mutual and syntrophic interactions of functionally distinct microorganisms (Akuzawa et al., 2011).

AD can take place under psychrophilic (below 20 °C) (Serrano León et al., 2018), mesophilic (25–40 °C) (Meneses-Reyes et al., 2018) or thermophilic (50–65 °C) (M Smith, Sharma, Lappin-Scott, Burton, & H Huber, 2013) conditions, although biodegradation under mesophilic conditions is most common (H. H. Chen & Lee, 2014). However, it has been reported that an AD process under thermophilic conditions produces higher yields using the same substrate. Sharma et al. (2013) reported Specific Methanogenic Activity (SMA) values in thermophilic conditions greater than 0.33 $m^3gk^{-1}d^{-1}$ from chicken litter anaerobic degradation, compared to that obtained by Meneses-Reyes et al. (2018) for a mesophilic process (maximum SMA obtained of 0.27 $m^3gk^{-1}d^{-1}$). However, it is well known that other process parameters, such as substrate type and pH, have a significative influence on the microbial community and the methane yield (Stolze et al., 2016).

3.3. Imbalance of the AD process

The growth rates and the sensitivity toward environmental changes differ widely between different microbial groups. As a consequence of this, an unrestrained reactor operation can lead to disturbances in the balance between the different microbial groups, which might lead to reactor failure(J. L. Chen et al., 2014). Normally, during a process imbalance, intermediates such as H_2, volatile fatty acids (VFA) and alcohols will accumulate, accompanied by fluctuations in gas production. In theory, this provides several parameters that can be used as

indicators of process instability (Nielsen et al., 2007). Volatile Fatty Acids (VFA), mainly acetic acid, propionic acid, butyric acid, and valeric acid, are the main intermediate products during AD of organic wastes (Cysneiros et al., 2012). These are not toxic themselves, however, they can accumulate at high organic loading, resulting in the decrease of pH and even the failure of AD (Pham et al., 2012). Among the four acids, acetic and propionic acids play a dominant role in biogas production (C. Zhang et al., 2013). For instance, Y. Wang et al. (2009) reported that acetic acid and butyric acid concentrations of 2400 and 1800 mg L^{-1}, respectively, resulted in no significant inhibition of the activity of methanogens, while a propionic acid concentration of 900 mg L^{-1} resulted in significant inhibition of the methanogens. It is worth mentioning that different systems have their own levels of VFA that can be considered 'normal' for the reactor, and conditions that cause instability in one reactor do not cause problems in another reactor (I. Angelidaki et al., 1993).

3.4. Role of propionate in anaerobic digestion process

Propionate is an important intermediate during anaerobic digestion of organic polymers. Its degradation into acetate and H_2/CO_2 (and then to CH_4) may contributes with 6 – 35 % of the total methane production (Glissmann & Conrad, 2000; Koch et al., 1983). However, propionate may have a detrimental impact on methanogenic microorganisms (Musa et al., 2018) at concentrations of 37 - 100 mM (Pullammanappallil et al., 2001), and modify the abundance of some microbial groups caused by growth inhibition (W. Kim et al., 2015).

Operational mismanagement (e.g., overloading) or inadequate substrate compositions (inhibitor substances, growth factor deficiencies) can hamper the process, leading to propionate accumulation (Karlsson et al., 2012). In addition, propionate degradation is a limiting factor of anaerobic fermentation (Ahlert et al., 2016). The accumulation of propionate is especially challenging, due to the thermodynamic constraints of its degradation. Under standard conditions, propionate degradation is an endergonic process (Thauer et al., 1977). The oxidation of propionate depends on stable hydrogen consumption (or respective

electrons) by associated processes (Stams & Plugge, 2009). Therefore it can only be oxidized if a syntrophic association occurs between propionate-oxidizing bacteria and H_2-consuming methanogens (McInerney et al., 2009; Bernhard Schink & Stams, 2013).

There are essentially two known pathways that are responsible for propionate metabolism. Most of the syntrophic propionate oxidation is accomplished via the Methylmalonyl-CoA (MMC) pathway, which is also referred to as the classical and common pathway (Kosaka et al., 2006). So far, at least ten syntrophic propionate-oxidizing bacteria (SPOB) species have been identified, isolated and metabolically analyzed. These belong to the phyla of gram-positive Firmicutes (*Desulfotomaculum*, *Pelotomaculum*) and gram-negative Deltaproteobacteria (*Smithella*, *Syntrophobacter*). (Jianzheng Li, Qiaoying Ban, Liguo Zhang, & Ajay Kumar Jha, 2012).

On the other hand, Yitai Liu et al. (1999) reported that, when methanogens establish synthopic relationships with a propionate-degrading syntroph of the genus *Smithella*, produce less methane and more acetate than the previously identified syntrophic propionate degraders such as *Syntrophobacter*. *Smithella* spp. utilize a pathway in which propionate is dismutated to acetate and butyrate via a six-carbon intermediate before being degraded via β-oxidation. This novel dismutation pathway is also known as the *Smithella* pathway (de Bok et al., 2001).

In 2013, Dolfing concluded that the *Smithella* pathway is less sensitive to H_2 than MMC pathway, and under a significant range of conditions propionate oxidation via the *Smithella* pathway is exergonic whereas the MMC pathway is endergonic. *Smithella* species have been detected in various anoxic environments, suggesting that this pathway is widespread (W. Liu et al., 2016).

3.5. Effects of Ammonia on AD

It is well known that ammonia plays an important role in balancing the C/N ratio which could affect the performance of AD significantly (Wang X. et al., 2012).

Many previous papers have reported that ammonia could enhance the buffer capacity of the AD, because VFA formed during digestion process could be neutralized by NH_3 (H. Wang et al., 2015).Ammonia could react with VFA, avoiding the inhibition from VFA and allowing enough VFA for biogas production. Despite its buffer capacity, NH_3 is known to be an inhibitor to a variety of bacteria at high concentrations (Fernandes T.V. et al., 2012). Many previous reports pointed out that the sensitivity to ammonia of acetoclastic methanogens, is much higher than hydrogenotrophic methanogens (Banks et al., 2012; A. Schnürer & Nordberg, 2008), and thus more likely to inhibit methane production, due to the accumulation of acetate up to inhibitory levels, thus contributing further to a negative feedback mechanism that eventually leads to complete reactor failure (H. Wang et al., 2015).

Among methanogens, *Methanosaeta concilii* and *Methanosarcina barkeri* showed higher sensitivity to increasing free ammonia concentrations (Sprott & Patel, 1986). The FA concentration increases with increasing temperature and pH value, e.g., at pH 7 and 35 °C, less than 1% of the TAN is in the form of FA. However, at the same temperature, the FA increases to 10% at pH 8 (Fernandes T.V. et al., 2012). Yenigün and Demirel (2013) stated that digestion failure could be caused by the ammonia concentration of 1.7–1.8 g L^{-1}. J. L. Chen et al. (2014) summarized the critical concentrations and pointed out that a 50% reduction in the methane yield will occur as a result of the ammonia concentration of 1.7 – 14 g L^{-1}. The wide range of ammonia concentration for causing inhibition depends on differences in feedstock, inoculums, environmental conditions (e.g., temperature and pH) (Yenigün & Demirel, 2013) and acclimatization periods (Y. Chen et al., 2008). High concentration of ammonia could not only lead to lower biogas production and even digestion failure, but also result in ammonia emission from effluent (J. Park et al., 2010).

3.6. Anaerobic co-digestion as an option to improve AD of CL

AcoD is an energy-efficient process that can improve fermentation performance by adding a secondary substrate that supplies nutrients that the initial substrate

is lacking or with complementary characteristics (Hagos et al., 2017). This lead to stable digestion, enhanced gas yields (AcoD can enhance biogas production from 25% to 400% over the mono-digestion of the same substrates), and improved economics due to higher methane production. In addition, AcoD can give the following important benefits: (i) improve process stabilization, (ii) dilution of inhibitory and/or toxic compounds, (iii) alleviate imbalance of nutrients, (iv) improve the required moisture contents in the digester feed, (v), reduce the emission of greenhouse gases to the atmosphere, (vi) facilitate synergistic effects of microorganisms, (vii) adjust the carbon-to-nitrogen (C/N) ratio and, (viii) increase the load of biodegradable organic matter (Shah et al., 2015).

3.7. Analysis of AD system

In order to know the changes in the microbial population and metabolic functions through all stages and caused by different environmental conditions, it has been decided to apply analysis that allows us to know the efficiency and performance of the system, as well as the structure of the microbial community and the way in which it relates to its environment. For this objectives, new technologies have been developed in recent years.

3.7.1. Biochemical methane potential (BMP)

The BMP experiments have been broadly used to determine the anaerobic biodegradability, potential efficiency, and concentration of organics in different feedstocks that can be converted by anaerobic digestion to methane (Labatut et al., 2011). BMP experiments also can give relevant information to estimate the parameters related to degradation and hydrolysis rates. In the ACoD process, BMP helps to assess the biodegradability and bioavailability of substrates and the biogas production performance (Hagos et al., 2017; Mou et al., 2014).

3.7.1. New technologies for AD analysis

The study of microbial communities has been revolutionized with the development of high-throughput sequencing technologies. Analysis of high-throughput

sequencing data and a suitable bioinformatics analysis approach therefore plays a very critical role in the investigation of microbiome related to the chicken litter anaerobic digestion process (L. Zhang, Loh, Lim, & Zhang, 2019). Genome sequencing analysis and 16S rRNA analysis can identify the keystone species and their interactions with other taxa. It helps to understand how synergistic biochemical reactions of AD microbiomes are affected by the different operational parameters. Identifying the microorganisms in AD systems has traditionally been accomplished by the construction of 16S rRNA gene clone libraries followed by sequencing Sanger process (Rivière et al., 2009), which in recent years has been replaced by high-throughput next-generation sequencing of 16S rRNA gene amplicons. However, this method focuses on taxonomic identification, and the metabolic pathways present can be determined only indirectly (Cai et al., 2016).

Shotgun metagenomic sequencing, which directly sequences microbiome DNA, can provide more detailed information on the identity of the microbes and their metabolisms as well as other biological information including novel genes (Ferrer et al., 2005). In addition to these advantages, metagenomic sequencing is gaining importance in the study of microbial communities because the decreasing cost and increasing sequencing depth have enabled high-resolution analysis of complex environmental samples, such as soils (Fierer et al., 2012), seawater (Tang et al., 2013b), human gut (Qin et al., 2010), and freshwater (Iliev et al., 2017). Several further studies have since examined AD metagenomes, with the focus mainly on taxonomy and gene-centric functional analyses (De Vrieze et al., 2018; Wandera et al., 2019).

Recently, metagenomic analysis has shifted toward reconstructing important metabolic pathways and genomes present in AD systems (Bedoya et al., 2019; Zhu et al., 2019). By investigating methanogenic communities with metagenomics, it has been possible to study multidimensional interspecies interactions that define composition and dynamics within syntrophic communities that play a key role in the global carbon cycle. It is necessary to remember that microorganisms live within complicated networks with a multitude of interactions,

such as mutualism and competition However, most of those interactions among microbial populations cannot be directly observed, representing a great challenge for studying population interactions in microbial communities (Wu et al., 2016). Network analysis has been used to deduce potential interactions among microbial populations by uncovering strong, non-random associations (Faust & Raes, 2012). It has been applied to examine complex microbial communities in various habitats, such as oceans (Chow, Kim, Sachdeva, Caron, & Fuhrman, 2014), soils (Barberán, Bates, Casamayor, & Fierer, 2012), human microbiomes (Faust & Raes, 2012) and bioreactors (Ju & Zhang, 2015). In addition, network analysis can reveal changes in the topology of microbial networks (Ye Deng et al., 2016). Therefore, network analyses have been considered as powerful tools for studying population interactions in complex microbial communities (Lupatini et al., 2014b).

3.8. Conclusions

Numerous studies have emphasized the huge potential of AD for chicken manure treatment as an ecofriendly stabilization method, allowing renewable energy generation. However, intrinsic difficulties, particularly high N content, has hindered the widespread use of this largely untapped resource. In recent years, several promising technical solutions for integrated ammonia removal that can successfully overcome the hindrances have been developed.

On the other hand, microbial communities involved in anaerobic digestion are one of the most phylogenetically and functionally diverse microbiomes among engineered environments. In order to fully understand these communities, new technologies for metagenome analysis need to be combined with computational methods and chemical analyses. Moreover, combining metagenomic sampling with network analysis is a powerful approach to elucidate the effects os substrates on AD microbiome performance.

This use of complementary techniques will allow the simultaneous identification of phylogeny, interspecies interactions and function, and improve the operation of anaerobic digesters to fully utilize their potential as an effective chicken litter

management strategy and resource recovery process, and for the production of high-value products.

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4. EMERGENCE OF MODULAR ASSOCIATION NETWORKS DURING FUNCTIONAL RESILIENCE IN A METHANOGENIC BIOREACTOR COMMUNITY

4.1. Abstract

Microbial diversity has been shown to play an important role in both natural and engineered ecosystem function. With knowledge on microbial composition and diversity, investigation of within-community interactions is a further step to elucidate microbial ecological functions. If the population of one species within a functional group is reduced or lost due to system perturbation, then another species from the same functional group, but higher resistance to the perturbation, may rapidly take its place if originally present in enough numbers. In this work, The main objectives were to evaluate at specie level, whether network structure is associated with methane production and, to illustrate the possible way in which the methanogenic community is correlated during each phase of the methane production process after a perturbation to the system. Metagenomic sequencing was used to characterized microbial community structure of the anaerobic digestion of chicken litter. Over 97 GBs of metagenomic sequence data were analyzed. In addition, microbial functional molecular ecological networks were studied. The results revealed structural changes of microbial communities, mainly methanogenic microbial community, during the three phases of methane production process (lag phase, methane log phase and stationary phase). In addition, we could conclude that both deterministic and stochastic process occurred simultaneously in the assembly of local communities.

Keywords: Anaerobic digestion, Methanoculleus marisnigri, methanogenic microbial community**,** network analysis, syntrophic interactions.

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4.2. Introduction/Rationale

Methane is the second most important anthropogenic greenhouse gas produced; it contributes about 10% to the annual CH_4 emissions (P. Liu, Klose, & Conrad, 2018). Methane and CO_2 are the end products of the complex process of degradation of organic matter under anaerobic conditions and are produced by a microbial community (Scavino et al., 2013). Methanogenic microbial communities consist of several guilds of microorganisms that perform the following functions: hydrolysis of proteins, fats, carbohydrates and some other biodegradable polymers; primary fermentation of the monomers (sugar, amino acids and fatty acids) to short-chain fatty acids and alcohols; secondary fermentation of these products to acetate, H_2 and CO_2; formation of acetate from sugars, organic acids or H_2 + CO_2 (acetogenesis). The last step is methanogenesis is the conversion of acetate, H_2, CO_2, formate and methyl compounds to CH_4 and CO_2 depending of which metabolic pathway is used (Conrad, Klose, & Noll, 2009; Leng et al., 2018; Venkiteshwaran et al., 2016). The bacteria involved in the three first steps of this process belong to *Firmicutes*, *Bacteroidetes, Proteobacteria, Chloroflexi* and *Actinobacteria.* On the other hand, the last step (methanogenesis) is carried out by methanogenic archaea, which are divided into two groups, hydrogenotrophic which use H_2, CO_2, C-1 compounds or formate and acetoclastic which use acetate. The most abundant genera of methanogens found in anaerobic digesters belong to acetoclastic methanogens. These are *Methanosarcina* which id facultative acetoclastic that can also utilize H_2/CO_2 and C-1 compounds (Yuchen Liu & Whitman, 2008)) and *Methanosaeta* which is an obligate acetoclastic methanogen (Venkiteshwaran et al., 2016)). *Methanoculleus, Methanospirillum, Methanoregula, Methanosphaerula, Metanobacterium, Methanobrevibacter* and *Methanothermobacter* are the most commonly observed hydrogenotrophic methanogens (Cai et al., 2016).

The methanogens are widespread in different habitats and show a high diversity in morphology and physiological parameters. Biological methanogenesis widely occurs in natural environments including soils, water, deep sea and digestive

systems of some animals (Jiang et al., 2013). However, in order to study the assembly of community structure in methanogenic food webs, methane-producing bioreactors (anaerobic digesters) have been established as a model system. These bioreactors are an excellent, tractable model for measuring the processes that control community structure and relating these processes to system functions. This food web is broadly defined as trophic groups and is thought to assemble from both deterministic and stochastic processes (Vanwonterghem et al., 2014). For instance, Chase and Myers (2011) reported that soil microbial community structure is shaped by a combination of deterministic and stochastic processes. According to Stegen, Lin, Konopka, and Fredrickson (2012), deterministic processes are niche-based, including environmental filtering, biotic interactions, and interspecific tradeoffs that enable species to co-exist within communities for long periods of time. Moreover, the resulting local communities generally have little site-to-site variation in species composition (low β-diversity) when environmental conditions are similar (Chase, Biro, Ryberg, & Smith, 2009). In contrast, the neutral theory hypothesizes that all species are ecologically equivalent and drift, i.e., stochastic processes such as, extinction, dispersal, colonization and speciation (Barber & Marquis, 2011; Hubbell, 2001), govern the diversity and species composition of local communities independent of their traits and niches. When stochastic processes are coupled with priority effects, local communities with greater site-to-site variations (high β-diversity) in species compositions can emerge under similar, even identical, environmental conditions (J. Zhou et al., 2014). However, due to the complexity of natural ecosystems it is very challenging to illustrate the existence of stochastic assembly and its relative roles in determining community composition. Nevertheless, in microbial communities, discerning the relationships between community assembly and ecosystem functioning is even more difficult compared with macro-ecological systems (J. Zhou et al., 2013). In these kind of systems, interaction networks and their topologies have revealed that community-wide interaction patterns maximize robustness and functionality (Saavedra, Stouffer, Uzzi, & Bascompte, 2011) and those can be either positive or negative (Faust & Raes,

2012). On the other hand, microorganisms also engage in a rich diversity of relationships. Interactions can be antagonistic (competition for a limiting resource or direct interference) or cooperative, such as transfer of complementary metabolites (e.g., interspecies H_2 transfer) or quorum sensing (Hibbing, Fuqua, Parsek, & Peterson, 2010). In addition, Barberán et al. (2012), assumed that microbial relationships can be depicted. When two species (or any operational taxonomic units) co-occur or show similar abundance patterns over multiple samples.

Knowledge about the composition of microbial communities from diverse environments is rapidly expanding due to tremendous advances in sequencing technologies (Berry & Widder, 2014). High-throughput sequencing technologies have opened new frontiers in microbial community analysis. The revolution in sequencing technology, combined with the development of advanced computational tools that exploit metadata can relate hundreds of samples to one another in ways that reveal clear biological patterns(Caporaso et al., 2011). Even though this information does not provide direct evidence of interactions between species, they are amenable to co-occurrence network construction using correlation coefficients or other association metrics (Barberán et al., 2012; Shokralla, Spall, Gibson, & Hajibabaei, 2012).

Network analysis has been used to explore interactions among microorganisms in various habitats, including soils (Khan et al., 2019; Lupatini et al., 2014a), lakes (Xue et al., 2018), the human gut (Faust et al., 2012) and anaerobic digesters (Campanaro et al., 2016; Wu et al., 2016). The topological properties of the networks reflect the interactions between microorganisms that traditional methods could not have predicted. For example, taxa in the co-occurrence network with high closeness centralization and low betweenness centrality can be considered as keystone taxa (Berry & Widder, 2014). The scores (network topology properties) also change as environmental conditions shift (Wu et al., 2016). Owing to such merits, network analysis has become a powerful tool in the study of microbial ecology.

Explaining and predicting such interactive network structures, dynamics, and the underlying mechanisms are essential parts of any study of biodiversity. However, these methods still do not capture the breadth of ecological interactions that occur in a complex microbial community. In addition, network analysis has still not been used extensively to explain the structure of microbial communities at the species level for the anaerobic digestion process, including how it is affected by different environmental conditions, such as disturbances. It is necessary to remember that most microbial communities are complex, consisting of many species potentially interacting with each other. Therefore, analysis at the species level may help to explain how the process of methane production is regulated by both syntrophic interactions and by the kind of microorganisms who participate in those interactions. The objectives of this study were to i) evaluate whether network structure is associated with methane production and ii) illustrate the possible way in which methanogenic community structure is correlated during each phase of the methane production process. Our design rationale was to begin with a stable AD community and disrupt it with dilution and a high level of substrate input. Dilution will disaggregate the syntrophic community and reduce populations, providing opportunity for substrate-induced increases in population size that can be detected in association networks. We further surmised that the large input of substrate will stimulate successive population increases as a pulse of activity through each trophic level. Furthermore, AD systems often display different performance, indicating that every iteration of a methanogenic network is not equally efficient at converting substrate into methane. Therefore, replication of reactor will be essential to measure stochastic and deterministic network assembly.

4.3. Materials and methods

4.3.1. Experimental set up.

This study was carried out in batch systems in bioreactors (digester) of 11 L capacity and a working volume of 10 L each (Meneses-Reyes et al., 2018). The experiment was performed in triplicate. The feedstock was a chicken litter slurry

with 3 % total solid (TS) from a commercial poultry farm in Tepetlaoxtoc, Mexico. The inoculum added was 5% (v / v). This was obtained from mesophilic digester of 10 L that had been fed with chicken litter (3% TS) and operated in stable operation for more than four years. The slurry was obtained by adding water to feedstock and filtering the resulting solution in order to remove large particles. The working temperature was 37 ± 2 °C.

4.3.2. Analytical methods.

For the substrate, TS, volatile solids (VS), fixed solids (FS), and chemical oxygen demand (COD) were determined using the standard methods (APHA, 2005), while ammonia nitrogen was determined with Method 10031 (HACH, 2004). pH was measured with a potentiometer (Thermo Scientific Orion 5 Star, Singapore). Profiles of volatile fatty acids (VFA) were determined as described by Meneses-Reyes, Hernández-Eugenio, Huber, Balagurusamy, and Espinosa-Solares (2017) and were obtained for acetate, propionate, butyrate, iso-butyrate, valerate, iso-valerate, caproate, isocaproate, and heptanoate acids using gas chromatography (GC) (Claurus 500, Perkin Elmer, USA) equipped with a flame ionization detector (FID) and a capillary column Elite-FFAP with a length of 30 m and 0.32 mm diameter (Perkin Elmer, USA). VFAs were determined using a sample of effluent which was acidified to pH 3 with HCl and centrifuged at 14,500 rpm for 10 min (MiniSpin Plus model micro-centrifuge Eppendorf, Germany). The supernatant was injected with an auto-sampler using 5 µL volume. Operation conditions were as follows: gas flow 5 mL min^{-1} at 10.6 psi, 150 °C injection port, 100 °C oven for 8 min with ramp of 160 °C for 8.5 min, 250 °C detector, and retention time of 16.5 min. Helium was used as the carrier gas. Free Volatile Acid Mix analytical grade (46975-U, Brand Sigma-Aldrich, USA) was used as a standard. This analysis was made each week throughout the experiment.

The biogas production was quantified by the displacement method in saline water 10% (v / v) with a flowmeter that uses the volume shift and a digital counter (LA8N-BN, Autonics, Korea). Methane content was determined by the procedure described by Meneses-Reyes et al. (2017) using a GC (Claurus 500, Perkin

Elmer, USA). 10 µL biogas samples were injected into the GC with the following conditions: flow 14 mL min^{-1} at 14 psi, 100 °C injection port, 70 °C oven, and 100 °C detector. The retention time was 4 min; helium was used as the carrier gas. The percentage of methane was obtained using a calibration curve with pure methane (HDSP No. P-4618-F, Praxair, Mexico). Methane percentage is reported with normal conditions of temperature and pressure.

4.3.3. Modeling and statistical analysis.

The methane production results were fitted to the modified Gompertz model (Zwietering, Jongenburger, Rombouts, & van 't Riet, 1990) which is presented in Equation 1.

$$AMY = BMP \cdot exp\left\{-exp\left[\frac{\mu_m \cdot e}{BMP}\right](\lambda - t) + 1\right\} \quad \text{Equation 2}$$

AMY is the accumulated methane yield (mL CH_4 $g_{VSfed}{}^{-1}$) at time t (d), BMP is the biochemical methane potential (mL CH_4 $g_{VSfed}{}^{-1}$), µm is the rate of methane production per day (mL NCH_4 $g_{VSfed}{}^{-1} d^{-1}$), e is the mathematical constant (2.718282), λ is the time lag phase (d), t is the time of digestion (d). All regression models were fitted using SigmaPlot 13.0 version (San Jose, California, USA).

A weighted principal component analysis (PCA) was used to evaluate the effect of the chemical parameters (such as pH, TS, VS, COD, $N\text{-}NH_3$ and mL of CH_4).over the microbial community (species level), and how it changed through the three phases of the methane production process. PCA was also used to evaluate the profiles of the four main VFA, propionate, acetate, butyrate and isobutyrate. PCA was performed with JMP software (V. !4.0).

4.3.4. Metagenomic analysis.

Cell samples were taken periodically through the experiment. To extract cell samples, 50 mL of sludge was centrifugated at 6700 rpm to obtain a cell pellet which was kept at -80 °C until DNA extraction. Genomic DNA was extracted using PowerSoil® DNA Isolation Kit (Qiagen N.V., Germany) according to the

manufacturer's protocol. Genomic DNA was quantified by standard measurements of light adsorption at 260 and 280 nm wavelength using a NanoDrop spectrophotometer (Thermo Fisher Scientific, Inc, Walthman, MA). Genomic DNA was stored at -80°C before sequencing. 18 genomic DNA samples were sent to Admera Health (New Jersey, USA) for library construction and sequencing. Genomic DNA samples were quantified with Qubit2.0 DNA HS Assay. Library preparation was performed using Nextera XT per manufacturer's recommendations. Library quality and quantity were assessed with Qubit 2.0 DNA HS Assay (ThermoFisher), Tapestation High Sensitivity D1000 Assay (Agilent Technologies, CA, USA), and QuantStudio ® 5 System (Applied Biosystems, USA). Genomic DNA libraries were paired end, which were loaded onto Illumina Hiseq 2x150bp format. Data analysis began with quality control using FastQC v.0.11.5 in order to know the quality of the sequences. All sequences were deposited in Metagenome Rapid Annotation using Subsystem Technology (MG-RAST) database (Meyer et al., 2008)).

4.3.5. Taxonomic and Functional Annotation of Metagenomes.

As recommended by the online metagenome analysis tool MG-RAST version 4.0.3, the reads were not assembled before submission for taxonomic and functional analyses. Artificial replicate sequences and irrelevant sequences (e.g., plant, human, or mouse) were removed automatically by MG-RAST and low-quality sequences were filtered out using default settings following a standard pipeline (Keegan, Glass, & Meyer, 2016). Reads were taxonomically annotated by comparison with the M5NR database using sBLAT (Kent, 2002). M5NR is a non-redundant (nr) protein database (Meyer et al., 2008) comprising the NCBI GenBank (Benson, Karsch-Mizrachi, Lipman, Ostell, & Wheeler, 2007), SEED (Overbeek et al., 2005), KEGG (Kanehisa & Goto, 2000) and eggNOGs (L. J. Jensen et al., 2008) databases.

To identify genes and their functions, the reads were additionally annotated via sBLAT (Kent, 2002) searches against the COG and SEED gene databases, both of which organize genes into nested hierarchies of groups (COG categories and

SEED subsystems) with related functional roles. Taxon abundance was normalized by dividing by the total number of hits in each metagenome (Mackelprang et al., 2011; Tang, Liu, Jiao, Zhang, & Chen, 2013a).

4.3.6. Association network construction.

Association networks were constructed using the relative abundance data of species for 18 samples (six from each replicate). Networks were constructed based on three phases of the methane production process: lag phase, rapid methane production phase and stationary phase. Therefore the 18 samples were divided into each of the phases. The relative abundances for the species, belong to *Bacteria*, *Archaea*, and *Eukarya*, were used to construct Spearman correlation coefficients between all possible pairs of species across samples. The analysis was performed in R environment using the package '*Hmisc*'. A correlation coefficient was considered statistically robust if a numeric value was either ≥ 0.9 or ≤ -0.9 with significance of $P \leq 0.001$ in order to reduce the chances of obtaining false positive results and to get the stronger correlations. When constructing networks, only significant pairwise relationships were used, with each node representing a species and each edge representing a significant pairwise association between them. Interacting nodes within networks represented co-occurrence across samples. In order to achieve our objectives, the most abundant methanogens (*Methanoculleus* marisnigri and *Methanosarcina mazei*) were selected and their primary and secondary correlations were analyzed separately.

To construct networks, the following topological features were measured using Cytoscape 3.7.2 (Cline et al., 2007), and used to describe network properties: (i) connectivity, which is the number of links (i.e. edges) of a node to other nodes; (ii) clustering coefficient, a measure of interconnectivity in the neighborhood of a node; (iii) path length, which is the average number of edges on the shortest path connecting any two nodes and (iv) betweenness centrality, which reflects the number of times a node plays a role as a connector along the shortest path between two other nodes. While average values of the indexes are generally used to describe the overall features of the network, the relative betweenness centrality

value of each node can indicate its relative importance in the network (Khan et al., 2019).Networks were visualized using the *edge-weighted spring embedded* layout to obtain an overall distribution pattern of the nodes, and *group attributes* layout, where nodes are separated into modules (Cline et al., 2007). In order to have structure and informative visualization for more efficient exploration and analysis of large biological network, the Clustermaker Cytoscape GLay was used (Su, Kuchinsky, Morris, States, & Meng, 2010). Sub-networks were created to visualize the correlations between methanogens, syntrophic bacteria, and sulfate reducing bacteria. The visualization of the first correlations of *Methanoculleus marisnigri* and *Methanosarcina mazei*, was performed using Circos Software (Krzywinski et al., 2009).

4.4. Results and discussion

4.4.1. BMP and physicochemical parameters.

The experiment was conducted for 191 days (time in which a steady stationary phase was reached). Table 6 shows the values for pH, sulfides, sulfates, N-NH_3 and COD at the beginning and at the end of the experiment. The tendency of the data shows a strong reduction in COD values, which demonstrates a clear degradation of organic matter, so that it can be used as a substrate in methane production through the anaerobic digestion process. On the other hand, the ammonia nitrogen levels increased. These results are in concordance with the values of N-NH_3, because it explains the degradation of proteins present in the substrate. It has been reported in previous studies (Fotidis, Karakashev, & Angelidaki, 2014; Sun, Müller, Westerholm, & Schnürer, 2014; Westerholm, Moestedt, & Schnürer, 2016), values greater than 0.14 g NH_3-N L^{-1}, such as those obtained in the present work, could indicate the dominance of the SAO (Syntrophic acetate oxidation) pathway at mesophilic temperatures. In the presence of high ammonium concentrations the acetate-utilizing methanogens were inhibited and acetate was, instead, converted to hydrogen and carbon dioxide by homoacetogenic bacteria (Anna Schnürer, Houwen, & Svensson, 1994; Anna Schnürer, Schink, & Svensson, 1996).

Table 6. Changes in physicochemical parameters during the experiment

Parameter	R1		R2		R3	
	Initial	Final	Initial	Final	Initial	Final
pH	6.82	8.05	6.86	8.01	6.84	7.94
Sulphide [mg L^{-1}]	37.67	16.25	36.68	13.61	36.98	12.73
Sulphate [mg L^{-1}]	4740.97	834.31	4825.78	945.63	4375.22	1003.94
N-NH_3 [mg L^{-1}]	483.80	1107.25	479.75	1302.35	501.20	1146.05
COD [mg L^{-1}]	27595.16	3959.78	25457.29	4316.09	26526.22	5503.80

The accumulated methane produced and VFA profiles in the three digesters is shown in Figure 14. In all cases, three typical periods can be defined: lag phase methane log phase and asymptotic methane accumulation or stationary phase (Meneses-Reyes et al., 2017), each of them contribute to the modified Gompertz model (Table 7) as λ, μm and BMP. The concentration of VFA initially increased and then, through the time, decreased drastically causing the pH at the end of the experiment to increase (Table 6). This as a result of oxidation process of VFA to be converted into acetate during acetogenesis step.

First phase (lag phase): the initial delay in methane accumulation could be attributed to the large dilution with the feeding, causing an environmental disturbance due to an increase of nutrient input with complex C substrate (J. Zhou et al., 2014). During this phase we could see a VFA accumulation mainly in R3, specifically acetate and butyrate. This could be derived both, 1) methanogenic network reassembly and, 2) degradation of complex polymers by hydrolytic bacteria (hydrolysis process). This VFA accumulation could contribute (in R3) with the higher value of λ, originated by an acidosis process (Vanwonterghem, Jensen, Rabaey, & Tyson, 2015), even if this this effect is not visible in a drop in pH values (Franke-Whittle et al., 2014). In addition, A kinetic imbalance between the production of VFA by fermentative and acidogenic bacteria and their consumption by a combined effort of acetogenic bacteria and methanogen is a direct reflection of the accumulation of these metabolites (Ahring et al., 1995). In accordance with Weiland (2010), due to their high sensitivity to the increased VFAs concentrations,

archaeal communities in AD reactors facing acidification are rapidly inhibited, leading to delay or decreased methane production.

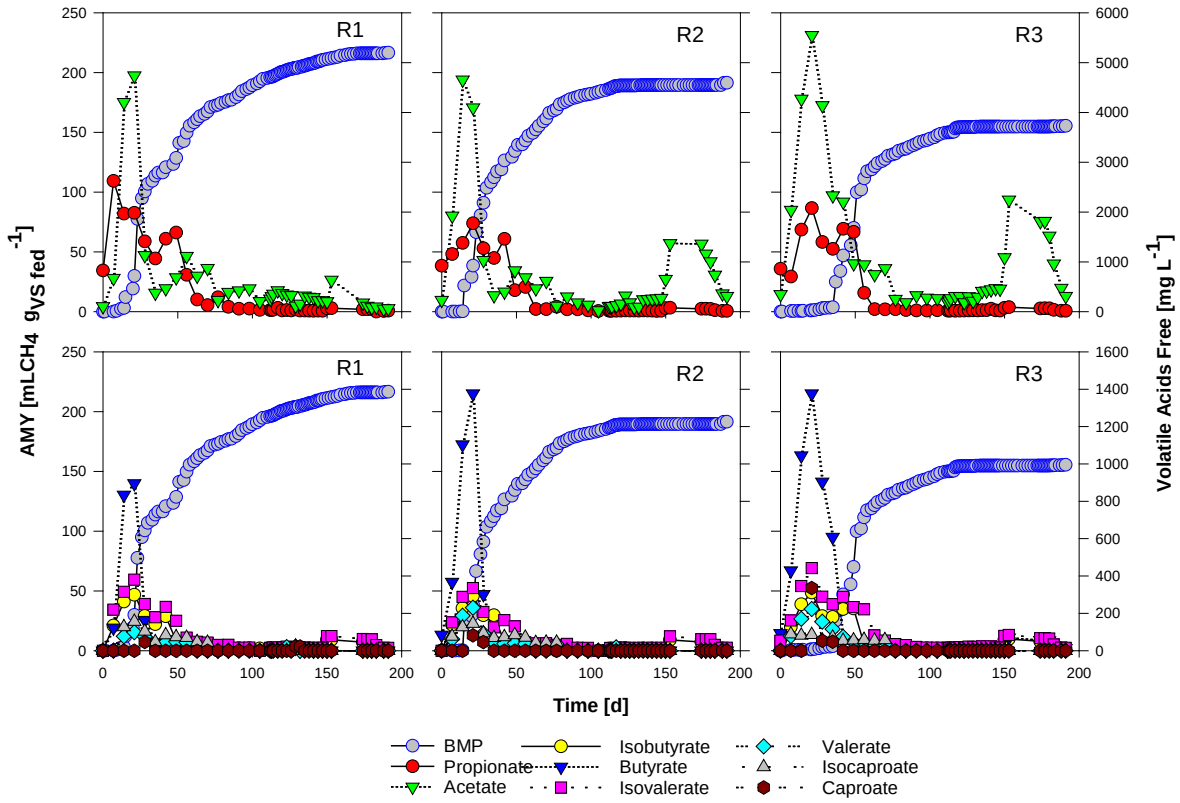

Figure 14. Accumulated methane yield and VFA profiles during experiment.

Second phase (methane log phase): This phase is characterized by a sudden increase of methane production and a gradual reduction in VFA levels. This, reflected the continual reinforcement of the activity of different groups of microorganism such as methanogens, syntrophic bacteria and sulfate reducing bacteria, which in addition to consuming H_2, CO_2 and other compounds degrade VFA to produce methane (Yin et al., 2019). Depending on methanogens diversity (acetoclastic and hydrogenotrophic), methane could be produced. In typical municipal anaerobic digesters, about 70 % of the CH_4 is produced from acetate, and the rest from H_2 and CO_2. Only a minimal amount of CH_4 is produced via methylotrophic methanogenesis (Ferry, 2012). The µm values showed that R3 produced a higher volume of methane in the same time compared with R1 and R2, even when its lag phase was longer than the other digesters.

Table 7. Modified Gompertz model parameters determined from the experimental data and previous studies.

Treatment	λ [d]	μ_m [mL CH_4 g_{svfed}^{-1} d^{-1}]	BMP [mL CH_4 g_{vsfed}^{-1}]	R^2
R1	4.52	3.07	210.03	0.9709
R2	8.10	3.68	188.52	0.9842
R3	31.96	4.35	153.06	0.9931
CL (Meneses-Reyes et al., 2017)	14.13	2.83	113.2	0.9917

Third phase (stationary phase): Around day 50, when the levels of VFAs started decreasing, methane production also began to decrease, possibly because of a depletion of the substrate (mainly composed of VFAs). Although around day 150 the acetate concentration increased, it had no significant effect on methane production, so it can be inferred that its degradation could be due to some other metabolic route such as production from Acetyl-CoA. The same behavior was reported by Meneses-Reyes et al. (2017) in a co-digestion process of chicken litter with oil-extracted microalgae.

The PCA applied to environmental parameters such as, VFA (acetate, propionate, butyrate and isobutyrate), pH, mL of methane, sulfates, sulfides, NH_3-N and COD is shown in Figure 15. The variation in the results are explained as 55.2 % by component 1 and 16.0 % by component 2. With respect to the phases, the fatty acids contributed more to phase 1 (P1) variation than to phase 2 and 3, while phase 2 (P2) was more affected by methane, COD, sulfates and sulfides. Which confirms that in phase two methane was produced exponentially, and is related to COD, since this parameter is an indicator of degradation of organic matter. Phase three was affected by pH and NH_3-N. This could explain why methane production decreased. These two parameters modified the environment in the digesters causing toxicity to the methanogenic community (Banks et al., 2012; Fernandes T.V. et al., 2012).

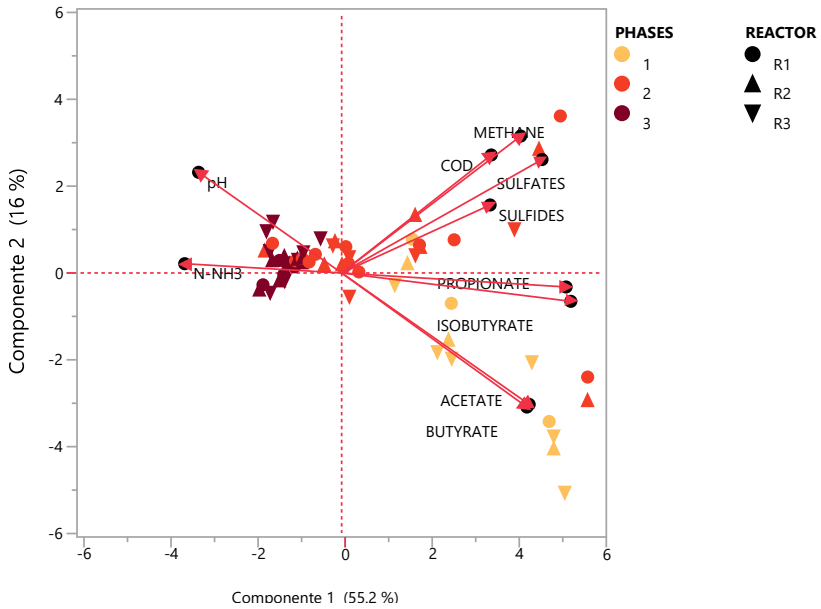

Figure 15. Principal component analysis base on chemical composition through the experiment.

4.4.2. Metagenomic Analysis.

Metagenomic DNA was obtained from 18 samples using Illumina HiSeq. This generated 97.62 billion bp of sequences. The microbial diversity was composed by 2427 species, grouped in 278 families, 76 classes, and 44 phyla. For a better understanding of the microbial community structure, taxonomic affiliations at different levels was analyzed. At the phylum level, the results indicated the most abundant phylum was *Proteobacteria* (41 % on P1), following by *Firmicutes* (~30 %), *Bacteroidetes* (~18 %), *Actinobacteria* (~7.5 %) *and Euryarchaeota* (0.7 %) (Figure 16). However, throughout the three phases these values were modified. Our results contrast with the findings of Treu et al. (2016), where *Firmicutes* was the most dominant phylum (60 % of the total community). This difference could be attributed to the invasion of feed bacteria, most of which are *Proteobacteria*. This is the most diverse bacterial phylum and commonly features in the fecal microbiota (Moon, Young, Maclean, Cookson, & Bermingham, 2018). Additionally, Shang, Kumar, Oakley, and Kim (2018) reported for chicken gut microbiota the next families: *Alcaligenaceae, Campylobacteraceae,*

Pasteurellaceae, and *Enterobacteriaceae*. These constituted 52 % of *Proteobacteria*.

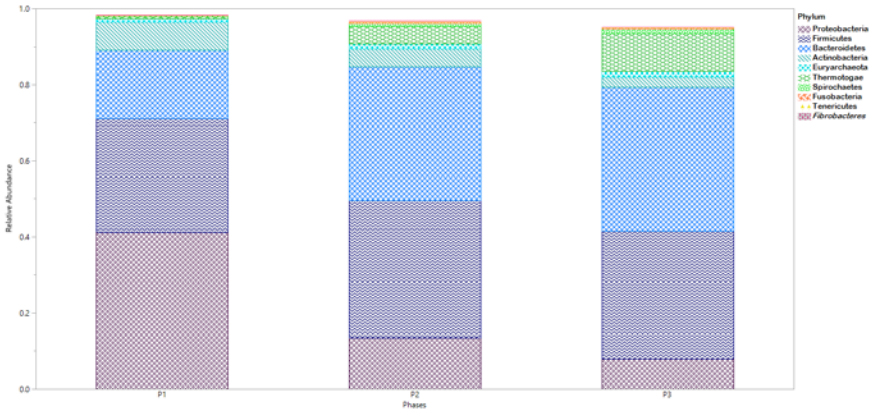

Figure 16. Abundance relative of the most abundant phyla in each phase of methane production process.

However, bacteria belonging to this phylum are important microbes in anaerobic digestion process because most of *Alpha*-, *Beta*-, *Gamma*-, and *Deltaproteobacteria* are well-known in glucose, propionate, butyrate, and acetate-utilizing microbial communities (Ariesyady, Ito, & Okabe, 2007). Most of the members belonging to the *Firmicutes* phylum are syntrophic bacteria that can degrade various VFAs, which were often detected in both activated sludge systems and anaerobic digesters (Garcia-Peña, Parameswaran, Kang, Canul-Chan, & Krajmalnik-Brown, 2011). In addition, *Firmicutes* has the capability of polysaccharide and oligosaccharide degradation. Therefore, it participates in an important way in the hydrolysis process together with *Fibrobacteres* (Treu et al., 2016). Within the phylum of *Firmicutes*, *Bacilli* and *Clostridia* are the most abundant, participating in degradation as fermenters (Guo et al., 2015; Wirth et al., 2012).

On the other hand, the main role of *Bacteroidetes,* together with *Firmicutes* is to contribute to the hydrolysis process, degrading complex carbohydrates (Guo et al., 2015). The archaea community presented a narrower phylogenetic diversity compared to the bacteria. Within *Euryarchaeota*, the most abundant species at

the beginning (P1) were *Methanosaeta thermophila, Methanoculleus marisnigri* and *Methanosarcina mazei.* Figure 17 shows the AMY curve with VFAs profile throughout the experiment and the relative abundance of methanogens, sulfate reducers and syntrophic bacteria at the species level. In the initial phase, the predominant methanogen was *Methanosaeta thermophila*, which is recognized as an obligate acetoclastic methanogen. However, when P2, started *Methanoculleus marisnigri* began to be the most abundant in R1 and R2, while in R3 *Methanosarcina mazei* was the methanogen which leads the beginning of phase 2. With this behavior, we can suppose that in R1 and R2, methane was produced by the hydrogenotrophic pathway, but in R3, *Methanosarcina mazei* may be primarilly responsible for methane. In addition, it is considered beneficial to AD systems, since this robust methanogen is able to use different substrates and thus multiple pathways, for methane production (acetoclastic and hydrogenotrophic) (De Vrieze, Hennebel, Boon, & Verstraete, 2012). Because of that we cannot speculate about what is the metabolic path by which methane was produced in R3.

Conrad et al. (2009) reported that metabolomic pathway of methane production in anoxic rice field soil over a wide temperature range up to 55 °C change from hydrogenotrophic plus acetoclastic methanogenesis to exclusively hydrogenotrophic methanogenesis. Simultaneously, the methanogenic community consisting of *Methanosarcinaceae*, *Methanoseataceae*, *Methanomicrobiales*, *Methanobacteriales* and Rice Cluster I (RC-1) changes to almost complete dominance of RC-1.

Figure 17 also showed changes in the relative abundance of SB and SRB. These groups increased in abundance while VFA concentration decreased. Proteins and carbohydrates degradation play an important role in VFA production. VFA can be converted into acetic acid by hydrogen-consuming bacteria and then turned into methane (Yue Li et al., 2019).

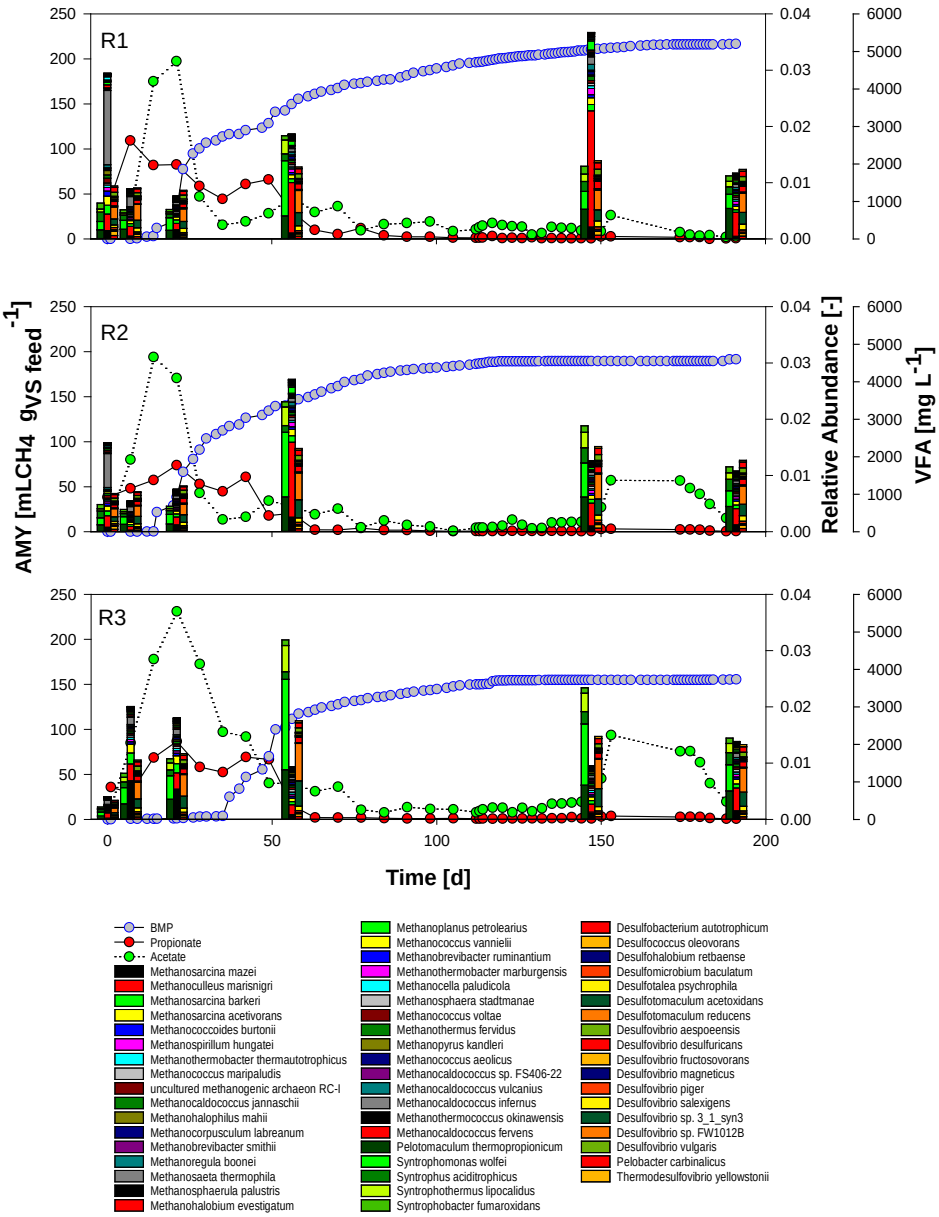

Figure 17. Methane production process, acetate and propionate profile vs relative abundance of methanogens, syntrophic bacteria (SB) and sulfate reducing bacteria (SRB).

Conversion of hydrolysis products implies that more short-chain fatty acids are generated indicating a higher substrate utilization of acidogens (Feng, Yan, & Chen, 2009; Yuan et al., 2006). This might indicate the presence of syntrophic relationships among a community of microbes, including fermenting bacteria, specialized acidogenic and acetogenic syntrophs, and methanogens (Werner et al., 2011; Yamada, Kato, Ueno, Ishii, & Igarashi, 2015).

4.4.3. Methanogenic microbial communities and association network construction

The AD community was derived from a single bioreactor and divided into three replicate batch reactors. We sampled community diversity and functions in each reactor during three distinct phases of system performance: 1) methane lag phase where hydrolysis and primary fermentation begin, fatty acid production rapidly rises, and methanogenesis is absent; 2) methane log phase where rapid methanogenesis occurs, and 3) stationary phase where methane production ceases. We hypothesized that these dramatically different functional phases would coincide with different community structures and give us insight into assembly and network processes (Figure 18). In addition, phase 2 would be bracketed by two periods of community functions where the methanogenesis food web is disrupted, presumably for different reasons (initially, disaggregation; later, environmental conditions deteriorate). This experimental design also provided the potential opportunity to observe whether community re-assembly yields different structures in the replicate reactors due to the large, potentially overwhelming, input of environmental microbes in the chicken litter substrate and stochastic assembly. In order to characterize and evaluate the co-occurrence interactions of the methanotrophic communities in each phase, we constructed co-occurrence networks. In Figure 18, changes in the structure of the microbial community through this process are evident, including their correlations, being different in each phase. One of the probable explanations for this kind of change is related to microbial community succession. Chase and Myers (2011), reported that two types of process (deterministic vs stochastic) influence the assembly of species into a local community. Considering the number of correlations, we found predominantly positive correlations in the second phase, which indicated that methanogenic microbial communities exhibited cooperative rather than competitive relationships (Oliveira, Niehus, & Foster, 2014).

Ecological succession refers to niche-based deterministic development of ecological community structure after perturbations (Kreyling, Jentsch, &

Beierkuhnlein, 2011). Although, by definition, deterministic succession is expected under identical or rather similar environmental conditions, very few studies have examined the roles of stochastic processes controlling the succession of ecological communities (Chase, 2010). Neutral theory predicts that chance, the stochasticity inherent in various probabilistic biological processes (such as dispersal, colonization, extinction, speciation, biotic interactions, and initial population heterogeneity) could lead to unpredictable variability in community composition (Chase, 2007) (i.e., compositional stochasticity). However, assessing the degree of stochasticity and its role in ecological succession in field studies is challenging (Farnon Ellwood, Manica, & Foster, 2009) because the three components of ecological succession (stages, trajectories, and mechanisms) depend on the individual characteristics of community members, environment, and perturbations (Prach & Walker, 2011). Thus, manipulative experiments under similar, if not identical, initial conditions are valuable for disentangling the drivers controlling the succession of natural communities in response to environmental perturbations (Kreyling et al., 2011).

4.4.3.1. Network P1. Overcoming disturbances

Taking into consideration the perturbation with large volumes of nutrient, the network in P1 behaved as a disorganized microbial community structure. Phase 1 is constituted by five big clusters, in which negative correlations were predominant. And only two of these were correlated with each other. One of the reasons could be the large dilution. This could separate the syntrophic relationship that were together in the original digester, breaking the prior correlations. In addition, as we discussed above, this allowed VFAs to accumulate.

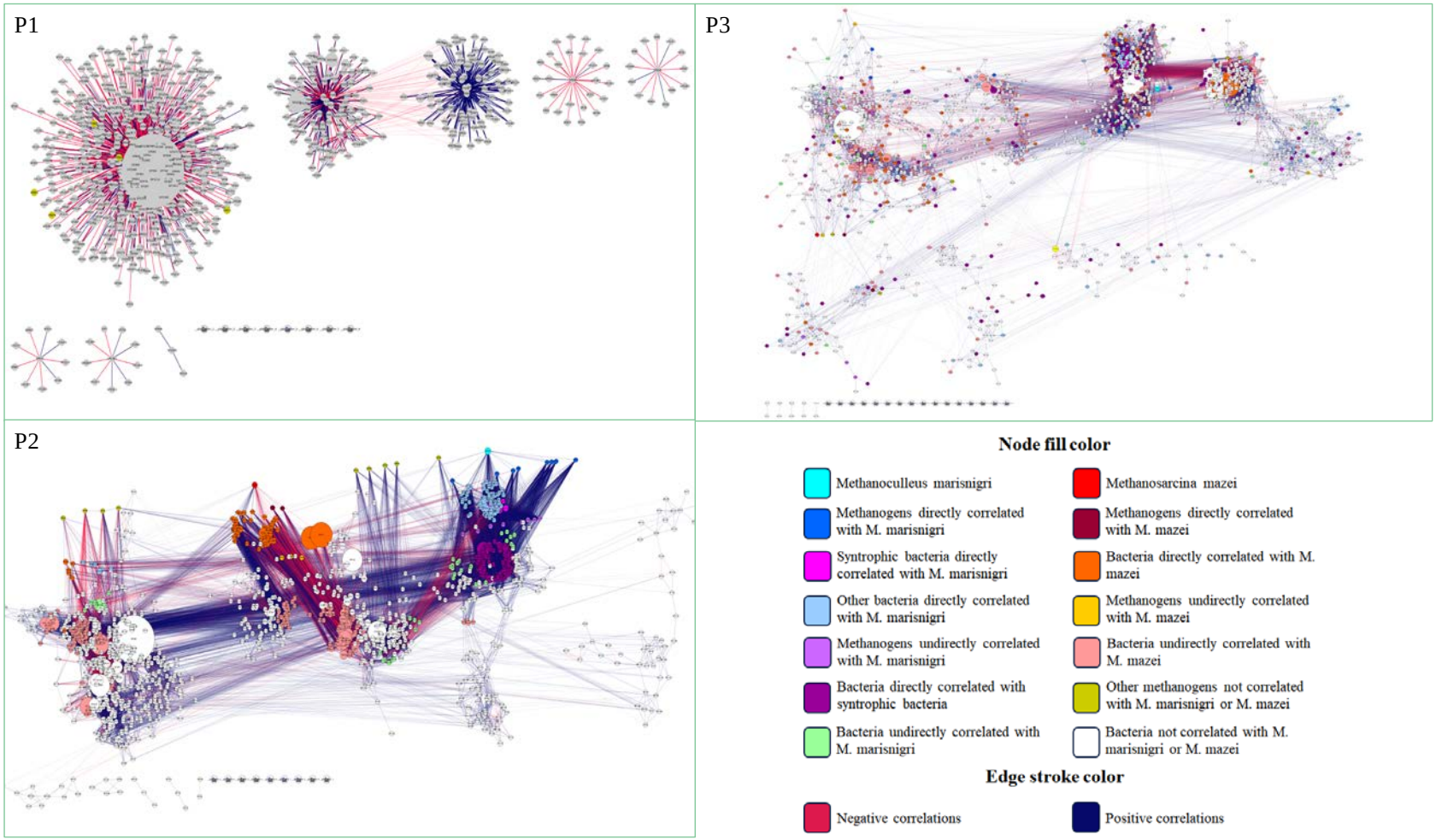

Figure 18. Associated network for three phases of methane production process. Each node represents an species, and each edge represents a significat pairwise association between them. The relative abundance is represented as bubble size.

According to J. Zhou et al. (2014), our methanogenic digester system can be considered to be a combination of a fluidic and non-fluidic ecosystem. This is because a batch reactor does not have flow-through but maintains the characteristics of a digester. However, in a system like this with a nutrient input of complex C substrates disturbance it is expected that compositional stochasticity increases after nutrient input primarilly due to three major processes. First, nutrients can be used by a variety of microorganisms as C and energy sources to stimulate growth, especially growth of less abundant microbial populations (e.g., the “rare biosphere”) that could physiologically respond differentially to nutrient addition. Second, such growth/stimulation could enhance stochastic processes of birth, death, colonization, extinction, and random changes in relative population abundance. Nutrient addition could also weaken niche selection by reducing competition for resources and by providing more diverse resources (C, energy, nutrients) to some species. Third, in addition, nutrient addition could strengthen priority effects (J. Zhou et al., 2014). Enhanced growth of early-arriving species could result in the exclusion of facilitation of later arriving species (Tuomisto, 2010)

4.4.3.2. Network P2. Stabilizing work correlations

Phase 2 is where methane was produced in an exponential way. We can say that this stage is the most important because it is where the correlations of the methanogenic microbial groups are created. In Figure 18 there is a large difference between P1 and P2, structurally speaking. While in P1 there are more negative correlations than positive, in P2 there are many positive correlations throughout the structure. Furthermore, P2 does not have separated groups as we saw in P1. In contrast, it has a complex structure divided into three big clusters.

Treu et al. (2016) reported that the composition of the archaea community is resilient because of its fundamental role in the biogas microbiome. They proposed that the bacterial community is deeply influence by the composition of the influent substrate, while archaea are not. On the contrary, a layer fraction of the bacteria remains unknown. In addition, syntrophic bacteria, specifically,

Syntrophobacterales and Synergistia, are also more stable and resilient due to their specialized functional roles in the AD system (Werner et al., 2011). Based on what was described above, as in this phase we were interested in the network positions of *Methanoculleus marisnigri* and *Methanosarcina mazei*, since they are the most abundant methanogens in P2. In Figure 18 (P2), both methanogens were pulled out of the network structure in order to better visualize the correlations. The original network is in supplementary data.

We hypothesized that both methanogens had to be correlated with at least bacteria belonging to syntrophs and sulfate reducers groups. However, when we modified the structure, moving out *M. marisnigri* and *M. mazei*, we realized that only *M. marisnigri* is part of a large group consisting of *Firmicutes, Bacteroidetes, Proteobacteria,* and others that carry out functions that contribute to methane production. On the other side, *M.mazei* seems not to be correlated with at least syntrophic bacteria or sulfate reducing bacteria (see supplementary data). Additionally, *M. marisnigri* has more positive connections compared with *M. mazei*. These methanogens were associated with different groups, divided into hydrogenotrophic and acetoclastic methanogens. *M. mazei* was correlated with other species belonging to its genus, like *Metanosarcina barkery* and *Methanosarcina acetivorans*. Also was correlated with *Methanosaeta thermophila* other acetoclasetic methanogen.

On the other hand, the primary correlations of *M. marisnigri* are with methanogens belonging to the genus *Methanocaldococcus, Methanoregula, Methanothermobacter, Methanocella* and *Methanococcus*, which are hydrogentrophic methanogens. These were directly correlated with syntrophic bacteria and indirectly correlated with sulfate reducing bacteria. Phase 2 could be explained as follow. After phase 1, deterministic processes gained in importance for several reasons. First, the effects of nutrient amendment on the local communities became diminished, and hence the relative roles of deterministic processes become stronger. Second, due to shorter fluid residence time and/or higher population dispersal rates, substantial portions of the populations in local

communities (feed microbiome) might be replaced by the populations from the common regional species pool (microbiome from inoculum). Third, the local environmental conditions could return to a state like the original levels in which deterministic factors play relatively bigger roles. All of these could decrease the relative importance of stochastic processes in controlling microbial community structure (J. Zhou et al., 2014).

The outcome of the succession with this scenario is an unpredictable divergence from original community structure following nutrient input, followed by convergence toward the original structure. Ultimately, these communities would show a high degree of resilience. Consequently, a community state, which is highly like the initial state, is expected. However, because deterministic processes (e.g., changes in abiotic environmental conditions and species interactions) still play roles in regulating community structure, especially in the late phase of succession, the recovered communities could be the same, very similar to, or statistically significantly different from the original communities. In addition, because nutrient input could potentially stimulate growth of a variety of populations and weaken competitions among different species, compared with the original communities before nutrient input, the average α-diversity of the local communities is expected to be higher during succession and similar at the end of succession (Chase, 2010).

4.4.3.3. Network P3. Surviving environmental stress

P3 (stationary phase) had a different microbial community structure (Figure 18) with more negative correlations in the whole system compared with P2. Additionally, as we can see in Figure 19, the number of correlations of *M. maraisnigri* and *M. mazei* were reduced drastically. In the same way, their correlations were not with the same microorganisms as in phase 2. In phase 3, when methane production is reduced, other metabolites could start accumulating causing disturbances in the system. In addition, because the most labile substrates were consumed first, competition will now increase. And seen in Figure

18, in the network structure, the microorganisms are more separated, hence, we have a greater number of clusters in this phase.

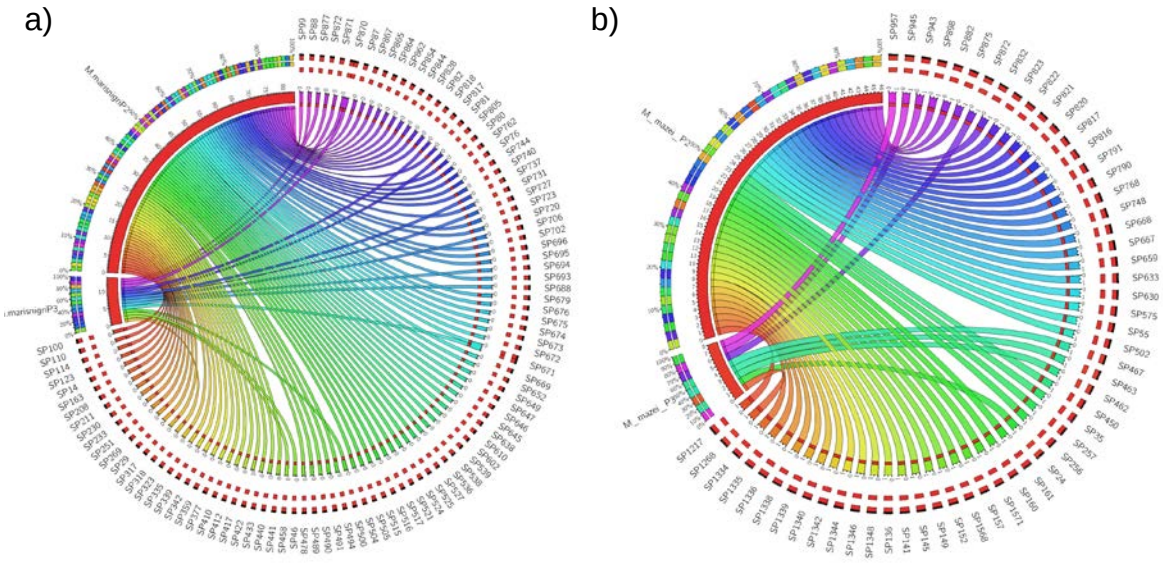

Figure 19. Circos plot showing the primary correlation of a) Methanoculeus marisnigri and b) Methanosarcina mazei during P2 and P3.

4.4.3.4. Analyzing the three main groups, methanogens, syntrophic bacteria and sulfate reducing bacteria.

In the methanogenesis process, three microbial trophic groups collaborate to mineralize organic waste to CH_4 and CO_2: 'fermenters', syntrophic metabolizers ('syntrophs') and 'methanogens' (B. Schink, 1997). However, one of the most important relationships is between syntrophic bacteria and methanogens. They form an essential group for producing methane. Figure 20 shows the network of the correlations between methanogens, syntrophic bacteria and sulfate reducing bacteria. During P1 and P2, almost all the correlations were positive while P3 has negative correlations in the network structure. Also, the number of correlations during the three phases are different, being larger on P2. Contrary to the findings of other studies it can see that methanogens, syntrophic bacteria and sulfate reducing bacteria are positively correlated. This could indicate syntrophic relationships between the first two with sulfate reducing bacteria. This is supported by the findings of (Muyzer & Stams, 2008). They explained that sulfate

reducing bacteria can grow without sulfate and in some cases, they grow only in syntrophic association with methanogens or other hydrogen-scavengers. In other words, sulfate reducing bacteria may either compete with methanogen or grow in syntrophy with methanogens depending on the prevailing environmental conditions.

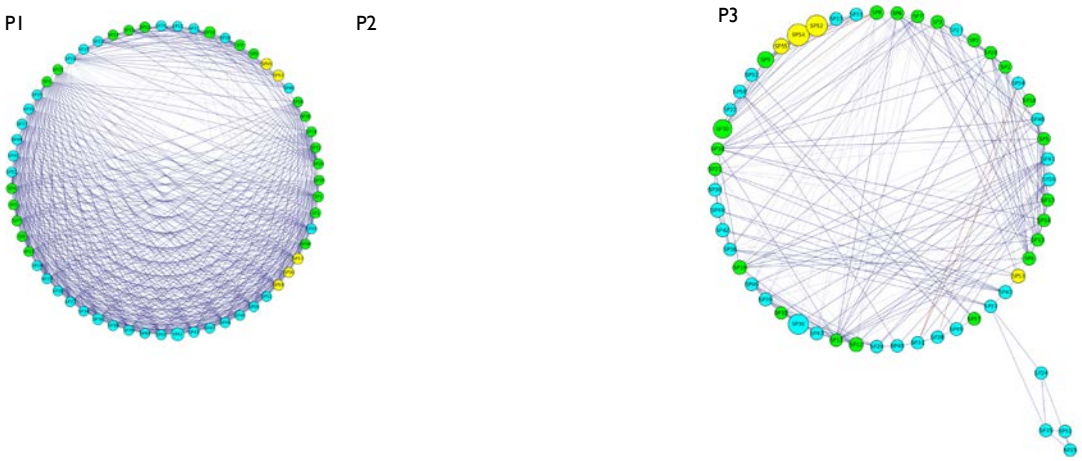

Figure 20. Nerwork plot of methanogens (blue), syntrophic bacteria (yellow) and sulfate reducing bacteria (green) per phases.

4.5. Conclusions

In short, metagenomic sequencing and network analysis allowed us to successfully dissect the detailed microbial community structure of a batch anaerobic digester. The knowledge garnered will facilitate the understanding of the methanogenic microbial community structure, and how this is affected by different environmental parameters. By combining associations networks and metagenomic analysis, we could determine the changes in community structure during three phases of methane production process following a large disturbance to the system. In addition, sampling efforts combined with laboratory experiments are required to further obtain fundamental insight into microbial ecological networks in complex environmental habitats.

Additionally, in this study, we describe a novel framework and approach for discerning network interactions using highthroughput sequencing-based metagenomic data. The approach developed would allow microbiologists to address research questions (network interactions) which could not be approached previously and thus should represent a research paradigm shift in metagenomic analysis.

4.6. Acknowledgement

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4.7. References

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5. BIOCHEMICAL METHANE POTENTIAL OF CHICKEN LITTER ADDED WITH PROPIONATE IN MESOPHILIC CONDITIONS

POTENCIAL BIOQUÍMICO DE METANO DE POLLINAZA ADICIONADA CON PROPIONATO EN CONDICIONES MESOFÍLICAS

5.1. Abstract / Resumen

El objetivo del trabajo fue determinar el potencial bioquímico de metano de pollinaza en combinación con una alta concentración de propionato, empleando un consorcio microbiano previamente adaptado a elevadas cantidades de este metabolito. La pollinaza al 3 % de sólidos totales (ST) con 4895 ppm de propionato fue degradada en condiciones mesofílicas empleando microcosmos con un volumen de trabajo de 250 mL. Los resultados del rendimiento de metano acumulado indicaron un comportamiento triple sigmoidal; lo cual podría atribuirse a la diferencia en las velocidades de degradación de los componentes, tales como macromoléculas y ácidos grasos volátiles. El potencial bioquímico de metano fue de 364,52 mL CH_4 g $_{SV\ alimentados}^{-1}$.

Palabras clave: biogás; digestión anaeróbica; modelo de Gompertz; Potencial Bioquímico de Metano

The objective of this work was to determine the biodegradability potential of chicken litter in combination with a high concentration of propionate, using a microbial consortium previously adapted to high amounts of this metabolite. The chicken litter 3 % of total solids (TS) with 4895 ppm of propionate was degraded under mesophilic conditions using microcosms with a working volume of 250 mL. The accumulated methane yield results indicated triple sigmoidal behavior, which could be attributed to the difference in the degradation rates of the components, such as macromolecules and volatile fatty acids. The biochemical potential of methane was 364,52 mL CH_4 g $_{VS\ fed}^{-1}$.

Keywords: anaerobic digestion; Biochemical Methane Potential; biogas; Gompertz model

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Thesis of Ph.D. in Ciencias Agroalimentarias, Doctorado en Ciencias Agroalimentarias, Departamento de Ingenieria Agroindustrial, Universidad Autonoma Chapingo
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5.2. Introducción

La digestión anaeróbica es una biotecnología empleada para el manejo de residuos que emplea un consorcio de microorganismos muy diverso para convertir materia orgánica en biogás rico en metano, este proceso tiene lugar a través de una compleja cascada de reacciones impulsadas por microorganismos que incluyen hidrólisis, fermentación (es decir, acidogénesis y acetogénesis) y metanogénesis (Alvarado, Montañez-Hernández, Palacio-Molina, Oropeza-Navarro, et al., 2014). La alta complejidad dentro del microbioma hace que el proceso sea vulnerable a la inestabilidad (perturbación) debido a la inhibición mediante la acumulación de ácidos grasos de cadena larga (AGCL), ácidos grasos volátiles (AGV), amoníaco libre y otros compuestos o condiciones operativas desfavorables, como la temperatura y el pH (Chen et al., 2014), estas perturbaciones pueden tener un impacto importante en las poblaciones microbianas, así como en el rendimiento del biorreactor. La presencia de altos niveles de ácidos grasos volátiles (AGV) disminuye el rendimiento en la producción de biogás; el propionato tiene un impacto perjudicial sobre los microorganismos metanógenos (Rétfalvi et al., 2011), modificando la abundancia de algunos grupos microbianos tales como bacterias sintróficas y géneros como *Acetanaerobacterium* y *Ruminococcus* (Tian et al., 2015). En este sentido, uno de los retos más importantes es el control de la concentración de propionato mediante la degradación del mismo (Ahlert et al., 2016). Una alternativa para evitar la inhibición de la metanogénesis es obtener un consorcio microbiano resiliente ante la acumulación de propionato. El objetivo de este trabajo fue evaluar el potencial de biodegradabilidad de pollinaza adicionada con un alto nivel de propionato utilizando un consorcio microbiano capaz de degradar propionato, en condiciones mesofílicas, mediante un ensayo de potencial bioquímico de metano (PBM) usando el Modelo modificado de Gompertz ya que, de acuerdo al estudio realizado por P. Li et al. (2019), este modelo tuvo un mejor ajuste de datos en la determinación del PBM de la digestión anaeróbica de residuos de cultivos vegetales, comparado con el modelo cinético de primer orden y el modelo de Chen y Hashimoto.

5.3. Metodología

5.3.1. Ensayo experimental.

Como sustratos se emplearon: pollinaza al 3 % ST (Tepetlaoxtoc, México) y 4 895 ppm de propionato suministrado como propionato de sodio (Sigma Aldrich, EE. UU.). El experimento se llevó acabo en microcosmos con un volumen de 250 mL; el inóculo añadido fue del 10 % (v/v), se obtuvo de un digestor que fue enriquecido durante 4 años con incrementos en la concentración de propionato en la alimentación (hasta 2 000 ppm de propionato de sodio). Los microcosmos fueron purgados con nitrógeno e incubadas a 37 °C ±1 °C durante 280 días.

5.3.2. Métodos analíticos.

Sólidos totales (ST), sólidos volátiles (SV) y la demanda química de oxígeno (DQO) fueron determinados empleando métodos estándares (APHA, 2005) mientras que, el nitrógeno amoniacal ($N\text{-}NH_3$) fue determinado mediante el método HACH 10031 (HACH, 2014). El pH fue medido con un potenciómetro (Thermo Scientific Orion 5 Star, Singapore), el perfil de AGV se determinó mediante el procedimiento descrito por Meneses-Reyes et al., (2017) utilizando un cromatógrafo de gases (Claurus 500, Perkin Elmer, E.U.A.) equipado con detector de ionización de flama (FID) y una columna capilar Elite-FFAP 30 m x 0,32 mm (Perkin Elmer, E.U.A.). Las condiciones de operación fueron las siguientes: flujo de gas acarreador (helio) 5 mL min^{-1} a 10,6 psi, temperatura del puerto de inyección de 150 °C, temperatura del horno de 100 °C y del detector de 250 °C. Se utilizaron estándares grado analítico de 9 ácidos grasos volátiles, acetato, propionato, isobutirato, butirato, isovalerato, valerato, isocaproato, caproato y heptanoato (46975-U, Brand Sigma-Aldrich, E.U.A.).

La producción de biogás fue cuantificada por el método de desplazamiento en agua salina 10 % (v/v). El contenido de metano se determinó usando un cromatógrafo de gases (Claurus 500, Perkin Elmer, E.U.A.) equipado con detector de ionización de flama (FID), se tomaron muestras de biogás de 10 µL para ser inyectadas en el cromatógrafo bajo las siguientes condiciones de

operación: se utilizó helio como gas acarreador a una velocidad de 14 mL min $^{-1}$ a 14 psi y 100 °C, la temperatura del horno fue de 70 °C y del detector de 100 °C. El porcentaje de metano se obtuvo utilizando una curva de calibración con metano puro (HDSP No. P-4618-F, Praxair, México). El porcentaje de metano se reporta en condiciones estándares de presión y temperatura.

5.3.3. Modelado de la cinética de producción de metano.

La producción de metano se ajustó al modelo modificado de Gompertz (Zwietering et al., 1990), el cual se puede observar en la ecuación 1:

$$RMA = PBM \cdot exp\left\{-exp\left[\frac{\mu_m \cdot e}{PBM}(\lambda - t) + 1\right]\right\} \qquad \text{Ecuación 1}$$

Dónde RMA, es el rendimiento de metano acumulado, y corresponde a la producción de metano acumulada (mL CH_4 $g^{-1}_{SV\ alimentados}$), a un tiempo t (d), el PBM, es el potencial bioquímico de metano (mL CH_4 $g^{-1}_{SV\ alimentados}$), μ_m es la tasa de producción de metano por día (mL NCH_4 $g^{-1}_{SV\ alimentados}$ d^{-1}), e es la base de logaritmos naturales (2,718282), λ es el tiempo de la fase de latencia o retraso (d), t es el tiempo de digestión (d). El modelo se aplicó a cada una de las etapas de la digestión, sin embargo, λ se calculó únicamente en la primera etapa debido a que en la segunda y tercera etapa los microorganismos ya estaban adaptados; y PBM, solamente se consideró en la tercera etapa pues representa la producción total del proceso de digestión. Todos los modelos tuvieron un ajuste de 0,99 ± 0,005. Los parámetros del modelado se obtuvieron con el paquete SigmaPlot versión 13,0.

5.4. Resultados y discusión

Las características químicas iniciales y finales para los microcosmos fueron las siguientes: C/N de 8,15 para A y B; pH inicial 8,12 para A y B, pH final 7,3 para A y 7.61 para B; DQO (mg L^{-1}) inicial de 30326,89 para A y de 29970,57 para B, final de 20706,45 para A y 14649,15 para B; y N-NH_3 (mg L^{-1}) incial de 267,45 para A y 267,80 para B, final de 475,95 y 680,30 para A y B respectivamente. El porcentaje de sólidos totales en la alimentación para A y B fue de 3,07 %,

mientras que la relación SV/ST (%) fue de 43,36 y 43,03 respectivamente. Con base en los resultados obtenidos se infiere que B tuvo una mayor degradación de sólidos totales ya que, presenta una mayor concentración de N-NH_3 (53,63 % más) y una mayor reducción de DQO (59,26 % más) en comparación con A. La producción de metano acumulado durante 280 días del experimento se muestra en la Figure 14, donde puede apreciarse un comportamiento de triple sigmoide. Cada sigmoide se analiza acontinuación.

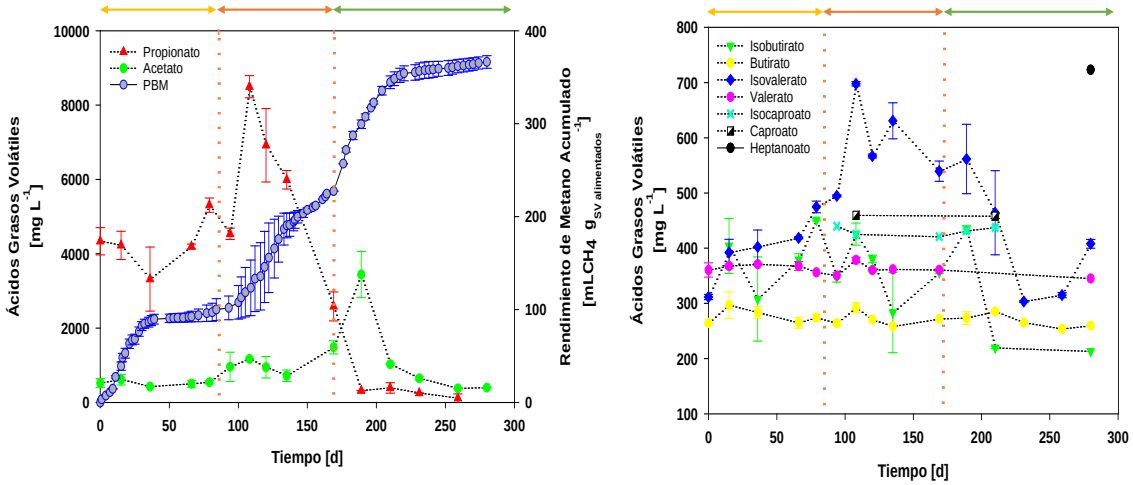

Figure 21. Comportamiento del rendimiento de metano acumulado y ácidos grasos volátiles de la digestión anaeróbica de pollinaza-propionato en función del tiempo. a) PBM - concentración de acetato y propionato. b) Comportamiento de AGVs encontrados en menor concentración.

Primera sigmoide: se pueden apreciar los tres periodos típicos de la curva de producción de metano: I) fase lag (tiempo necesario para obtener un 10 % de degradación del sustrato); II) fase exponencial (expresada como la pendiente máxima de la curva de producción de metano) y III) fase estacionaria (acumulación asintótica de metano, rendimiento máximo de biodegradabilidad del sustrato); Con base en los resultados observados en la Figura 1 y el valor de μ en la primera etapa (λ = 4,33; μ = 3,86) se puede atribuir la primera sigmoide a una reducción en la concentración de propionato, acompañada de un aligera degradación del butirato e isobutirato, dando lugar a un incremento en la producción de metano. Además, en la fase estacionaria se observa que la concentración de propionato y de isovalerato se eleva, dando lugar a la segunda sigmoide.

Segunda sigmoide: en esta etapa solo se aprecian las fases exponencial y estacionaria (Figura 1). Esta etapa tiene un valor menor de μ (2,70), que puede ser atribuída a una inhibición en el proceso de producción de metano debido a la alta concentración de propionato, sin embargo, posiblemente este mismo es convertido a acetato por los microorganismos acetogénicos, por lo que se logra apreciar en la Figura 1a una disminución de este AGV acompañado de un incremento en la concentración de acetato, además, en la Figura 1b se observa que durante esta etapa existe una acumulación de isovalerato y en menor medida butirato y valerato, los cuales pueden ser convertidos a acetato, lo que permite que la producción de metano se incremente, dando origen a la tercera sigmoide.

Tercera sigmoide: En la Figura 1 se pueden apreciar la fase exponencial y la fase estacionaria de esta etapa, las cuales fueron originadas en primer lugar por un incremento en la concentración de acetato (proveniente de la degradación de algunos AGVs en la segunda etapa) y, en segundo lugar por su evidente degradación así como del resto de los ácidos grasos volátiles originando que se alcance la mayor producción de metano. En esta etapa existe un incremento en μ_m de 168% y 241% respecto a la primera y segunda sigmoide (valor de μ = 6,50) lo que implica que en esta etapa la velocidad de producción de metano fue mayor posiblemente debido a la disminución en la concentración de propionato en el sistema, lo que pudo ocasionar que se reestableciera la abundancia y diversidad de algunos grupos microbianos tales como bacterias sintróficas y géneros como *Acetanaerobacterium* y *Ruminococcus* de acuerdo con lo reportado por Tian et al. (2015).

De acuerdo con Yang et al., (2015), la concentración de ácidos grasos volátiles es un parámetro importante durante el proceso de digestión anaeróbica. Como se observa en la Figura 1, la concentración de AGVs cambia durante todo el experimento; estos cambios pueden deberse principalmente a la transformación de los compuestos orgánicos a ácidos grasos de cadena corta por microorganismos acidogénicos. Posteriormente, el acetato y los AGVs de cadena de más de 2 carbonos que son transformados a acetato por microorganismos

acetogénicos, son convertidos a CH_4 y CO_2 por microorganismos metanogénicos. Por tanto, en la Figura 1 se puede observar que cuando la concentración de AGVs es elevada comienza a producirse una mayor cantidad de metano dando lugar a la siguiente sigmoide.

Los resultados obtenidos en trabajos similares como, Meneses-Reyes et al., (2017) (duración del experimento 90 días) y Jing et al., (2017) (30 días de experimentación), se analizaron el PBM de pollinaza pura y propionato respectivamente. Se observó que el rendimiento de metano obtenido en esta investigación (PBM = 364,52 mL CH_4 g $_{svalimentados}^{-1}$) es 340 % mayor que el alcanzado con pollinaza pura como sustrato por Meneses-Reyes et al., (2017) y 832,24% mayor que el obtenido a partir de la degradación de propionato de acuerdo a lo reportado por Jing et al., (2017). Este contraste en los resultados posiblemente sea consecuencia de las diferencias en la composición de la comunidad microbiana del inóculo utilizado en el proceso de digestión anaeróbica, lo que genera resultados diferentes en la biodegradabilidad de un sustrato como lo concluyó Raposo et al., (2012) al analizar y comparar diversos trabajos sobre biodegradabilidad anaeróbica de sustratos orgánicos. Así mismo, en un estudio realizado por Rodrigues, Rodrigues, Klepacz-Smolka, Martins, and Quina (2019) al comparar el PBM de diferentes fuentes de sustratos concluyeron que, aquellos provenientes de plantas, vegetales, cereales y estiércol muestran mejores rendimientos en la producción de metano (110 – 450 mL CH_4 g $_{svalimentados}^{-1}$), debido a su contenido de materia orgánica y fracción biodeegradable. Por otro lado, Acharya et al., (2015) al analizar el empleo de cultivos enriquecidos degradadores de propionato, acetato y butirato en el proceso de DA sugiere que aumentan la estabilidad del sistema, lo que se reflejaría en mejores rendimientos en la producción de metano. Esto demuestra que un cultivo microbiano adaptado a condiciones de estrés (resiliente) tiene una mayor capacidad de bioconvertir material orgánico en metano. Varios estudios encontraron que la adición de cultivos que utilizan propionato (Schauer-Gimenez et al,. 2010; Tale et al., 2015) o cultivos de degradación de AGVs (Acharya et al., 2015) podría reducir la acumulación de propionato y mejorar el proceso de

digestión anaeróbica. Por otro lado, la tasa de producción de metano por día (μ_m) se ha identificado como un parámetro apropiado para evaluar la idoneidad del inóculo (Moreno-Andrade y Buitrón, 2004) de tal manera que la μ_m obtenida en el presente estudio es 42 % mayor que la obtenida por Meneses-Reyes et al., (2017) y 34 % mayor que la obtenida en el trabajo de Jing et al., (2017), lo que implica que se pueden obtener mejores resultados en la degradación del mismo sustrato (pollinaza o propionato) empleando un proceso en co-digestión y un inóculo adapatado a altas concentraciones de propionato, lo que también, se ve reflejado en un menor tiempo de latencia en comparación con los trabajos de pollinaza y propionato.

5.5. Conclusiones

Se determinó el potencial bioquímico de metano de pollinaza enriquecida con propionato, obteniéndose 364,52 mL CH_4 g $_{svalimentados}^{-1}$,; el tiempo de latencia fue de sólo 4,33 d, lo cual podría indicar que el consorcio microbiano involucrado en el proceso de digestión anaeróbica estaba adaptado a altas concentraciones de propionato, por lo que es capaz de biodegradar este sustrato. Como recomendación para futuros trabajos, se sugiere hacer pruebas del inóculo utilizando diferentes sustratos y diferentes concentraciones de propionato.

5.6. Reconocimientos

Esta investigación fue apoyada por la Universidad Autónoma Chapingo. El primer autor agradece al CONACYT por la beca otorgada para la realización de estudios de Doctorado.

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6. GENERAL CONCLUSIONS

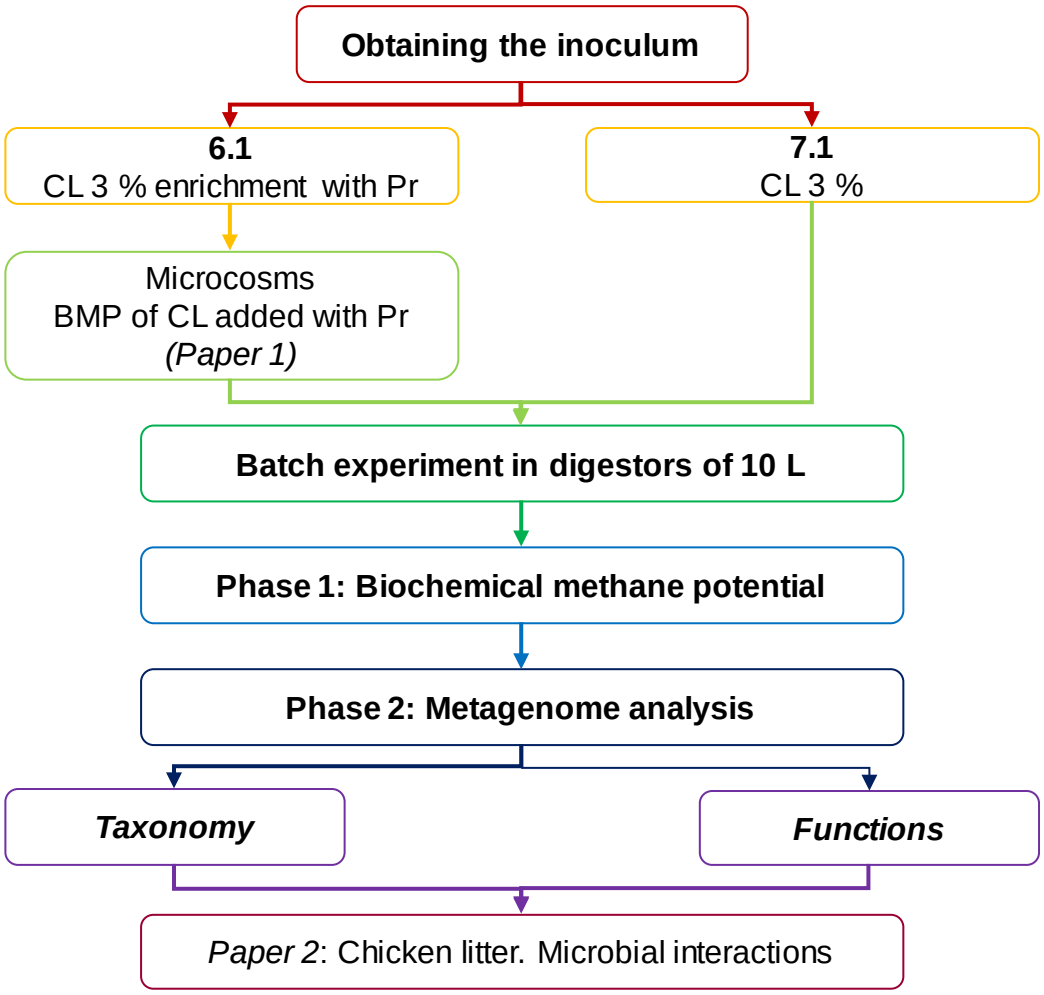
The result of the project shown interesting results. The enriched inoculum used to propionate degradation in microcosmos, not only can it survive high concentrations of propionate, but it also takes advantages of propionate and increase methane yield. Taking in consideration the problem propionate accumulation could causes on AD process, using adapted microbial cultures to degrade propionate is a useful way to avoid the process fail and improving methane production in AD process.

On the other hand, identify key populations in a community is a challenge, because of the high diversity. In addition, microorganisms do not exist in isolation but form complex ecological interaction webs with several interaction types

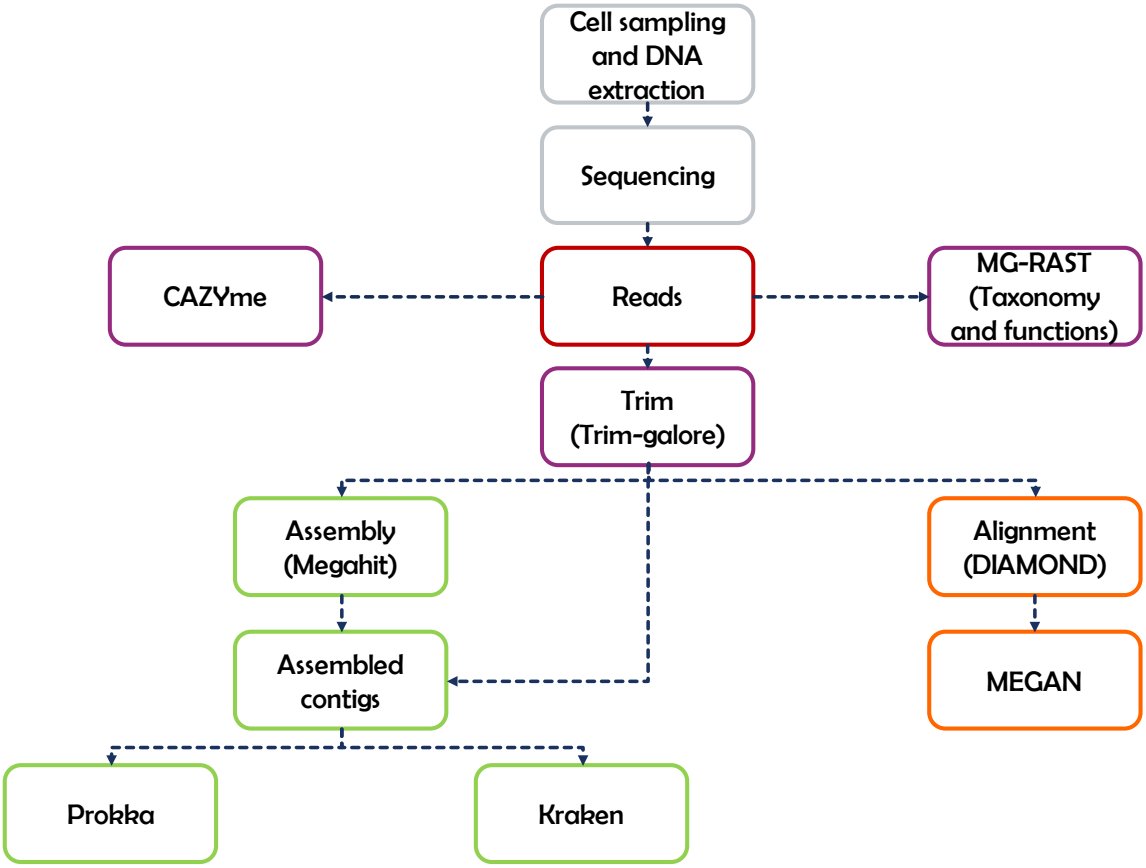
Comprehensive information on microbial species or taxonomic units across relatively large numbers of samples was essential in detecting the co-occurrence relationships among microbial communities using network analysis. Using a simulation approach it has shown that co-occurrence networks can indeed identify putative interactions between microorganisms in the environment, but that the performance of networks is highly dependent on several factors and are therefore a powerful tool for generating hypotheses about interactions that can then be tested in targeted experiments.

7. ANNEXES

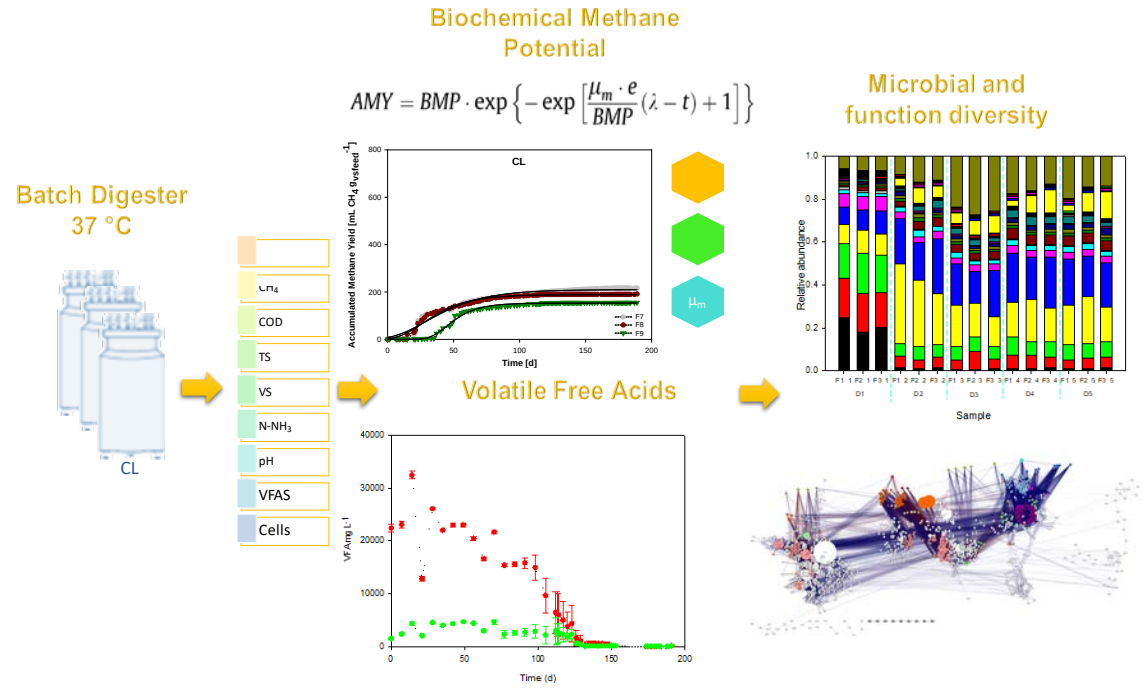
7.1 General work scheme



7.2. Metagenome analysis workflow



7.3. General graphical abstract



7.4. Data not presented in the text

Table 8.Number of sequences obtained by Illumina HiSeq paired-end format.

Sample	Raw reads	Sample	Raw reads	Q30(%)
s19045D_01_31_R1	15982669	s19045D_01_31_R2	15982669	93.86
s19045D_01_32_R1	17444122	s19045D_01_32_R2	17444122	93.93
s19045D_01_33_R1	19283866	s19045D_01_33_R2	19283866	94.37
s19045D_01_34_R1	16253340	s19045D_01_34_R2	16253340	93.95
s19045D_01_35_R1	18514290	s19045D_01_35_R2	18514290	93.85
s19045D_01_36_R1	15517473	s19045D_01_36_R2	15517473	94.16
s19045D_01_37_R1	19077745	s19045D_01_37_R2	19077745	94.09
s19045D_01_38_R1	17774890	s19045D_01_38_R2	17774890	94.06
s19045D_01_39_R1	16216965	s19045D_01_39_R2	16216965	93.32
s19045D_01_40_R1	21317120	s19045D_01_40_R2	21317120	93.69
s19045D_01_41_R1	20287397	s19045D_01_41_R2	20287397	93.91
s19045D_01_42_R1	18344247	s19045D_01_42_R2	18344247	93.85
s19045D_01_43_R1	19574099	s19045D_01_43_R2	19574099	93.91
s19045D_01_44_R1	17554675	s19045D_01_44_R2	17554675	93.68
s19045D_01_45_R1	19945994	s19045D_01_45_R2	19945994	93.68
s19045D_01_46_R1	16687092	s19045D_01_46_R2	16687092	92.52
s19045D_01_47_R1	17121002	s19045D_01_47_R2	17121002	93.43
s19045D_01_48_R1	18493236	s19045D_01_48_R2	18493236	93.85

Note: Each sample has two reads, forward and revers, because of this, is shows two columns with the same number of sequences.

Table 9. Number of sequences after trimming

Sample	Raw reads	Sample	Raw reads
s19045D_01_31_R1	15978673	s19045D_01_31_R2	15978673
s19045D_01_32_R1	17440385	s19045D_01_32_R2	17440385
s19045D_01_33_R1	19279906	s19045D_01_33_R2	19279906
s19045D_01_34_R1	16249846	s19045D_01_34_R2	16249846
s19045D_01_35_R1	18510295	s19045D_01_35_R2	18510295
s19045D_01_36_R1	15514477	s19045D_01_36_R2	15514477
s19045D_01_37_R1	19073064	s19045D_01_37_R2	19073064
s19045D_01_38_R1	17770816	s19045D_01_38_R2	17770816
s19045D_01_39_R1	16213623	s19045D_01_39_R2	16213623
s19045D_01_40_R1	21312107	s19045D_01_40_R2	21312107
s19045D_01_41_R1	20283264	s19045D_01_41_R2	20283264
s19045D_01_42_R1	18340491	s19045D_01_42_R2	18340491
s19045D_01_43_R1	19569179	s19045D_01_43_R2	19569179
s19045D_01_44_R1	17550283	s19045D_01_44_R2	17550283
s19045D_01_45_R1	19941414	s19045D_01_45_R2	19941414

Sample	Raw reads	Sample	Raw reads
s19045D_01_46_R1	16683017	s19045D_01_46_R2	16683017
s19045D_01_47_R1	17116813	s19045D_01_47_R2	17116813
s19045D_01_48_R1	18488880	s19045D_01_48_R2	18488880

7.5. Network analysis

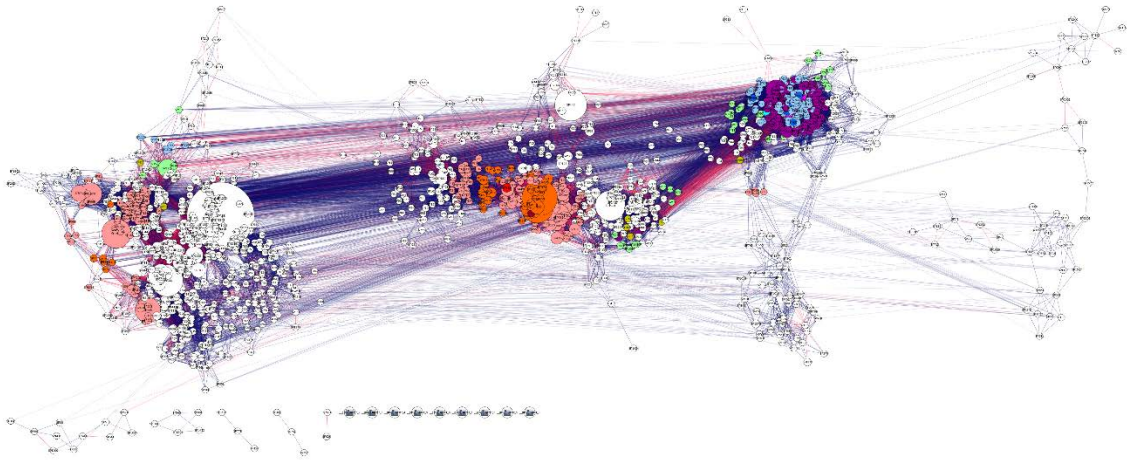


Figure 22. Orginial network structure for phase 2 (P2)