UNIVERSIDAD AUTÓNOMA CHAPINGO

DEPARTAMENTO DE IRRIGACIÓN E INGENIERÍA MECÁNICA Y AGRICOLA

POSGRADO EN INGENIERÍA AGRÍCOLA Y USO INTEGRAL DEL AGUA

CHARACTERIZATION OF ENRICHED DIGESTERS WITH HIGH CONCENTRATIONS OF PROPIONATE AND CHICKEN LITTER IN ANAEROBIC DIGESTION

THESIS

In partial fulfillment of the requirements for the degree of:

DOCTOR EN INGENIERÍA AGRÍCOLA Y USO INTEGRAL DEL AGUA

By:

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APROBADA

Chapingo, Estado de México, October 2024

CHARACTERIZATION OF ENRICHED DIGESTERS WITH HIGH CONCENTRATIONS OF PROPIONATE AND CHICKEN LITTER IN ANAEROBIC DIGESTION

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DEDICATION

To my father, Juan Jose Silvestre Ochoa Garcia, who left this life, and I cannot say goodbye to him the best way.

ACKNOWLEDGEMENTS

To Consejo Nacional de Humanidades Ciencias y Tecnologías (CONAHCYT) for the scholarship received for postgraduate studies.

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RESUMEN GENERAL

Caracterización de digestores enriquecidos con altas concentraciones de propionato y pollinaza en la digestión anaeróbica

El proceso biológico de la digestión anaeróbica involucra cuatro pasos metabólicos principales: 1) hidrolisis, 2) acidogénesis, 3) acetogénesis y 4) metanogénesis. Estos pasos son impulsados por una compleja red de microorganismos que trabajan al unísono para descomponer completamente los compuestos orgánicos en biogás. El propionato representa un intermediario clave en la digestión anaeróbica, aproximadamente del 20 % al 43 % de la producción de metano proviene de su oxidación completa. Las sobrecargas orgánicas inducen concentraciones de propionato altas que superan los 2 g L^{-1}. En esas condiciones, los procesos metanogénicos fallan y generan desequilibrios en el proceso. El objetivo de este trabajo fue caracterizar un digestor en estado continuo en condiciones mesofílicas (35±5 °C), alimentado con pollinaza al 3 % de solidos totales y enriquecimientos graduales con alimentación de 0.5, 0.8, 1.0, 1.2, 1.4, 1.6, 1.8, 2.0, 6.0, 12.0, 18.0 y 24.0 g L^{-1} de propionato de sodio, para el estudio de los cambios en las poblaciones microbianas. El consorcio microbiano desarrollado fue tolerante a altas concentraciones de propionato. Para dilucidar su papel en la degradación del propionato, se tomaron 18 muestras de ADN en puntos estratégicos del enriquecimiento y se sometieron a análisis bioinformático. Este análisis reveló la presencia de especies que no actúan en sintrofía y facilitan la degradación de propionato. Además, hubo un aumento sustancial del 50 % en las arqueas metanogénicas. El microbioma desarrollado permitió el mantenimiento estable de la digestión anaeróbica, asegurando una producción constante de metano sin inhibición de la metanogénesis a pesar de las altas concentraciones de propionato.

Palabras clave: Propionato, Pollinaza, Digestión anaeróbica, Metagenómica, Taxonomía

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GENERAL ABSTRACT

Characterization of digesters enriched with high concentrations of propionate and chicken litter in anaerobic digestion

The process of anaerobic digestion (AD) involves four key metabolic steps: 1) hydrolysis, 2) acidogenesis, 3) acetogenesis, and 4) methanogenesis. These steps are driven by a complex network of microorganisms working in unison to fully break down organic compounds into biogas. Propionate serves as a crucial intermediate in anaerobic digestion, with about 20 % to 43 % of methane production stemming from its complete oxidation. High concentrations of propionate, exceeding 2 g L^{-1}, can result from organic overloads, leading to the failure of methanogenic processes and process imbalances. This study aimed to characterize a digester under continuous mesophilic conditions (35 ± 5 °C), fed with chicken litter 3 % of total solids and gradual enrichments with 0.5, 0.8, 1.2, 1.4, 1.6, 1.8, 2.0, 6.0, 12.0, 18.0, and 24.0 g L^{-1} of sodium propionate, to analyze changes in microbial populations. The microbial consortium that developed exhibited tolerance to high propionate concentrations. To elucidate its role in the degradation of propionate, 18 DNA samples were taken at strategic points of the enrichment and subjected to bioinformatic analysis. This analysis revealed the presence of species that do not act in syntrophy and facilitate propionate degradation. Furthermore, there was a substantial 50 % increase in methanogenic archaea. The developed microbiome enabled the stable maintenance of anaerobic digestion, ensuring consistent methane production without inhibition of methanogenesis despite high propionate concentrations.

Keywords: Propionate, Chicken litter, Anaerobic digestion, Metagenomics, Taxonomy

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1 GENERAL INTRODUCTION

An estimated 11.2 billion tons of solid waste is collected worldwide each year, while the disintegration of the organic proportion of this solid waste contributes approximately 5% of global greenhouse gas emissions (ONU, 2023), and 33 % are not appropriately managed (Shah et al., 2021). In this sense, biomass is a general term used to describe waste derived from animals and plants (Ma et al., 2018), such as agricultural waste, food and industrial waste, and forest waste, each with its own characteristics and biodegradability that can be used. as a mono-substrate or by co-digestion method (Guan et al., 2019).

Due to the constant growth in the volume and complexity of urban and industrial waste, waste management has been identified as one of society's current and future problems (Shah et al., 2021). Anaerobic digestion (AD) offers an alternative for treating, stabilizing, minimizing, and recovering nutrients from waste through the application of digesters (Capson-Tojo et al., 2018). Additionally, it is a widely applied technology to generate renewable energy (Guan et al., 2019) that can effectively reduce our dependence on fossil fuels and mitigate greenhouse gas emissions (Wang et al., 2023).

AD is powered by an intricate network of microorganisms from the domains of bacteria and archaea, collaborating for the complete degradation of organic compounds. Either in the form of heat, electricity, and/or biofuels such as methane (CH_4), carbon dioxide (CO_2), and hydrogen (H_2) (Shah et al., 2024). Four main metabolic steps are involved in this network: 1-hydrolysis, 2-fermentation, 3-acetogenesis, and 4-methanogenesis (Zinder, 1984). Various microbial groups are involved in these steps, each specializing in different processes.

Various hydrolytic and fermentative bacteria are involved in the initial two steps, while the oxidation of fermentation intermediate products to acetate ($[C_2H_3O_2]^-$) is carried out by acetogens producing hydrogen (H_2) or formiate (HCOO). Methane is then exclusively produced from acetate ($[C_2H_3O_2]^-$) and H_2/CO_2 by methanogenic archaea (Stams & Plugge, 2009). However, when overloaded with

biomass, digesters generally suffer from acidification and foaming, consequently clogging pumps, breakage of digesters, environmental problems derived from sludge overflow, and financial losses due to reduced biogas production and additional costs of maintenance (Mutungwazi et al., 2023).

One of the main precursors of the instability of the process is the acidification of the medium generated by the accumulation of short-chain fatty acids, mainly propionate (Ahring et al., 1995; Boe et al., 2010; Gallert & Winter, 2008; Ma et al., 2019; Wang et al., 2006). It has been reported that concentrations greater than 900 mg L^{-1} of propionic acid generate disturbances in the system, affecting the performance and inhibition of methanogens. It is difficult to degrade, and thermodynamically it is an extremely endergonic reaction with a change in Gibbs energy (ΔG°') of +76 kJ per mole of reaction, with a partial pressure of hydrogen less than 10 Pa and $[C_2H_3O_2]^-$ concentration less than 0.8 g L^{-1} (Wang et al., 2009). However, in methanogenic environments, propionate degradation is based on syntrophic associations between propionate-oxidizing bacteria (SPOB) and methanogenic archaea that allow a favorable energy balance, allowing its conversion to $[C_2H_3O_2]^-$, H_2, and CO_2. These substrates produce methane (CH_4) during methanogenesis (Dyksma & Gallert, 2019).

Propionate represents a key intermediate in anaerobic digestion; approximately 20 % to 43 % of CH_4 production comes from its complete oxidation (Bottausci et al., 2022). This substrate serves as a valuable model for investigating the interconnected, mutually beneficial relationships in energy-constrained methanogenic ecosystems. These ecosystems can be found in various settings, ranging from natural environments such as freshwater sediments to human-made systems like anaerobic bioreactors (Chen et al., 2008). From 1980 to 2006, only 10 SPOB were isolated, and in 2019, *Candidatus Syntrophosphaera thermopropionivorans* were identified (Dyksma & Gallert, 2019).

This study aimed to assess the modifications in the structure and function of the microbial communities acquired during the gradual enrichment process spanning 13 stages. The process involved varying propionate concentrations ranging from

0.5 to 30.0 g L-1, combined with chicken litter at 3% total solids for co-digestion, in order to elucidate the adaptive response of the microbiome to a highly stressed environment.

Chapter 1: The global problem of organic waste is briefly described, using anaerobic digestion as an alternative for transforming organic waste. The obstacles that AD presents by overloading the system with these wastes, mainly propionate, as an inhibitor of the methanogenic process, are considered. Bioinformatics and omics sciences are considered tools to advance the black box of anaerobic digestion.

Chapter 2: Describes the changes in microbial communities in mesophilic digesters enriched with high concentrations of propionate in co-digestion with chicken litter. Agricultural residues and wastewater are used for methane production via anaerobic digestion. One of the main problems is the variation in the organic loading rate during feeding, which could lead to unstable processes. As a result, fatty acids accumulate, which are propionate and have the most inhibitory effect. This study applied 3 % manure and gradual enrichments in the feed for 1,200 days using stepwise propionate concentrations from 1.0 up to 24.0 g L^{-1} under mesophilic conditions. The operating volume was 10 L with a semi-continuous CSRT. The hydraulic retention time was 30 days. This paper aimed to evaluate the microbial community changes along the propionate stepwise enrichment process to elucidate the microbiome response to high levels of propionate concentration. Taxonomic and metabolic functions were used as the leading indicators of the adaptation process. Physicochemical analyses, as well as methane and fatty acid concentrations, were recorded during the process. Eleven sampling points were used for shotgun metagenomic analysis. The results showed that the microbiome degraded propionate without inhibition of the process. Methane concentration ranged from 50 to 60 %. At high propionate levels, species from the *Brachymacterium, Corynebacterium, Cloacimonas, Vibrio*, and *Treponema* genera were found. It revealed that the degradation of high propionate concentration levels could be attributed more to propionate-degrading

bacteria via ackA and pta genes than to propionate-oxidizing bacteria; the lactate pathway was also detected via the pct gene. Archaea populations increased their relative abundance during the enrichment process, mainly with the *Methanoculleus, Methanospirillum*, *Methanococcus,* and *Methanocella* genera. Synthesizing methane through CO_2 and acetoclastic were the principal pathways observed.

2 GENERAL LITERATURE REVIEW

2.1 Problem of Agricultural Waste

Agricultural and livestock waste, also known as agricultural waste, is experiencing exponential growth (Sheer et al., 2024). Globally, it is estimated that an approximate 998 million tons of agricultural waste, including by-products from farming and livestock production, are generated annually (Parlato et al., 2022). By 2050, the world is expected to generate 3.4 billion tons of waste annually, a dramatic increase from 2.01 billion tons today (Kaza et al., 2018). Agricultural waste, such as crop residues (horticulture, stubble, husks), and livestock waste, such as manure (chicken, cow, pig, horse, wild animals) are the types of waste that constitute an important part of these materials (Nagendran, 2011). Livestock waste is dominated by manure, with 20 million tons per day of production (Chávez-Fuentes et al., 2017). Figure 1 shows the types of agricultural waste (Sheer et al., 2024).

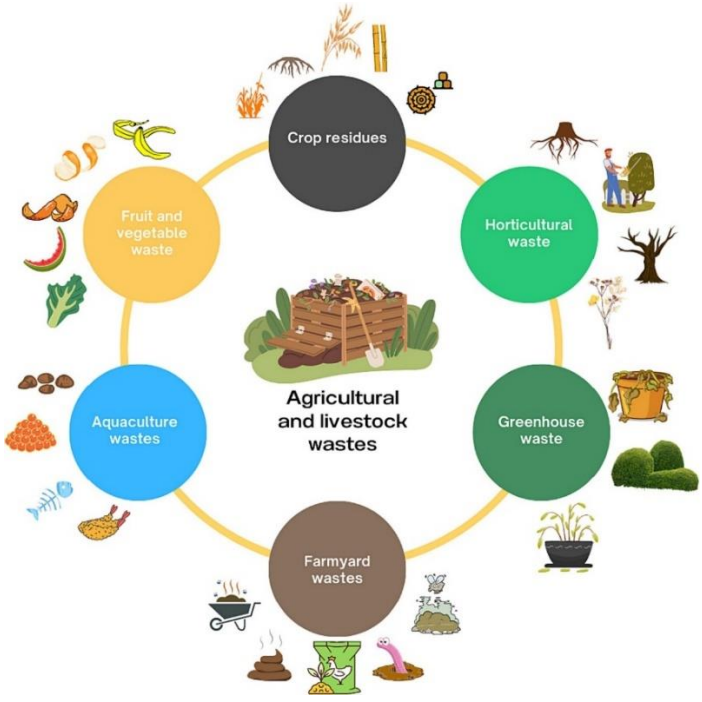

Figure 1. Multiple sources and categories of agricultural and livestock waste material. Taken from Shee et al., 2024.

Poor management of agricultural waste creates several critical environmental problems (Parlato et al., 2022), ecological nuisances (van Zanten et al., 2015), environmental damage and water pollution such as nitrate leaching (Forge et al., 2016), soil with heavy metals (Li et al., 2018), and of the air with the production of greenhouse gas emissions, with serious consequences for the life of our planet (Parlato et al., 2022).

Agricultural animals are responsible for about 12 % of greenhouse gas emissions. Cattle are the main source of emissions (62 %), followed by pigs (14 %), chickens (9 %), buffaloes (8 %), and sheep and goats (7 %). From the production of feed for livestock to the arrival of the food to the stores, 6.2 gigatons of CO_2 equivalent were generated (FAO, 2023). Additionally, farm animals contribute to 35-40 % of yearly human-caused CH_4 emissions due to digestive fermentation in ruminants and manure. Livestock manure also accounts for over 60 % of anthropogenic nitrous oxide (N_2O) emissions when it is deposited in the soil (Steinfeld et al., 2006); N_2O and CH_4 are greenhouse gases with a global warming potential 310 and 25 times greater than CO_2, respectively (EC, 2024; Masaka et al., 2016). In addition, emissions of ammonia (NH_3), hydrogen sulfide (H_2S), sulfur dioxide (SO_2), and volatile organic compounds contribute to the soil acidification process, water eutrophication, and tropospheric ozone pollution (Chávez-Fuentes et al., 2017).

First, efforts must be made to minimize waste production. If its generation cannot be avoided, the second option is to recover it and not dump it into the environment (Parlato et al., 2022; Sheer et al., 2024). The action for sustainable global development is the recovery, reuse, and recycling of waste to obtain new suitable products, materials, and energy (Fetting, 2020). Agricultural waste can be handled, used, and transformed as mulching (Erenstein, 2003), composting (Harindintwali et al., 2020), fertilizer (Oo et al., 2019), animal bedding (Shinde et al., 2022), biomass for the creation of bioenergy (Cu et al., 2015), and biogas through AD (Aravani et al., 2022).

Europe promotes anaerobic digestion of manure and the use of agricultural waste for bioproducts and biofuels (Rekleitis et al., 2020). South America emphasizes using biomass from agricultural waste for bedding and animal feed, the production of biofuels, and the generation of biogas from manure (Bottausci et al., 2022). Minimizing greenhouse gas emissions, promoting sustainability in the energy sector, and reducing dependence on fossil fuels are advantages of using agricultural waste for energy generation (Saleem, 2022).

2.2 Chicken litter

Chicken litter is a mixture of manure and bedding materials (wood shavings, rice husks, and straw) generated from broiler chicken farms (Zahan & Othman, 2019). It contains, expressed as a percentage in dry base, protein 24.70 ± 2.13, extract ether 1.81 ± 0.15, ash 20.13 ± 0.12, carbohydrates 19.65 ± 3.52, crude fiber 16.25 ± 0.37. In the case of elements, expressed as a w / w percentage, carbon .34.86 ± 1.28, hydrogen 4.80 ± 0.19, nitrogen 4.28 ± 0.08, sulfur 0.44 ± 0.04, C: N 8.15 ± 0.21 (Meneses-Reyes et al., 2017). For other compounds in [mg L^{-1}], chicken litter as ammonium (NH_4) 2.96 ± 0.06, total phosphorous 0.60 ± 0.01, volatile acids 3.56 ± 1.12, glucans 12.6 %, xylans 5.1 %, lignin 34.4 %. The total crystallinity index is 1.89 (Zahan & Othman, 2019).

Intensive chicken farming generates enormous amounts of manure biomass. It has been estimated that around 20,708 million tons are produced globally (Jurgutis et al., 2020), and only 30-40 % of this manure is converted into biogas (Bayrakdar et al., 2017). Chicken manure is traditionally disposed of as organic fertilizer for soil amendment (Lee et al., 2023). However, excessive use of chicken litter as fertilizer can enrich water nutrients, resulting in the eutrophication of water bodies, the spread of pathogens, the production of phytotoxic substances, air pollution, and greenhouse gas emissions (Kelleher et al., 2002).

Chicken litter can be converted into renewable energy through AD (Tawfik et al., 2023). However, biogas plants limit their use for renewable energy generation (Jurgutis et al., 2020). This is due to the fact that chicken litter is difficult to degrade

(Molaey et al., 2019). Chicken litter has a lignocellulosic nature, characterized by a resistant and crystalline structure that makes it difficult for bacteria to break down (Mohsenzadeh et al., 2012). Additionally, when a high organic loading level of proteins and amino acids in the manure. These proteins and urea are degraded to NH_3 (Niu et al., 2013). The accumulation of NH_3 can become toxic to anaerobic microorganisms once it reaches a certain concentration (Borja et al., 1996; Niu et al., 2013). Compared to cow manure, pig manure, food waste, and waste-activated sludge, chicken litter has a higher nitrogen content (Qiao et al., 2011). Other limiting factors in the use of chicken litter include the content of antibiotics, the production of fatty acids, and the existence of trace elements and organic compounds. Figure 2 shows the characteristics of chicken manure (Tawfik et al., 2023). It is crucial to treat chicken litter before AD to enhance the breakdown of lignocellulose, reduce its crystallinity, and eliminate its network structure by removing amorphous components (Salehian et al., 2013). AD is a sustainable solution for chicken litter management technology from the point of view of energy recovery, biofertilizer production, and greenhouse gas management (Jurgutis et al., 2020).

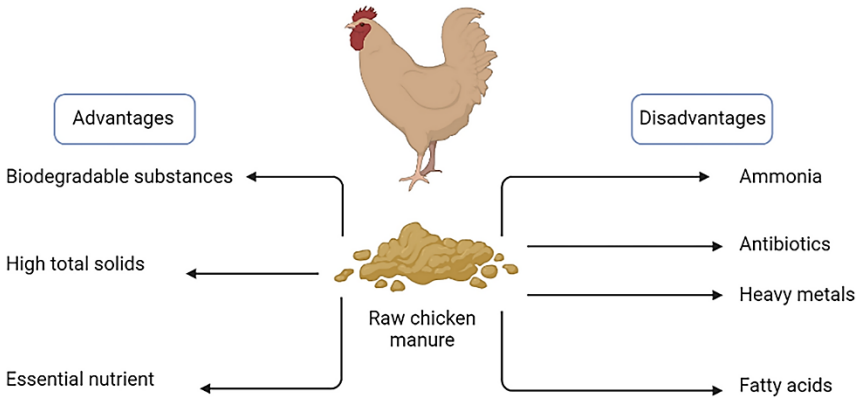

Figure 2. Characteristics of chicken litter play a key role in selecting effective management strategies. Chicken manure has an abundance of biodegradable materials, high total solids, and essential nutrients, which make it advantageous for the AD process. However, there are also unfavorable components in chicken manure, such as NH_3, antibiotics, heavy metals, and fatty acids, which may hinder the AD process. Taken from Tawfik et al., 2024.

2.3 Anaerobic Digestion

2.3.1 What is it?

Anaerobic digestion is a biological process (Leng et al., 2018b) and technology (Wang et al., 2023) used for the treatment of a wide variety of agricultural, livestock, municipal, food, biochemical, and industrial wastes; in an oxygen-free environment (Rekleitis et al., 2020) in which microorganisms mineralize organic materials in the absence of molecular oxygen (Leng et al., 2018b). It aims to convert organic waste into two categories of valuable products: 1. Biogas, a renewable fuel used to produce green electricity, heat, or vehicle fuel. 2. Digestate is used as fertilizer in agriculture. The digestate can be further refined into concentrated fertilizers, fiber products, and clean water, all suitable for recycling (Holm-Nielsen et al., 2009). Additionally, it has proven to be effective in avoiding risks associated with uncontrolled emissions of greenhouse gases (Papageorgiou et al., 2009). AD has generally been perceived as a green, competitive, promising biotechnology to address energy and environmental problems (Zhang et al., 2018).

2.3.2 Transformation Process

The basis of this technology involves the concerted action of several complex and microbial consortia (Appels et al., 2008) of hydrolytic bacteria, fermentative bacteria, sulfate-reducing bacteria, ammonifying bacteria (Salama et al., 2019), syntrophic bacteria, and methanogenic archaea; that allows the anaerobic degradation of organic matter (Kleinsteuber, 2018) and its conversion. AD is metabolically interconnected (Kundu et al., 2017), and it has its optimal working conditions (Appels et al., 2008). It involves distinctive and consecutive biochemical phases of hydrolysis, acidogenesis, acetogenesis, interspecies electron transfers (IET), and methanogenesis (Tremblay et al., 2017) (Figure 3).

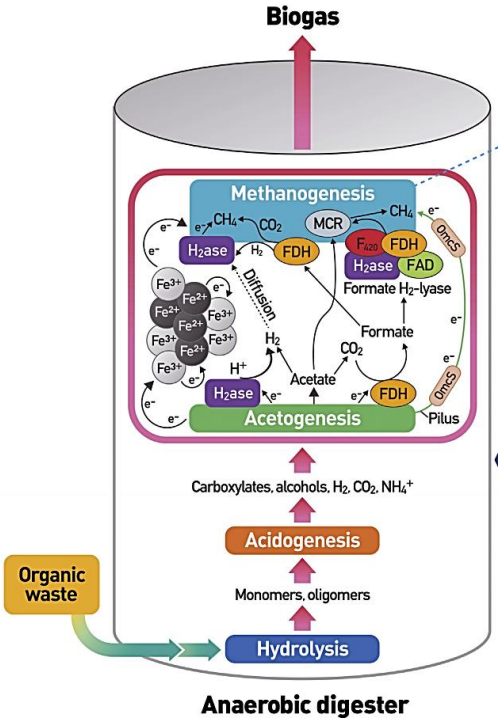

Figure 3. Anaerobic digestion process. Taken from Saha et al., 2020.

Hydrolysis

Hydrolysis is the first stage of the AD process (**Error! Reference source not found.**) and is carried out by hydrolytic microorganisms such as *Anaerosalibacter, Bacillus, Bacteroides, Butyrivibrio, Clostridium, Eubacterium, Fusobacterium, Hydrogenoanaerobacterium, Lachnoclostridium, Micrococcus, Paraclostridium,* and *Prevotella* the which secrete several hydrolytic enzymes, such as amylase, cellulase, cellobiose, lipase, protease, and xylanase (Chang et al., 2018; Saha et al., 2019). This causes insoluble organic polymers such as carbohydrates, proteins, and lipids to hydrolyze to generate monomers and soluble or insoluble oligomers. Sugars, glycerol, amino acids, and long-chain fatty acids (LCFAs) are formed at this stage (Saha et al., 2020). This critical step is known as the primary rate-limiting step in anaerobic digestion. The pace of hydrolysis directly affects the availability of the substrate for subsequent stages. If these substrates are converted too rapidly, it can lead to the swift production of volatile fatty acids (VFAs) and other potentially harmful byproducts, ultimately impeding the efficient progress of biomethanization (Appels et al., 2008).

Acidogenesis

Acidogenesis is the second stage (**Error! Reference source not found.**). It is u sually the fastest in AD's anaerobic conversion of complex organic matter (Karthikeyan et al., 2016). It is produced through hydrogenation and dehydrogenation depending on microbial metabolism (Salama et al., 2019). The products generated in hydrolysis are used as substrates by fermentative bacteria such as *Bacillus, Clostridium, Eubacterium, Hydrogenoanaerobacterium, Lachnoclostridium, Lactobacillus, Paraclostidium, Prevotella, Streptococcus,* and *Tissierella* for acid formation, i.e. acidification (Chang et al., 2018; Saha et al., 2019). The products of this stage are organic, acetic, propionic, butyric acids, short-chain fatty acids (SCFAs), alcohols (methanol, ethanol), H_2, and CO_2 (Kleinsteuber, 2018).

Acetogenesis

Acetogenesis is the third stage (Figure 4). LCFAs such as C_{13}-C_{22} are oxidized to $[C_2H_3O_2]^-$ and H_2 by acetogenic bacteria and by syntrophic fatty acid oxidants (SFAO) (Saha et al., 2020). LCFAs produced during hydrolysis are also oxidized through β-oxidation to $[C_2H_3O_2]^-$ and H_2 during acetogenesis by acetogenic proton-reducing bacteria belonging to the families *Bacteroidaeceae, Clostridiaceae, Enterobacteriaceae, Syntrophomonadaceae,* and *Dyntrophaceae* (Ziels et al., 2016). In homoacetogenesis, two moles of CO_2 are reduced with H_2 to one mole of $[C_2H_3O_2]^-$ via the Wood-Ljungdahl (WL) pathway (Schuchmann & Müller, 2014). H_2 and CO_2, which serve as electron donors and acceptors, respectively, are used by organisms to produce $[C_2H_3O_2]^-$ as an end for ATP formation (Diekert & Wohlfarth, 1994; Wood, 1991). Syntrophic acetate oxidation (SAO) is a process in which methyl groups of acetate are converted to CO_2 with the generation of H_2 (Barker, 1936) by syntrophic acetate-oxidizing bacteria (SAOB) such as *Clostridium ultunense, Syntrophomonas,* and *Syntrophus* (Amha et al., 2017).

Methanogenesis

Methanogenesis is the fourth stage of the AD process (**Error! Reference source not found.**). $[C_2H_3O_2]^-$, H_2, CO_2, and alcohols, produced during acidogenesis and acetogenesis, serve as substrates to produce CH_4 (Liu & Whitman, 2008). Through bacteria in conjunction with acetoclastic methanogens (dismutate $[C_2H_3O_2]^-$ into CH_4 and CO_2), hydrogenotrophic methanogens (reduce H_2, formate ($[HCOO]^-$), and CO_2/bicarbonate as electron acceptor) and methylotrophic methanogens (dismutate C_1 methylated compounds to CH_4 and CO_2) (Sorokin et al., 2017).

Syntrophism

Utilization of H_2 and $[HCOO]^-$ as electron carriers during interspecies electron transfer (IET) is an important means of electron exchange between syntrophic microorganisms (Shrestha & Rotaru, 2014) for the oxidation of organic matter and the reduction of CO_2 to CH_4 in the AD (Tremblay et al., 2017).

In AD, a syntrophic association (Figure 3) exists between exoelectrogenic and hydrogenogenic bacteria and acetoclastic and hydrogenotrophic methanogens. This relationship ensures the stable functioning of AD by regulating acidity and H_2 pressure within the digesters (Sorokin et al., 2017). Exoelectrogens are acidogenic and acetogenic bacteria that produce and transfer electrons, H_2, and/or $[HCOO]^-$ out of cells to insoluble metallic electron acceptors or to electrotrophic microorganisms such as methanogens. This process helps in maintaining thermodynamic stability (Kouzuma et al., 2015; Logan & Rabaey, 2012). Any slight change in the rate of H_2 consumption by methanogens can disrupt the syntrophic metabolism, leading to the accumulation of SCFAs and disturbance of the digesters (Zhao et al., 2015).

For the growth and metabolism of syntrophic microorganisms through H_2, $[HCOO]^-$, interspecies formate transfer (IFT), and interspecies hydrogen transfer (IHT) are rapidly consumed by methanogens as reducing equivalents.

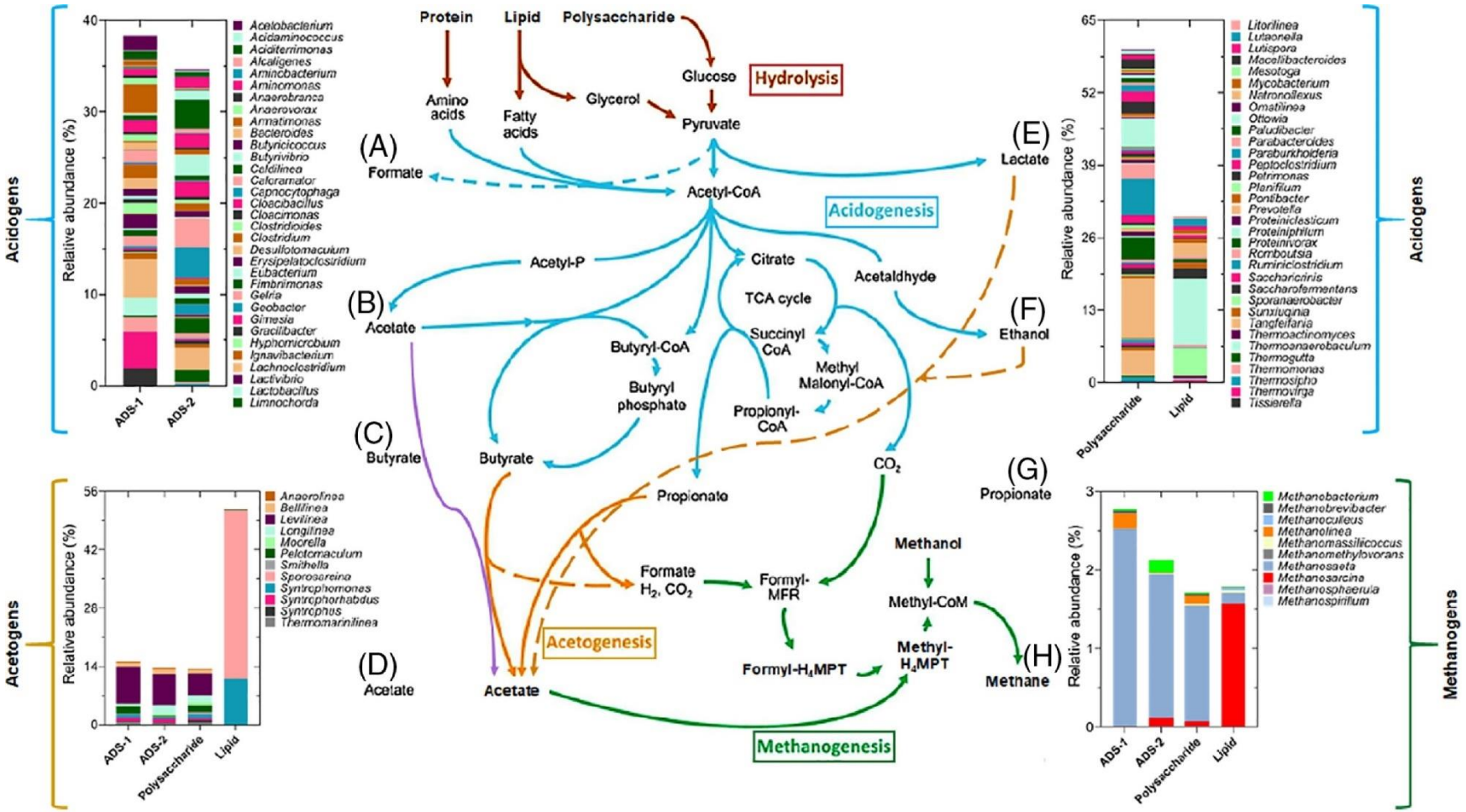

Figure 4. Metabolic reactions of AD hydrolysis, acidogenesis, acetogenesis, and methanogenesis; and the most abundant genera (> 3 %). Letters A-H are the products obtained. Taken from Saha et al., 2020.

Methanogens obtain energy from the reduction of CO_2 to CH_4 by using H_2 as an electron donor (Shrestha & Rotaru, 2014; Tremblay et al., 2017).

$[HCOO]^-$ is an important diffusive redox mediator in the methanogenic community due to its higher diffusion rate than H_2. H_2-absorbing hydrogenase is used as an electron carrier to reduce coenzyme F_{420} and oxidize ferredoxin. Reduced flavin adenin dinucleotide (FAD) and F_{420} (F_{420}-H_2) act as direct electron donors in IHT. Formate dehydrogenase (FDH) oxidizes $[HCOO]^-$ to CO_2 and further reduces it to CH_4 (Liu & Whitman, 2008).

2.3.3 End Products of Anaerobic Digestion

The final products of AD are: 1-Biogas, which is a mixture of gases that contains CH_4 (50 to 70 %), CO_2 (30 to 45 %), H_2 (1 to 3 %), oxygen (O_2) (0.5 to 1 %), various gases (1 to 5 %), and traces of SO_2 (Varnero-Moreno, 2011). CH_4 and CO_2 constitute most of the biogas produced by bacteria during digestion (Carrillo-Reyes et al., 2021). The conversion efficiency of these organic resources into biomethane is relatively high lipids 94.8 %, proteins 71 %, and carbohydrates 50.4 % contribute to 69 % of atmospheric CH_4 (Karmee, 2016). This biogas is collected and cleaned to remove contaminants and can then be used to generate energy or heat (Al Mamun & Torii, 2017). 2- Obtain bio digestate and/or fertilizers.

On the other hand, previous studies show that agricultural waste converted to bioenergy leads to reduced environmental impact (Duque-Acevedo et al., 2020), environmental sustainability (Khoshnevisan et al., 2021), sustainable agricultural practices (Koul et al., 2022), improved soil health (Khatoon et al., 2020), business creation, and opportunities within the agricultural industry. A high loading rate, low nutrient demands, and minimal operational control and maintenance costs are further advantages (Wijekoon et al., 2011).

2.3.4 Process Failures

Several factors inhibit CH_4 production during the AD process. Heavy metals, hydrocarbon compounds, H_2S, NH_3, and one of the most influential factors is the

production of VFAs. These problems lead to a significant reduction in the CH_4 production rate and digester performance.

Heavy Metals

Cobalt (Co), copper (Cu), cadmium (Cd), chromium (Cr), mercury (Hg), nickel (Ni), and zinc (Zn) are some of the main heavy metals that influence AD (Hickey et al., 1988; Zayed & Winter, 2000). They act as micronutrients in low concentrations and improve CH_4 production (Uemura, 2010). However, at high concentrations, the toxic effect of heavy metals lies in their enzymatic disruption activity, which operates either by binding to thiol and other groups in a protein molecule or by replacing natural metals in enzymatic prosthetic groups (Salama et al., 2019). The level of inhibition depends on its concentration, chemical form, redox potential, and hydrogen potential (pH) (Chen et al., 2008). Inhibition concentrations for Cu, Hg, and Zn 50 μM (Pankhania & Robinson, 1984), Cd 36 mg L^{-1}, Cr 27 mg L^{-1}, Ni 35 mg L^{-1}, and Zn 7.5 mg L^{-1} (Altas, 2009).

Hydrocarbon Compounds

Compounds such as halogenated alcohols (Blum & Speece, 1991), amines, amides, carboxylic acids, nitriles (Stergar et al., 2003), chlorophenols, benzenes, methylcyclohexane, toluene, xylenes (Sherry et al., 2014), acetylenes, chloroform, dichloromethane, ethylene, hexane, isoprene, nitrophenols, perchloroethylene, and trichloroethylene (McCue et al., 2003) are toxic to methanogens. Inhibition is affected by various factors, including exposure time, biomass load, inhibitor concentration, temperature, cell age, and methanogens' acclimatization capacity (Salama et al., 2019).

Sulfide

Sulfide is produced by sulfate-reducing bacteria (SRB), which utilize organic compounds as electron donors and convert sulfate to sulfide, a substance highly detrimental to methanogens (Karhadkar et al., 1987). Sulfide toxicity increases with an increase in pH (Koster et al., 1986). Sulfide reacts with H_2 to form H_2S,

which then diffuses directly into the cell membrane. Inside the cell, it denatures proteins and coenzymes by forming sulfide cross-links, and interferes with sulfur assimilation metabolism (Chen et al., 2008). Dissociated H_2S is more toxic than dissolved sulfur. The toxicity levels of sulfide range between 100-800 mg L^{-1} for dissolved sulfide and 50-400 mg L^{-1} for undissociated H_2S. The IC_{50} values of sulfide for acetoclastic methanogens are 160 mg L^{-1}, and for hydrogenotrophic methanogens, 220 mg L^{-1}(Salama et al., 2019).

Ammonia

During the AD of nitrogen-rich compounds such as proteins, urea, and chicken litter, NH_3 is formed. NH_3 is toxic to methanogens as it passively diffuses into cells, alters intracellular pH, increases cellular maintenance energy, causes potassium deficiency, and inhibits enzymatic activity (Chen et al., 2008). In aqueous solution, free NH_3 is more toxic than NH_4^+ (Yenigün & Demirel, 2013). Toxicity increases with increasing pH, temperature, and organic loading rate as the degradation of protein substrates accumulates more NH_3 and NH_4^+ (Sánchez et al., 2000). The IC_{50} value of NH_3 in hydrogenotrophic methanogens is 520 mg L^{-1}; for acetoclastic methanogens, 280 mg L^{-1} (Koster et al., 1986). NH_3 also inhibits anaerobic bacteria at high concentrations (Yenigün & Demirel, 2013). Calli et al. (2005) reported the inhibition of acetogenic bacteria that degrade propionate at a concentration greater than 200 mg L^{-1}.

Volatile Fatty Acids

The accumulation of VFAs can be caused by organic overloads and different inhibitors such as heavy metals, sulfides, NH_3, and other toxic substances (Kleybocker et al., 2012). When the concentration of an inhibitor exceeds a critical level, methanogens are usually the first to be inhibited. This leads to an increase in acetic acid, a rise in the partial pressure of H_2, and a decrease in methane content. If the partial pressure of H_2 exceeds 0.1 mbar, acetogenic bacteria are also inhibited, leading to the accumulation of propionic acid (Harper & Pohland, 1986). Propionic acid then further inhibits methanogens. As a result of the

inhibition of both acetogenesis and methanogenesis, products of hydrolysis and acidogenesis begin to build up. Therefore, the pH and gas production rate decrease even further, which causes over-acidification of the medium, inhibiting the AD process (Kleybocker et al., 2012).

2.4 Propionate

2.4.1 What is propionate?

Propionate is a VFA (Figure 5) and is the conjugate base of propionic acid (CH_3CH_2COOH), It is colorless, soluble in water, and corrosive, with an odor similar to vinegar, with a molecular weight of 76 g mol^{-1}, and pKa 4.87 (Rahimieh & Nosrati, 2024). It is abundant in nature because it is the final product of the fermentation of a variety of microorganisms (Simonte et al., 2017). It is used in applications as a food preservative, antimicrobial, and for the production of biopolymers (Jiang et al., 2011). In AD, propionate is one of the main intermediates of organic waste to CO_2 and CH_4 (Chen et al., 2020). Its degradation into $[C_2H_3O_2]^-$ and H_2/CO_2 represents 6 % to 35 % of total methanogenesis (Li et al., 2012).

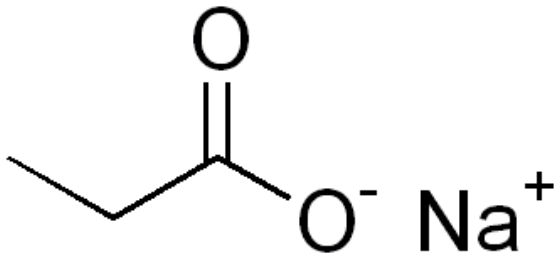

Figure 5. Chemical structure of sodium propionate. Source: Google Images.

2.4.2 Propionate degradation

Propionate degradation involves multiple syntrophic organisms, pathways, interactions, and preferences toward formate-based electron transfer to methanogenic partners. In methanogenic environments, propionate is degraded through a mutualistic interaction between symbiotic propionate oxidants and methanogens (Chen et al., 2020). And it is only possible through syntrophic

relationships (Leng et al., 2018b). Defined as a thermodynamically interdependent lifestyle in which none of the members can operate without the other (Morris et al., 2013), that is, between bacteria and methanogenic archaea (Li et al., 2012) so that the reaction general is thermodynamically feasible, and can only be carried out when the concentrations of the products, H_2 and/or $[HCOO]^-$, are maintained at low concentrations caused by their consumption by methanogens (McInerney et al., 2009). However, propionate oxidation is energetically unfavorable with a standard change in Gibbs free energy ($\Delta G°'$) of +76 kJ per mole of reaction, as illustrated in Table 1 (Li et al., 2012). In that sense, propionate is challenging to degrade compared to other fatty acids, causing its accumulation in anaerobic digesters. In engineered biogas systems, it is especially important to address propionate degradation. This is because the buildup of propionate, often along with high levels of $[C_2H_3O_2]^-$, commonly occurs when there is a disturbance in the process. This buildup can significantly reduce CH_4 productivity (Westerholm et al., 2015) and can inhibit the overall process.

Table 1. Reactions Involved in the syntrophic metabolism of propionate.

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

Standard Gibbs Free Energy Change ($\Delta G°'$). Concentrations of 1 M, pH 7.0, and T= 25°C. Taken from (Li et al., 2012).

Since anaerobic bioprocesses occur in an energy-scarce environment where substrate concentrations remain relatively low, metabolic pathways occur very close to thermodynamic equilibrium with minimal energy dissipation. Therefore, a slight change in substrate/product concentrations or environmental conditions can alter the direction of the pathway (Leng et al., 2018b). Propionate production and consumption are influenced by thermodynamic constraints, microbial competition, and metabolic inhibition (Rahimieh et al., 2024). Investigation of propionate

oxidation pathways generally provides detailed information on syntrophic microorganisms associated with their functions (Li et al., 2012).

2.4.3 Propionate degradation pathways

Four routes have been proposed for the syntrophic oxidation of propionate. 1) methylmalonyl-CoA (MMCoA), 2) C_6 dismutation, 3) the putative lactate, and 4) hydroxypropionyl (Figure 6) (Jin & Lu, 2023; Paton et al., 2020). Table 2 shows the thermodynamics of these metabolic pathways (Jin & Lu, 2023).

Table 2. Reactions related to syntrophic propionate oxidation and methanogenesis. Taken from Jin & Lu, 2023.

Reaction	ΔG0′ (kJ per reaction) [a]
Syntrophic propionate oxidation through the MMC pathway	
$Propionate^- + 2H_2O \rightarrow acetate^- + CO_2 + 3H_2$	+ 73.5
Syntrophic propionate oxidation through the C6 dismutation pathway	
$2Propionate^- \rightarrow acetate^- + butyrate^-$	+ 2.7
$Butyrate^- + 2H_2O \rightarrow 2acetate^- + H^+ + 2H_2$	+ 49.4
$Propionate^- + H_2O \rightarrow 1.5acetate^- + 0.5H^+ + H_2$	+ 26.1
Sulfate-reducing combined with propionate oxidation	
$Propionate^- + 0.75SO_4^{2-} + 1.5H^+ \rightarrow acetate^- + 0.75H_2S + H_2O + CO_2$	−44.6
Methanogenesis	
$4H_2 + CO_2 \rightarrow CH_4 + 2H_2O$	−130.6
$Acetate^- + H^+ \rightarrow CH_4 + CO_2$	−35.7
Methanogenesis from propionate oxidation	
$Propionate^- + 0.5H_2O + H^+ \rightarrow 1.75CH_4 + 1.25CO_2$	−60.2

a Values of Gibbs free energy ($\Delta G^o f$) and enthalpy ($\Delta H^o f$) for formate, acetate, and propionate. The Gibbs free energy change ($\Delta G^{o\prime}$) of each reaction under standard conditions (1 M concentration,1 atm for gasses, pH 7.0, and temperature of 25°C).

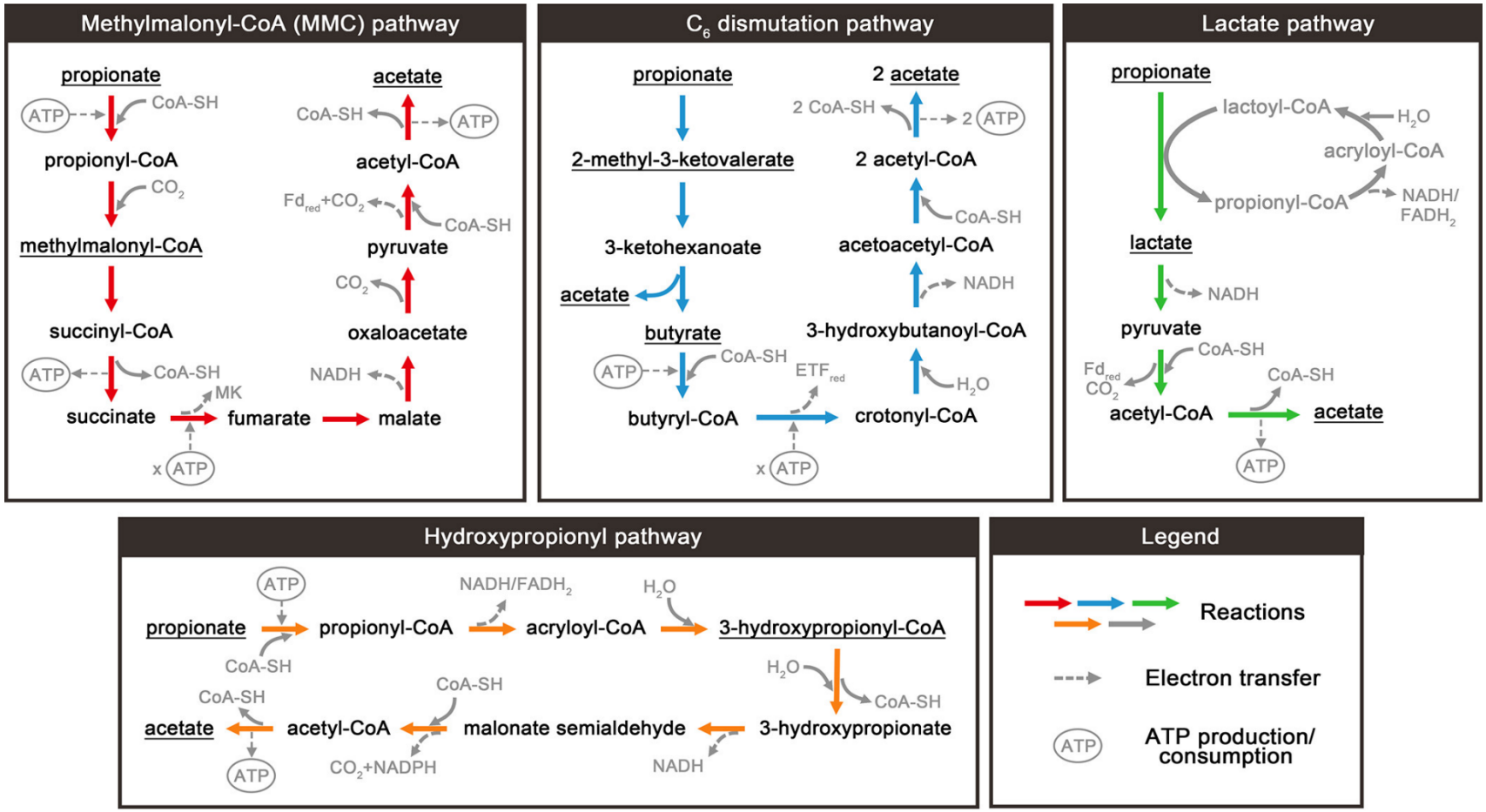

Figure 6. Catabolic pathways of syntrophic propionate oxidation. The MMCoA pathway by Syntrophobacter fumaroxidans and the C_6 dismutation pathway by Smithella propionica. The two hypothetical pathways were referenced by Paton (2020). Underlining represents representative substrates, products, and intermediates in the corresponding pathways. Taken from Jin et al., 2023.

Methylmalonyl CoA

The methylmalonyl-CoA (MMCoA) pathway consists of 11 steps. It forms intermediates such as methylmalonyl-CoA, malate, and pyruvate (Figure 7) (Westerholm et al., 2022). It is the most studied and known pathway for the degradation of propionate and is carried out by most syntrophic microorganisms. The steps are the following:

1. Propionate to propionyl-CoA. Propionate is activated by the enzyme propionyl-CoA transferase (EC 2.8.3.1) (Kato et al., 2009). Alternatively, it has been proposed that propionate activation is driven as an autonomous reaction by an AMP (adenosine monophosphate) generating acetyl-CoA synthetase, also called CoA ligase (EC 6.2.1.1) (Hao et al., 2020).
2. Endergonic carboxylation of propionyl-CoA to (S)-methylmalonyl-CoA by the enzyme methylmalonyl-CoA carboxyltransferase. It is driven by the decarboxylation of oxaloacetate to pyruvate (Stams et al., 1993).
3. Conversion of (S)-methylmalonyl-CoA to (R)-methylmalonyl-CoA by the enzyme methylmalonyl-CoA epimerase.
4. Conversion of (R)-methylmalonyl-CoA to succinyl-CoA by the enzyme methylmalonyl-CoA mutase.
5. Conversion of succinyl-CoA to succinate by the enzyme succinyl-CoA synthetase.
6. Oxidation of succinate to fumarate by a membrane-bound succinate dehydrogenase is the most energetically unfavorable step, requiring an energy input from reverse electron transport (Van Kuijk et al., 1998). It is estimated that 0.66 ATP (adenosine triphosphate) must be invested for this step to be energetically feasible, provided that the methanogen maintains the hydrogen partial pressure below 1 Pa and the formate concentration remains under 10 µM (Schink, 1997). The oxidation of succinate (+30 mV) and the reduction of menaquinone (-80 mV) by membrane-bound succinate dehydrogenase requires input of chemiosmotic energy to overcome the highly endergonic nature of the reaction (Plugge et al., 2012). The reoxidation of reduced

menaquinone by membrane-bound hydrogenase or formate dehydrogenase, leading to the release of H_2 (−414 mV) or $[HCOO]^-$ (−432 mV), also remains endergonic. This process requires the concentrations of H_2 or $[HCOO]^-$ to be maintained at low enough levels for the reaction to be feasible (Jin & Lu, 2023). This indicates that the oxidation of succinate to fumarate requires a proton gradient across the membrane and the inversion of ATP to form H_2 and/or $[HCOO]^-$ on the outside of the cytoplasmic membrane (Stams & Plugge, 2009).

7. Oxidation of fumarate to malate by the enzyme fumarate hydratase.
8. Convert malate to oxaloacetate by the enzyme malate dehydrogenase. They are NADH-dependent reactions. NADH oxidation is coupled via ferredoxin to hydrogenase to form H_2. It is energetically challenging to reduce protons with NADH, which is essentially generated in this step. (Stams & Plugge, 2009). NADH (−320 mV) formed from malate oxidation is re-oxidized to H_2 or $[HCOO]^-$ formation in the cytoplasm, which is also endergonic under standard conditions, but can become exergonic if the concentration of H_2 or $[HCOO]^-$ is maintained at a sufficiently low level. It has been proposed that some cytoplasmic hydrogenases or formate dehydrogenases perform FBEB/C, allowing this reaction at a relatively higher H_2 or $[HCOO]^-$ concentration than non-bifurcating enzymes (Buckel & Thauer, 2018).
9. Decarboxylation of oxaloacetate to pyruvate by the enzyme pyruvate carboxylase (Stams et al., 1993).
10. Conversion of pyruvate to acetyl-CoA by the enzyme pyruvate-ferredoxin reductase.
11. Conversion of acetyl-CoA to $[C_2H_3O_2]^-$ by the enzyme acetate-CoA ligase.

Generally, in the process of propionate oxidation, reduced electron carrier groups such as FADH, NADH, and ferredoxin are formed at steps succinate dehydrogenation (6), malate oxidation to oxaloacetate (8), and oxidation of pyruvate to acetyl-CoA (10), respectively. Reoxidation of these electron carrier groups is essential for syntrophy in methanogenic communities (Stams & Plugge, 2009).

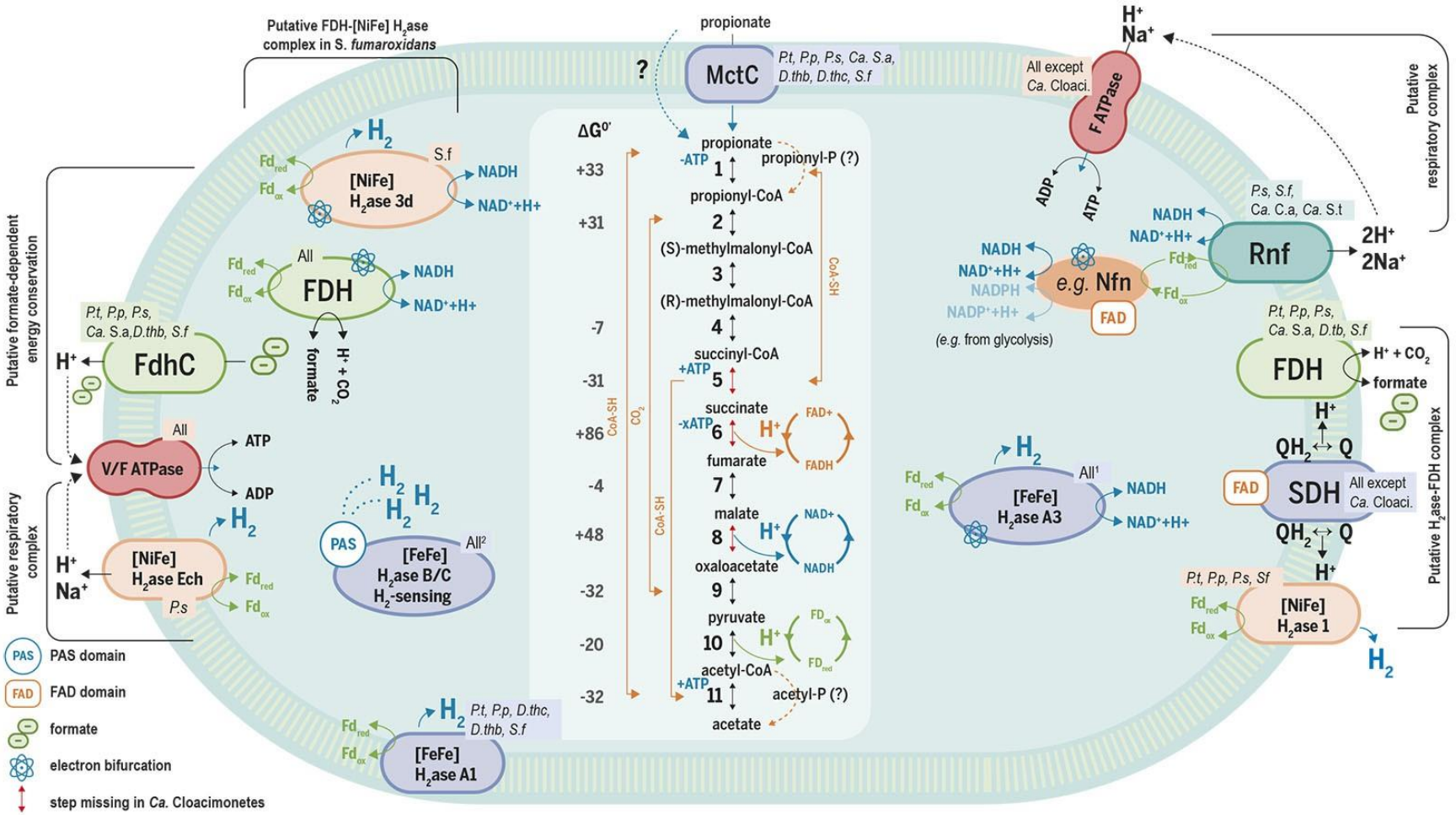

Figure 7. The illustration depicts the methylmalonyl-CoA pathway and the primary enzyme complexes responsible for the reoxidation of reduced electron transporters FADH, NADH, and ferredoxin (Fd). The numbers 1 to 11 represent the different

steps of the routes. Red arrows highlight steps that are presumably absent in *Ca. Cloacimonetes*. Dashed arrows in orange and blue indicate putative reactions. Solid orange arrows indicate interconnected reactions. NiFe and FeFe H_2ase refer to NiFe and FeFe hydrogenase, respectively. SDH stands for succinate dehydrogenase, FDH denotes formate dehydrogenase, FdhC represents formate transporter, MctC signifies propionate transporter, Rnf refers to ferredoxin:NADH reductase, Nfn stands for ferredoxin-dependent transhydrogenase, and PAS (Per-Arnt-Sim) is a signaling domain acting as a molecular sensor. This information is sourced from Westerholm et al., 2022.

C_6 dismutation

Little is known about the enzymes involved in the C_6 dismutation pathway. However, the first step is the formation of a C_6 intermediate from two propionates (Jin & Lu, 2023), to form $[C_2H_3O_2]^-$ and butyrate, while butyrate is finally oxidized to H_2 (Dyksma & Gallert, 2019). Butyrate metabolism involves the transient formation of C_6 electron transfer flavoproteins (ETFs), which act as electron acceptors for the oxidation of butyryl-CoA to crotonyl-CoA (-10 mV). The electrons are then transferred to menaquinone (-80 mV) through membrane complexes, with the likely input of chemiosmotic energy (Figure 6) (Jin & Lu, 2023). *Smithella propionica* is the only species known to degrade propionate through the C_6 dismutation pathway (Figure 8) (de Bok et al., 2001).

Although the C_6 dismutation pathway remains poorly understood, *Smithella*-like organisms appear widespread (Jin et al., 2021). Generally, only one H_2 molecule is produced per oxidized propionate compared to three H_2 molecules or $[HCOO]^-$ in the MMCoA pathway. The C_6 dismutation pathway is considered less sensitive to H_2 accumulation and provides a broader range of admissible H_2 concentrations for propionate oxidation (Dolfing, 2013). The thermodynamic calculations suggest that the dismutation pathway is more tolerant of changes in temperature and H_2 concentration compared to the MMCoA pathway (Wresta et al., 2021). Based on a systematic modeling methodology, propionate oxidation via the C_6 dismutation pathway produces the maximum ATP among all probable metabolisms under optimal environmental conditions (Paton et al., 2020).

Lactate and Hydroxypropionyl-CoA

The putative lactate and hydroxypropionyl-CoA pathways involve the use of electron transfer flavoproteins (ETFs) as electron carriers. These proteins facilitate the oxidation of propionyl-CoA and the reduction of menaquinone (Liu & Lu, 2018). In this process, the reoxidation of reduced menaquinone can be achieved by membrane-bound hydrogenase or formate dehydrogenase, which are necessary in the MMCoA pathway and require a low concentration of H_2 or

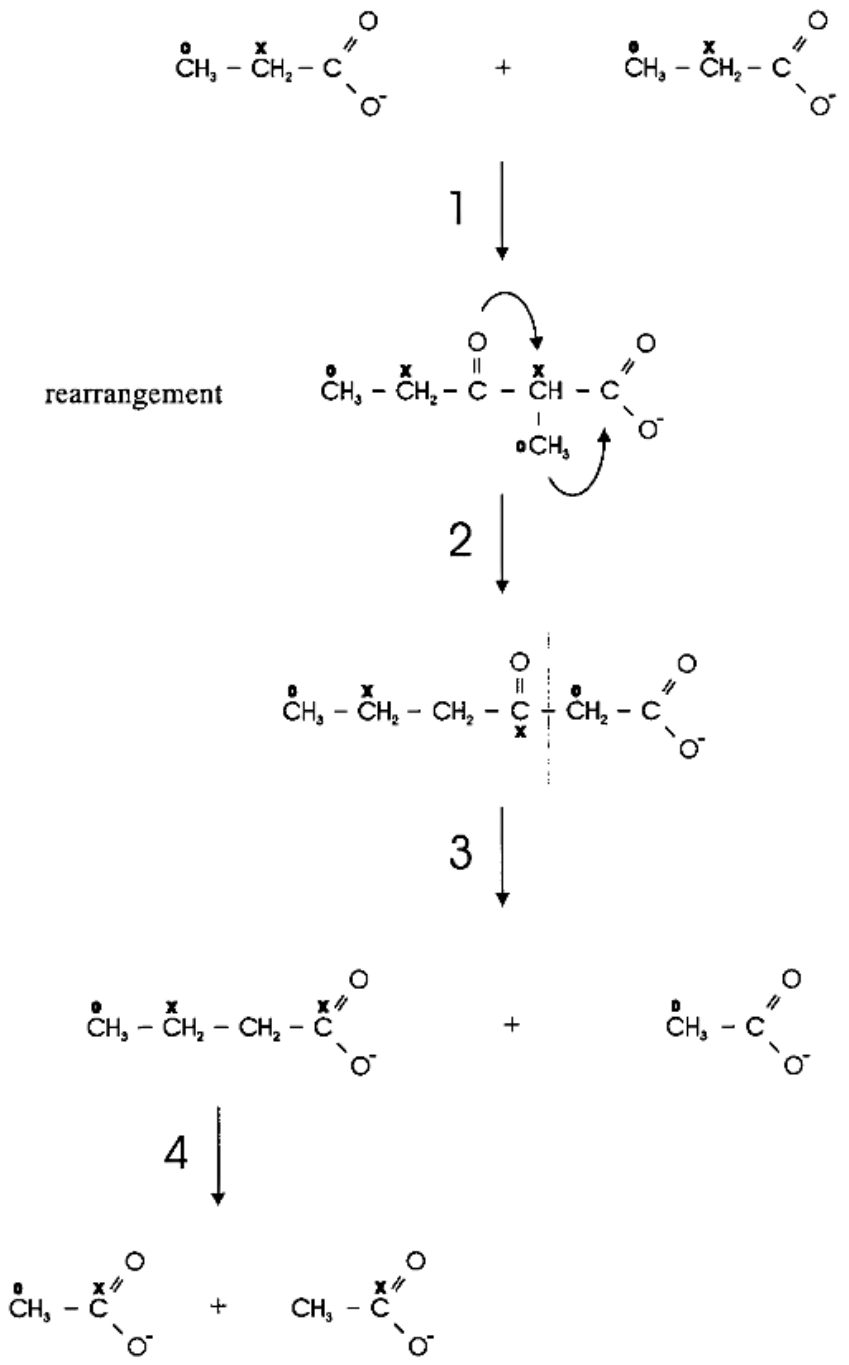

Figure 8. The proposed pathway for the conversion of propionate by *Smithella propionica* involves several steps. Step 1: The C_2 of propionate condenses with the carboxyl of another molecule or propionate derivative. Step 2: The methyl group rearranges, and oxygen is transferred to C_3 of the intermediate. Step 3: 3-ketohexanoate undergoes cleavage, yielding butyrate and $[C_2H_3O_2]^-$. Step 4: Butyrate undergoes syntrophic β-oxidation to form $[C_2H_3O_2]^-$. Taken from De Bok et al., 2001.

[HCOO]$^-$. Additionally, an alternative mechanism, the flavin-based electron confurcation (FBEC) mechanism, may also be at play. This mechanism allows for the endergonic reoxidation of butyryl-CoA or propionyl-CoA to be coupled to the exergonic reduction of NAD^+ to NADH by the reduced Fd if available (Sieber et al., 2015) (Figure 6).

2.5 Propionate-degrading bacteria

Syntrophic propionate-oxidizing bacteria (SPOB) are part of the rare biosphere and are found on the edge of the thermodynamic limit in most natural environments (Jin & Lu, 2023). It provides scarce energy for the microorganisms involved, so the degradation of propionate often becomes a bottleneck in methanogenic systems (Westerholm et al., 2022). SPOB are found in various phyla and exhibit a wide range of metabolic capabilities including syntrophic, fermentative, sulfidogenic, and acetogenic metabolism. They also show variations in their interaction with cooperating partners, indicating differences in their syntrophic lifestyle (Westerholm et al., 2022). They use different metabolisms for the oxidation of propionate (methylmalonyl-CoA, C_6 dismutation, lactate, and hydroxypropionyl-CoA pathways) (Jin & Lu, 2023). So far, only 10 bacterial species capable of syntrophic propionate oxidation have been identified. Seven mesophilic syntrophic propionate-oxidizing bacteria: *Syntrophobacter wolinii, S. pfennigii, S. fumaroxidans, S. sulfatireducens, Smithella propionica, Pelotomaculum schinkii and P. propionicum* and three thermophilic SPOB *P. thermopropionicum, Desulfotomaculum thermocisternum,* and *D. thermobenzoicum subsp. thermosyntrophicum* as seen in the Table 3 (Dyksma & Gallert, 2019).

Isolated or cocultured bacteria exhibiting syntrophic propionate oxidation traits in biochemical (Table 4), methanogenic, genomic, and transcriptomic cultures (Table 5) characterized to date utilize the MMCoA pathway, except for species in the genus *Smithella*, which use the C_6 dismutation (Westerholm et al., 2022). Figure 9 shows some of the bacteria syntrophic in propionate degradation (Embree et al., 2015).

Table 3. comprises a list of characterized bacteria that have been grown either in pure culture or co-culture. These bacteria possess the capability to degrade propionate in syntrophic association with a methanogen.

Genus (Family)[1]	Species	Temperature optimum [°C] (range)	Salt tolerance	Isolation source[2]	Metabolic pathway[3]	pH optimum (range)	Substrate		Electron acceptor in pure culture
							Pure culture	Co-culture	
Desulfofundulus (Peptococcaceae)	*D. thermocisternus*	62 (41–75)	0.8 M NaCl	Oil reservoir	mmc	6.7 (6.2–8.9)	H_2/CO_2, lactate, pyruvate, ethanol, propanol, butanol and medium-chain fatty acids (e.g. pentanoate-decanoate)	Propionate	Sulfate, sulfite and thiosulfate[4]
	D. thermobenzoicus subsp. *thermosyntrophicus*	55 (45–62)	nd	UASB fed mixture of VFAs	mmc	7.2 (6–8)	Benzoate, fumarate, H_2/CO_2, pyruvate, lactate and glycine	Propionate	Sulfate[5]
Pelotomaculum (Peptococcaceae)	*P. propionicicum*	37 (25–45)	0.09 M NaCl	UASB fed sucrose, propionate, acetate and yeast	mmc	6.5–7.2 (6.5–7.5)	Obligate syntroph	Propionate	-
	P. schinkii	37 (nd-44)	nd	UASB fed sugar beet wastewater	mmc	nd	Obligate syntroph	Propionate	-
	P. thermopropionicum	55 (45–65)	0.07 M NaCl	UASB fed sucrose, propionate, acetate and yeast	mmc	7.0 (6.7–7.5)	Pyruvate and fumarate	Propionate, ethanol, lactate, butanol, pentanol, propanediol, propanol and ethylene glycol	Fumarate[6]
Syntrophobacter (Syntrophobacteraceae)	*S. wolinii*	35 (25–40)	0.09 M NaCl or KCl	AD of municipal sewage	na	7.0 (6.0–7.5)	Pyruvate, fumarate and malate	Propionate	Sulfate[7]
Syntrophobacterium (Syntrophobacteraceae)	*S. pfennigii*	37 (20–37)	nd	AD of municipal sewage	na	7.0–7.3 (6.2–8.0)	nd	Propionate, lactate and propanol	Sulfate, sulfite and thiosulfate [8]
	S. fumaroxidans	37 (20–40)	nd	Anaerobic granular sludge	mmc	7.0–7.6 (6.8–8.0)	Fumarate, malate, aspartate and pyruvate	Propionate	Sulfate and fumarate[9]

Genus (Family)[1]	Species	Temperature optimum [°C] (range)	Salt tolerance	Isolation source[2]	Metabolic pathway[3]	pH optimum (range)	Substrate		Electron acceptor in pure culture	References
							Pure culture	Co-culture		
	S. sulfatireducens	37 (20–48)	0.1 M NaCl	UASB fed brewery/bean curd production wastewater	na	7.0–7.6 (6.2–8.8)	Pyruvate	Propionate	Sulfate, sulfite and thiosulfate[8]	Chen, Liu and Dong (2005)
Smithella (Syntrophaceae)	*S. propionica*	35 (20–40)	0.2 M NaCl	Up-flow anaerobic filter fed propionate	Dis-mutating	6.5–7.5 (6.5–7.5)	Crotonate	Propionate, butyrate, malate, crotonate and fumarate	nd	Liu *et al.* (1999); de Bok *et al.* (2001)

[1]Taxonomy using the National Center for Biotechnology Information (NCBI) database: phylum Firmicutes, class Clostridia, order Clostridiales and family Peptococcaceae. Corresponding taxonomy when using the Genome Taxonomy Database (GTDB (Parks *et al.* 2018): phylum Firmicutes, class Desulfotomaculia, order Desulfotomaculales and family Pelotomaculaceae. nd—not determined. Validation of the genus *Syntrophobacterium* (Oren and Garrity 2021).
[2]UASB -upflow anaerobic sludge bed, VFA—volatile fatty acids.
[3]Confirmed metabolic pathway used in syntrophic propionate oxidation, mmc—methylmalonyl-CoA pathway, na—sequenced genome not available
[4]H_2/CO_2, lactate, pyruvate, ethanol, propanol, butanol C_3-C_{10} and C_{14}-C_{17} carboxylic acids as electron donor/carbon source.
[5]Propionate, lactate, pyruvate and H_2/CO_2 as electron donor/carbon source.
[6]Propionate, ethanol and lactate as electron donor.
[7]Lactate as electron donor.
[8]Propionate as electron donor.
[9]Propionate, formate, succinate and H_2 as electron donor.

Take from Westerholm et al., 2022.

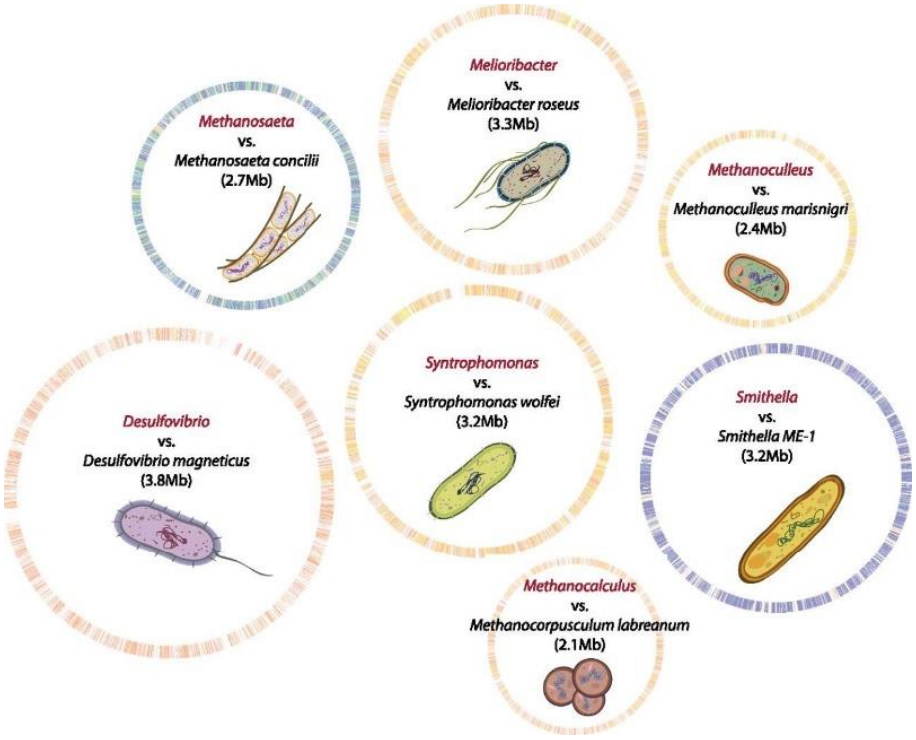

Figure 9. Species of bacteria and archaea present in anaerobic digestion. Taken from Embree et al., 2015.

Table 4. Characterized bacteria were grown in pure culture or co-culture, with the ability to degrade propionate in syntrophic association with a methanogen. Taken from Westernholm et al., 2022 with modifications.

Phyla	Genus (Family)	Species	Metabolic pathway
Firmicutes	*Desulfofundulus* (Peptococcaceae)	*D. thermocisternus*	MMCoA
		D. thermobenzoicus subsp. *thermosyntrophicus*	
	Pelotomaculum (Peptococcaceae)	*P. propionicicum*	MMCoA
		P. schinkii	
		P. thermopropionicum	
Deltaproteobacteria	*Syntrophobacter* (Syntrophobacteraceae)	*S. wolinii*	na
	Syntrophobacterium (Syntrophobacteraceae)	*S. pfennigii*	na
		S. fumaroxidans	MMCoA
	Smithella (Syntrophaceae)	*S. sulfatireducens*	na
		S. propionica	C_6

2.5.1 Desulfofundulus

Desulfofundulus species characteristically use sulfate as an electron acceptor coupled to propionate oxidation (Watanabe et al., 2018). They also use less common substrates such as benzoate and medium-chain fatty acids. They have the capacity for autotrophic growth in H_2/CO_2 with $[C_2H_3O_2]^-$ production (Singh et al., 2021). They have discovered a full set of genes that encode enzymes involved in the WL pathway, including the important enzyme formyltetrahydrofolate synthetase. This indicates that they are likely acetogens. In addition to their diverse fermentative abilities, sulfate reduction, and capacity for symbiotic growth on propionate, this suggests that they have a high level of metabolic versatility, more so than other known SPOBs (Singh et al., 2019). *Desulfofundulus* species thrive in a pure culture environment at a thermophilic temperature range. They utilize substrates such as pyruvate and lactate, which are commonly employed by syntrophic bacteria. (Watanabe et al., 2018). *D. thermocisternus* can also use sulfite and thiosulfate as electron acceptors (Bertran et al., 2020).

2.5.2 Pelotomaculum

The group consists of two mesophilic species and one thermophilic species. The mesophilic species *Pelotomaculum schinkii* and *Pelotomaculum propionicicum* are the only SPOBs that exhibit obligate syntrophic characteristics because they cannot be cultured in isolation (de Bok et al., 2005). However, it has been shown that the thermophilic species *Pelotomaculum thermopropionicum* can be cultured independently using pyruvate and fumarate. Notably, this thermophilic SPOB can degrade not only propionate but also various alcohols and lactate in partnership with a hydrogenotrophic methanogen (Imachi, 2002).

2.5.3 Syntrophobacter

The current composition contains a mesophilic SPOB, *Syntrophobacter wolinii* (Galushko et al., 2020). *S. wolinii* holds the distinction of being the first organism to have been isolated as a symbiotic partner in co-culture with a hydrogenotrophic

methanogen (Boone & Bryant, 1980). Additionally, *Syntrophobacter* has the capability to undergo fermentative growth on pyruvate, fumarate, and malate when in pure culture. Moreover, propionate has the potential to undergo incomplete oxidation to $[C_2H_3O_2]^-$, and CO_2 when in the presence of sulfate, resulting in sulfide formation (Westerholm et al., 2022).

2.5.4 Syntrophobacterium

In a controlled environment, the organism can thrive on organic compounds such as pyruvate. Additionally, it has the capability to convert propionate into $[C_2H_3O_2]^-$ and CO_2, provided that sulfate, sulfite, thiosulfate, or fumarate are present to act as the final electron acceptor. Notably, the genome of *S. fumaroxidans* contains a complete set of genes required for dissimilatory sulfate reduction (Plugge et al., 2012).

2.5.5 Smithella

Smithella propionica stands as the sole known species of the SPOB (strictly anaerobic propionate-oxidizing bacteria) within the genus Smithella. This unique species utilizes a specific metabolic pathway, commencing with the dismutation of propionate to $[C_2H_3O_2]^-$ and butyrate. Subsequently, butyrate undergoes β-oxidized to $[C_2H_3O_2]^-$, and H_2 (de Bok et al., 2001). While the exact growth substrates suitable for pure culture have yet to be fully identified, it has been documented that only crotonate promotes fermentative growth (Liu et al., 1999).

2.5.6 Candidate SPOB identified by omics studies

Several recent studies have provided genomic information on the metabolic potential of candidate SPOBs that are taxonomically different from previously known species (Table 5). These SPOB candidates have been identified through transcriptomic responses to feeding propionate or a selective propionate enrichment technique. This technique involves continuously washing unfed microorganisms to create a minimal consortium from a complex inoculum (Westerholm et al., 2022).

Table 5. Candidate SPOB identified with omics studies. Taken from Westernholm et al., 2022 with modifications.

***Candidatus* epithet**	**Taxonomic position**	**Indicated metabolic Pathway**
Ca. Cloacamonas acidaminovorans	*Uncultured phylum Cloacimonetes*	MMCoA
Ca. Propionivorax syntrophicum F70	*Family Peptococcaceae*	MMCoA
Ca. Syntrophopropionicum ammoniitolerans	*Family Peptococcaceae*	MMCoA
Ca. Syntrophosphaera thermopropionivorans	*Uncultured Cloacimonadaceae W5*	MMCoA (not all genes were identified)

Ca. Cloacimonetes

Genomic analysis of the species provisionally named *Ca. Syntrophosphaera thermopropionivorans* indicates that propionate oxidation is likely carried out through the MMCoA pathway, while a complete set of genes was not identified (Dyksma & Gallert, 2019). Genomic and/or transcriptomic properties suggest that other members of *Ca. Cloacimonetes*, such as *Ca. Cloacamonas acidaminovorans*, also possess the capability for syntrophic propionate degradation. These findings have been observed in mesophilic wastewater sludge digesters at 33°C (Pelletier et al., 2008) and in biofilms of *Ca. Cloacimonetes* in hypermesophilic bioreactors at temperatures ranging from 46-50°C (Nobu et al., 2015).

Ca. Propionivorax syntrophicum

Hao et al. (2020) found a complete set of genes for sulfate reduction of this SPOB.

Ca. Syntrophopropionicum ammoniitolerans

Belonging to the *Peptococcaceae* family. It can tolerate ammonia > 5 g of NH_4^+-N/L and NH_3 > 0.9 g of NH_3/L. Genomic analyses of this candidate indicated the

presence of most of the genes necessary to degrade propionate via the MMCoA pathway (Singh et al., 2021).

2.6 Methane synthesis

Methanogenesis, the process of producing methane, is catalyzed by a specialized group of microorganisms called methanogens. These methanogens are classified into three main groups based on their carbon sources: acetoclastic methanogens use acetate, hydrogenotrophic methanogens use hydrogen and carbon dioxide, and methylotrophic methanogens use methylated compounds like methanol and methylamines (Figure 10) (Amin et al., 2021). Acetoclastic methanogens transform $[C_2H_3O_2]^-$ into CH_4 and CO_2 (Wang et al., 2018). Hydrogenotrophic methanogens use H_2, CO_2, and $[HCOO]^-$, to produce CH_4. Methylotrophs take up methyl compounds, including methyl sulfides, methylamines, and methanol, to produce CH_4 (Lloyd, 1996).

Acetoclastic methanogens are strictly anaerobic (Wang et al., 2018). They are particularly susceptible to lower pH levels in comparison to hydrogenotrophic methanogens, which are capable of functioning even at a pH lower than 5. At reduced pH values, acetate-oxidizing syntrophs and hydrogenotrophic methanogens have the ability to outcompete acetoclastic methanogens, as highlighted in the study by Kim et al. (2004). Certain types of microorganisms known as methanogens utilize two distinct pathways for producing methane: acetoclastic and methylotrophic. Examples of methanogens that use these pathways include *Methanosarcina flavescens, Methanosarcina barkeri*, and *Methanosarcina mazei* (Kern et al., 2016).

The role of hydrogenotrophic methanogens is essential to keep the partial pressure of H_2 low $<10^{-4}$ is a vital factor, which describes the stability or failure of the process. The growth rate of hydrogenotrophic methanogens is 4-12 hours, much faster than that of acetoclastic methanogens, which is 4-9 days (Amin et al., 2021).

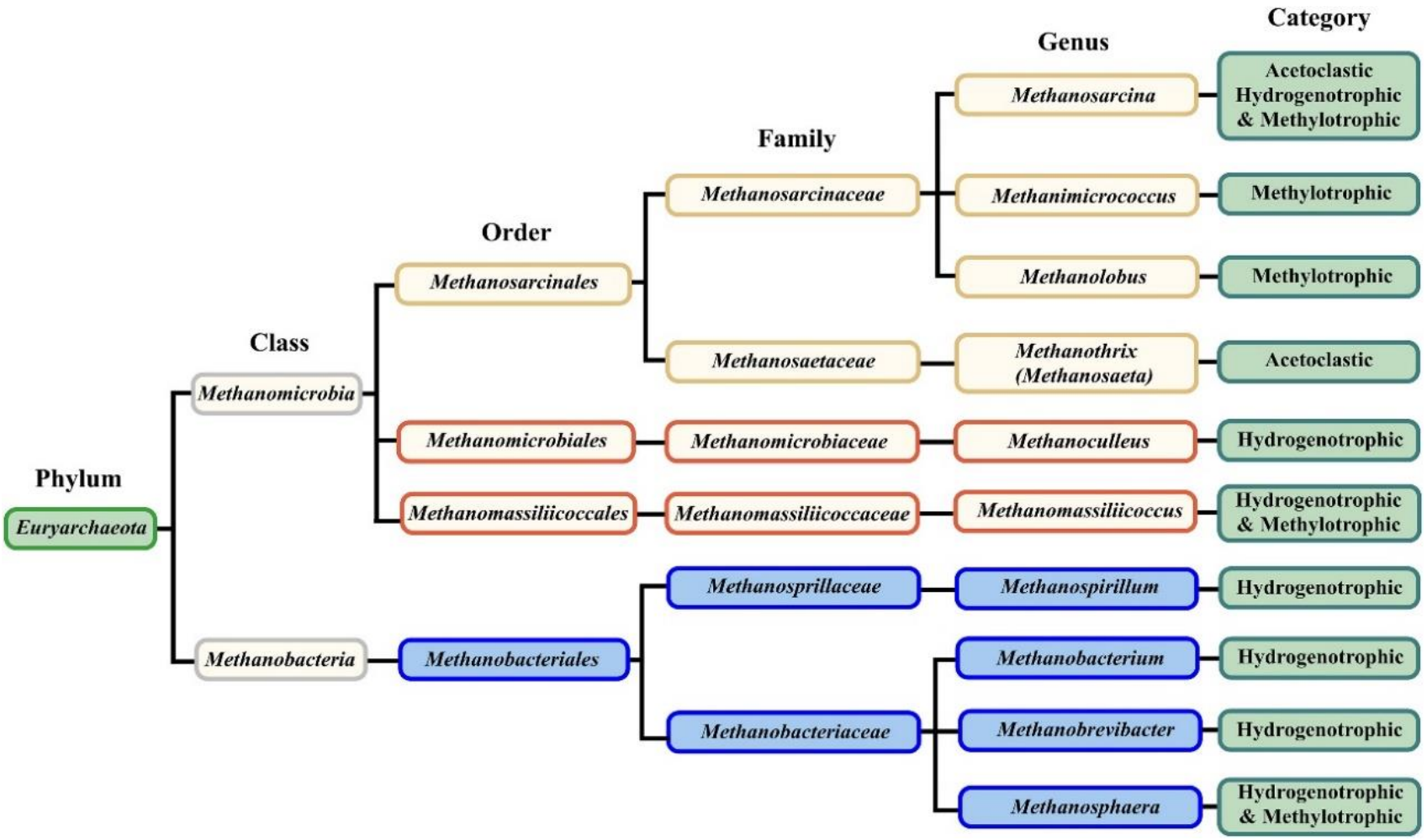

Figure 10. Taxonomic dendrogram of acetoclastic, hydrogenotrophic, and methylotrophic methanogens at phylum, class, order, family, and genus level. Taken from Amin et al., 2021.

Methanomassiliicoccus luminyensis is a methylotrophic archaea with H_2 as an electron donor and produces CH_4 by reducing methanol with a growth yield of 2.4 g cells/mol CH_4 (Kroninger et al., 2017). Most methanogens, with a specific focus on species like *Methanosarcina barkeri* and *Methanosarcina mazei*, have been demonstrated to carry out the transfer of methyl groups from methanol or methylamines to coenzyme M (HS-CoM), thus producing methyl-coenzyme M (methyl CoM), an indispensable intermediate in the process of methanogenesis. (Amin et al., 2021). At the same time, *Methanimicrococcus blatticola* produces CH_4 by reducing methylated amines and methanol with H_2 (Sprenger et al., 2000).

In classic AD digesters, approximately 70-74 % of CH_4 is produced from $[C_2H_3O_2]^-$ (acetoclastic pathway) and the remainder from H_2 and CO_2 (hydrogenotrophic pathway) (Lieber et al., 2014; Liu & Whitman, 2008). A very small amount of CH_4 is generated by methylsulfides, methylamines, and methanol (methylotrophic pathway) (Liu & Whitman, 2008; Venkiteshwaran et al., 2015). Figure 11 shows the paths of CH_4 synthesis (Liu & Whitman, 2008).

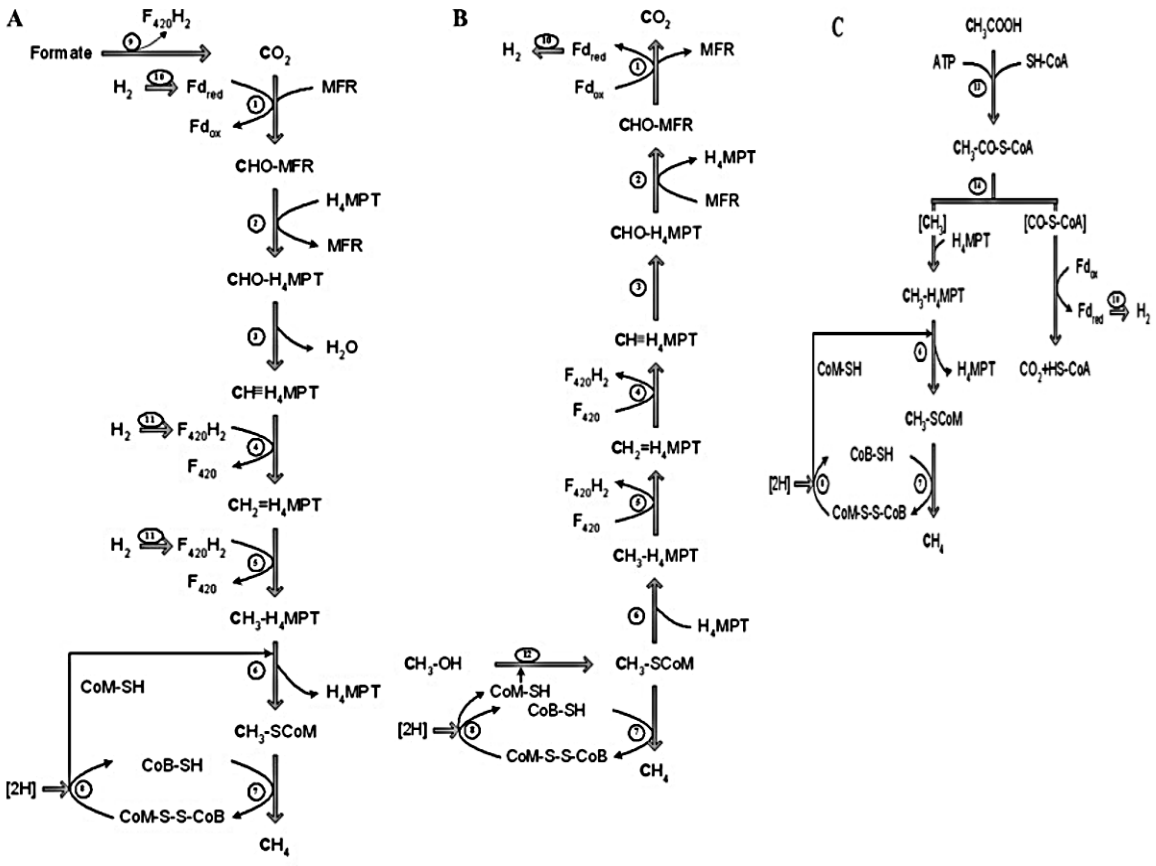

Figure 11. Summary of methanogenesis pathways: A) Methanogenesis from H_2/CO_2 or $[HCOO]^-$. B) Methanogenesis from methanol. C) Methanogenesis from $[C_2H_3O_2]^-$. Key components and enzymes involved: Fd_{red}: reduced form of ferredoxin, Fd_{ox}: oxidized form of ferredoxin, $F_{420}H_2$: reduced form of coenzyme F_{420}, MFR: methanofuran, H_4MPT: tetrahydromethanopterin, CoM-SH: coenzyme M, CoB-SH: coenzyme B, CoM-S-S-CoB: heterodisulfide of CoM and CoB, CoASH: coenzyme A. Enzymes: 1. formyl-MFR dehydrogenase (Fmd), 2. formyl-MFR: H_4MPT formyltransferase (Ftr), 3. methenyl-H_4MPT cyclohydrolase (Mch), 4. methylene-H_4MPT dehydrogenase (Hmd), 5. methylene-H_4MPT reductase (Mer), 6. methyl-H_4MPT: HS-CoM methyltransferase (Mtr), 7. methyl-CoM reductase (Mcr), 8. heterodisulfide reductase (Hdr), 9. formate dehydrogenase (Fdh), 10. energy-conserving hydrogenase (Ech), 11. Reducing hydrogenases F_{420}, 12. Methyltransferase, 13. acetate kinase (AK)-phosphotransacetylase (PTA) system in *Methanosarcin*; AMP-forming acetyl-CoA synthetase in *Methanosaeta*, 14. CO dehydrogenase/acetyl-CoA synthase (CODH/ACS).

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3 ACETATO QUINASA COMO FACTOR CLAVE PARA LA DEGRADACIÓN DEL PROPIONATO EN UN MICROBIOMA OBTENIDO MEDIANTE PRESIÓN DE SELECCIÓN GRADUAL DE PROPIONATO EN CONDICIONES MESÓFILOS

3.1 Resumen

Los residuos agrícolas y las aguas residuales se utilizan para la producción de metano mediante digestión anaeróbica. Uno de los principales problemas es la variación en la tasa de carga orgánica durante la alimentación, lo que podría provocar procesos inestables. Como resultado, se produce una acumulación de ácidos grasos, siendo el propionato el que tiene mayor efecto inhibidor. En este estudio, se aplicaron estiércol al 3% y enriquecimientos graduales en el alimento durante 1200 días utilizando concentraciones de propionato gradual desde 1.0 hasta 24.0 g L^{-1} en condiciones mesófilas. El volumen operativo fue de 10 L con un CSRT semicontinuo. El tiempo de retención hidráulica fue de 30 días. El objetivo de este artículo fue evaluar los cambios en la comunidad microbiana a lo largo del proceso de enriquecimiento gradual de propionato, para dilucidar la respuesta del microbioma a los altos niveles de concentración de propionato. Se utilizaron funciones taxonómicas y metabólicas como principales indicadores del proceso de adaptación. A lo largo del proceso se registraron análisis fisicoquímicos, concentraciones de metano y ácidos grasos. Se utilizaron once puntos de muestreo para el análisis metagenómico de escopeta. Los resultados mostraron que el microbioma degradaba el propionato sin inhibir el proceso. La concentración de metano osciló entre el 50 y el 60%. En niveles altos de propionato se encontraron especies de los géneros *Brachymacterium, Corynebacterium, Cloacimonas, Vibrio* y *Treponema*. Reveló que la degradación de niveles elevados de concentración de propionato podría atribuirse más a bacterias que degradan propionato, a través de los genes ackA y pta, que a bacterias que oxidan propionato; La vía del lactato también se detectó a través del gen pct. Durante el proceso de enriquecimiento, las poblaciones de arqueas aumentaron su abundancia relativa, principalmente con los géneros *Methanoculleus, Methanospirillum, Methanococcus,* y *Methanocella*. La síntesis de metano a través del CO_2 y el acetoclástico fueron las principales vías observadas.

Palabras clave: Propionato, Pollinaza, Digestión anaeróbica, Metagenómica, MAGs, Taxonomía

Thesis of Ph.D. in Ingeniería Agrícola y Uso Integral del Agua, Departamento de Irrigación e Ingeniería Mecánica Agrícola, Universidad Autónoma Chapingo
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4 ACETATE KINASE AS A KEY FACTOR FOR PROPIONATE DEGRADATION IN A MICROBIOME OBTAINED BY STEPWISE PROPIONATE SELECTION PRESSURE UNDER MESOPHILIC CONDITIONS

4.1 Abstract

Agricultural residues and wastewater are used for methane production via anaerobic digestion. One of the main problems is the variation in the organic loading rate during feeding, which could lead to unstable processes. As a result, fatty acids accumulate, which are propionate and have the most inhibitory effect. This study applied 3% manure and gradual enrichments in the feed for 1,200 days using stepwise propionate concentrations from 1.0 up to 24.0 g L^{-1} under mesophilic conditions. The operating volume was 10 L with a semi-continuous CSRT. The hydraulic retention time was 30 days. This paper aimed to evaluate the microbial community changes along the propionate stepwise enrichment process, to elucidate the microbiome response to the high levels of propionate concentration. Taxonomic and metabolic functions were used as the main indicators of the adaptation process. Physicochemical analyses, as well as methane and fatty acid concentrations, were recorded during the process. Eleven sampling points were used for shotgun metagenomic analysis. The results showed that the microbiome degraded propionate without inhibition of the process. Methane concentration ranged from 50 to 60%. At high propionate levels, species from the *Brachymacterium, Corynebacterium, Cloacimonas, Vibrio*, and *Treponema* genera were found. It revealed that the degradation of high propionate concentration levels could be attributed more to propionate-degrading bacteria via ackA and pta genes than to propionate-oxidizing bacteria; the lactate pathway was also detected via the pct gene. Archaea populations increased their relative abundance during the enrichment process, mainly with the *Methanoculleus, Methanospirillum, Methanococcus*, and *Methanocella* genera. Synthesizing methane through the CO_2 and acetoclastic were the principal pathways observed.

Keywords: Chicken litter, *Methanoculleus, Corynebacterium, Brachybacterium*, *Proteinipilum, Petrimomas*, enrichment, mesophilic, anaerobic digestion.

Thesis of Ph.D. in Ingeniería Agrícola y Uso Integral del Agua, Departamento de Irrigación e Ingeniería Mecánica Agrícola, Universidad Autónoma Chapingo
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4.2 Introduction

In anaerobic digestion, methane is a key product. In that process, the trophic net starts with high molecular-weight compounds up to the complete degradation of the organic products, even with thermodynamically non-favorable reactions via syntrophic relationships (Leng et al., 2018a). One of the main problems in the industrial operation of anaerobic digesters is related to failure by organic overloading. Mills et al. (2023) reported that environmental disturbances, like changes in the organic loading rate (OLR), could lead to process failure by volatile fatty acids (VFAs) accumulation in the system. Vera-Perez et al. (2023) have reported that during controlled overloading events the propionate could reach concentrations above 4.3 g L^{-1} for mesophilic anaerobic digestion. Other authors, having also disturbances in similar digesters, have reported high concentrations of propionate as a response to that type of event; He et al. (2017) reported propionate levels up to 2.76 g L^{-1}. Berninghaus and Radniecki (2022) indicated that shock loads could modify the resistance, resilience, and productivity of digesters. The interaction of bacteria and archaea under anaerobic conditions allows to establish complex relationships among them.

He et al. (2017), working with food waste digesters, reported shifts in bacterial and archaeal communities during organic overloading events; *Proteiniphilum*, *Syntrophomonas*, *Thermovirga*, *Methanoculleus*, and *Methanospirillum* genera changed their relative abundance along with the volatile fatty acids (VFA) concentration. Lv et al. (2019) reported the influence of high ammonia levels on methane production. They have found microbial community shifts as the ammonia concentration rises. Under anaerobic conditions, methane is produced by both hydrogenotrophic and acetoclastic pathways at high ammonia levels. Li et al. (2017) reported that high ammonia concentrations induced just the syntrophic pathway. Wang et al. (2019), working with inhibitory conditions (acetate, propionate, sulfide, and ammonium addition), reported that *Smithella* has more resistance to sulfite and/or ammonia then *Syntrophobacter*, while the latter had more resistance to acid inhibition conditions than the former. Zhang et al. (2022)

conducted an AD study under ammonia stress, they reported that *Methanotrix* presented an inhibitory effect at low ammonia concentration, while *Methanosarcina* was capable of deal with high ammonia levels by the acetoclastic methanation. These authors reported that syntrophic propionate-oxidizing bacteria (SPOB) were the most affected by high levels of ammonia.

One of the mechanisms for propionate degradation, which has been associated to methane production is the participation of SPOB. (Y. D. Jin & Y. H. Lu, 2023) indicated that only 10 species have been identified or candidate SPOB; which are as follows: six of them are Protobacteria, *Syntrophobacter wolinii, Syntrophobacter pfennigii, Syntrophobacter fumaroxidans*, *Syntrophobacter sulfatireducens*, *Smithella–propionica*, and "Candidatus *Desulfonatronobulbus propionicus*"; four of them are *Firmicutes*, *Pelotomaculum schinkii*, *Pelotomaculum propionicicum*, *Pelotomaculum thermopropionicum*, and *Desulfotomaculum thermobenzoicum* subsp. *thermosyntrophicum*. Most of these bacteria have facultatively syntrophic lifestyle, while just two of them have obligately syntrophic lifestyle. It is important to notice that just one specie is capable to follow the dismutation pathway (*Smithella propionica*).

Additionally, it has been reported that *Corynebacterium* is also capable for propionate degradation. Claes et al. (2002) indicated that *Corynebacterium glutamicum* in a mutational analysis is capable of growing in presence of propionate, the gene prpD2BEC2 was essential for propionate oxidation to pyruvate. Hüser et al. (2003), working with *Corynebacterium glutamicum* and propionate as a carbon source, reported an induction of the prpD2BEC2 gene cluster, which is the responsible for propionate degradation by the 2-methylcitrate cycle. This pathway was confirmed by metabolic profiling (Plassmeier et al., 2007).

Since high propionate levels in anaerobic digestion can lead to reduce methane production, several research groups have carried out enrichment processes based on high propionate concentration levels. Cao et al. (2021), conducting an enrichment, using just propionate as a sole feed, for 420 days, indicate that

Syntrophobacter was the most abundant syntrophic bacteria, while *Methenoculleus* could deal with unfavorable environmental conditions. Jannat et al. (2021), working with an enrichment for two years, found that four microorganisms are involved in propionate degradation *Smithella*, *Metanobacterium*, *Methanosaeta*, and *Syntrophomonas*. These results indicated that enrichment conduced to stablish synergistic and syntrophic relations.

Shi et al. (2022) indicated that when a group of microorganisms is subject to a strong selective pressure, the adaptative evolution by natural selection takes place. These authors reported that several processes can occur during the adaptative evolution such as follows: horizontal gene transfer, plasmid and phages as vectors for host evolution, coevolution of the bacteria in the same community, gene duplication and amplification, insertion sequences, specific gene mutations promote adaptative evolution, and bacterial parallel evolution. Kehila et al. (2023) have proposed that horizontal gene transfer plays an important role in the called intermediate states of the pathway, since the metabolic genes are distributed in co-occurring microbes; this guarantees that the microbial community generates collectively the metabolic flux.

The objective of this paper was to evaluate the microbial community changes along the propionate stepwise enrichment process, to elucidate the microbiome response to the high levels of propionate concentration, taxonomic and metabolic functions were used as the main indicators of the adaptation process.

4.3 Materials and Methods

4.3.1 Experimental setup

The anaerobic digestion experiment started on December 10, 2014, under mesophilic conditions (37 ± 5 °C). The stainless-steel digester had a working volume of 10 L. The hydraulic retention time (HRT) was 30 d. The enrichment process was conducted for around 41 HRT. The adaptation process (data not shown) took 90 days (3 HRT). The initial inoculum was taken from a lagoon digester fed with cow manure from an organic farm, “La Hondonada”, which was

located in Colón, Querétaro, México. Chicken litter for this experiment was provided by a local farm located in Tepletlaoxtoc, Estado de México, México. The digester was fed with chicken litter with a target of 3.0 % concentration during the entire enrichment process, while the added sodium propionate (Sigma Aldrich, Saint Louis, MO, USA) was fed stepwise, accordingly a selection pressure process, as it is shown in Table 6. Summary of the experimental conditions of phased co-digestion. the strategy for the propionate concentration feed rise was based on the propionate response of the digester in each step. In the beginning, the changes in concentration were low, it was considered that the system was not able to manage the high concentration of propionate. In the later steps, the changes in concentration were high.

Table 6. Summary of the experimental conditions of phased co-digestion.

Enrichment phase	Starting day	Ending day	Sodium Propionate [g L^{-1}]	Chicken litter [%]
0	0	199	0.0	3.0
1	200	259	0.5	3.0
2	260	319	0.8	3.0
3	320	362	1.0	3.0
4	363	422	1.2	3.0
5	423	552	1.4	3.0
6	553	613	1.6	3.0
7	614	669	1.8	3.0
8	670	727	2.0	3.0
9	728	798	6.0	3.0
10	799	883	12.0	3.0
11	884	980	18.0	3.0
12	981	1220	24.0	3.0

4.3.2 Physicochemical parameter evaluation

Methane concentration and volatile fatty acids (VFAs) were determined by gas chromatography as reported by our research team (Meneses-Reyes et al., 2018). pH was measured with a potentiometer (Thermo Scientific Orion 5 Star, Singapore). Temperature was evaluated using a thermocouple.

4.3.3 DNA isolation and sequencing

Eleven DNA samples were taken for shotgun metagenomic analysis during the stepwise enrichment process, based on sodium propionate, as follows: two at 1.0, three at 1.2, one at 6.0, two at 12.0, two at 18.0, and one at 24.0 g L^{-1}. Total DNA was isolated using a DNA kit from MoBio Power Soil (MoBio Laboratories, Carlsbad, CA, USA). The concentration and quality of the DNA were measured using a UV Spectrophotometer Q3000 (Quawell, Saint Joseph, CA, USA). Metagenomic libraries were prepared by Nextera XT and sequenced using the Illumina HiSeq platform according to the manufacturer's protocol.

4.3.4 Metagenomic analysis

Raw data sequences were quality checked using FastQC (v0.12.1). RQCFilter (v38.22) was used for general cleaning of the sequences. Kaiju (v1.9.0) was used for taxonomic classification. Clean reads were used to perform metagenomic assembly using MEGAHIT (v1.2.9) and metaSPAdes (v3.15.3). DRAM (v0.1.2) was used for predicting metabolic functions. KEGG (Kyoto encyclopedia of genes and genomes) was used to clarify metabolic functions.

4.3.5 Statistics analysis

Two input matrices were made for Canonical Correlation Analysis (CCA). Matrix 1 included the physicochemical parameters along the enrichment process (pH, temperature, acetate, propionate). Matrix 2 was constructed using the 25 most abundant species of both bacteria and archaea, in the entire enrichment process. Canonical correlation analysis was performed using the CCA package (v1.2.2) of Rstudio.

4.4 Results and Discussion

4.4.1 Physicochemical changes and digester performance during the enrichment process

Figure 12 shows the changes in temperature, pH, methane, acetate, propionate, and specific methanogenic activity (SMA) during the experimentation time, starting on day 0 and ending on day 1220. Gray boxes show sodium propionate concentration added to the feed; arrows indicate DNA sampling points. The system was kept under mesophilic conditions at 37 ± 5 °C. The pH was in the alkaline region at 7.5 ± 0.5, which indicated that the VFAs were in the ion form. Methane concentration was above 50-60%. Thus, the parameters in Figure 12 indicate that anaerobic conditions were satisfied during the entire enrichment process. Chicken litter and propionate are substrates on their own that are difficult to degrade and form toxic compounds in typical anaerobic digestion systems. In this work, it can be seen that the developed microbiome was able to process chicken litter in codigestión with propionate (up to 24 g L^{-1} in the feed) , thus the methane can be produced. Mosche and Jordening (1998) pointed out that the saturation constant of propionate obtained ranged from 0 to 0.003 g L^{-1} in the treatment of sugar factory.

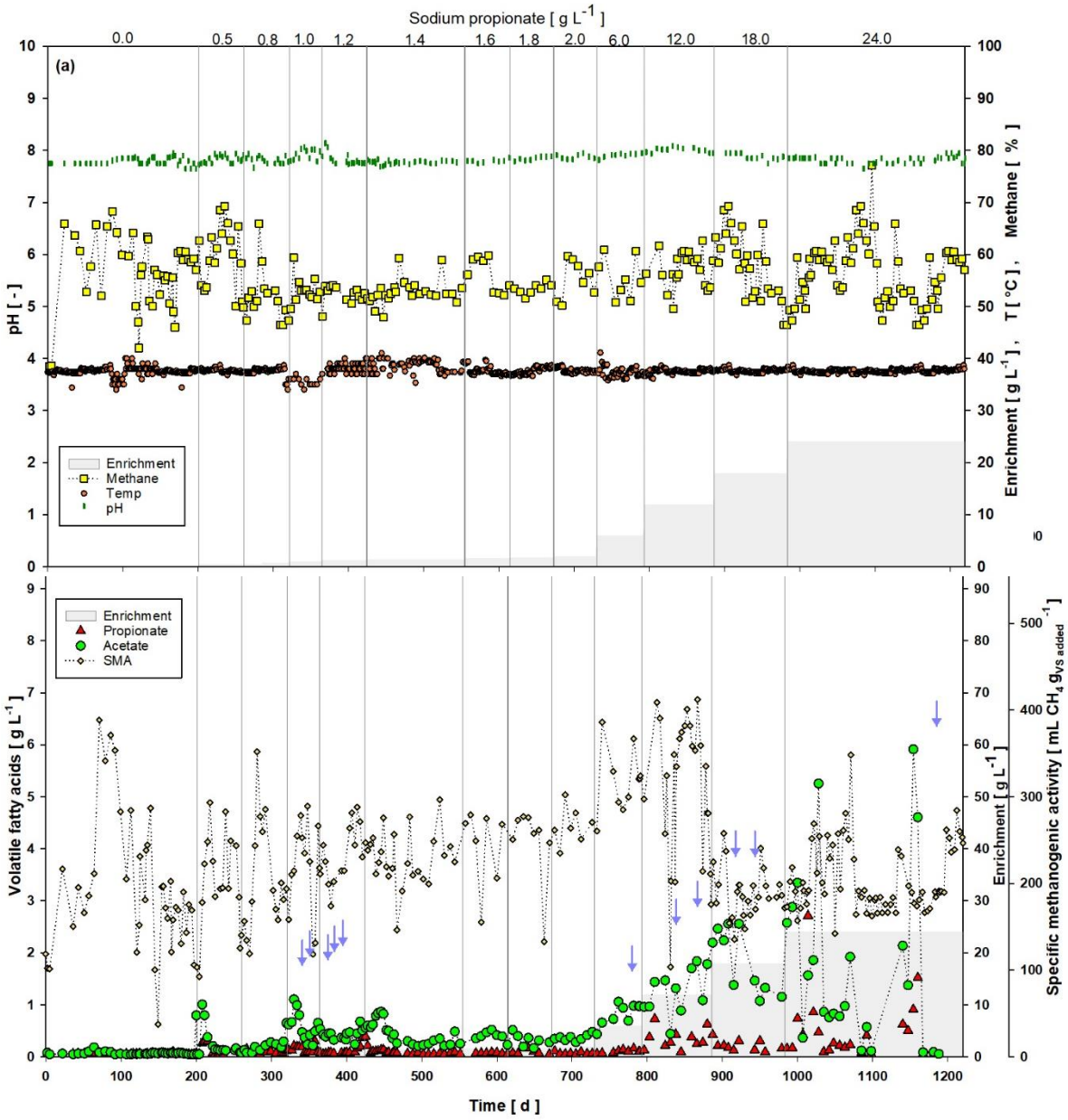

Figure 12. Physicochemical parameters changes during the enrichment process. a) Methane, Temperature, and pH. b) Propionate, acetate, and specific methanogenic activity. Gray shadows indicate the stepwise propionate enrichment, while purple arrows show the DNA sampling points.

Stage one. From 200 up to 554 d (0.5 to 1.4 g L^{-1} of added propionate). In this stage, the rise in the propionate concentration in the feed produced an acetate peak in the digester, followed by a small propionate peak. The maximum concentrations for acetate and propionate in the digester were, respectively, 1.10 and 0.36 g L^{-1}. Even though methane production was kept by the system.

Stage two. From 555 to 798 d (1.6 to 6.0 g L^{-1} of added propionate), The stepwise fed propionate rise produced changes in the acetate, while the propionate was kept practically constant in the system. In this case, the maximum concentrations for acetate and propionate were, respectively, 1.05 and 0.15 g L^{-1}. It is important to note that the methane production was kept with small variations in the SMA.

Stage three. From 799 to 1220 d (12.0 to 24.0 g L^{-1}). In this stage, once the propionate in the feed rises, the acetate increases in the digester higher than the propionate does. It is important to mention that in this stage, the acetate reduces slower than in the previous stages. In the case of propionate, it oscillates along the entire stage. The digester showed resilience to the disturbance by the high propionate concentration in the feed. It is surprising that even having acetate and propionate concentrations of 5.92 and 2.72 g L^{-1}, respectively, the levels reduced as low as 0.06 and 0.05 g L^{-1} once the system was able to deal with that type of feed. The methane production had higher variations in the SMA than in the second stage.

It is important to notice that, in **Stage one**, during the increase of the propionate concentration in the feed, the acetate and propionate concentrations have risen. This behavior is like the one reported by Vera-Perez et al. (2023). These authors made a disturbance above the six-fold organic loading rate (OLR) of chicken litter. The digester perturbation had a methane percent reduction, as well as a rise in acetate, propionate, and butyrate concentrations. Similar behaviors have been reported by Bi et al. (2019); (Bi et al., 2020), working with chicken manure; He et al. (2017) for food waste. These behaviors could be attributed to a rearrangement of the microbiome during the enrichment process. In fact, Ma et al. (2023) reported

that the accumulation of acetate and propionate is the result of diminished their rate of degradation when the feed has a high OLR.

In **Stage two**, there were no changes in terms of the rise of VFAs. It could be possible since the microbiome was adapted to manage such VFA concentrations in the feed. Thus, it was capable of dealing with the propionate and acetate degradation; the perturbance was practically not perceptible for the microbiome.

In the case of **Stage Three**, the changes in propionate and acetate varied in different manners. This perturbance was attributed to the adaptation of the digester to high propionate concentrations in the feed. Even though the methane production was kept during the high disturbance of the system.

4.4.2 Microbial community structure changes during enrichment

Taxonomic changes

To explain the changes in the microbial distribution along the entire enrichment process, there were selected only one sample per each propionate concentration in the feed to avoid bias.

Figure 133 shows that the unclassified fraction reads ranged from 46.9 to 61.8 %. There were identified 39 phyla along with 10 phyla candidates. The most abundant phyla were as follows: *Bacteroidetes, Firmicutes, Euryarchaeota*, *Actinobacteria*, and *Proteobacteria*. Their relative abundance ranged from 92.8 to 96.1 % of the total reads along the enrichment process. It is important to note that the two top *Bacteroidetes* and *firmicutes* (bacteria) phyla diminished their relative abundance from 86.0 to 36.4 %, while *Euryarchaeota* (archaea) went from 5.1 to 51.2 %. *Actinobacteria and Proteobacteria* had small changes, together changed from 5.0 to 8.5 %. Chen et al. (2024) analyzed the bacteria-archaea interaction to better understand pig gut microbial changes during diet variations, showing bacterial phyla such as *Bacteroidetes, Firmicutes, Proteobacteria, Actinobacteria, Spirochaetae, Verrucomicrobia,* and *Euryarchaeota.* Atasoy and Cetecioglu (2022) mentioned that the production of propionate was mainly influenced by

alkali and neutral pH, with the *Firmicutes* and *Bactorides* phylum being the most dominant.

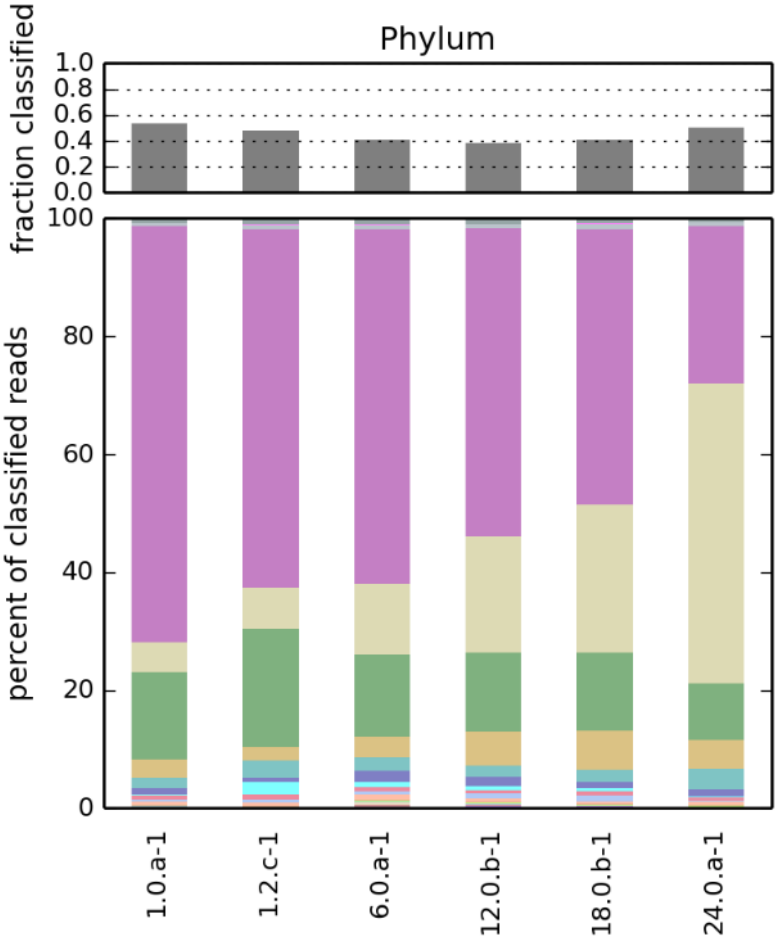

Figure 13. Relative abundance phyla of the digester microbial community at each propionate enrichment change.

Figure 14 shows the species present in the system during the enrichment. The unclassified reads had an average of 55.0 %. The classified reads average was as follows: bacteria 66.8 %, archaea 18.8 %, 14.4 % could not be assigned to a taxon, and viruses 0.05 %. For the analysis of the consortia changes during the enrichment process, there were considered just the identified bacteria (5279 species) and archaea (133 species) populations. Thus, it is important to stress that bacteria changed from 94.7 % to 46.0 %, while archaea did from 5.3 % to

54.0 %. In terms of the detected phyla, the *Euryarchaeota* (51.2 %), *Bacteroidetes* (26.7 %), *Firmicutes* (9.6 %), *Proteobacteria* (4.9 %), *Actinobacteria* (3.6 %), and *Spirochaetes* (1.2%). Guo et al. (2024) evaluated the propionate degradation under mesophilic conditions, in a batch process using sludge as a substrate; the initial concentration varied as follows: 0, 1, and 4 g L^{-1}. The data showed that, in the presence of propionate, archaea were the most abundant phylum. The most representative phyla were, in decreasing order, as follows: *Euryarchaeota, Bacteroidetes, Chloroflexi, Proteobacteria, Firmicutes,* and *Synergistetes.*

At the beginning of the process (1.0.a g L^{-1} of propionate), the most abundant bacterial, with above 1 %, were just 3 species as follows: *Proteiniphilum saccarofermentans, Petrimonas mucosa* and *Proteiniphilum propionicum sp. JNU-WLY501*. Their relative abundance together was 61.8 %. It is important to note that their abundance was reduced at the end of the process (24.0.a g L^{-1} of propionate) to 14.4 %. There was a group of bacteria (*Vibrio cincinnatiensis, Corynebacterium stationis, Brachybacterium faecium,* and *Corynebacterium casei*) with a relative abundance of 0.5 % at the beginning of the enrichment that ended up with a 3.6 %. It is important to highlight that archaea with the higher relative abundance were *Methanoculleus marisnigri, Methanoculleus bourgensis,* and *Methanoculleus chikugoensis*; they changed their relative abundance from 1.2 % to 53.0 % along the enrichment process. The results have shown that, in all the sampling points along the enrichment, there were several types of bacteria, having a relative abundance lower than 0.05 %, such as sulfate-reducing, acetate-oxidating, propionate-oxidizing, and ammonia-oxidizing. Table **5** and **Table** 6 show bacteria and archaea in this study. Substrates that catabolize and their fermentation.

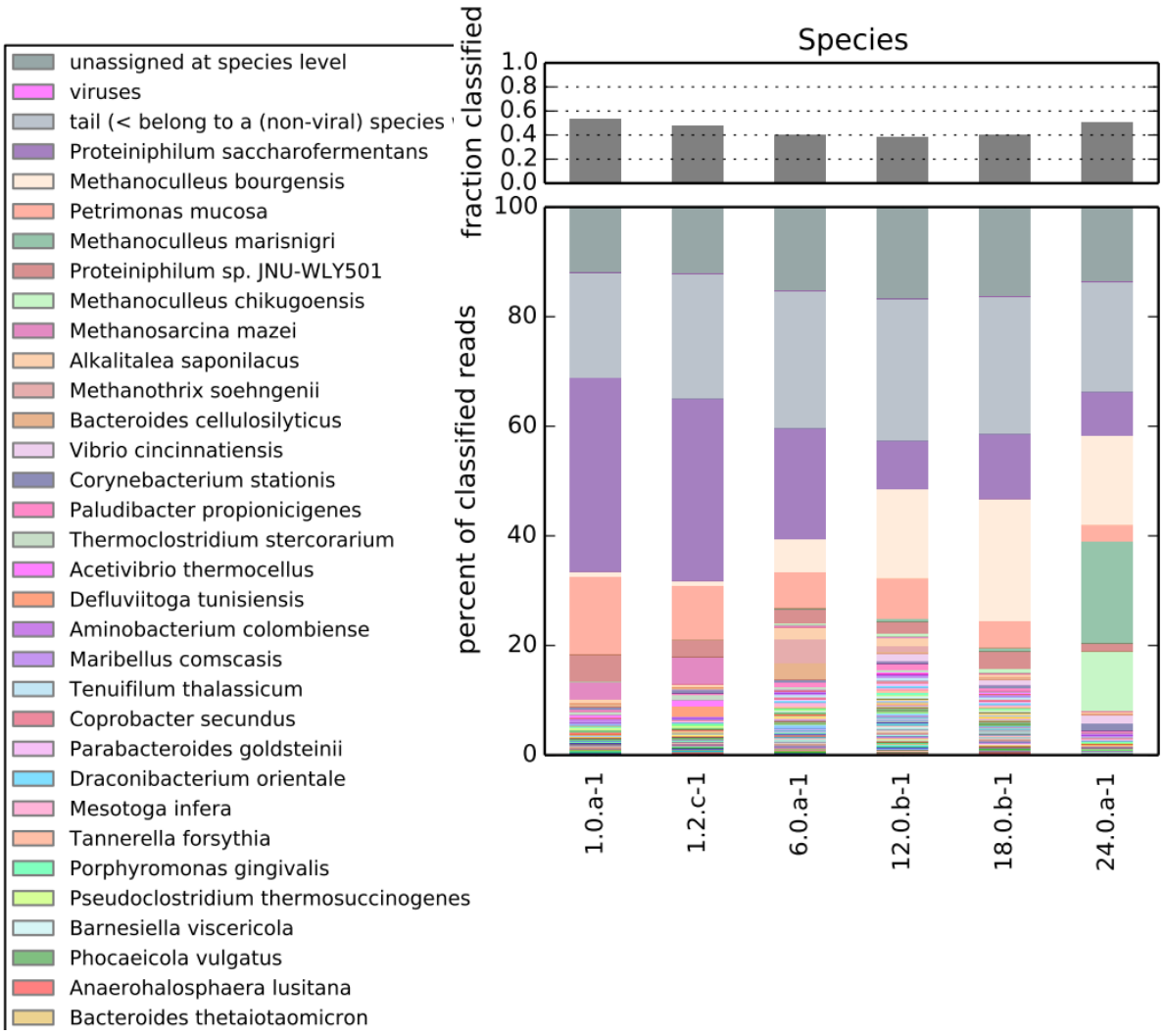

Figure 14. Relative abundance species of the digester microbial community at each propionate enrichment change.

Environment influence on microbial communities

Figure 15 shows the results of applying the CCA to bacterial and archaeal relative abundances to physicochemical parameters along the enrichment process. The results were split into two figures for a better display. The full description of the colored letters, for bacteria and archaea, is presented in Table 7. The analysis agreed with the results presented in **Figure 12** *i.e.*, the concentrations were distributed in different quadrants. Starting in the second quadrant, the analysis gave the group of the initial concentrations (1.0.a to 1.2.c) where the predominant bacteria were as follows: *Acetivibrio saccincola, Aminobacterium colombiense,* and *Petrimonas mucosa*. Following the process, the third quadrant includes treatment 6.0.a. While 12.0.a to 18.b were in the fourth quadrant. At the end of the process (24.0.a) the archaea *Methanospirillum sp. J361F273, Methanocella paludicola,* and *Methanoculleus marisnigri* were the most abundant species. The proximity between physicochemical parameter vectors and species reflects their correlation. In the first quadrant is the vector corresponding to the pH, which indicates that the microorganisms located in this quadrant have an affinity to high pH.

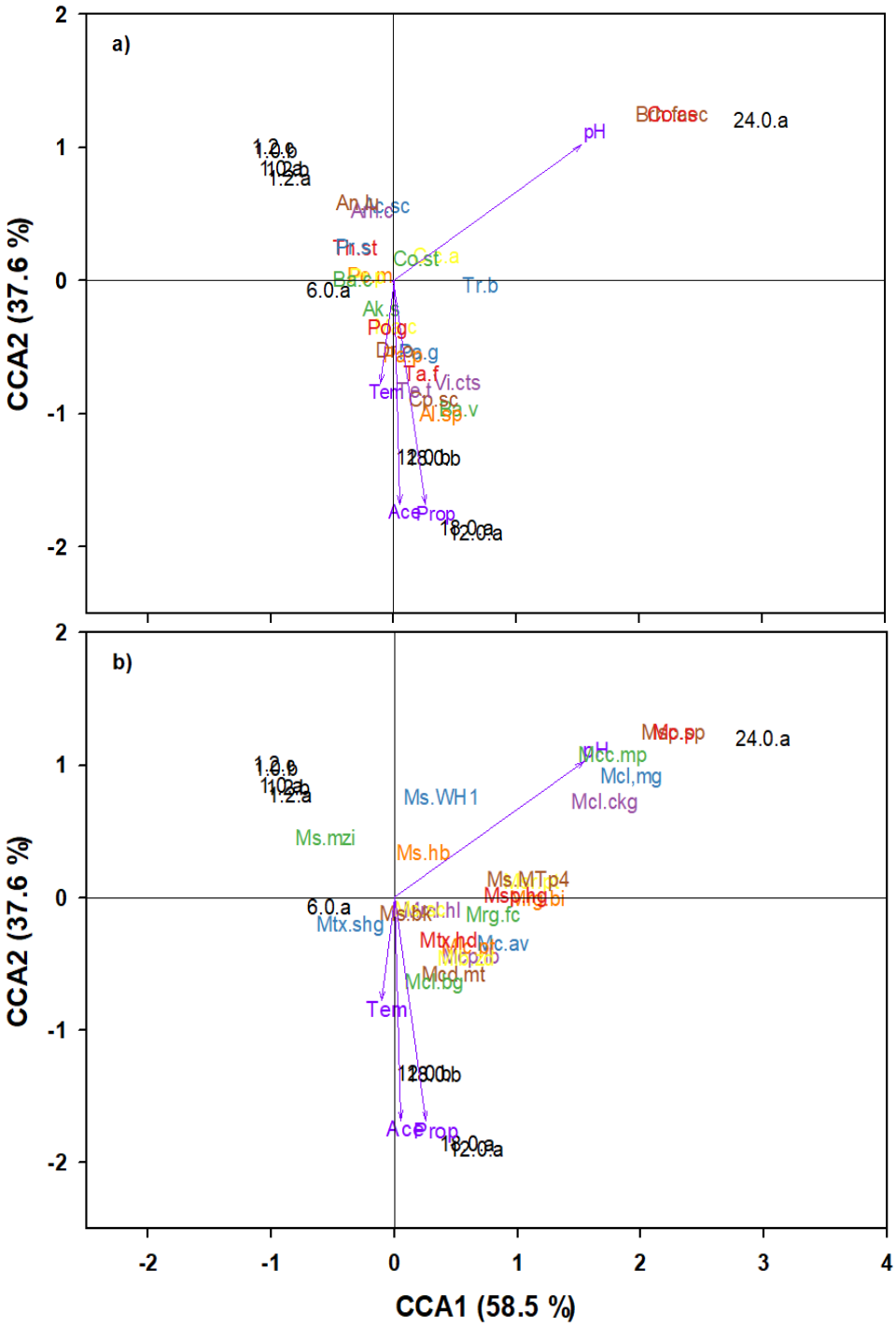

Figure 15. Canonical correlation analysis (CCA) between physicochemical parameters. a) Bacteria, b) archaea (the name of species are shown in Table 2). The label description is as follows: pH: potential hydrogen. Tem: Temperature. Ace: Acetate. Pro: Propionate. The physicochemical parameters are shown in purple color. 1.0.a, 1.0.b, 1.2.a, 1.2.b, 1.2.c, 6.0.a, 12.0.a, 12.0.b, 18.0.a, 18.0.b, y 24.0.a are the different sampling points, the propionate concentrations are expressed in g L-1.

Table 7. Detailed description of the CCA (Fig. 19) of the most abundant bacteria and archaea in the system.

Concentration	Code	a) Bacteria	CCA1	CCA2	Code	b) Archaea	CCA1	CCA2
	Ac.sc	*Acetivibrio saccincola*	-0.059	0.563	**Ms.mzi**	*Methanosarcina mazei*	-0.559	0.450
	Am.c	*Aminobacterium colombiense*	-0.172	0.521				
	Pe.m	*Petrimonas mucosa*	-0.194	0.034				
1.0 g L-1	**Pr.p**	*Proteiniphilum propionicum sp. JNUWLY501*	-0.215	0.032				
1.2 g L-1	**An.lu**	*Anaerohalosphaera lusitana*	-0.286	0.583				
	Th.st	*Thermoclostridium stercorarium*	-0.317	0.242				
	Pr.s	*Proteiniphilum saccharofermentans*	-0.331	0.251				
	Ba.c	*Bacteroides cellulosilyticus*	-0.334	0.004				
	Po.g	*Porphyromonas gingivalis*	-0.047	-0.356				
6.0 g L-1	**Ak.s**	*Alkalitalea saponilacus*	-0.099	-0.216	**Mtx.shg**	*Methanothrix soehngenii*	-0.360	-0.202
	Tr.b	*Treponema brennaborense*	0.708	-0.037	**Mrg.bi**	*Methanoregula boonei*	1.163	-0.011
	Ba.v	*Barnesiella viscericola*	0.526	-0.970	**Mc.av**	*Methanocella arvoryzae*	0.884	-0.345
	Vi.cts	*Vibrio cincinnatiensis*	0.520	-0.770	**Mrg.fc**	*Methanoregula formicica*	0.800	-0.133
	Al.sp	*Alistipes sp. dk3624*	0.388	-1.005	**Mcp.lb**	*Methanocorpusculum labreanum*	0.619	-0.441
	Cp.sc	*Coprobacter secundus*	0.325	-0.891	**Mlc.pt**	*Methanolacinia petrolearia*	0.602	-0.369
12.0 g L-1	**Ta.f**	*Tannerella forsythia*	0.222	-0.700	**Mlb.zd**	*Methanolobus zinderi*	0.584	-0.462
18.0 g L-1	**Pa.g**	*Parabacteroides goldsteinii*	0.203	-0.539	**Mcd.mt**	*Methanococcoides methylutens*	0.476	-0.584
	Te.t	*Tenuifilum thalassicum*	0.164	-0.825	**Mtx.hd**	*Methanothrix harundinacea*	0.442	-0.319
	Pa.p	*Paludibacter propionicigenes*	0.079	-0.564	**Mcl.bg**	*Methanoculleus bourgensis*	0.321	-0.641
	Ma.c	*Maribellus comscasis*	0.021	-0.349	**Mml.hl**	*Methanomethylovorans hollandica*	0.308	-0.101
	Dr.o	*Draconibacterium orientale*	0.010	-0.526	**Ms.sc**	*Methanosarcina siciliae*	0.209	-0.093
					Ms.bk	*Methanosarcina barkeri*	0.094	-0.122
	Brh.faec	*Brachybacterium faecium*	2.267	1.246	**Msp.sp**	*Methanospirillum sp. J361 F273*	2.267	1.246
24.0. g L-1	**Co.cs**	*Corynebacterium casei*	2.267	1.246	**Mc.p**	*Methanocella paludicola*	2.267	1.246
	C.c.a	*Candidatus Cloacimonas acidaminovorans*	0.349	0.185	**Mcl,mg**	*Methanoculleus marisnigri*	1.922	0.919
	Co.st	*Corynebacterium stationis*	0.183	0.162	**Mcc.mp**	*Methanococcus maripaludis*	1.772	1.081

Mcl.ckg	*Methanoculleus chikugoensis*	1.709	0.722
Msr.pt	*Methanosphaerula palustris*	1.113	0.120
Ms.MTp4	*Methanosarcina sp. MTP4*	1.087	0.133
Msp.hg	*Methanospirillum hungatei*	0.986	0.012
Ms.WH1	*Methanosarcina sp. WH1*	0.380	0.755
Ms.hb	*Methanosarcina horonobensis*	0.235	0.334

4.4.3 Prediction of functions

Pathways

Figure 16 shows the functional units of the set of genes and molecular complexes in the metabolic pathways in a simplified way. It includes the coverage of modules, the coverage of electron transport chain components, and the presence of selected metabolic functions. The discussion will be centered on the two main metabolic pathways of the microbiomes, propionate degradation and methane synthesis.

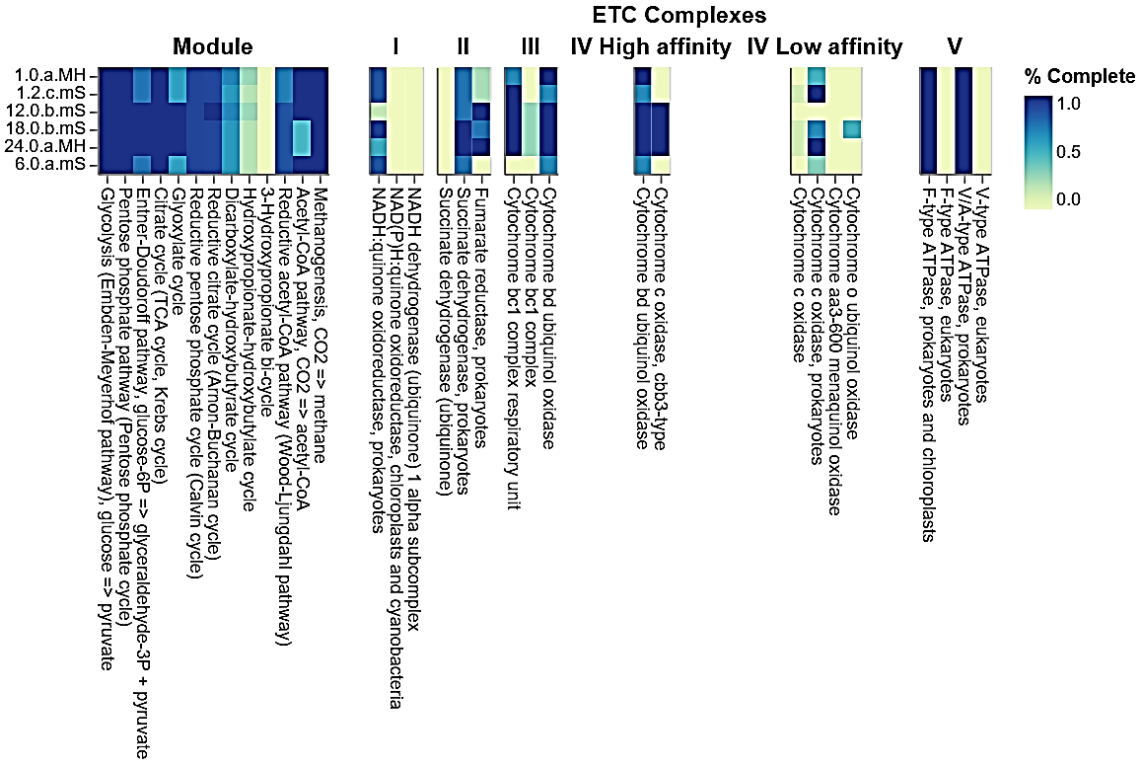

Figure 16. Modules present in the system during enrichment with propionate g L-1, electron transport chain (ETC), and carbohydrate active enzymes (CAZy).

Propionate degradation

Figure 17 shows the predicted pathways based on seventeen microbial species that were present along the enrichment process. The predicted enzymes and the corresponding hits are presented in Table 8 was obtained based on literature and the Kyoto Encyclopedia of Genes and Genomes (KEGG) database. The Table 9 shows pH, temperature, growth, substrates, and fermentation of the most abundant bacteria in this study.

Table 8. Enzymes and hits present in the degradation of Propionate.

KO	Enzyme	Symbol	Reaction	Distilled and Refined Annotation of Metabolism (DRAM)	Hits					
					1.0.a	1.2.c	6.0.a	12.0.b	18.0.b	24.0.a
Propionate metabolism										
K01026	2.8.3.1	pct		propionate CoA transferase	1	1	4	1	1	0
K00925	**2.7.2.1**	**ackA**	**R00315**	**acetate kinase**	**25**	**34**	**33**	**26**	**28**	**28**
K00625	**2.3.1.8**	**pta**	**R00230**	**phosphate acetyltransferase**	**10**	**10**	**8**	**6**	**6**	**4**
K13788	**2.3.1.8**	**pta**	**R00230**	**phosphate acetyltransferase**	**1**	**0**	**1**	**1**	**0**	**2**
K15024	**2.3.1.8**	**K15024**	**R00230**	**putative phospho-transacetylase**	**8**	**13**	**8**	**9**	**8**	**8**
K00101	1.1.2.3	lldD	R00196	L-lactate dehydrogenase (cytochrome)	1	0	0	1	2	1
K00016	1.1.1.27	ldh	R00703	L-lactate dehydrogenase	9	11	5	5	5	4
K01966	6.4.1.3	pccB	R01859	propionyl-CoA carboxylase beta chain	14	16	13	11	11	7
K05606	5.1.99.1	MCEE	R02765	methylmalonyl-CoA/ ethylmalonyl-CoA epimerase	12	14	13	11	10	9
K01847	5.4.99.2	MUT	R00833	methylmalonyl-CoA mutase	10	11	11	8	8	7
K01848	5.4.99.2	mcmA1	R00833	methylmalonyl-CoA mutase N-terminal domain	1	4	1	1	2	1
K01849	5.4.99.2	mcmA2	R00833	methylmalonyl-CoA mutase C-terminal domain	2	4	2	1	2	3
K01902	6.2.1.5	sucD	R00405	succinyl-CoA synthetase alpha subunit	9	9	7	8	8	9
K01903	6.2.1.5	sucC	R00405	succinyl-CoA synthetase beta subunit	9	10	6	6	8	6
Pyruvate metabolism										
K00239	1.3.5.1	sdhA		succinate dehydrogenase flavoprotein subunit	8	8	6	5	7	5
K00240	1.3.5.1	sdhB		succinate dehydrogenase iron-sulfur subunit	12	13	15	11	14	9

K00241	1.3.5.1	sdhC	R02164	succinate dehydrogenase / fumarate reductase	12	9	8	8	9	7
K00244	1.3.5.1	frdA	R02164	succinate dehydrogenase / fumarate reductase	7	2	0	1	0	1
K01676	4.2.1.2	fumB	R01082	fumarate hydratase class I	12	9	11	9	9	8
K01677	4.2.1.2	fumA	R01082	fumarate hydratase subunit alpha	9	14	13	9	12	11
K01678	4.2.1.2	fumB	R01082	fumarate hydratase subunit beta	11	14	11	10	14	11
K01679	4.2.1.2	fumC	R01082	fumarate hydratase class II	10	8	7	4	6	6
K00024	1.1.1.37	mdh	R00342	malate dehydrogenase	6	7	7	4	3	5
K01596	4.1.1.32	pckA	R00431	phosphoenolpyruvate carboxykinase (GTP)	10	11	18	13	10	9
K01596	4.1.1.32	pckA	R00726	phosphoenolpyruvate carboxykinase (GTP)	0	11	18	13	10	9
K01610	4.1.1.49	pckA	R00341	phosphoenolpyruvate carboxykinase (ATP)	13	15	17	14	16	13
K00873	2.7.1.40	pyk	R00200	pyruvate kinase	26	33	28	22	22	25
K00163	1.2.4.1	aceE	R00209	pyruvate dehydrogenase E1 component	2	2	1	1	3	4
K00382	1.8.1.4	lpd	R00209	dihydrolipoyl dehydrogenase	30	31	30	20	27	22
K00169	1.2.7.1	porA	R01196	pyruvate ferredoxin oxidoreductase alpha subunit	4	8	6	6	5	7
K00170	1.2.7.1	porB	R01196	pyruvate ferredoxin oxidoreductase beta subunit	4	8	4	6	4	7
K00171	1.2.7.1	porD	R01196	pyruvate ferredoxin oxidoreductase delta subunit	5	8	5	5	3	8
K00172	1.2.7.1	porC,G	R01196	pyruvate ferredoxin oxidoreductase gamma subunit	5	8	6	5	5	7
K03737	1.2.7.1	por	R01196	pyruvate-ferredoxin/ flavodoxin oxidoreductase	27	33	41	32	32	29
K00625	2.3.1.8	pta	R00230	phosphate acetyltransferase	10	10	8	6	6	4
K13788	2.3.1.8	pta	R00230	phosphate acetyltransferase	1	0	1	1	0	2
K15024	2.3.1.8	K15024	R00230	putative phospho-transacetylase	8	13	8	9	8	8
K00925	2.7.2.1	ackA	R00315	acetate kinase	25	34	33	26	28	28

Table 9. pH, temperature, growth, substrates, and fermentation products of the most abundant bacteria in this study. The letter remarked in black, indicates information on this work.

Relative abundance % of this study	Bacteria	g L-1		Growth T °C	Optimal T °C	Growth pH	Optimal pH	Growth	Substrates			Ferment (produce)	References
		1.0	24.0										
	Acetivibrio saccincola	0.20	0.22	45-65	55-60	6.5-9.0	7.0	xylan xylose glucose	glucose, galactose xylose, cellobiose cellulose		xylan sorbitol	acetate H2 ethanol	(Koeck et al., 2016)
	Aminobacterium colombiense	0.50	0.36	20-42	37	6.6-8.5	7.3	yeast extract serine glycine threonine pyruvate		serine, alanine threonine, leucine glycine, isoleucine glutamate, valine aspartate, methionine	pyruvate α-Ketoglutarato	acetate propionate H2	(Baena et al., 1998) (Chertkov et al., 2010)
	Petrimonas mucosa	16.1	3.50	20-50	45 **37-42**	9.3-9.1	7.5-7.8 **7.5-8.0**	catalase oxidase peptone peptidoglycan chitin	monosaccharides disaccharides polysaccharide carbohydrates sugar residues cellulose hemicellulose	proteins proline, glycine serine, threonine glutamine, glutamate	**acetate** **acetyl-phosphate** **propionate** **propionyl-phosphate** **propionyl-CoA** **(S)-2 / (R)-methyl-malonyl-CoA** **succinyl-CoA**	acetate propionate isovaleric butyric H2 / CO2	(Hahnke et al., 2016) (Maus et al., 2020) This study
1.0 g L-1 **1.2 g L-1**	***Proteiniphilum propionicum sp. JNU-WLY501***	5.54	1.77	20-40	37 **37-42**	5.4-9.1	7.5 **7.5-8.0**	tryptone peptone yeast extract			**acetate** **acetyl-phosphate** **propionate** **propionyl-phosphate** **propionyl-CoA** **(S)-2 / (R)-methyl-malonyl-CoA** **succinyl-CoA**	acetate propionate	(Liu et al., 2022) This study
	Anaerohalosphaera lusitana	0.30	0.15	25-45	37	6.5-8.5	7.0	vitamina B12 D-galactose D-mannose	sucrose D -galactose D -glucose D -mannose		N -acetylglucosamine	acetate ethanol H 2 / formate lactate	(Pradel et al., 2020)
	Thermoclostridium stercorarium				60				saccharides, pentose hexose, cellobiose celullose hemicellulose lignocelullose		xylan pectin	ethanol lactate acetate formate	(Wang et al., 2022) (Bing et al., 2023)
	Proteiniphilum saccharofermentans	40.1	9.16	15-45	35-40 **37-42**	6.3-9.1	7.5-7.8 **7.5-8.0**	catalase oxidase peptone arabinose amino acids yeast extract	disaccharides polysaccharides carbohydrates organic compounds cellulose, arabinan hemicellulose arabinogalactan B-glucans, mannan	protein	xylan, pectin chitin, starch fructan **acetate** **acetyl-phosphate** **propionate** **propionyl phosphate** **propionyl-CoA** **(S)-2 / (R)-methyl-malonyl-CoA** **succinyl-CoA**	acetate propionate isovaleric H2 / CO2 formate	(Hahnke et al., 2016) (Tomazetto et al., 2018) This study

	Bacteroides cellulosilyticus	0.77	0.15	30-39	37	6.5-7.2	6.8	lactose raffinose arabinose cellobiose aesculin xylan salicin	glucose, sucrose fructose, maltose xylose, galactose ribose, melibiose mannose, lactulose cellulose		xylan pectin starch galacturonic acids	acetate propionate succinate	(Robert et al., 2007)
	Porphyromonas gingivalis	0.25	0.16		**37-42**		**7.5-8.0**				**propionate**		This study
6.0 g L-1	*Alkalitalea saponilacus*	0.89	0.70	8-40	35-37 **37-42**	7.5-10.5	9.7 **7.5-8.0**	L-arabinose cellobiose maltose trehalose	maltose, sucrose D-fructose, D-ribose hemicellulose lignocellulose		malate yeast extract **acetate** **acetyl phosphate** **propionate** **propionyl-phosphate** **(R)-methyl-malonyl-CoA** **succinyl-CoA**	acetate **propionate** butyrate iso-valerate ethanol	(Zhao & Chen, 2012) This study
	Treponema brennaborense	0	0.16		37 **37-42**		**7.5-8.0**				**acetate** **acetyl phosphate** **propionate** **propionyl-phosphate** **(S)-Lactate**	raffinose mannose	(Schrank et al., 1999) This study
	Barnesiella viscericola	0	0.20		37 **37-42**		**7.5-8.0**		glucose, maltose sucrose cellobiose D-cellobiose D-mannose		**acetate** **acetyl phosphate** **propionate** **propionyl-phosphate** **(S)-2 / (R)-methyl-malonyl-CoA**	acetic propionic succinic formiac, isovaleric	(Sakamoto et al., 2007) (Morotomi et al., 2008) This study
12.0 g L-1 **18.0 g L-1**	*Vibrio cincinnatiensis*	0	1.77	25-35	37 **37-42**	10.0	7.8-8.2 **7.5-8.0**	peptone yeas extract N2	D-xylose D-cellobiose	amino acids L-citrulline	acetate**, acetyl phosphate** **propionate** **propionyl phosphate** **(S)-Lactate** butyrate, fumarate citrate, ethanol, salicin	sucrose glucose salicin varbutin N-inositol L-mannose L-arabinose	(Brayton et al., 1986) (Urdaci et al., 1988) (Jackel et al., 2020) This study
	Alistipes sp. dk3624	0	0.17	32-45	37 **37-42**		**7.5-8.0**	glucose		leucyl glutamyl glycine arylamidase alanine arylamidase glutamine arylamidase	**acetate** **acetyl phosphate** **propionate** **propionyl phosphate** **propionyl-CoA** **(R)-methyl-malonyl-CoA**	succinic acetic propionic fumaric indole	(Shkoporov et al., 2015) This study.
	Coprobacter secundus	0	0.27	32-45	37 **37-42**	6.2-8.0	7.0 **7.5-8.0**	glucose	lactose, maltose mannose, raffinose sucrose, trehalose D-glucose L-rhamnose cellobiose		**acetate** **acetyl phosphate** **propionate** **propionyl phosphate** **propionyl-CoA** **(R)-methyl-malonyl-CoA**	acetate succinate fumarate lactate	(Shkoporov et al., 2015) (Sakamoto et al., 2021) This study
	Tannerella forsythia	0.22	0.21					peptides amino acids		peptides amino acids proteins		amino acids	(Sharma, 2010)

	Parabacteroides goldsteinii	0.24	0.21						glucose, rhamnose sucrose, trehalose xylose, cellobiose carbohydrates		peptone yeast	acetate propionic succinic isovaleric formic	(Sakamoto & Benno, 2006)
	Tenuifilum thalassicum	0	0.22	20-65	50	4.0-9.0	6.5-7.0	yeast extract carbohydrates peptide proteins	glucose carbohydrates			acetate H2 / CO2	(Podosokorskaya et al., 2020)
	Paludibacter propionicigenes	0.37	0.33	15-35	33 **37-42**	5.0-7.6	6.6 **7.5-8.0**	sugars	glucose, arabinose xylose, cellobiose fructose, galactose mannose, maltose melibiose		glycogen, starch **acetate** **acetyl phosphate** **propionate** **propionyl phosphate** **(S)-2 / (R)-methyl-malonyl-CoA** **succinyl-CoA**	acetate propionate succinate	(Ueki et al., 2006) This study
	Maribellus comscasis	0.26	0.28	28-37	30	6.0-8.0	7.0	cellulose pectin xylan	glycosides fucoidan hydrolases polysaccharides mannan, cellulose		xylan, starch pectin	amino acids	(Zheng et al., 2021) (Zheng & Sun, 2021)
	Draconibacterium orientale	0.26	0.17	20-40	28-32	5.5-9.0	7.0-7.5	yeast extract peptone NaCl	glucose, trehalose sucrose, turanose raffinose, melezitose gentibiose, melobiose inulin, cellobiose D-galactose D-mannose D-xylose	L-serine acetylglucosamine	gelatin, starch glycogen, potassium esculin ferric citrate **propionic acid** **acetic acid**	indol	(Jun Du et al., 2014) This study
	Brachybacterium faecium	0	0.17	25-30	28 **37-42**		7.2 **7.5-8.0**	starch yeast extract triptone	sucrose, glucose sugar, maltose mannose, cellobiose	arginine casein hippurate **L-serine**	uric acid, urea nitrate, esculin starch, **acetate** **acetyl phosphate** **propionate** **propionyl-CoA** **succinyl-CoA** **formate** **coenzyme F420**	acids	(Collins et al., 1988) (Lapidus et al., 2009) This study KEGG 2024
24.0 g L-1													
	Corynebacterium casei	0	0.14		**37-42**		**7.5-8.0**		glucose, ribose mannose, fructose	**L-serine**	nitrate, **acetate** **acetyl phosphate** **propionate** **propionyl phosphate** **propionyl-CoA** **3-oxopropanoate** **3-hydroxy-propinyl-CoA** **acryloyl-CoA** **2-hydroxybutanoate** **2-oxobutanoate** **(S)-Lactate** **succinyl-CoA** **formate**	acids	(Brennan et al., 2001) This study KEGG 2024

Candidatus Cloacimonas acidaminovorans	0	0.19		**37-42**	**7.5-8.0**		protein amino acids **L-serine**	**acetate** **acetyl phosphate** **propionate** **propionyl phosphate** **(S)-2-methylmalonyl-CoA** **(R)-methylmalonyl-CoA** **formate** **(2R)-3-sulfolactate**	H2 / CO2	(Solli et al., 2014) This study
Corynebacterium stationis	0.53	1.57	25-42	**37-42**	**7.5-8.0**	glucose fructose ribose	**L-serine**	**acetate** **acetyl phosphate** **propionate** **peopionyl phosphate** **propionyl-CoA** **3-oxopropanoate** **3-hydroxy-propinyl-CoA** **acryloyl-CoA** **2-hydroxybutanoate** **2-oxobutanoate** **L-Lactate** **succinyl-CoA** **formate**	nucleotides nucleosides inosinic acid guanylic acid vitamin B12 citrate acids	(Bernard et al., 2010) (Liu et al., 2016) (Jiang et al., 2024) This study KEGG 2024

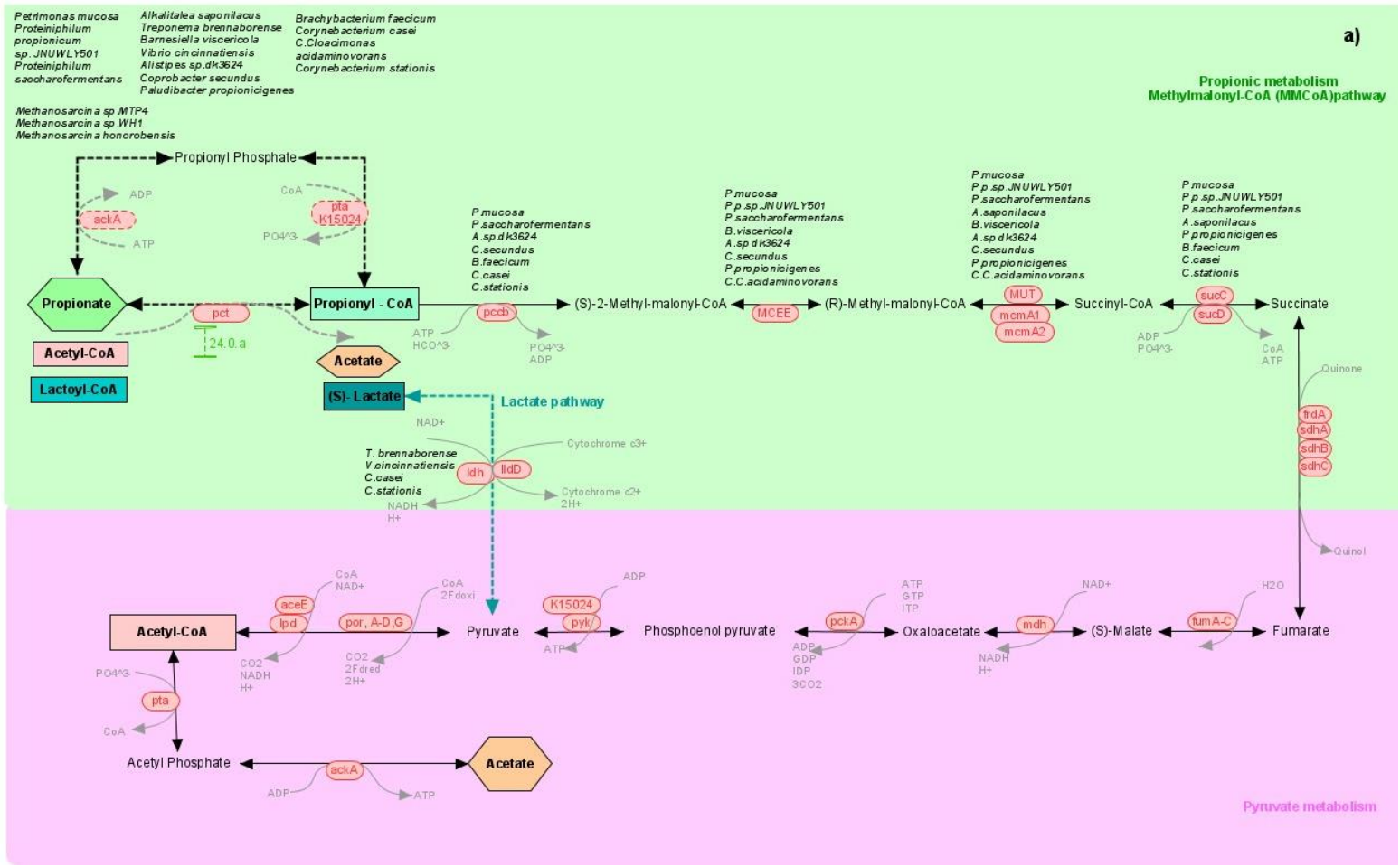

Figure 17. Metabolic pathways of propionate degradation during enrichment in the system. The bar indicate that there was no prediction of genes in concentrations 24.0.a. The dotted line indicates the possible mechanism of propionate degradation in this system. And the microorganisms that carried out the degradation process.

It is important to notice that in the entire enrichment process, as it can be seen in Figure 17, propionate was transformed to acetate by two pathways as follows: mainly by methyl malonyl-CoA (MMC) with modification and putative lactate. Both pathways were present practically along the enrichment; however, the enzyme **pct** was not detected at the highest propionate concentration (24 g L^{-1}).

Based on these results, the potential pathway could be as follows: Propionate was converted to propionyl phosphate by **ackA** (acetate kinase) enzyme**.** Propionyl phosphate was transformed to propionyl-CoA by **pta (**phosphate acetyltransferase), and **K15024** (putative phosphotransacetylase) enzymes. It was infer that propionate was degraded by *Petrimonas mucosa, Proteiniphilum propionicum sp. JNUWLY501, Proteiniphilum saccharofermentans, Alkalitalea saponilacus, Treponema brennaborense, Barnesiella viscericola, Vibrio cincinnatiensis, Alistipes sp. dk3624, Coprobacter secundus, Paludibacter propionicigenes, Brachybacterium faecium, Corynebacterium casei, Candidatus Cloacimonas acidaminovorans, Corynebacterium staionis, Methanosarcina sp. MTP4, Methanosarcina sp. WH1,* and *Methanosarcina horonobensis* metabolize propionate.

Palacios et al. (2003) mentioned that **ackA** and **pta (K15024)** can synthesize propionyl phosphate and propionyl -CoA, respectively. Ingram-Smith et al. (2005) mentioned that the sequence alignments of **PduW** (propionate kinase), and **ackA** have similar catalytic mechanisms and binding sites. These authors strongly supported this mechanism based on modeling the coupling of the substitution at the position equivalent to Val^{93}, which allows the accommodation of propionate in **ackA**. It has been reported that *Corynebacterium glutamicum* activates propionate within the cell through **ackA** and **pta,** which is essential if propionate is the only carbon source (Veita et al., 2009).

The change from propionyl-CoA to (S)-2-methyl-malonyl-CoA by the **ppcb** enzyme could be attributed to the following bacteria: *P. mucosa, P. saccharofermentans, A. sp. dk3624, C. secundus, B. faecicum, C. casei,* and *C. stationis*. While, the change from (S)-2-methyl-malonyl-CoA to (R)-methyl-

malonyl-CoA through the **MCEE** enzyme by bacteria *P. mucosa, P. p. sp. JNUWLLY501, P. saccharofermentans, B. viscericola, A. sp. dk3624, C. secundus, P. propionicigenes,* and *C. C. acidaminovorans.* In the change from (R)-methyl-malonyl-CoA to succinyl-CoA by the **MUT** enzyme could be produced by the bacteria *P. mucosa, P. p. sp. JNUWLLY501, P. saccharofermentans, A. saponilacus, B. viscericola, A. sp. dk3624, C. secundus, P. propionicigenes,* and *C. C. acidaminovorans.* The change from succinyl-CoA to succinate through the **suc** enzyme was attributed to the bacteria *P. mucosa, P.p.sp.JNUWLLY501, P. saccharofermentans, A.saponilacus, P. propionicigenes, B. faecicum, C. casei,* and *C. stationis.*

The change from succinate to fumarate is a critical step in the degradation of propionate because it is a highly endergonic reaction. In order to drive these reactions, it is needed the input of metabolic energy, using a mechanism called reverse electron transport (A. J. M. Stams & C. M. Plugge, 2009); however, there was not a limitation along the enrichment process. The transformation from succinate to pyruvate was present along the six sampling points, which indicates that there were no limitations of this pathway. The step from pyruvate to acetyl-CoA has two pathways that were predicted along the enrichment process; both had small changes. For the acetyl-CoA conversion to acetate, it was observed a reduction of the hits of the phosphate acetyltransferase (**pta**), while the putative phosphotransacetylase (**K15024**) and the acetate kinase (**ackA**) had oscillation values along the enrichment process.

On the other hand, in the lactate pathway, the step from (S)-Lactate to pyruvate by the **ldh** enzyme was attributed to bacteria *T. brennaborense, V. cincinnatiensis, C. casei,* and *C. stationis.*

KEGG shows the metabolic pathways of these bacteria and archaea that have enzymes that participate in the degradation of propionate. Thus, the findings indicate that propionate degradation is not limited to a specific group of syntrophic bacteria. Since Claes et al. (2002) reported gene transfer in *Corynebacterium glutamicum*. This gene transfer between these microorganisms has been

documented in other environments (Frigaard et al., 2006; Fuchsman et al., 2017). It was inferred that there may have been gene transfer among these detected bacteria and archaea. According to our findings, it seems that the digester was subjected to an extreme enrichment that caused the selection of bacteria and methanogens with well-suited metabolic capabilities as well as environmental traits that enable proliferation in the digester environment. The enrichment could cause a rare population to become quite abundant. It is needed additional work to define that Proteiniphilum *saccharofermentans, Petrimonas mucosa, Proteiniphilum propionicum sp. JNUWLY501* are responsible for transferring their genes and the activation of others (which allowed the degradation of propionate) to other species in this system. There are several studies that report that there is gene transfer when microorganisms are subjected to the same environmental conditions (Barkay et al., 2010; Brochier-Armanet et al., 2011; Frigaard et al., 2006; Guo et al., 2015; Mongodin et al., 2005; Rhodes et al., 2011). After the enrichment with high levels of propionate, it was observed that the microbiome could reduce the propionate levels in the digester.

Methane production

In **Figure 18** it is possible to see the predicted pathways for methane production along the enrichment process. Table 10 shows the hits for the corresponding predicted enzymes. There were identified four pathways for methane production along the enrichment as follows: 1) acetoclastic, 2) CO_2 metabolism, also known as hydrogenotrophic, 3) methanethiol, and 4) trimethylamines pathways.

As can be seen in Table 10, from the beginning to the end of the enrichment process, there was an increase in enzyme acetate kinase (**ackA**) responsible for converting the acetate to acetyl phosphate for the acetoclastic pathway. In the same manner, there was an increase in enzyme formylmethanofuran dehydrogenase (**fwh**) responsible for converting the CO_2 to formyl-MFT for the pathway CO_2 metabolism. In the methanethiol and trimethylamines decreases the **mta** and **mtt** enzymes. These changes indicate that among the four pathways for

methane production, at the end of the enrichment process, the acetoclastic and hydrogenotrophic pathways were the predominant pathways.

Table 10. Enzymes and hits present in Methane synthesis.

KO	Enzyme	Symbol	Reaction	Distilled and Refined Annotation of Metabolism (DRAM)	Hits					
					1.0.a	1.2.c	6.0.a	12.0.b	18.0.b	24.0.a
Methane metabolism Acetoclastic										
K00925	2.7.2.1	ackA	R00315	acetate kinase	25	34	33	26	28	28
K00625	2.3.1.8	pta	R00230	phosphate acetyltransferase	10	10	8	6	6	4
K13788	2.3.1.8	pta	R00230	phosphate acetyltransferase	1	0	1	1	0	2
K01895	6.2.1.1	acs	R00235	acetyl-CoA synthetase	4	2	10	8	4	7
K00193	2.3.1.169	cdhC	R09096	acetyl-CoA decarbonylase/ synthase complex subunit beta	1	1	3	2	1	0
K00194	2.3.1.169	cdhD	R09096	acetyl-CoA decarbonylase/ synthase complex subunit delta	1	1	3	2	1	1
K00197	2.3.1.169	cdhE	R09096	acetyl-CoA decarbonylase/ synthase complex subunit gamma	2	2	3	1	1	7
Methane metabolism CO2										
K00200	1.2.7.12	fwdA	R03015	formylmethanofuran dehydrogenase subunit A	5	5	5	3	2	5
K00201	1.2.7.12	fwdB	R03015	formylmethanofuran dehydrogenase subunit B	6	6	8	5	4	7
K00202	1.2.7.12	fwdC	R03015	formylmethanofuran dehydrogenase subunit C	5	3	4	3	3	7
K00203	1.2.7.12	fwdD	R03015	formylmethanofuran dehydrogenase subunit D	4	6	4	3	3	8
K11261	1.2.99.5	fwdE	R03015	formylmethanofuran dehydrogenase subunit E	8	4	14	5	4	9
K00205	1.2.7.12	fwdF	R03015	4Fe-4S ferredoxin	3	2	4	2	1	2
K11260	1.2.7.12	fwdG	R03015	4Fe-4S ferredoxin	4	3	2	1	1	2
K00672	2.3.1.101	ftr	R03390	formylmethanofuran tetrahydromethanopterin N-formyltransferase	2	1	2	1	1	2
K01499	3.5.4.27	mch	R03464	methenyltetrahydro-methanopterin cyclohydrolase	2	2	3	2	1	3
K00319	1.5.98.9	mtd	R04456	methylenetetrahy-dromethanopterin dehydrogenase	2	2	2	2	1	2

K00320	1.5.98.2	mer	R04464	coenzyme F420-dependent N5,N10 methenyltetrahydromethanopterin reductase	3	2	4	2	1	2
Methane metabolism Methanethiol										
K14080	2.1.1.246	mtaA	R09098	coenzyme M methyltransferase	4	3	0	0	0	1
K04480	2.1.1.90	mtaB	R09098	methanol 5-hydroxybenzimidazolylcobamide Co-methyltransferase	3	4	1	1	1	0
K14081		mtaC	R09098	methanol corrinoid protein	3	3	0	1	0	1
Methane metabolism Me/Di/Tri-methylamine										
K16176	2.1.1.248	mtmB	R09998	corrinoid protein Co-methyltransferase	0	0	0	0	0	4
K14082	2.1.1.247	mtbA	R09998	coenzyme M methyltransferase	1	1	0	0	0	0
K16178	2.1.1.249	mtbB	R09999	dimethylamine corrinoid protein Co-methyltransferase	2	2	0	0	0	0
K16179		mtbC	R09999	dimethylamine corrinoid protein	3	3	0	0	0	0
K14083	2.1.1.250	mttB	R09124	trimethylamine corrinoid protein Co-methyltransferase	4	3	0	0	0	3
K14084		mttC	R09124	trimethylamine corrinoid protein	2	1	0	0	0	0
Methane metabolism in comun										
K00577	2.1.1.86	mtrA	R04347	tetrahydromethanopterin S methyltransferase subunit A	3	6	4	3	2	0
K00578	2.1.1.86	mtrB	R04347	tetrahydromethanopterin S methyltransferase subunit B	2	2	2	2	1	1
K00579	2.1.1.86	mtrC	R04347	tetrahydromethanopterin S methyltransferase subunit C	2	2	2	2	1	1
K00580	2.1.1.86	mtrD	R04347	tetrahydromethanopterin S methyltransferase subunit D	2	2	2	2	1	2
K00581	2.1.1.86	mtrE	R04347	tetrahydromethanopterin S methyltransferase subunit E	2	2	2	2	1	2
K00582	2.1.1.86	mtrF	R04347	tetrahydromethanopterin S methyltransferase subunit F	1	2	2	2	1	0

K00583	2.1.1.86	mtrG	R04347	tetrahydromethanopterin S methyltransferase subunit G	1	1	1	1	0	0
K00584	2.1.1.86	mtrH	R04347	tetrahydromethanopterin S methyltransferase subunit H	3	2	3	2	1	1
K00399	2.8.4.1	mcrA	R04541	methyl-coenzyme M reductase alpha subunit	2	1	2	2	1	5
K00401	2.8.4.1	mcrB	R04541	methyl-coenzyme M reductase beta subunit	2	1	3	2	2	5
K00402	2.8.4.1	mcrG	R04541	methyl-coenzyme M reductase gamma subunit	3	1	3	2	2	5
K22480	1.8.7.3	hdrA1	R11943	heterodisulfide reductase 1	1	1	0	0	0	0
K03388	1.8.7.3 1.8.98.4 1.8.98.5 1.8.98.6	hdrA2		heterodisulfide reductase 2	4	3	4	3	5	6
K22481	1.8.7.3	hdrB1	R11931	heterodisulfide reductase 1	2	2	1	1	1	1
K03389	1.8.7.31.8.98.41.8.98.51.8.98.6	hdrB2		heterodisulfidereductase 2	3	1	3	1	2	2
K22482	1.8.7.3	hdrC1	R11931	heterodisulfide reductase 1	1	1	0	0	0	0
K03390	1.8.7.3 1.8.98.4 1.8.98.5 1.8.98.6	hdrC2		heterodisulfide reductase 2	6	4	7	4	4	5
K08264	1.8.98.1	hdrD	R4540	heterodisulfide reductase	1	2	1	0	0	0
K08265	1.8.98.1	hdrE	R4540	heterodisulfide reductase	1	1	1	0	0	0
K14126	1.8.98.5	mvhA	R11943	F420-non-reducing hydrogenase	1	1	2	1	2	3
K14127	1.12.99 1.8.98.5 1.8.98.6	mvhD		F420-non-reducing hydrogenase iron-sulfur subunit	1	1	3	2	4	4
K14128	1.8.98.5	mvhG	R11943	F420-non-reducing hydrogenase	1	1	1	1	1	2
K22516	1.8.98.6	fdhA	R11944	formate dehydrogenase (coenzyme F420) alpha subunit	2		3	2	3	7
K00125	1.8.98.6	fdhB	R11944	formate dehydrogenase (coenzyme F420) beta subunit	2	1	3	2	3	10

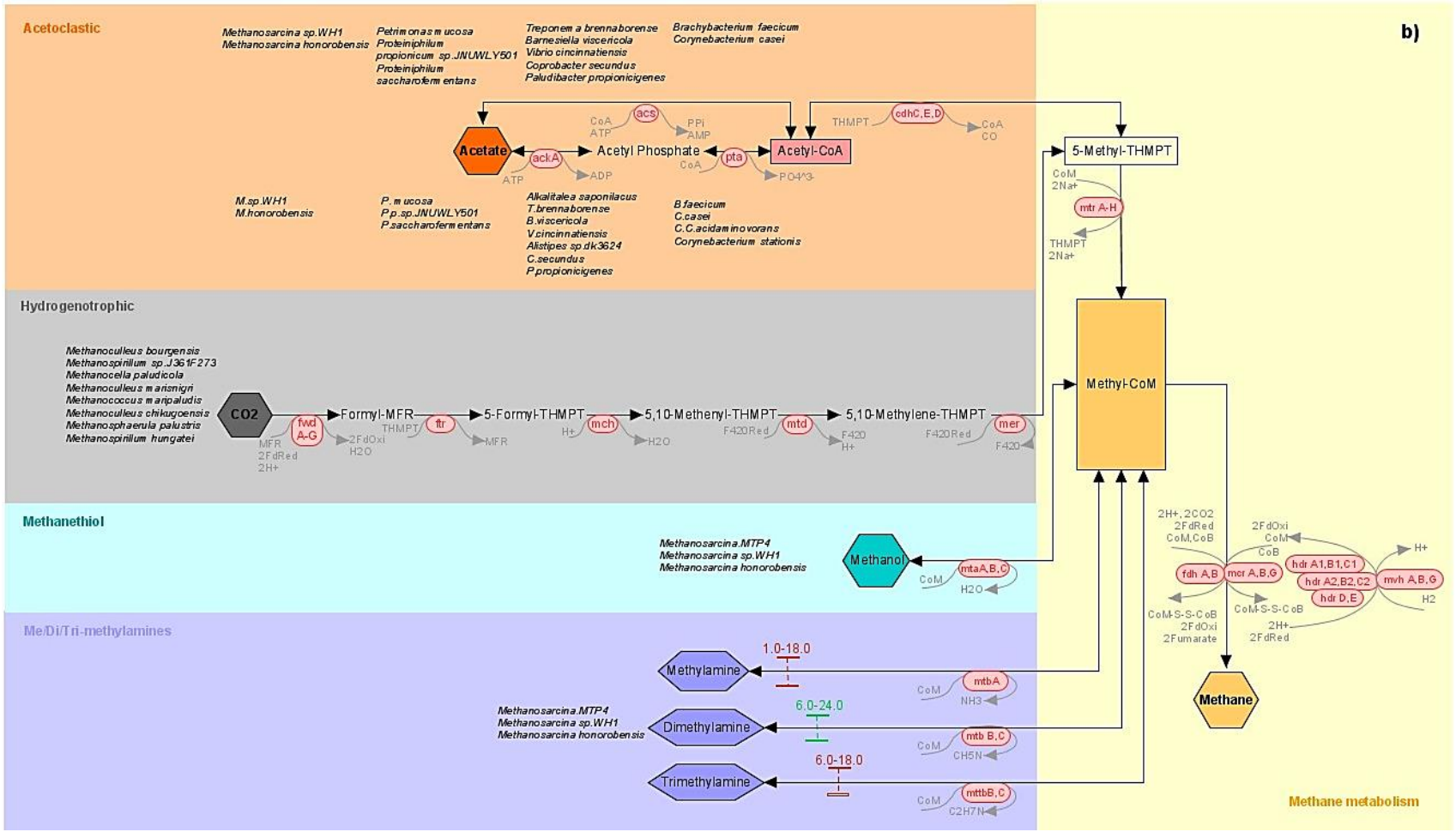

Figure 18. Methane synthesis during enrichment in the system. The bars indicate that there were no prediction of genes in concentrations 6.0.a, 12.0.b, 18.0.b, and 24.0.a. And the microorganisms that carried out the degradation process.

Table 11 shows pH, temperature, growth, substrates, and fermentation of the most abundant methanogenic archaea in this study. We speculate that the acetoclastic pathway was performed by bacteria and archaea such as *P. mucosa, P. p. sp. JNUWLY501, P. saccharofermentans, A. saponilacus, T. brennaborense, B. viscericola, V. cincinnatiensis, A.spdk3624, C. secundus, P. propionicigenes, B. faecicum, C. casei, C. C. acidaminovorans, C. stationis, Methanosarcina sp. WH1,* and *M. honorobensis.* While the hydrogenotrophic pathway by *Methanoculleus bourgensis, Methanospirillum sp. J361F273, Methanocella paludicola, Methanoculleus marisnigri, Methanoculleus maripaludis, Methanoculleus chikugoensis, Methanosphaerula palustris,* and *Methanospirillum hungatei.* The Methanethiol and Me/Di/Tri-methylamines pathways by *Methanosarcina.MTP4, Methanosarcina sp. WH1,* and *M. honorobensis.*

We infer that methane production in the system was influenced by a hydrogenotrophic environment, with production and consumption of H_2 and CO_2. As it is shown in **Table 10**, the experimental conditions of pH and temperature were the optimal for *Methanoculleus* methanogens. The difference among the relative abundance of *Methanoculleus marisnigri, Methanoculleus bourgensis,* and *Methanoculleus chikugoensis* could be attributed to the growing source. At the 24 g L-1 additional methanogens were detected among others *Methanospirillum sp. J361 F273, Methanocella paludicola, Methanococcus maripaludis, Methanosphaerula palustris,* and *Methanospirillum hungatei.*

Table 11. pH, temperature, growth, substrates, and fermentation products of the most abundant methanogenic archaea in this study. The letter remarked in black, indicates information on this work.

Relative abundance % of this study	Archaea	g L-1		Growth T°C	Optimal T °C	Growth pH	Optimal pH	Growth			Substrates to produce methane				References
		1.0	24.0												
1.0 g L-1 1.2 g L-1	*Methanosarcina mazei*	3.68	0.06	32-37	35	6.2-7.4	6.0-7.0	methanol trimethylamine			acetate	methanol	methylamines trimethylamine		(Robinson, 1986) (Mei-Chin et al., 1999) (Deppenmeier et al., 2002) (Shimizu et al., 2011)
6.0 g L-1	*Methanothrix soehngenii*	0.09	0.04	3-45	37	6.8-8.2	7.4-7.8	acetate			acetate				(Huser et al., 1982) (Jetten et al., 1992)
	Methanoregula boonei	0	0.03	10-40	35-37	4.5-5.5	5.1	acetate coenzyme M	H2 / CO2						(Cadillo-Quiroz et al., 2009) (Yashiro et al., 2011) (Brauer et al., 2015)
	Methanocella arvoryzae	0	0.01	37-55	45	6.0-7.8	7.0	acetate H2 / CO2 formate yeast extract	H2 / CO2	formate					(Sakai et al., 2010)
	Methanoregula formicica	0	0.08	10-40	30-33	7.0-7.6	7.4	acetate H2 / formate yeast extract coenzyme M	H2	formate					(Yashiro et al., 2011)
	Methanocorpusculum labreanurn	0	0.03	25-40	37	7.0	6.5-7.5	H2 / CO2 formate yeast extract trypticase peptone	H2 / CO2	formate					(Zhao et al., 1989)
12.0 g L-1 18.0 g L-1	*Methanolacinia petrolearia*	0	0.08	17-43	37-40	5.3-8.2	7.0	acetate	H2 / CO2	formate				2-propanol	(Goker et al., 2014) (Cadillo-Quiroz et al., 2009)
	Methanolobus zinderi	0	0.02	25-50	40-50	6.0-9.0	7.0-8.0	methanol N-methylamines				methanol	methylamines dimethylamine trimethylamine		(Doerfert et al., 2009)
	Methanococcoides methylutens	0	0.01	15-36	30-35	7.0-7.5	7.0-7.5	methanol N-methylamines yeast extract tripticase				methanol	methylamine diethylamine trimethylamine		(Sowers & Ferry, 1983)
	Methanothrix harundinacea	0	0.04	25-45	34-37	6.5-9.0	7.2-7.6	acetate yeast extract peptone			acetate				(Ma et al., 2006)
	Methanoculleus bourgensis	**1.13**	**18.89**	15-45	37 **37-42**	6.7-7.5	6.7 **7.5-8.0**	acetate	H2 / CO2	formate				2-propanol 1,2-propanol	(Maestrojuan et al., 1990) (Schnurer et al., 1999) (Manzoor et al., 2016) This study

	Methanomethylovorans hollandica	0	0.02	12-40	34-37	6.0-8.0	6.5-7.0	methanol N-methylamines				methanol methanethiol	methylamine dimethylamine trimethylamine	dimethyl sulfide		(Lomans et al., 1999)
	Methanosarcina siciliae	0.01	0.04	35-40	35	6.0-7.7	6.5-6.8	acetate methanol methanethiol N-methylamines	H2 / CO2		acetate	methanol	methylamines trimethylamine			(Ni et al., 1994) (Elberson & Sowers, 1997) (Shimizu et al., 2011)
	Methanosarcina barkeri	0.02	0.02	20-45	37-40	5.0-8.0	6.0-7.0	acetate N-methylamines	H2 / CO2		acetate	methanol	methylamine dimethylamine trimethylamine	ethyl-dimethylamine		(Weimer & Zeikus, 1978) (Hippe et al., 1979) (Zellner & Winter, 1987) (Schnurer et al., 1999) (Shimizu et al., 2011)
	Methanospirillum sp. J361F273	0	0.05	30-37	35 **37-42**	6.5-7.4	6.6-7.4 **7.5-8.0**	H2 / CO2 formate yeas extract trypticase	H2 / CO2	formate						(Ferry et al., 1974) This study
	Methanocella paludicola	0	0.01	25-40	35-37 **37-42**	6.5-7.8	7.0 **7.5-8.0**	acetate	H2 / CO2	formate						(Sakai et al., 2008) This study
	Methanoculleus marisnigri	**0.04**	**21.45**	15-45	40 **37-42**	6.0-8.0	8.0 **7.5-8.0**	formate 2-propanol/CO2	H2 / CO2	formate					2-propanol 2-butanol	(Romesser et al., 1979) (Zellner & Winter, 1987) (Maestrojuan et al., 1990) (Anderson et al., 2009) This study
	Methanococcus maripaludis	0	0.03	18-47	35-39 **37-42**	6.4-8.2	6.8-7.2 **7.5-8.0**	H2 / CO2 formate	H2 / CO2	formate						(Jones et al., 1983) This study
24.0 g L-1	***Methanoculleus chikugoensis***	**0.04**	**12.63**	15-40	25-30 **37-42**	6.7-8.0	6.7-7.2 **7.5-8.0**	acetate 2-propanol/CO2	H2 / CO2	formate					2-propanol 2-butanol ciclopentanol	(Dianou et al., 2001) This study
	Methanosphaerula palustris	0	0.10	14-35	30 **37-42**	4.8-6.5	5.3-5.5 **7.5-8.0**	acetate formate coenzyme M	H2 / CO2	formate						(Cadillo-Quiroz et al., 2009) (Yashiro et al., 2011) This study
	Methanosarcina sp. MTP4	0	0.04	28-30	**37-42**		**7.5-8.0**	MTP4 dimethylsulfide			acetate		3-S methyl-mercapto-propionate (MTP4)	demethylates	**propionate** **propionyl-phophate** **propionyl-CoA**	(Van Der Maarel et al., 1995) This study
	Methanospirillum hungatei	0	0.07	20-40	37 **37-42**	6.5-10.0	6.6-7.4 **7.5-8.0**	acetate	H2 / CO2	formate						(Zellner & Winter, 1987) (Cadillo-Quiroz et al., 2009) (Gunsalus et al., 2016) This study
	Methanosarcina sp. WH1	0.01	0.02		**37-42**		**7.5-8.0**		H2 / CO2	formate	acetate	methanol	methylamine dimethylamine trimethylamine		**propionate** **propionyl-phophate**	This study KEGG., 2024

															propionyl-CoA	
	Methanosarcina honorobensis	0.01	0.03	20-42	37 **37-42**	6.0-7.7	7.0-7.2 **7.5-8.0**	acetate methanol N-methylamines dimethylsulfide		acetate	methanol	trimethylamine	dimethyl sulfide	**propionate** **propionyl-phophate** **propionyl-CoA**	(Shimizu et al., 2011) This study

4.5 Conclusions

In this work, after subjecting the microbiome to gradual stress and selection pressure for 1200 d, fed with chicken litter and the stepwise propionate concentration increase, important changes were observed in the microbial community as follows: 1. The propionate degradation was attributed to the genes **ackA** and **pta**, through the MMCoA pathway, having the acetate as a main product, while the **pct** gene for the lactate pathway. 2. The microorganisms responsible for propionate degradation are not necessarily syntrophic. 3. Many bacteria species and methanogens, as a response to the enrichment process, possibly activated and/or transferred genes that degrade propionate. 4. It was observed an increase in the abundance and diversity of methanogenic archaea at the end of the enrichment process. The synthesis of methane were acetoclastics, CO_2, methanethiol, and trimethylamines pathways generated by a variety of methanogens. 5. The decrease in bacteria and the increase in archaea at the end of the enrichment, it was inferred that the favorable environment of pH, temperature, and substrates aimed more at the group of methanogens. Changing the substrate source of the bacteria triggered gene activation and transfer.

4.6 Acknowledgement

The authors would like to thank the Consejo Nacional de Ciencia, Humanidades y Tecnología (CONACHYT) (México) for the scholarship (number 862782) granted to Thalia Guadalupe Ochoa-Bernal and the Bioprocesses Laboratory Team of the Universidad Autónoma Chapingo for their comments concerning this manuscript.

4.7 CRediT authorship contribution statement

Thalia Guadalupe Ochoa-Bernal: Bioinformatics, Data Analysis, Data Curation, Discussion, and Writing – Original Draft Preparation. **Guadalupe Hernández-Eugenio:** Conceptualization, Methodology, Data Collection & Curation, and Discussion. **David H. Huber**: Discussion, Review & Editing. **Teodoro Espinosa-**

Solares: Conceptualization, Methodology, Founding, Bioinformatics, Data Collection, Analysis, & Curation, Discussion, Writing & Editing.

4.8 Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

4.9 Funding

The present work was supported by the Dirección General de Investigación y Posgrado, from Universidad Autónoma Chapingo, through the project 24020-EI. This work was also supported by the USDA National Institute of Food and Agriculture, Evans-Allen project Accession Number: 1021122. The content of this publication does not represent the opinion of the U.S. Department of Agriculture and should not be interpreted as representing any agency determination or policy.

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5 GENERAL CONCLUSION

Through the gradual enrichment of propionate in co-digestion with chicken litter, a resilient microbial consortium tolerant to high concentrations of propionate was developed. This consortium demonstrated the capability to degrade concentrations of up to 30 g L^{-1} of propionate in mesophilic digesters (35 ± 5 °C), indicating the presence of species that do not operate in syntrophy and facilitate propionate degradation. These conditions also fostered a microbial community with up to 50% relative abundance of methanogenic archaea. Despite the high propionate concentrations, the anaerobic digestion remained stable, ensuring consistent methane production without inhibiting methanogenesis. Functional analysis suggested that ackA (acetate kinase) could play a significant role in propionate degradation for methane production.