Characterisation of the African elephant (*Loxodonta africana*) gut microbiome for bio-energy applications

by

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DECLARATIONS

I, Elzette van der Walt, declare that I submit this thesis in accordance with the requirements of a Master of Science qualification (Magister Scientiae) at the University of the Free State, Bloemfontein, South Africa. I, Elzette van der Walt, declare that this work is solely my own and that I have not previously submitted it for a qualification at another institution of higher education.

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Signature

11/11/2020

Date

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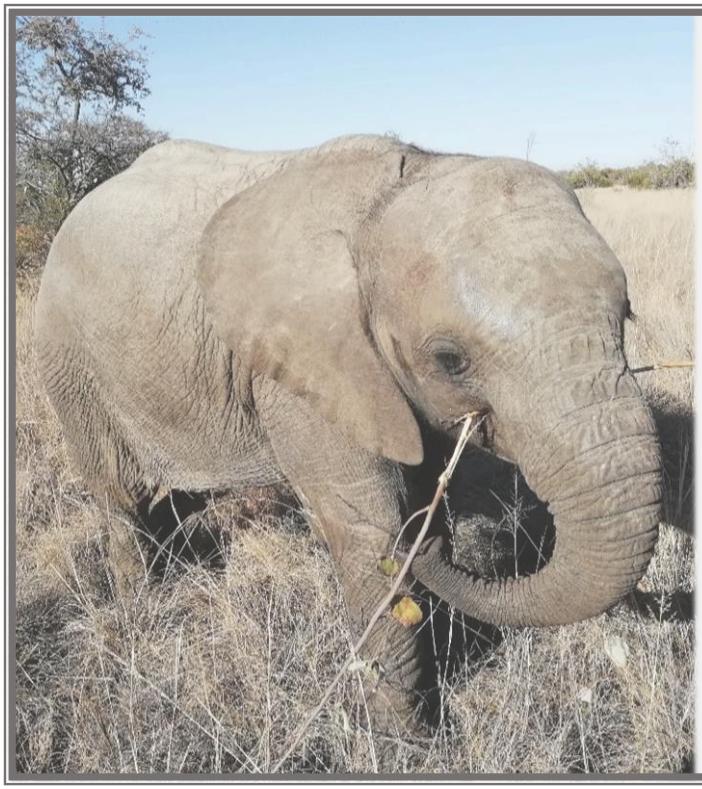
LIST OF ABBREVIATIONS AND SYMBOLS

%	Percentage
<	Less than
>	Greater than
≤	Less than or equal to
≥	Greater than or equal to
®	Registered trademark
™	Unregistered trademark
ºC	Degrees Celsius
ng	Nanogram
µg	Microgram
mg	Milligram
µL	Microlitre
mL	Millilitre
µM	Micromolar concentration
ATP	Adenosine triphosphate
bp	Base pairs
CoA	Coenzyme A
CoB	Coenzyme B
CoM	Coenzyme M
DNA	Deoxyribonucleic acid
gDNA	Genomic deoxyribonucleic acid
e.g.	exempli gratia/for example
et al.	et alia/and others
EtBr	Ethidium Bromide

GHG	Greenhouse gas
HRT	Hydraulic retention time
Min	Minutes
NCBI	National Center for Biotechnology Information
OLR	Organic loading rate
PCR	Polymerase chain reaction
RNA	Ribonucleic acid
rRNA	Ribosomal ribonucleic acid
Sec	Seconds
SA	South Africa
RSA	Republic of South Africa
sp.	Species (singular)
spp.	Species (plural)
V	Volt
VFA	Volatile fatty acid
USA	United States of America
w/v	Weight per volume
C/N	Carbon/Nitrogen
$Ca(OH)_2$	Calcium hydroxide
CH_4	Methane
CO_2	Carbon dioxide
H_2	Hydrogen
H_2S	Hydrogen sulphide
KOH	Potassium hydroxide
NaOH	Sodium hydroxide
NH_4OH	Aqueous ammonia



CHAPTER 1

LITERATURE REVIEW

1. Introduction

The human population currently exceeds seven billion individuals and continues to grow nearly unconstrained due to advances in medicine and technology (Coyle *et al.*, 2014; US Census Bureau, 2019). All organisms modify their environment and humans are no exception, yet the nature of these modifications change drastically as the population grows and evolves (Vitousek, 1997). This growth along with the escalation of global energy demands and a continued reliance on fossil-based fuels for energy generation and transportation has evolved into a growing global energy crisis (Friedmann, 2007; Coyle *et al.*, 2014). The combustion of fossil fuels like coal, crude oil and natural gas for energy generation releases greenhouse gases (e.g., carbon dioxide) into the atmosphere through the conversion of fixed carbon found within the Earth's crust (Berner, 2003). These fixed carbon reservoirs are the fossilised remains of fauna and flora, exposed to heat and pressure in the Earth's crust for millions of years (Al Seadi *et al.*, 2008). This non-renewable resource is thus being depleted faster than it can be replaced. The resulting imbalance in carbon fixation and utilisation is a driving force for the gradual increase of greenhouse gas (GHG) within the Earth's atmosphere, ultimately resulting in a cascade of environmental (climate change) and economic complications (Kurian *et al.*, 2013).

Over the years, rising global GHG emissions were largely attributed to industrialised, high-income nations (e.g., United States, China) (Arto and Dietzenbacher, 2014). However the contributions of developing countries (e.g., South Africa, India) have been steadily increasing and were estimated at around 55 % of global emissions by 2000 (Amer and Daim, 2010; Pegels, 2010). The burning of coal is the primary energy source (Figure 1.1) utilised for electricity generation in South Africa (SA) (Winkler, Spalding-Fecher and Tyani, 2002). This resource is abundant and relatively inexpensive to come by, as SA is home to one of the largest coal deposits in the world (BP Statistical Review of World Energy, 2017). By 2005, SA contributed to approximately 1.1 % of global GHG emissions and around 40 % of the total emissions in sub-Saharan Africa (Pegels, 2010). However, continued efforts to provide universal access to electricity within SA inevitably led to an increase in carbon emissions (Winkler, 2005). According to the latest BP Statistical Review estimates, SA has become the leading carbon dioxide (CO_2) emitter in all of Africa (35 %) and the 14th-largest in the world.

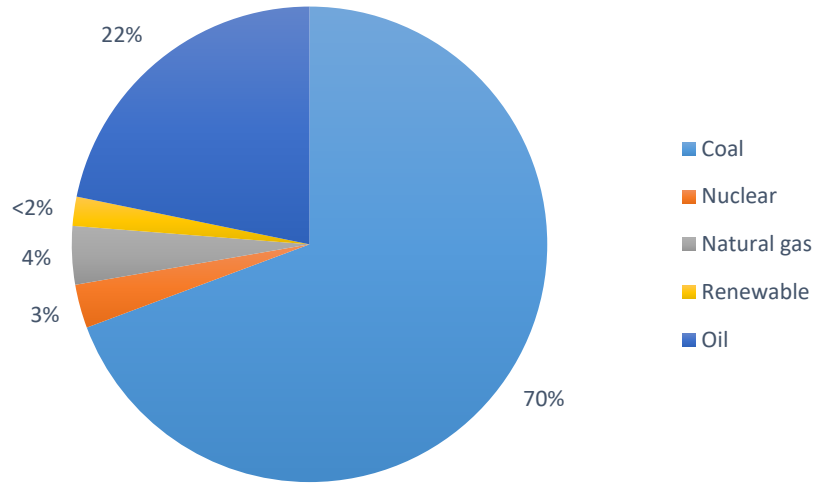


Figure 1.1. Primary sources of energy consumption in South Africa (2016). *In 2016, coal (70 %) and oil (22 %) were the main sources of energy consumption in South Africa according to the BP Statistical Review of World Energy 2017. Less than 2 % of the total consumption is derived from renewable energy sources like wind-, hydro- and solar power.*

Yet, South Africa's main electricity supplier is under immense pressure and already operating at nearly full capacity (Pegels, 2010). In 2008, this situation led to significant power shortages, resulting in massive financial losses (Inglesi and Pouris, 2010). Accordingly, the focus has shifted toward the use of renewable energy sources to address these challenges in an environmentally friendly manner (Owusu and Asumadu-Sarkodie, 2016). The incorporation of green energy within society may alleviate dependence on traditional fuels for electricity generation, heating and transport (Quintana *et al.*, 2011). As of 2016, less than 2 % of the total energy consumption in SA (Figure 1.1) originated from renewable sources (BP Statistical Review of World Energy, 2017). However, renewable sources like wind and solar power are inherently fluctuating, resulting in periods with an imbalance between power production and demand (Agneessens *et al.*, 2018). These fluctuations sprout predominantly from a dependence on seasonal weather patterns, jeopardising operation of the power grid and decreasing the economic viability of renewable power producers (Coker *et al.*, 2020). An urgent need exists for alternative sources of renewable energy that circumvent these energy fluctuations. Bio-energy, generated from organic matter (biomass), can play a critical role in promoting renewable energy alternatives due to a wide array of applications and multiple environmental advantages (Weiland, 2010). Furthermore, energy generation from biomass is considered carbon neutral, since the release of CO_2 during biomass combustion is balanced by CO_2 fixation during photosynthesis (Ingrao *et al.*, 2019).

1.1. Anaerobic digestion of organic material

Anaerobic digestion is considered the most successful, versatile and energy-efficient path for bio-energy production (Werner *et al.*, 2011; Paul and Dutta, 2018). This is attributed to factors like the ability to drastically reduce GHG emissions, organic waste disposal, pathogen reduction, scalability and because the digesters (Section 1.4) are largely independent of geographical location and weather (Pöschl, Ward and Owende, 2010; Abdelgadir *et al.*, 2014; Mao *et al.*, 2015). The anaerobic digestion of biomass aided by microorganisms yields a renewable green energy source, known as biogas (Figure 1.2) (Weiland, 2010).

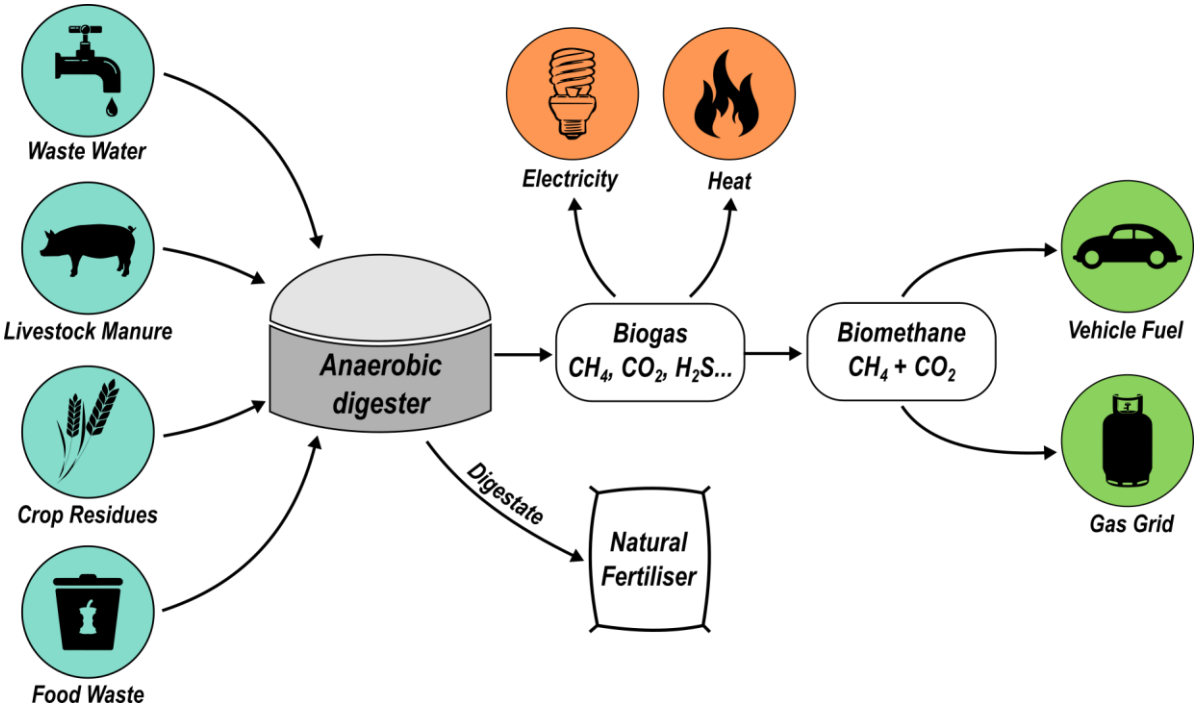


Figure 1.2. Anaerobic digestion of organic biomass. *Unrefined biogas, produced through the anaerobic digestion of organic biomass (e.g., livestock manure, crop residues) is a versatile green energy source suitable for electricity and heat generation. Upgraded biogas (biomethane) serves as a source of green vehicle fuel and can be directly introduced into existing gas grid infrastructure as an alternative to non-renewable natural gas.*

Raw/unrefined biogas is a mixture of methane (CH_4) and CO_2, along with other trace elements, but biogas composition may vary depending on feedstock (biomass) properties and operational parameters (Zheng *et al.*, 2014). The purification of biogas yields a methane-rich gas, known as biomethane (Figure 1.2), typically containing 95 – 97 % CH_4 and 1 – 3 % CO_2 (Ryckebosch, Drouillon and Vervaeren, 2011; Sun *et al.*, 2015). Non-renewable natural gas can be substituted for biomethane by feeding it into existing gas distribution networks for household use, as well as industrial chemical synthesis (Welte and Deppenmeier, 2014).

This multilateral energy source could be used to mitigate global energy needs by replacing conventional fuels for heat and power generation or as gaseous fuel in automotive applications (Yadvika *et al.*, 2004; Achinas, Achinas and Euverink, 2017). Adding to its versatility, nearly any organic material can serve as feedstock for biogas production, these include waste materials like manure and crop residues, energy crops like maize, cellulose rich biomass and the by-products of ethanol and bio-diesel production (Amon *et al.*, 2007; Igoni *et al.*, 2008; Al Seadi *et al.*, 2008; Zhang *et al.*, 2014).

1.2. Overview of anaerobic digestion

During anaerobic decomposition an intricate community of symbiotic microorganisms transform organic material into biogas, nutrients and cell matter (Merlin Christy, Gopinath and Divya, 2014). This time-consuming and complex process is divided into four main stages: hydrolysis, acidogenesis, acetogenesis and methanogenesis (Jingura and Kamusoko, 2017). Figure 1.3 offers a broad overview of anaerobic digestion and the increasing specialisation of the microbes involved in each stage (Campanaro *et al.*, 2016).

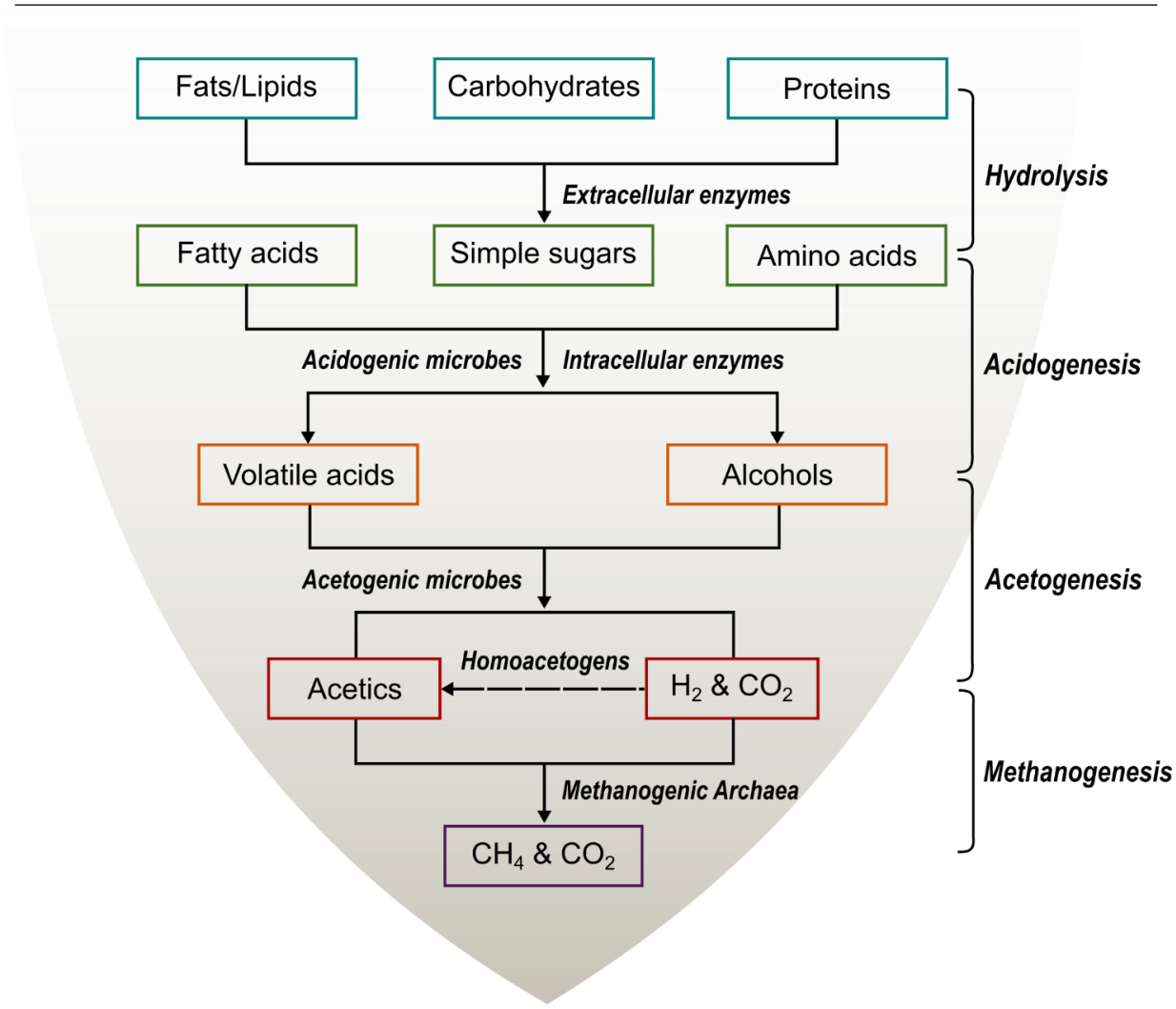


Figure 1.3. Summary of the anaerobic digestion process. *Anaerobic digestion proceeds via four sequential phases: hydrolysis, acidogenesis (fermentation), acetogenesis and methanogenesis. Microbial specialisation represents a funnel shape, with the final step restricted to a single microbial group, methanogenic Archaea. Substrate-specific enzymes secreted by a complex community of microorganisms facilitate each phase ultimately yielding methane gas. Initially, hydrolysing and fermenting microorganisms convert complex polymers to their constituent monomers. The products produced during the first stages of anaerobic digestion provide methanogenic Archaea with the components required for methanogenesis. Adapted from Mao et al., 2015.*

Initially, soluble derivatives produced from the digestion of complex organic polymers like carbohydrates, proteins and lipids undergo fermentation to yield volatile fatty acids (VFAs), acetate and hydrogen (H_2) (Figure 1.3) (Weiland, 2010; Maus *et al.*, 2016). Acetate and H_2 are direct pre-cursors of methanogenesis, however some VFAs must undergo further conversion via acetogenesis (Meegoda *et al.*, 2018). Highly specialised, methanogenic archaea, utilise the compounds produced during the previous phases of anaerobic digestion to produce CH_4 and CO_2. Strikingly, these reactions are owed to a small number of cooperating functional groups, including primary fermenters, secondary fermenters (e.g., syntrophic-, acetogenic bacteria) and methanogenic archaea (Ziemiński and Frąc, 2012).

1.2.1. The stages and taxonomy of anaerobic decomposition

1.2.1.1. Hydrolysis

During hydrolysis, hydrolysing and fermenting microorganisms utilise hydrolytic enzymes like cellulase, amylase, lipase and xylanase to facilitate the breakdown of polymers (e.g., carbohydrates) to their constituent monomers (e.g., simple sugars) (Figure 1.2) (Biely, 1985; Yadvika *et al.*, 2004). The generalist (non-specialised) nature of this stage supports the participation of various microbial groups, including the bacterial phyla, Firmicutes and Bacteroidetes (Campanaro *et al.*, 2018). Members of these bacterial phyla aid fermentation and polysaccharide digestion during hydrolysis (Vanwonterghem *et al.*, 2014). The general abundance and common occurrence of these phyla in anaerobic digesters, treating numerous substrates, further highlights their involvement in anaerobic digestion (Kampmann *et al.*, 2012; Hanreich *et al.*, 2013). However, the exact abundance of each microbial group is dependent on the feedstock (Cirne *et al.*, 2007).

Hydrolytic reactions comprise two phases: first, bacteria colonise the surface of solid organic material, once complete, the bacteria release extracellular enzymes, which enable the production of soluble compounds utilised directly by hydrolytic bacteria or made available to other bacteria within the system (Merlin Christy, Gopinath and Divya, 2014). Complex polymers like cellulose, proteins and fats can take several days to separate into their corresponding monomers. These complex polymers restrict the penetration of microorganisms and extracellular enzymes to substrates, thereby decreasing the efficiency of hydrolysis (Jeihanipour, Niklasson and Taherzadeh, 2011). During the digestion of solid waste, only approximately 50 % of organic compounds undergo degradation (Ziemiński and Frąc, 2012). The remaining fraction remains in the primary state due to a lack of sufficient access or the necessary enzymes to partake in their biodegradation. One strategy to combat these limitations involves substrate pre-treatment (Section 1.6) to break apart the complex polymers and increase the available surface area (Ariunbaatar *et al.*, 2014; Meegoda *et al.*, 2018).

1.2.1.2. Acidogenesis (fermentation)

During acidogenesis, fermentative microorganisms (e.g., *Streptococcus* spp., *Lactobacillus* spp., *Clostridium* spp.) utilise long chain fatty acids and amino acids generated during hydrolysis to produce volatile fatty acids (VFAs), alcohols, H_2 and CO_2 (Figure 1.2) (Spector, 2009; Sträuber, Schröder and Kleinsteuber, 2012; Saha *et al.*, 2019). Volatile fatty acids refer to a class of short-chain organic acids like acetic acid, but may also refer to larger organic acids such as propionic- and butyric acid (Meegoda *et al.*, 2018). The accumulation of these VFAs yield direct pre-cursors for methanogenesis. Two types of acidogenic microbial communities participate during anaerobic digestion (Shin *et al.*, 2011). Facultative anaerobic acidogens (tolerate low levels of oxygen) are initially dominant, whereas obligatory anaerobic acidogens become active during the later stages of anaerobic digestion. These acidogenic microorganisms are generally members of the phyla, Firmicutes, Bacteroidetes, Proteobacteria and Actinobacteria (De Vrieze *et al.*, 2018; Laiq Ur Rehman *et al.*, 2019). Equations (1) to (3) illustrate typical fermentative reactions. All three equations illustrate the conversion of glucose to various products including ethanol (Eq. 1), propionate (Eq. 2) and acetic acid (Eq. 3) (Merlin Christy, Gopinath and Divya, 2014).

$$C_6H_{12}O_6 \leftrightarrow 2\,CH_3CH_2OH + 2\,CO_2 \quad (1)$$

$$C_6H_{12}O_6 + 2\,H_2 \leftrightarrow 2\,CH_3CH_2COOH + 2\,H_2O \quad (2)$$

$$C_6H_{12}O_6 \leftrightarrow 3\,CH_3COOH \quad (3)$$

Reactions that facilitate the conversion of glucose to a specific product commonly result in the production of pyruvic acid (a.k.a., pyruvate) as an intermediate (Spector, 2009). Pyruvic acid fermentation yields numerous C_1 to C_4 compounds including VFAs such as acetic-, propionic- and butyric acids, alcohols, ketones and aldehydes (Joubert and Britz, 1987; Zhou *et al.*, 2018). This multifaceted role makes pyruvate an essential intermediate of the anaerobic digestion process (Agler *et al.*, 2011). Furthermore, during the digestion of protein-rich substrates, amino acids resulting from protein hydrolysis can serve as a carbon source for strict or facultative fermentative bacteria (Ziemiński and Frąc, 2012). The production of short chain VFAs ($C_1 – C_5$) from proteins can occur via (1) the deamination of non-polar amino acids, (2) the fermentation of specific individual amino acids and (3) oxidation-reduction reactions between pairs of amino acids (Stickland fermentation) (Gavala, Angelidaki and Ahring, 2003; Merlin Christy, Gopinath and Divya, 2014; Chen *et al.*, 2018).

1.2.1.3. Acetogenesis

Acetogenesis is the conversion of organic acids, including propionic-, butyric- and pentatonic acid predominantly through the acetyl-CoA pathway to the primary metabolic end-product, acetate (Enrich-Prast *et al.*, 2009). Acetogenic bacteria (e.g., acetogens, homoacetogens) display incredible metabolic diversity and are considered the most metabolically diverse group of anaerobes (Schuchmann and Müller, 2016). Homoacetogenic bacteria produce acetate as the sole metabolic end-product during the reduction of H_2 and CO_2 via the Wood-Ljungdahl pathway (Laiq Ur Rehman *et al.*, 2019). Acetogenic microbes belong to numerous phyla and utilise a vast number of electron donors in addition to organic acids, including hexoses (e.g., glucose, fructose) and pentoses (e.g., xylose) (Ragsdale, 2008; Schuchmann and Müller, 2016). Nonetheless, acetogenic microbes are slow growing and sensitive to environmental shifts; as such, these organisms require extended lag periods to adapt to changing environmental conditions (Xing, Criddle and Hickey, 1997).

During acidogenesis, H_2 accumulation can increase the number of electrophilic atoms capable of attracting an electron from another area within the molecular system (Ungerfeld, 2015). These electrophilic atoms, described as electron sinks, promote bond formation and electron pairing within the system (Merlin Christy, Gopinath and Divya, 2014). Methanogens are unable to utilise the products in these sinks directly, which is why they require the obligate hydrogen producing acetogenic bacteria to convert these products to acetate, CO_2, and H_2 (pre-cursors for methanogenesis) (Meegoda *et al.*, 2018). However, the accumulation of H_2 increases the H_2 partial pressure within the system, inhibiting acetogen metabolism (product inhibition) (Al Seadi *et al.*, 2008). To counteract this effect, acetogens form syntrophic associations with hydrogen-consuming methanogens (Zhu *et al.*, 2011). Syntrophy refers to a form of symbiosis between metabolically distinct groups of organisms, enabling the combined decomposition or synthesis of certain compounds (Schink, 1997; Manzoor *et al.*, 2016). Hydrogen-consuming archaeons therefore utilise the accumulating H_2 for methanogenesis, thereby maintaining a low hydrogen pressure for their acetogenic counterparts (Laiq Ur Rehman *et al.*, 2019).

1.2.1.4. Methanogenesis

A wide spectrum of microorganisms partake in the initial stages of anaerobic digestion, however the final phase, methanogenesis, remains the limiting factor, as this process is performed exclusively by a group of methanogenic archaea (Figure 1.3) (Pyzik *et al.*, 2018). Methanogenesis proceeds via one of three pathways: the hydrogenotrophic-, acetoclastic- or methylotrophic pathway (Figure 1.4) (Galagan *et al.*, 2002). These processes involve a number of enzymes and prosthetic groups unique to methanogens (Ziemiński and Frąc, 2012).

Hydrogenotrophic methanogens reduce CO_2 to CH_4 in a stepwise manner via carrier-bound C_1 intermediates with H_2 acting as electron donor (Figure 1.4) (Blaut, 1994). Select hydrogenotrophs are capable of substituting H_2 for formate as the terminal electron donor during this pathway (Alvarado *et al.*, 2014; Sikora *et al.*, 2019). Carbon transfer proceeds via six steps utilising specialised coenzymes including, methanofuran, tetrahydromethanopterin and coenzyme M (CoM) as carriers (Merlin Christy, Gopinath and Divya, 2014; Pyzik *et al.*, 2018).

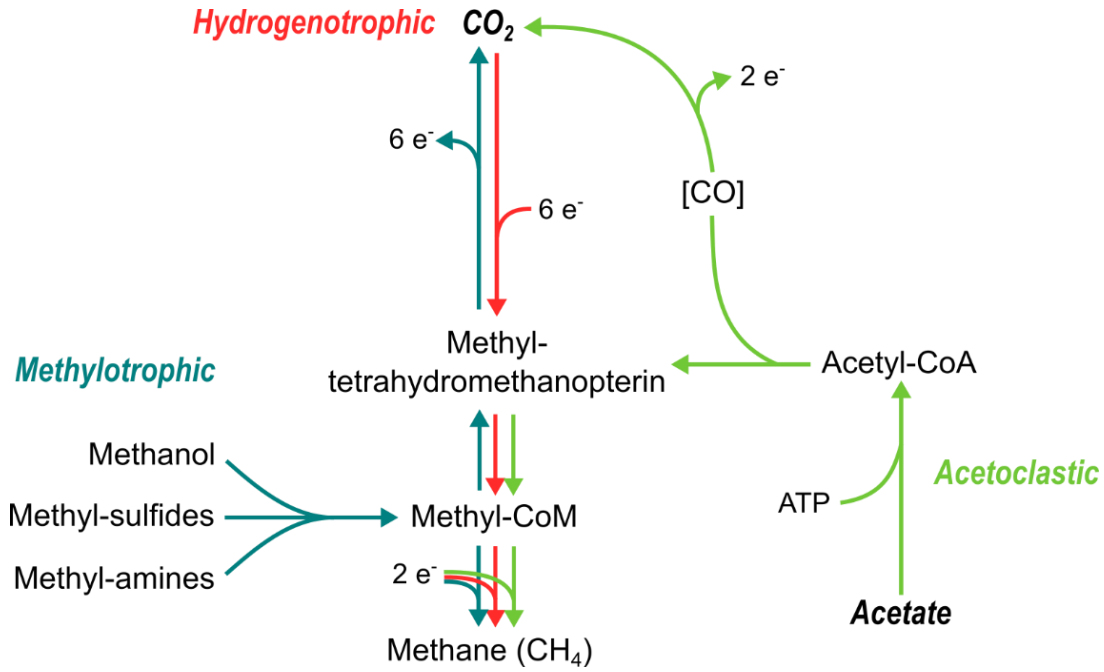


Figure 1.4. The pathways of methanogenesis. *Methanogenesis refers to a form of anaerobic respiration, where one-carbon (C_1) compounds or acetic acid act as a terminal electron acceptor. The hydrogenotrophic pathway (red) involves the reduction of carbon dioxide (CO_2) to methane using electrons derived by oxidising hydrogen (H_2). The methylotrophic pathway (blue) involves the utilisation of C_1 compounds like methanol and methylamines. The direct conversion of acetate to ultimately yield methane refers to the acetoclastic pathway (green). All three these pathways ultimately converge on the reduction of methyl-CoM to methane. Adapted from Galagan et al., 2002.*

Acetoclastic methanogens dismutate acetate to CH_4 and CO_2, preceded by the ATP-dependent activation of acetate to acetyl-CoA (Figure 1.4) (Galagan *et al.*, 2002). The cleavage of acetyl-CoA into a methyl moiety and a carbonyl group (CO) enables oxidation of the carbonyl group, thereby producing reducing equivalents for the reduction of the methyl moiety to methane (Blaut, 1994; Berger, Welte and Deppenmeier, 2012). Methylotrophic methanogens utilise methyl-containing compounds like methanol and methyl-amines (C_1 compounds) for energy conservation (Nettmann *et al.*, 2010).

A portion of the methyl groups are oxidised to CO_2 to facilitate reduction of the remaining methyl groups to methane (Figure 1.4) (Merlin Christy, Gopinath and Divya, 2014). A single class of enzymes, methyltransferases, is required during the reduction of such methylated compounds. These methanogens belong to one of two groups depending on the presence or absence of cytochromes (Vanwonterghem *et al.*, 2016). Those lacking cytochromes are obligately H_2-dependent and lack the central pathway for CO_2 reduction. Methylotrophs with cytochromes can also oxidise methyl groups to CO_2 via a membrane-bound electron transport chain, similar to hydrogenotrophic methanogens (Lang *et al.*, 2015; Vanwonterghem *et al.*, 2016). Nonetheless, the last common step of any methanogenesis pathway involves the reduction of methyl-coenzyme M (methyl-CoM) to methane (Figure 1.4) (Ferry, 2011). The key enzyme during this step, methyl-CoM reductase, reduces methyl-CoM to methane, enabling oxidised coenzyme M (CoM) to form a heterodisulfdie complex with coenzyme B (CoB).

According to the currently available data, all methanogens belong to the archaeal phylum, Euryarchaeota (Paul *et al.*, 2012). Hydrogenotrophic methanogens include members from the orders, Methanobacteriales, Methanomicrobiales, Methanocellales, Methanopyrales, Methanoplasmatales and Methanococcales (Welte and Deppenmeier, 2014; Lang *et al.*, 2015). To date, members from the orders Methanosarcinales, Methanobacteriales and Methanomassiliicoccales have shown the ability to convert methylated compounds to methane (Enzmann *et al.*, 2018). Yet, only members of the Methanosarcinales utilise acetate as the sole carbon source (Vanwonterghem *et al.*, 2016; Pyzik *et al.*, 2018). *Methanosarcina* species, from the order Methanosarcinales, are extremely versatile and the only currently known methanogens to possess the metabolic capability of all three methanogenesis pathways (Thauer *et al.*, 2008; Lang *et al.*, 2015). Most methanogens possess a single methanogenesis pathway and utilise as few as two substrates (Galagan *et al.*, 2002). Yet, our knowledge regarding methanogenic substrate utilisation and energy conservation is still incomplete and continues to grow with the development of new molecular techniques (Vanwonterghem *et al.*, 2014).

1.3. Feedstock for bio-energy production

One of the major advantages of anaerobic digestion is that nearly any biomass consisting of carbohydrates, proteins and lipids is suitable for biogas production, thus enabling the use of a large variety of substrates (Laiq Ur Rehman *et al.*, 2019). Anaerobic digestion is commonly associated with the treatment of cow manure and sewage sludge from wastewater treatment plants (Weiland, 2010).

Nonetheless, modern reactors employ numerous substrates including pig manure (Bruni, Jensen and Angelidaki, 2010), chicken manure, municipal solid waste (Mao *et al.*, 2015), agricultural waste (Momayez, Karimi and Taherzadeh, 2019), energy crops (Weiland, 2010) and food waste (Zhang *et al.*, 2014). First-generation biofuels produced from energy crops (suitable for human consumption) raised concerns as to their sustainability and possible threat to food security, especially in developing countries (Momayez, Karimi and Taherzadeh, 2019). These concerns led to the development of second-generation biofuels, mainly produced from lignocellulosic biomass that would not affect food sustainability (Morrison *et al.*, 2009; Lin *et al.*, 2010).

1.3.1. Feedstock types and characteristics

1.3.1.1. Municipal solid waste

The management and disposal of municipal solid waste is under pressure due to the swift expansion of industrialisation and urbanisation (Mao *et al.*, 2015). Municipal or domestic waste refers to common items discarded on a daily basis, usually kept separate from food waste (Firoz, 2017). This waste can be used to generate biogas in a much more controlled manner than the common practice of collecting methane produced naturally during the breakdown of waste in landfills (Weiland, 2010). This method of biofuel production is already a common practice throughout Germany (Al Seadi *et al.*, 2008). The food service industry, corporate companies and households are the main producers of food waste, a rapidly increasing section of municipal solid waste (Mao *et al.*, 2015; Singh and Kumar, 2019). Discarded, uneaten and spoiled food along with kitchen waste is well suited to anaerobic digestion, however pre-treatment is required since most of the carbohydrates and proteins present in food waste are insoluble (Zhang *et al.*, 2014; Zhou *et al.*, 2018). Anaerobic digestion offers efficient disposal of these compounds in a manner which reduces odours and insect activity, yet still creates organic fertiliser, which can be used in the agricultural industry (Al Seadi *et al.*, 2008; Firoz, 2017).

1.3.1.2. Livestock manure

Livestock manure (dung and urine) is rich in organic matter and nitrogen, making it an attractive natural plant fertiliser (Leip *et al.*, 2019). However, due to the continued expansion of livestock farming in various countries, manure availability now exceeds demand (Mao *et al.*, 2015). Untreated animal manure can become a major contributor to air and water pollution (Holm-Nielsen, Al Seadi and Oleskowicz-Popiel, 2009). Potential risks include pathogen contamination, nutrient leaching and ammonia evaporation (Díaz-Vázquez *et al.*, 2020). The use of livestock manure for anaerobic digestion is fairly common since manure is a natural source of anaerobic bacteria, easily accessible, cheap and has a high water content, aiding in the solubility of co-substrates (Al Seadi *et al.*, 2008).

Anaerobic digestion of livestock manure not only reduces pathogens and odours, but also decreases the total solids by converting a fraction of the volatile solids into biogas (Divya, Gopinath and Merlin Christy, 2015). The digestion residues (incompletely digested remnants) are a more efficient, high-value biofertiliser, capable of replacing widely used mineral fertilisers, which have been associated with nitrogen build-up in ground and water sources (Achinas, Achinas and Euverink, 2017; Singh and Kumar, 2019). However, the digestion of livestock manure ultimately has a low methane yield (Mao *et al.*, 2015). Co-digestion of animal manure and high-yield substrates (e.g., energy crops, food industry waste) to boost the final biogas production is therefore common practice (Amon *et al.*, 2007).

1.3.1.3. Lignocellulosic biomass

Biomass offers a living storage of solar energy, harnessed via photosynthesis, consisting of carbohydrates, proteins, cellulose, lipids and hemicellulose (Al Seadi *et al.*, 2008; Achinas, Achinas and Euverink, 2017). Lignocellulosic biomass accounts for up to half of the yearly global biomass yield and is a desirable potential source of renewable energy (Mutschlechner, Illmer and Wagner, 2015). Lignocellulosic plant biomass refers to a complex mix of cellulose (40 - 50 %), hemicellulose (20 - 40 %) and lignin (5 - 30 %), bound in a recalcitrant matrix, resistant to chemical and biological hydrolysis (Figure 1.5) (Saritha and Arora, 2012; Karimi and Taherzadeh, 2016).

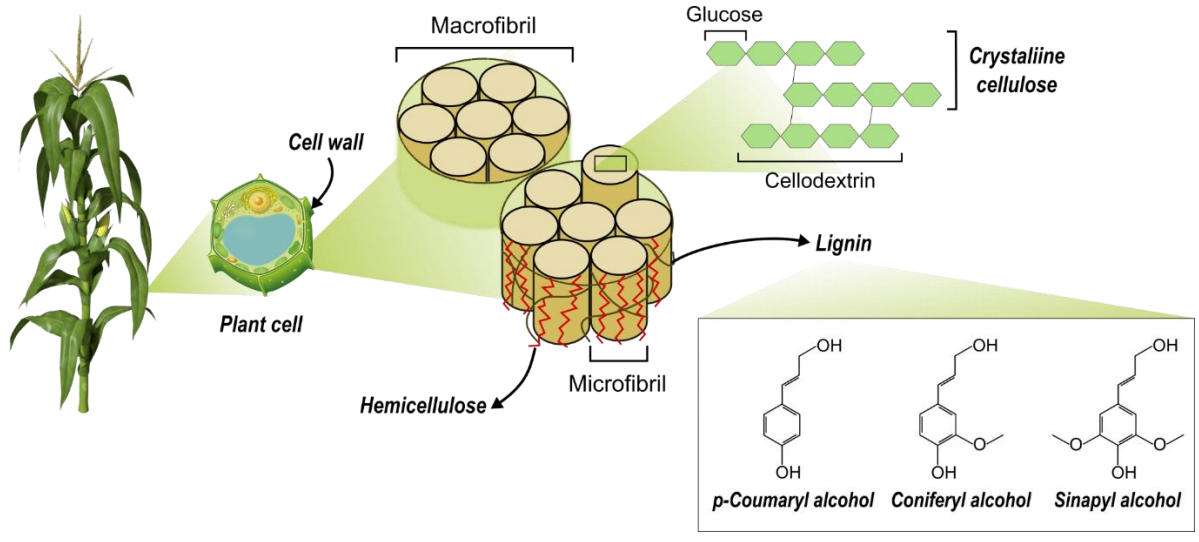


Figure 1.5. Interconnected structure of lignocellulose. *Cellulose, hemicellulose and lignin intertwine to form a recalcitrant matrix chemically bonded by non-covalent forces, covalent cross linkages, hydrogen bonding and van der Waals forces. This recalcitrant matrix restricts the access of enzymes and microorganisms to the digestible components of lignocellulose, preventing digestion. Adapted from Nunes and Kunamneni, 2018.*

Cellulose is a linear polymer consisting of glucose linked by numerous α- and β-1,4-glycosidic bonds (Malherbe and Cloete, 2002). Durable microfibrils result from cellulose chains interlinked via hydrogen bonds and van der Waals forces (Figure 1.5) (Zheng *et al.*, 2014). Hemicellulose is a less well-organised, branched polysaccharide consisting of various sugars combined into easily hydrolysed polymers (Hendriks and Zeeman, 2009; Achinas, Achinas and Euverink, 2017). The branched chains of hemicellulose provide points of connection between cellulose microfibrils and lignin resulting in an extremely rigid matrix (Zheng *et al.*, 2014). Lignin is an amorphous and insoluble polymer of three phenyl propane units, specifically, sinapyl-, p-couramyl- and coniferyl alcohol (Agbor *et al.*, 2011). Lignin limits the conversion of the carbon-rich components, cellulose and hemicellulose, to fermentable sugars by creating a physical barrier that limits permeability, reducing enzyme binding efficiency and protecting against microbial attack and oxidative stress (Figure 1.5) (Lin *et al.*, 2010). Furthermore, since lignin is insoluble in water it renders lignocellulosic biomass insoluble, limiting degradation efficiency (Hendriks and Zeeman, 2009; Agbor *et al.*, 2011).

Due to this unique structure, pre-treatment is required to alter or remove structural and compositional barriers to hydrolysis and facilitate conversion of lignocellulose to fermentable sugars (Malherbe and Cloete, 2002; Saritha and Arora, 2012). The ability of hydrolytic microorganisms to access solid lignocellulosic material is an important rate-limiting step of anaerobic digestion (Meegoda *et al.*, 2018). Lignocellulose hydrolysis without proper pre-treatment can limit the extraction of fermentable sugars to only 20 % of the true potential, whilst pre-treatment can push this number up to 90 % (Sindhu, Binod and Pandey, 2016). Anaerobic digestion could convert lignocellulosic hydrolysates and fermentable sugars to biogas, however the limitations caused by the structural components of lignocellulose require special attention prior to anaerobic digestion (Gonçalves *et al.*, 2015). Studies have shown that the biodegradability of lignocellulose and biogas yield increases with a decrease in lignin content (Hendriks and Zeeman, 2009). However, several other properties may also influence the biodegradability of lignocellulosic biomass, including the accessible surface area, cellulose crystallinity and degree of cellulose polymerisation (Zheng *et al.*, 2014).

1.4. Natural and engineered anaerobic digesters

Swamps, marshes, landfills, marine sediments and the rumen of animals are niches where biogas formation can occur naturally (Bassey *et al.*, 2013). However, the process can be artificially simulated through the use of bioreactors or digesters. Historical evidence indicates the use of biogas to heat bathing water from as early as the 10th century B.C., although the first use of an anaerobic reactor/digester is linked to a leper colony in Bombay, India during 1859 (Abdelgadir *et al.*, 2014). Since then bioreactors have changed and evolved as anaerobic digestion gained academic recognition through scientific inquiry.

1.4.1. Artificial digester design

All anaerobic digestion systems adhere to the same basic principles regardless of the feedstock within each system (Abdelgadir *et al.*, 2014). A basic digester generally consists of a digestion chamber with a dome-like structure, an inlet for substrate loading, an outlet for biogas and an outlet for the digestion residues (also known as slurry/digestate) (Kumar, Mandal and Sharma, 2015). Biogas trapped within the dome-like structure flows through the outlet as pressure builds within the reactor. Batch digesters lack a continuous supply of feedstock (finite amount) and are generally designed for singular applications, like determining the biomethane potential of a feedstock (Van Foreest, 2012). In contrast, continuous style digesters rely upon a constant inflow of substrate and removal of digestion residues to reach a steady state, resulting in a stable, efficient process and better yield (Van Foreest, 2012; Jingura and Kamusoko, 2017). Developments in process engineering and digester design continue to improve the industrial applications of this technology (Khanal *et al.*, 2017).

1.4.2. Animal digestive strategies

Regardless of the technological advancements made within the biogas industry, the effectiveness of animal digestive systems still remain superior to artificial bioreactors (Bayané and Guiot, 2011). This is especially true in terms of the digestion of complex plant polymers, like lignocellulose. Herbivorous mammals rely greatly on their gastrointestinal microbiomes for the hydrolysis and fermentation of plant biomass (Morrison *et al.*, 2009). Such mammals have developed specialised anatomical structures to enhance fermentation efficiency and are natural sources of anaerobic microbial communities capable of producing biogas (Al Seadi *et al.*, 2008; Shapira, 2016). The location of the main fermentation chamber divides herbivorous mammals into two groups, hindgut- (e.g., cattle, sheep) and foregut fermenters (e.g., elephant, rhinoceros, horse) (Ilmberger *et al.*, 2014). The foregut rumen and hindgut caecum may differ considerably in their anatomical and chemical composition, but they share a remarkably similar microbial composition (Shapira, 2016). Yet, only the ruminant digestive system has been thoroughly studied as a model for anaerobic digestion (Weimer, Russell and Muck, 2009).

Herbivorous diets consist largely of plant cell wall polysaccharides that cannot be digested by the host, which is why about 70 % of the total gut volume is devoted to microbial fermentation (Flint *et al.*, 2008). However the efficiency of this process is directly correlated to fermentation time or passage rate (Clauss *et al.*, 2003). In the animal kingdom, a larger body size can provide numerous ecological advantages. The two most often discussed include a reduced relative energy requirement for maintenance and an increase in gastrointestinal capacity in relation to energy requirements (Parra, 1978).

This means that larger animals can utilise forage of lower quality due to longer passage rates and specialised gastrointestinal microbiomes, which ensure more thorough digestion (Demment and Van Soest, 1985; Clauss *et al.*, 2003). South Africa is home to numerous indigenous large herbivores (e.g., rhinoceros, elephant) adapted to surviving on diets consisting of low quality feed due to arid environmental conditions and habitats with limited food supply (Myers, 1990). Previous characterisation of the mammalian gastrointestinal microbiome indicated that faecal microbiome composition is clustered according to diet, more so than host phylogeny (Muegge *et al.*, 2011). However, the gastrointestinal composition of these indigenous herbivores are poorly characterised. These herbivores may host microbial gut communities uniquely suited to extracting energy from abundant, but poorly digestible complex plant polymers like lignocellulose (Codron *et al.*, 2006). Improved insight into the digestive strategies and gastrointestinal microbiomes employed by these herbivores could aid biogas production from lignocellulosic compounds not only in South African, but globally.

1.5. Parameters that influence microbial ecology and biogas yield

Each step of the anaerobic digestion process is facilitated by a select community of microorganisms with specific capabilities and as such is dependent on various external and internal factors (Yadvika *et al.*, 2004). Changes in internal parameters are closely controlled within mammals, however knowledge of the parameters involved and their effect during anaerobic digestion is crucial to understanding the digestive strategies and predicting possible alterations to the gastrointestinal microbiome (Kartzinel *et al.*, 2019). Such knowledge could prove beneficial during the transition from a natural community to an artificial setting. Since, if improperly managed, the process may become unstable, resulting in a decrease or complete suspension of biogas production (Mao *et al.*, 2015).

1.5.1. Temperature

Due to its multi-layered effect, temperature is one of the most influential parameters with regards to anaerobic digestion (Khanal *et al.*, 2017). The digestion temperature affects conversion rates, kinetics and process stability, ultimately altering methane production (Sánchez *et al.*, 2001). Anaerobes grow at psychrophilic (10 – 30 °C), mesophilic (30 – 40 °C) and thermophilic (50 – 60 °C) temperatures, but the efficiency of anaerobic digestion improves as temperatures rise (El-Mashad *et al.*, 2004; Arikan, Mulbry and Lansing, 2015). This phenomenon is attributed to a change in the duration of fermentation brought about by an increase in metabolic rate and higher specific growth rates (Yadvika *et al.*, 2004; Zhang *et al.*, 2014). The advantages of thermophilic digestion include improved pathogen removal, higher biogas yields, faster nitrogen degradation and the use of smaller digestion tanks due to the increased digestion rate (Sánchez *et al.*, 2001; Wei *et al.*, 2009).

The solubility of gaseous compounds (e.g., CO_2, H_2S, H_2, CH_4) is also altered by temperature changes. The increased solubility of such compounds at lower temperatures could prove inhibitory by altering the internal operating conditions of a digester (Khanal *et al.*, 2017). However, several disadvantages limit the use of thermophilic digesters, such as an increased sensitivity to environmental changes and possible acidification (Kim, Ahn and Speece, 2002). In order to maintain a stable thermophilic digestion, temperatures should fluctuate less than 0.6 °C/day, with changes in excess of 1 °C/day leading to bioreactor failure (Appels *et al.*, 2011). Alternative concerns include increased compound toxicity, increased net energy input and depleted methanogenesis. Ideally the optimal anaerobic system would consist of a thermophilic hydrolysis/acidogenesis phase and mesophilic methanogenesis (Mao *et al.*, 2015).

1.5.2. Acid-alkaline fluctuations (pH) and volatile fatty acid production

During anaerobic digestion, pH generally refers to the acidity/alkalinity of the feedstock solution (Al Seadi *et al.*, 2008; Abdelgadir *et al.*, 2014). Fluctuations in pH influence microbial growth rates and compound separation, which in turn directly affects biogas formation (Szewczyk and Bukowski, 2008; Cioabla *et al.*, 2012). A neutral pH is ideally suited for the anaerobic digestion of biomass, however pH optimisation is hampered due to varying requirements among the numerous anaerobic microorganisms involved (Zhang *et al.*, 2014). Acidogenic and acetogenic microbes function optimally in a slightly acidic environment (pH 4.5 to 5.5), whereas methanogens prefer a neutral to slightly alkaline pH range (Pore *et al.*, 2015; Khanal *et al.*, 2017). Methanogens are extremely sensitive to changes in pH, operating between a narrow interval of 6.5 to 8.5 (Weiland, 2010). However, hydrogenotrophic methanogens are more resistant to environmental fluctuations than their acetoclastic counterparts and have a doubling time of only six hours compared to 2.6 days for acetoclastic methanogens (Merlin Christy, Gopinath and Divya, 2014).

During the acidification of sugars, the products of hydrolysis are used as substrates for fermentative microorganisms to produce various organic products (Franke-Whittle *et al.*, 2014). The accumulation of organic acids (e.g., acetate, propionate, butyrate) during the initial phases of anaerobic digestion may result in a decrease in pH throughout the entire system (Divya, Gopinath and Merlin Christy, 2015). This shift is advantageous for the acidogenic and acetogenic microbes, however if the pH dips below 5.0, methanogen growth is severely affected (Merlin Christy, Gopinath and Divya, 2014). The concentration of intermediates like VFAs is often used as an indication of the stability of the anaerobic digestion process (Al Seadi *et al.*, 2008). Volatile fatty acid accumulation is hastened by the fast growth rate and subsequent increased digestion rate of hydrolytic and acidogenic microbes, especially in relation to methanogens (Divya, Gopinath and Merlin Christy, 2015).

Yet, studies have shown that anaerobic digesters may react differently to varying VFA concentrations. A specific VFA concentration may be optimal for one digester, but inhibitory for another (Al Seadi *et al.*, 2008). In contrast, the build-up of ammonia during protein digestion may increase the overall pH, affecting digestion efficiency (Chen, Cheng and Creamer, 2008). In order to maintain pH balance during anaerobic digestion, the rate of degradation in each phase must remain near identical, making careful balance and close monitoring of pH essential.

1.5.3. Toxic compounds

Toxic compounds may refer to substances already present in the feedstock solution or substances produced via the metabolic activities of the microbial community (Khanal *et al.*, 2017). The digestion of organic matter produces light metal ions like magnesium (Mg), potassium (K), sodium (Na) and calcium (Ca), but these metals are occasionally also introduced as pH adjustment chemicals. Although these metals are necessary for microbial growth and therefore affect growth rates, in too high quantities they can inhibit microbial growth or cause severe toxicity (Chen, Cheng and Creamer, 2008). Municipal sewage and sludge are rich in heavy metals such as chromium (Cr), iron (Fe), cobalt (Co), copper (Cu), zinc (Zn), cadmium (Cd) and nickel (Ni) (Soto, Méndez and Lema, 1993). Unlike other toxic compounds, these metals are not biodegradable and accumulate over time. Studies have shown that heavy metal toxicity is one of the leading causes of digester disturbance or failure due to the disruption of enzyme structure and therefore function (Chen, Cheng and Creamer, 2008).

1.5.4. Retention time and organic loading rate

Hydraulic retention time (HTR) refers to the mean time soluble compounds (liquids) remain inside a digester to facilitate the degradation of organic matter (Abdelgadir *et al.*, 2014; Meegoda *et al.*, 2018). The HRT is therefore not only associated with the microbial growth rate, but process temperature, organic loading rate (OLR) and substrate composition (Mao *et al.*, 2015). Under mesophilic conditions, the average retention time is about 15 - 30 days (Meegoda *et al.*, 2018). Equation 6 illustrates that HRT (expressed in hours or days) is the volume of the anaerobic reactor divided by the volume of substrate fed into the reactor over time (loading rate) (Al Seadi *et al.*, 2008; Abdelgadir *et al.*, 2014).

$$HRT = \frac{Biological\ reactor\ volume\ [m^3]}{Influent\ flow\ rate\ over\ time\ [m^3/d]} = \frac{V}{Q} \quad (6)$$

During the operation of continuous style digesters, the loading rate refers to the volume of organics fed into a digester per day (Meegoda *et al.*, 2018). High loading rates correspond to shorter HRTs. However, microbial growth rates limit loading rate, as an excessively high loading rate could result in the washout of cells (Khanal *et al.*, 2017).

Shorter retention times face the possible loss of the active microbial community or VFA acidification, whereas longer retention times require larger digestion chambers and therefore more capital investment (Yadvika *et al.*, 2004; Meegoda *et al.*, 2018). The optimal HRT depends on the decomposition rate of the introduced substrate and is optimised according to each substrate (Al Seadi *et al.*, 2008).

1.5.5. Carbon/Nitrogen ratio

The Carbon/Nitrogen (C/N) ratio is a measure of the nutrient levels in a digestion substrate (Mao *et al.*, 2015). Carbohydrates and lipids provide the main source of carbon for digestion, whereas nitrogen derived from protein digestion is essential for microbial protein synthesis (Meegoda *et al.*, 2018). Since mammalian gut microbiomes are linked to diet composition, nutrient levels are expected to influence the intestinal gut microbiome. Generally, microbes utilise carbon at a much quicker pace than nitrogen. Knowledge about substrate composition is essential to maintain the correct ratio for efficient reactor operation (Yadvika *et al.*, 2004). A ratio of 25:1 (C:N) is commonly accepted, but the optimal range for a substrate may vary from 20 to 30 or 35 to 1 in some cases (Puñal *et al.*, 2000). Substrates with an overly high C/N ratio provide insufficient nitrogen to maintain cell biomass. The available nitrogen is degraded swiftly, leading to a decrease in biogas production (Mao *et al.*, 2015). The use of substrates with a low C/N ratio increases the risk of ammonia inhibition (Mao *et al.*, 2015). Methanogens are particularly susceptible to the toxic effects of ammonia inhibition, ultimately leading to insufficient utilisation of carbon sources (Al Seadi *et al.*, 2008).

1.5.6. Antibiotics

Antibiotics are widely used on livestock farms for the prevention of disease, infection or in some cases to promote growth (Álvarez *et al.*, 2010). Antibiotics ail microbial survival by targeting several key areas of the prokaryotic system including blocking cell wall synthesis, interfering with membrane functionality and inhibiting protein biosynthesis (Sanz, Rodríguez and Amils, 1996). Once ingested, these compounds are metabolised via a number of metabolic pathways; however, approximately 17 – 76 % of antibiotics administered to livestock retain their original form or a metabolite thereof when excreted via urine and faeces (Álvarez *et al.*, 2010). A study by Zarrinpar and co-workers (2018) illustrates that the microbiome of mice underwent a significant shift following antibiotic-induced microbiome depletion (AIMD). Prior to antibiotic exposure, the phyla, Firmicutes and Bacteroidetes dominated the microbiome, however after exposure the number of species from these phyla decreased significantly with dominancy shifting towards Proteobacteria (Zarrinpar *et al.*, 2018). These phyla represent some of the major complex plant-carbohydrate degraders and primary producers of VFAs, acetic acid and CO_2/H_2 (Maus *et al.*, 2016).

A significant shift in the ratios of these phyla could potentially alter the efficiency and biogas yield during anaerobic digestion, especially when using animal manure as the main substrate (Li *et al.*, 2017).

1.6. Pre-treatment methods

The effectiveness of mammalian digestive strategies is the result of cooperative action between enzymes targeting the structural components of plant biomass and in some cases additional chemical or mechanical action like repeated chewing (ruminants) (Bayané and Guiot, 2011). However, outside of mammalian guts, treatment to enhance digestion is a separate process preceding anaerobic digestion. Pre-treatment originated from wood science for use by pulp and paper manufacturers (Rabemanolontsoa and Saka, 2016). The goal of pre-treatment is to alter properties that affect lignocellulose biodegradability, thereby making the biomass more susceptible to enzymatic and microbial digestion. This ultimately improves the rate of enzyme hydrolysis and increases the conversion of cellulose and hemicellulose to fermentable sugars (Mosier *et al.*, 2005; Zheng *et al.*, 2014). However, undesired compounds or inhibitors (e.g., furfural) produced during these pre-treatment strategies may affect the growth of fermentative microorganisms, interfering with hydrolysis and acidogenesis (Hassan, Williams and Jaiswal, 2018). The model pre-treatment method would increase lignocellulose hydrolysis susceptibility without the need for biomass size reduction, whilst producing fermentable sugars and limiting inhibitor production, cost and time (Sindhu, Binod and Pandey, 2016). Pre-treatment methods can be grouped into physical-, chemical-, biological- or combinatorial pre-treatment (co-digestion) (Agbor *et al.*, 2011). Table 1.1 summarises common pre-treatment methods and their effect on factors that affect lignocellulose biodegradability.

Table 1.1. *Summary of the commonly used pre-treatment methods and their effect on factors that contribute to lignocellulose digestibility. Adapted from Zheng et al., 2014.*

Classification	Pre-treatment	Increase of accessible surface area	Decrystallisation of cellulose	Solubilisation of hemicellulose	Solubilisation of lignin	Alteration of lignin structure	Formation of furfural/ hydroxymethylfurfural (HMF)
Physical	Mechanical	+	+				
	Irradiation	+	-	-			-
	Steam-explosion	+		+	-	+	+
	Liquid hot water (LHW)	+	ND	+	-	-	-
Chemical	Catalysed steam-explosion	+		+	+/-	+/-	+
	Acid	+		+	-	+	+
	Alkaline	+		-	+/-	+	-
	Oxidative	+	ND		+/-	+	-
	Ionic liquids	+	+	-			
	Thermal acid	+	ND	+			+
	Thermal alkaline	+	ND	-	+/-	+	-
	Thermal oxidative	+	ND	-	+/-	+	-
	Ammonia fiber explosion	+	+	-	+	+	-
Biological	Fungal	+	ND	+	+	+	
	Microbial community*	+	ND	+	+	+	
	Enzymatic	+	ND	+	+	+	

+ = major effect, - = minor effect, ND = not determinded, blank = no effect, *Generally includes fungi and bacteria

1.6.1. Mechanical pre-treatment

Mechanical or physical pre-treatment refers to methods that disintegrate or grind solid substrate particles with the aim of degrading cellular components and increasing the available surface area without the use of chemicals or microorganisms (Ariunbaatar *et al.*, 2014; Zheng *et al.*, 2014). Some notable physical pre-treatment methods include comminution, steam-explosion, liquid hot water pre-treatment, extrusion and irradiation (ultrasound and microwave) (Zheng *et al.*, 2014).

1.6.1.1. Comminution

Comminution refers to mechanical milling or grinding of compounds with the aim of reducing the substrate particle size (Zheng *et al.*, 2014). These systems are often directly coupled to the feeding system of anaerobic digesters (Weiland, 2010). An increase in available surface area improves contact between the substrate and anaerobic microbes, thus enhancing anaerobic digestion (Ariunbaatar *et al.*, 2014). Smaller substrate particles may increase the overall digestion speed, yet may not alter the final methane yield (Weiland, 2010).

1.6.1.2. Steam explosion and liquid hot water

Steam explosion and liquid hot water treatments utilise heat and pressure to not only increase the available surface area, but also improve the overall solubility of lignocellulose (Rabemanolontsoa and Saka, 2016). Both treatments have a major effect on the solubility of hemicellulose, however their effect on lignin is limited (Zheng *et al.*, 2014).

1.6.1.3. Irradiation

Irradiation refers to the use of sound or light waves, including microwaves, ultrasound and gamma rays (Thostenson and Chou, 1999). Of these, microwave processing is widely used as a rapid heat treatment method to improve solubility and decrease treatment time. Most physical pre-treatment strategies result in the production of inhibitors (Table 1.1, HMF), that can negatively affect enzymatic hydrolysis to varying degrees. Furthermore, these methods require specialised equipment and consume a great deal of energy (Sindhu, Binod and Pandey, 2016).

1.6.2. Chemical pre-treatment

Chemical pre-treatment involves the use of acids, alkalis or oxidants to alter the tertiary structure of lignocellulose (Agbor *et al.*, 2011). The effectivity of chemical pre-treatment depends on the method used and specific substrate characteristics (Ariunbaatar *et al.*, 2014). The two chemical pre-treatment strategies employed most often include alkaline- and acid pre-treatment.

1.6.2.1. Alkaline pre-treatment

Alkaline pre-treatment induces swelling of solid biomass resulting in the increase of the internal surface area, increased porosity and the breakage of lignin-carbohydrate linkages (Tarkow and Feist, 1969). This method also decreases both the degree of substrate polymerisation and cellulose crystallinity (Agbor *et al.*, 2011). Cellulose consists of crystalline (organised) regions and amorphous (poorly organised) regions of low crystallinity (Hendriks and Zeeman, 2009). The crystallinity index characterises the level of cellulose crystallisation. This roughly means that cellulose with a high crystallinity index is less biodegradable, thereby adding another level of complexity to pre-treatment (Zheng *et al.*, 2014). Commonly utilised alkalis include calcium hydroxide ($Ca(OH)_2$), sodium hydroxide (NaOH), aqueous ammonia (NH_4OH) and potassium hydroxide (KOH). Alkaline pre-treatment is conducted at lower temperatures than other chemical pre-treatment methods, however the reaction time is quite lengthy (several hours to weeks) (Rabemanolontsoa and Saka, 2016).

1.6.2.2. Acid pre-treatment

Acid pre-treatment offers two treatment options: concentrated acid (30 – 70 %) at relatively low temperatures (40 °C) or diluted acid (0.1 %) and high temperatures (230 °C) (Monlau *et al.*, 2013). Organic and inorganic acids have proven suitable for acid pre-treatment, however sulphuric acid (H_2SO_4) remains the most widely utilised (Liu *et al.*, 2009; Monlau *et al.*, 2012). Dilute acid treatment is one of the most common chemical pre-treatment strategies and generally favoured due to the numerous drawbacks of concentrated acid treatment (e.g., toxicity, highly corrosive, costly) (Agbor *et al.*, 2011). Acid pre-treatment mainly hydrolyses hemicellulose to its component sugars and effectively disrupts lignin, but does not improve the solubility of lignin (Ariunbaatar *et al.*, 2014; Zheng *et al.*, 2014). This strategy therefore renders cellulose more susceptible to microbial and chemical digestion (Hendriks and Zeeman, 2009).

1.6.3. Biological pre-treatment

Biological pre-treatment employs fungi, enzymes that target specific components of plant biomass or microbial consortia (Ariunbaatar *et al.*, 2014). Compared to physical and chemical pre-treatment methods, biological pre-treatment generally requires a far lower energy input and no chemicals (Kalyani *et al.*, 2013). Furthermore, the process takes place under much milder environmental conditions resulting in fewer anaerobic inhibitors (Mosier *et al.,* 2005; Saritha and Arora, 2012). However, pre-treatment duration is generally lengthy, limiting commercial or industrial use thus far (Zheng *et al.*, 2014). Table 1.2 summarises the effect biological pre-treatment has on various feedstock types, the reaction conditions for treatment and how pre-treatment altered the final biogas yield.

1.6.3.1. Fungal pre-treatment

Lignocellulose consists of three components, lignin, hemicellulose and cellulose (Lin *et al.*, 2010). The carbohydrate components, hemicellulose and cellulose, are a rich source of fermentable sugars. Fungi capable of producing enzymes that selectively degrade lignin, hemicellulose and polyphenols, but utilise as little as possible cellulose are therefore the main focus of biological pre-treatment (Agbor *et al.*, 2011; Zheng *et al.*, 2014). Fungal pre-treatment predominantly utilises white- and soft-rot fungi, with white rot fungi aiding delignification, thereby increasing the rate of reducing sugar extraction (Chen *et al.*, 2010). White-rot fungi degrade all the components of lignocellulose either simultaneously or in a sequential pattern. Brown- and soft-rot fungi on the other hand target cellulose and hemicellulose, but mostly leave lignin nearly unaltered (Vaz *et al.*, 2017). However, these digestions are usually carried out under sterile conditions over the course of multiple days or even weeks and have thus far not proven commercially viable (Zheng *et al.*, 2014). As indicated in Table 1.2, fungal pre-treatment has proven highly effective to increase the biogas yield, however of the discussed methods, fungal pre-treatment requires the longest digestion period (up to 8 weeks in some cases).

1.6.3.2. Enzymatic pre-treatment

Biological saccharification is the microbial extraction of reducing sugars from lignocellulosic biomass (Marriott, Gómez and McQueen-Mason, 2016). The concern that microbes utilised during pre-treatment could digest the produced monosaccharides during pre-treatment has garnered interest in enzymatic saccharification (Rabemanolontsoa and Saka, 2016). This process involves the usage of purified hydrolytic enzymes extracted from lignocellulolytic microbes. Lignolytic enzymes identified as key players in lignin digestion include phenol oxidase (laccase) and peroxidases (lignin peroxidase and manganese peroxidase) (Saritha and Arora, 2012). Enzyme systems targeting cellulose (cellulases) are generally also effective on hemicellulose as these enzymes are commonly co-produced by the numerous fungi and bacteria capable of digesting cellulose and hemicellulose (Lynd *et al.*, 2002). The effect of purified enzymes on the final biogas yield has thus far proven minimal, yet the reaction duration is considerably quicker than other biological pre-treatment methods (Table 1.2). Nonetheless, the high cost of enzymes and specific reaction conditions required for the enzymes to function optimally limit industrial use (Zheng *et al.*, 2014).

1.6.3.3. Microbial consortia

The synergistic actions of microbial communities, which include fungi and bacteria, have shown great potential for the effective biodegradation of lignocellulose (Tsegaye, Balomajumder and Roy, 2019). This type of treatment generally involves microbes screened and enriched from natural environments rich in lignocellulose. Numerous advantages support the use of microbial consortia for lignocellulose pre-treatment including the increased adaptability of the consortia, improved substrate utilisation, improved reducing sugar extraction and better pH control (Kalyani *et al.*, 2013; Mishra *et al.*, 2018). Treatment with microbial consortia is highly effective, increasing the final biogas yield nearly a 100 % in some cases (Table 1.2). Furthermore, the optimal temperature range for this method of treatment is quite broad, which lessens the need for constant monitoring and temperature management (Table 1.2).

Currently, a need exists for more diverse, complex microbial communities suitable for biological pre-treatment (Zheng *et al.*, 2014). Indigenous South African herbivores could represent a possible source of novel microbial communities suitable for the simultaneous production of biogas and lignocellulose digestion, circumventing the need for treatment prior to digestion.

Table 1.2. *Summary of widely used biological pre-treatment methods, the agents involved, previously used substrates and the final effect of the treatment on the biogas yield. MSW – municipal solid waste. Adapted from Zheng et al., 2014.*

Pre-treatment	Microbes and enzymes	Feedstock type	Conditions	Yield	References
Fungal	White-, brown-, soft-rot and basidiomycete fungi	**Agricultural:** Sweet chestnut leaves/hay, sisal leaf decortications residue (SLDR) **Hardwood:** Japanese cedar wood chip	Mesophilic (28 – 37 °C) 12 to 56 days incubation Autoclaving or no autoclaving of feedstock before inoculation Aerobic	15 % to 5 fold increase of methane yield	Amirta *et al.*, 2006; Take *et al.*, 2006; Muthangya *et al.*, 2009; Makculak *et al.,* 2012
Microbial	Complex microbial agents containing yeast and cellulolytic bacteria, and a mixture of fungi and composting microbes	**Agricultural:** Corn straw, corn stalks, cotton stalks, cassava residues, and manure biofibers	Mesophilic and thermophilic (20 – 55 °C) 12 h - 20 days incubation Autoclaving or no autoclaving of feedstock before inoculation Aerobic	25 - 96.63 % yield increase	Lu *et al.*, 2009; Bai *et al.*, 2010; Zhang *et al.*, 2011
Enzymatic	Laccase; pectinase; mixture of cellulase, hemicellulases and β-glucosidase; crude Trichoderma enzyme complex	**Agricultural:** Sugar beet pulp, spent hops, manure biofibers, Jose tall wheatgrass **MSW:** Pulp and paper sludge	Mesophilic (37 °C) 4 - 24 h incubation No sterilisation Aerobic (treatment is separated from digestion), Anaerobic (enzymes added directly to reactor)	0 – 34 % yield increase	Bruni, Jensen and Angelidaki, 2010; Gerhardt, Pelenc and Bauml, 2007; Lin, Wang and Wang, 2010

1.7. Conclusions

Electricity generation from coal is highly effective and a well-established practice throughout various regions of the world. However, the burning of this non-renewable resource releases harmful GHGs like CO_2 into the earth's atmosphere. The continued reliance on fossil fuels could lead to resource depletion resulting in major power shortages, economic crisis and disastrous environmental pollution. However, the human population is expanding at an alarming rate, necessitating the need for alternative energy sources that meet growing demands in an environmentally friendly manner.

The production of biogas via anaerobic digestion produces a multifaceted renewable energy source. This green energy source can mitigate numerous energy needs as a replacement for natural gas, via conversion to electricity and for the production of biofuels. Furthermore, any substrate consisting of proteins, carbohydrates and lipids is suitable for anaerobic digestion, including numerous waste streams. Lignocellulosic biomass is an abundant, inexpensive, under-utilised source of potential energy. Lignocellulosic waste material from large industries like forestry and agriculture could provide an alternative source of feedstock for green energy production mitigating the overuse of fossil fuels. Yet, these lignocellulosic materials require timely and sometimes costly pre-treatment to harness all the energy locked within the material.

Biological pre-treatment does not require the use of harmful chemicals and is more favourable than chemical or mechanical pre-treatment methods. Nonetheless, biological pre-treatment methods are time-consuming and could take up to months to complete. The use of microbial consortia for lignocellulose pre-treatment has proven highly effective, however diverse novel communities are required to aid in the treatment of lignocellulose. South Africa is a diverse country home to numerous large indigenous herbivores that flourish in arid habitats with limited nutritional options. Characterisation of their gastrointestinal microbiomes could provide novel insight into these remarkable herbivores and provide a source of untapped microbial potential for the biogas industry.

1.8. References

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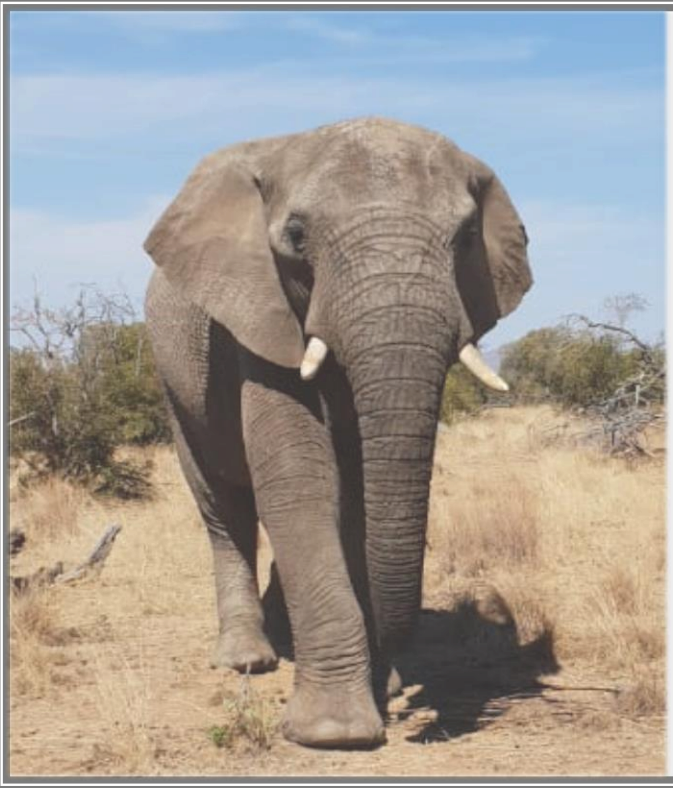

CHAPTER 2

INTRODUCTION TO THE PRESENT STUDY

2. Introduction

2.1. South Africa: Potential untapped microbial diversity

The combustion of coal is the primary source of energy for both industrial and domestic activities within South Africa (SA) (Coyle *et al.*, 2014). However, the burning of fossil fuels, like coal, releases greenhouse gas (GHG) into the atmosphere, contributing to potentially catastrophic global warming (Myhrvold and Caldeira, 2012). As a developing nation, SA's industries are continually expanding resulting in higher energy demands and subsequent higher carbon emissions (Walker and Jourdan, 2003). Industrialisation of developing countries has raised concerns surrounding the effect of increased annual GHG emissions (Winkler, Spalding-Fecher and Tyani, 2002). These concerns have renewed efforts toward finding alternative, renewable and low-emission energy sources. Biogas is a renewable energy source that offers a viable substitute to non-renewable fossil fuels for power-, heat-, and vehicle fuel generation (Weiland, 2010).

Biogas is produced via the anaerobic digestion of organic material (biomass), facilitated by a specialised community of microorganisms (Schlüter *et al.*, 2008). Lignocellulosic biomass (consisting of lignin, cellulose and hemicellulose) from agricultural residues and food industry waste offers an abundant, easily accessible substrate for biogas production (Pore *et al.*, 2016). Rural communities in developing countries, such as SA, often have access to various such sources of cellulosic biomass, including cattle dung and agricultural residues (Yadvika *et al.*, 2004). The anaerobic digestion of lignocellulosic biomass could offer an attractive route to address many of the social-economic needs of rural communities by providing clean inexpensive energy, thereby improving the overall health of these communities and addressing rising GHG emissions (Gautam, Baral and Herat, 2009). However, pre-treatment to enable the conversion of lignocellulose to usable energy presents a critical challenge (Hassan, Williams and Jaiswal, 2018). The microorganisms traditionally utilised for biogas production are unable to directly ferment the polymeric lignocellulose, which is why pre-treatment is essential to enable the hydrolysis of lignocellulose into fermentable sugars (Rabemanolontsoa and Saka, 2016). Pre-treatment aims to alter the structural components of lignocellulose and improve the ability of microorganisms to access the substrate (Liu *et al.*, 2017). Nonetheless, traditional physical- and chemical pre-treatment strategies often require significant capital investment and/or harsh chemicals (Kurian *et al.*, 2013; Bhutto *et al.*, 2017). Biological pre-treatment, utilising enzymes or microbial communities, is environmentally friendly, but difficult to apply on an industrial scale (Philbrook, Alissandratos and Easton, 2013).

A community of microbes capable of both, producing biogas and hydrolysing lignocellulose to fermentable sugars, could prove revolutionary to the production of biogas from lignocellulosic materials. Microorganisms inhabit nearly the entire mammalian body, however the area with the richest diversity and density is found within the mammalian gastrointestinal tract (Flint *et al.*, 2008). This close association enables host organisms to extract energy and nutrients from their diets using metabolic functions provided by the gastrointestinal microbiome (Ley, Hamady, *et al.*, 2008). South Africa is one of the most ecologically diverse regions in the world, containing seven major terrestrial biomes confined in a relatively small geographical area (Myers, 1990; Turpie, 2003). It is therefore not surprising that SA hosts indigenous fauna and flora, which have adapted to thrive in these diverse habitats (Myers, 1990). However, the microbial communities that co-exist in close relationships with these fauna and flora, affecting host health and nutrition, are poorly characterised (Bengmark, 2013; Kabat, Srinivasan and Maloy, 2014). The inability to cultivate these complex cultures in the laboratory is a major constraint to proper characterisation (Bodor *et al.*, 2020).

Yet, continued advances in molecular sequencing technologies have enabled scientists to closely study these previously neglected relationships without the need for laboratory cultivation (Ley, Hamady, *et al.*, 2008; Ley, Lozupone, *et al.*, 2008). The gastrointestinal microbiomes of large indigenous herbivores (e.g., elephant, giraffe, buffalo) is of particular interest, as they have adapted to unique SA ecological niches that tend to be arid and offer limited feed choices (Codron *et al.*, 2006). Despite this, these herbivores are capable of maintaining their large body mass from food sources that offer little nutritional value (Senft *et al.*, 1987). This suggests that these mammals may host a gastrointestinal microbiota capable of facilitating nutrient release from otherwise nearly indigestible food sources (McNaughton and Georgiadis, 1986). The potential capability of these microorganisms to breakdown resistant biological material could be the key to improving the production of biogas from low-yield energy sources. The focus of this study was therefore to explore the gastrointestinal microbiota of large endemic herbivores adapted to living on lignocellulose-rich diets with the aim of potentially uncovering such a community.

Broad aims of the study

- To assess, *in silico*, the taxonomic diversity of the African elephant gastrointestinal microbiota and evaluate the potential for methane-gas production and lignocellulose digestion.
- To identify key metabolic pathways and genes linked to the anaerobic digestion of biomass.
- To evaluate the metabolic repertoire of the African elephant gastrointestinal microbiota, focusing on methane-gas production and lignocellulose digestion.

2.2. Outline of the thesis

Chapter 3 outlines the sampling of African elephant (*Loxodonta africana*) faecal matter from seven elephants of varying age and sex. The chosen sample group is located at Adventures with Elephants, Bela-Bela, South Africa. Literature suggests that due to the location of the main fermentation chamber in hindgut fermenters (e.g., elephants), gastrointestinal microbes are likely excreted in the faeces (Ley, Hamady, *et al.*, 2008). The microbial diversity in the faeces was assessed using molecular methods (targeted sequencing of 16S rRNA genes and ITS regions) focusing on three groups: Bacteria, Archaea and Fungi. Taxonomic classification was based on the SILVA (Bacteria and Archaea) and UNITE (Fungi) sequence collection databases. The potential functional capacity of the prokaryotic community was derived from taxonomy using FAPROTAX.

In *Chapter 4*, the genetic diversity and metabolic pathways utilised by the gut community was assessed via shotgun metagenomic sequencing. Metagenomics is the culture-independent genomic analysis of a community of organisms, generally from environmental samples, that inhabit a common environment (Handelsman, 2004; Hugenholtz and Tyson, 2008). Genomics is a powerful tool employed to evaluate the phylogenetic composition and functional capabilities of a microbial community (Schloss and Handelsman, 2003; Dunham, 2005). This type of analysis provides a way of not only viewing the community structure in terms of species richness and diversity, but also the functional potential of the community (Hugenholtz and Tyson, 2008; Ley, Hamady, *et al.*, 2008). Analysing the genomes of a dominant microbial group within the population provides information on the metabolic potential of that group of microbes by inferring the absence or presence of specific metabolic pathways. Pathways were assessed according to the participation in anaerobic digestion and degradation of lignocellulosic compounds. The genes of interest were identified and biochemical pathways created to map the flow of the different phases of anaerobic digestion.

Chapter 5 encompasses the final conclusions and summary pertaining to the results obtained throughout the present study.

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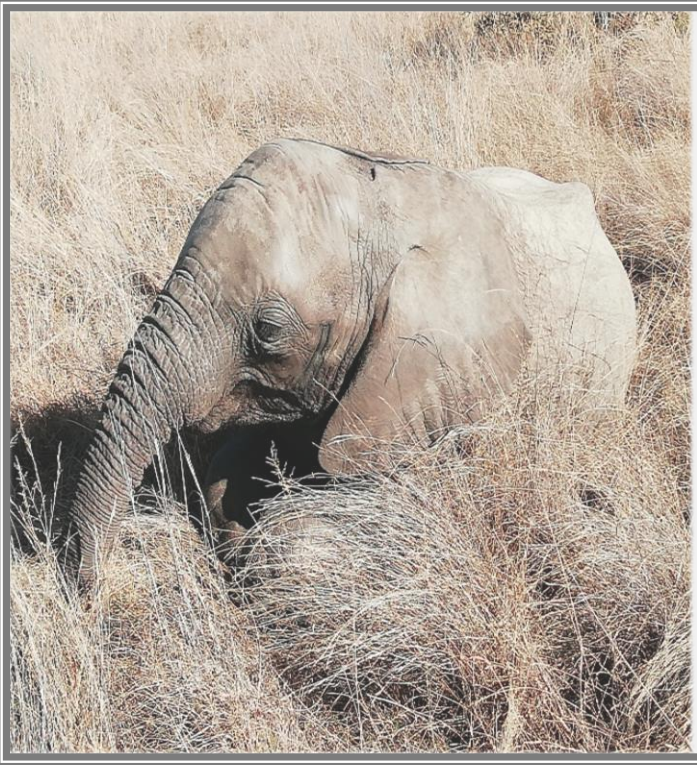

CHAPTER 3

TARGETED SEQUENCING OF THE AFRICAN ELEPHANT *(Loxodonta africana)* GASTROINTESTINAL MICROBIOME

3. Introduction

3.1. Addressing the global energy crisis

The world's continued reliance on fossil based fuels has led to the depletion of fuel deposits, increased greenhouse gas emissions, pollution and unbalanced supply-demand relations (Sindhu, Binod and Pandey, 2016). These factors have boosted research efforts into the search for alternative energy from renewable bioresources (Ahorsu, Medina and Constantí, 2018). Bio-energy produced from biomass is estimated to be one of the top five largest energy resources in the world and offers a nearly greenhouse gas neutral alternative to traditional fossil fuels (Mao *et al.*, 2015). The anaerobic digestion of biomass by a specialised community of microorganisms converts biodegradable organic materials into biogas (mainly methane and carbon dioxide) (Cysneiros *et al.*, 2012). Biogas is a multifaceted renewable energy source, which can be used to replace natural gas for heat and power production including utilisation in biofuel production (Simate *et al.*, 2011; Merlin Christy, Gopinath and Divya, 2014). Compared to other paths for bio-energy generation, the biogas production process is more energy-efficient, environmentally friendlier and also offers a source of alternative fuel, high-quality fertiliser (digestion residues), greenhouse gas reduction and environmental protection from pollutants (Weiland, 2010; Hublin, Schneider and Džodan, 2014).

Lignocellulose is the world's most abundant biomass and represents an enormous source of renewable organic matter for the production of second-generation biofuels and -energy (Glass *et al.*, 2013). Lignocellulosics are abundantly available and produced as waste product by a number of industries, including agriculture, forestry and municipal activity (municipal solid waste) (Yarkwan and Anyanwu, 2016). However, lignocellulose consists of three polymers (cellulose, hemicellulose and lignin) interlaced to form a recalcitrant matrix resistant to biodegradation (Lange, 2007). This recalcitrant matrix is a major hurdle toward the conversion of lignocellulosics to fermentable sugars, essential to its utilisation for bio-energy (Tian *et al.*, 2009). The inability to properly convert insoluble polysaccharides like cellulose to fermentable sugars poses a barrier to the commercial production of biofuels from lignocellulosics (Glass *et al.*, 2013). Pre-treatment offers a way to increase accessibility to the digestible fractions of lignocellulose, however most methods use harsh chemicals and are time and energy intensive, often constituting 40 % of the total processing cost (Sindhu, Binod and Pandey, 2016). Biological pre-treatment utilises microorganisms or their metabolites to degrade lignin and hemicellulose in a simple, energy-efficient and environmentally friendly manner that does not require major capital investment (Muthangya, Mshandete and Kivaisi, 2009).

Filamentous fungi are the main degraders of lignocellulose, although some bacteria and other fungi are capable of hydrolysing cellulose and hemicellulose to their monosaccharide units (Rabemanolontsoa and Saka, 2016; Yarkwan and Anyanwu, 2016). Among non-filamentous fungi, white- and soft-rot fungi produce extracellular enzymes capable of degrading all or some of the components of lignocellulose (Vaz *et al.*, 2017). Cellulolytic and hemicellulolytic bacteria have been found among the Firmicutes (e.g., *Bacillus* spp.), Proteobacteria (e.g., *Pseudomonas* spp.) and Actinobacteria (Jurado *et al.*, 2014). Lignin degrading bacteria are less well studied, but examples have been found among Proteobacteria (e.g., *Pseudomonas* spp.) and Actinobacteria.

3.1.1. Mining novel microbial communities

Microbial communities present in the mammalian gut enable hosts to extract energy and nutrients from their diets using functions outside of their own metabolic repertoire (Ley, Hamady, *et al.*, 2008). One such example is the association of mammalian species with microbial communities that enable these animals to catabolise plant fibre (Akin, 1988). Plant fibre, the structural component of plant cell walls consists primarily of insoluble polysaccharides of which cellulose is the most abundant. Since mammals lack the genes for the enzymes linked to structural polysaccharide degradation, the microbiota of the mammalian intestine is the driving force for plant cell wall degradation (Ilmberger *et al*., 2014; Madigan *et al*., 2012). African bush elephants (*Loxodonta africana*) consume large amounts of fibrous woody vegetation (e.g., grass, tree bark, roots, twigs) rich in lignocellulose and as such are high-impact mega-herbivores of the savannah (Codron *et al.*, 2006). Yet, information on the gastrointestinal microbiome of these mammals is sorely lacking and to date no comprehensive overview of the potential functional capabilities of the African elephant gut microbiome exists. Considering their diet, these mammals could host a microbial gut community specialised in the art of degrading indigestible lignocellulosic biomass. Given the need to improve biogas production from lignocellulosic materials for industrial application, such a community could prove invaluable to the bio-energy industry (Rabemanolontsoa and Saka, 2016).

Chapter aims

- To investigate the African elephant gut microbiome focusing on Bacteria, Archaea and Fungi.
- To determine the functional potential of the African elephant gastrointestinal prokaryotic community from taxonomic diversity.

3.2. Material and Methods

3.2.1. Reagents

All reagents, excluding primers were obtained from the Sigma-Aldrich Corporation (USA) unless otherwise stated. Primers were supplied by Whitehead Scientific (Pty) Ltd, Integrated DNA technologies (RSA).

3.2.2. Sample collection and preservation

The sample group includes seven African elephants (*Loxodonta africana*), four females and three males, ranging between the ages of two and twenty-three (Table 3.1) residing at the Adventures with Elephants Reserve, Bela-Bela, Limpopo, RSA (24°46'53.8"S; 27°57'03.3"E). Manure samples were collected from each individual elephant in sterile Falcon tubes (Lasec, RSA) and stored at -20 °C for downstream analysis. Care was taken to collect the manure samples immediately following defecation. Samples were collected from the center of the manure mounds to limit environmental contamination and refrigerated within 30 minutes of collection.

Table 3.1. *Summary of essential information regarding the African elephant test group for this study, including the sex and age of each elephant. Juvenile < 18 years of age, adult > 18 years of age.*

Identifier (name)	Sex	Age (years)	Maturity	Parentage
Bela	Female	2	Juvenile	Mussina and Chova
Mussina	Female	17	Juvenile	Unknown
Nuanedi	Female	17	Juvenile	Unknown
Shan	Female	18	Adult	Unknown
Zambezi	Male	3	Juvenile	Shan and Chova
Chova	Male	23	Adult	Unknown
Chisuru	Male	21	Adult	Unknown

3.2.3. Genomic DNA extraction and ribosomal DNA amplification

Prior to DNA extraction, each sample was treated with liquid nitrogen and grinded to a fine powder using a mortar and pestle. Genomic DNA (gDNA) was extracted using the NucleoSpin® Soil kit (Macherey-Nagel, Germany) with minor modifications made to the manufacturer's instructions. These modifications include the use of lysis buffer SL1 and only 50 µL enhancer SX, which was combined with 250 - 300 mg sample matter. Cell lysis was increased to 10 minutes of horizontal shaking. The extracted gDNA was visualised on a 0.8 % (w/v) agarose gel containing 3 x 10^{-4} mg/mL ethidium bromide (4 µL 10 mg/mL stock in 120 mL gel). Undiluted (5 µL) gDNA mixed with 2 µL 6X DNA loading dye (Thermo Scientific, USA) was loaded onto the gel alongside 2 µL GeneRuler™ DNA ladder mix (0.5 µg/µL; Thermo Scientific, USA) as reference.

The gel was resolved at 90 V for 1 hour and visualised with a ChemiDoc XRS (Bio-Rad, USA). The DNA concentration and purity were confirmed on the NanoDrop One^{C} spectrophotometer (Thermo Scientific, USA). NanoDrop results are available in Supplementary data A (Table A1). The remaining purified gDNA was stored at -20 °C until required.

Amplification of the 16S ribosomal RNA gene was employed to identify the bacterial and archaeal communities, whereas the internal transcribed spacer (ITS) region 2 served to elucidate the composition of the fungal community. This was achieved by polymerase chain reaction (PCR) in a thermal cycler T100™ (Bio-Rad, USA) according to the cycling conditions (Table 3.4; Table 3.5; Table 3.7) and reaction setup (Table 3.3; Table 3.6) to follow. Table 3.2 contains a list of the primers designed and utilised for the characterisation of the microbial community.

Table 3.2. *List of primers and corresponding sequences for the amplification of bacterial- (BacF and BacR), archaeal- (Arch2A519F and Arch1017R) and fungal (ITS3 and ITS4) targeted sequences respectively.*

Primer	Sequence	Reference
16S BacF Illumina	5' – TCG TCG GCA GCG TCA GAT GTG TAT AAG AGA CAG CCT ACG GGN GGC WGC AG – 3'	Klindworth *et al.*, 2013
16S BacR Illumina	5' – GTC TCG TGG GCT CGG AGA TGT GTA TAA GAG ACA GGA CTA CHV GGG TAT CTA ATC C – 3'	Klindworth *et al.*, 2013
Arch2A519F	5' – TCG TCG GCA GCG TCA GAT GTG TAT AAG AGA CAG CAG CMG CCG CGG TAA – 3'	Fischer *et al.*, 2016
Arch1017R	5' – GTC TCG TGG GCT CGG AGA TGT GTA TAA GAG ACA GGG CCA TGC ACC WCC TCT C – 3'	Fischer *et al.*, 2016
ITS3_KYO2_F	5' – TCG TCG GCA GCG TCA GAT GTG TAT AAG AGA CAG GAT GAA GAA CGY AGY RAA – 3'	Bokulich and Mills, 2013
ITS4_KYO3_R	5' – GTC TCG TGG GCT CGG AGA TGT GTA TAA GAG ACA GCT BTT VCC KCT TCA CTC G – 3'	Bokulich and Mills, 2013

Multiple rounds of PCR were performed to establish the optimal reaction setup and cycling conditions for each primer pair, thereby minimising non-specific binding and increasing the overall yield.

Table 3.3. *Polymerase chain reaction setup (25 µL) for bacterial and archaeal primer pairs. Genomic DNA stocks were diluted to a working solution of 10 ng/µL with nuclease-free water (WhiteSci, RSA).*

Component	25 µL reaction	Final concentration
PCR-grade water	3 µL	N/A
2X KAPA HiFi HotStart ReadyMix	12.5 µL	1X
10 µM Forward Primer	0.75 µL	0.3 µM
10 µM Reverse Primer	0.75 µL	0.3 µM
10 ng/µL Template DNA	8 µL	80 ng

Table 3.4. *Final polymerase chain reaction cycling conditions for the amplification of the bacterial 16S rRNA gene region, following annealing temperature selection and optimisation.*

Initial denaturation	Denaturation	Annealing	Extension	Final extension
95°C 5 min	95°C 30 sec	72.5°C 45 sec	72°C 30 sec	72°C 5 min
	32 cycles			

Table 3.5. *Final polymerase chain reaction cycling conditions for the amplification of the archaeal 16S rRNA region, following optimisation.*

Initial denaturation	Denaturation	Annealing	Extension	Final extension
95°C 5 min	95°C 30 sec	75°C 30 sec	72°C 30 sec	72°C 5 min
	32 cycles			

Table 3.6. *Polymerase chain reaction (25 µL) setup for ITS region amplification with fungal primer pair following optimisation. Genomic DNA stocks were diluted to 50 ng/µL working solutions with nuclease-free water.*

Component	25 µL reaction	Final concentration
PCR-grade water	9.5 µL	N/A
2X KAPA HiFi HotStart ReadyMix	12.5 µL	1X
10 µM Forward Primer	0.5	0.2 µM
10 µM Reverse Primer	0.5	0.2 µM
50 ng/µL Template DNA	2 µL	100 ng

Table 3.7. *PCR cycling conditions for the amplification of the fungal ITS region following annealing temperature selection and optimisation.*

Initial denaturation	Denaturation	Annealing	Extension	Final extension
95°C 5 min	98°C 20 sec	57°C 15 sec	72°C 1 min	72°C 2 min
	35 cycles			

Amplified fragments were visualised on a 0,8 % (w/v) agarose gel containing 3 x 10^{-4} mg/mL ethidium bromide (3 µL 10 mg/mL stock in 100 mL gel). The PCR product (4 µL) mixed with 2 µL 6X DNA loading dye (Thermo Scientific, USA) was loaded onto the gel alongside 2 µL GeneRuler™ DNA ladder mix (0.5 µg/µL; Thermo Scientific, USA) as reference. The gel (Supplementary Figure A1) was resolved at 90 V for 30 minutes and visualised with a ChemiDoc XRS (Bio-Rad, USA). The amplified archaeal-targeted PCR product was approximately 600 - 700 base pairs (bp) (Supplementary Figure A1). Both the bacterial- and fungal-targeted PCR products were approximately 500 - 600 bp (Supplementary Figure A1). Following PCR amplification, 30 – 40 µL of the PCR products were shipped to the Next Generation Sequencing (NGS) unit at the University of the Free State (RSA). Fragments were sequenced on an Illumina MiSeq with a MiSeq reagent kit v3 (600-cycle).

3.2.4. Sample preparation for sequencing

Impurities introduced during the initial amplification of the genes of interest were removed by using AMPure XP beads (Beckman Coulter, USA). Negatively charged nucleic material binds to the magnetic beads, enabling easy separation from impurities within the supernatant. An index PCR was performed to introduce dual indices (8 nucleotide oligos), which uniquely mark and distinguish samples from one another. The indexed PCR samples were subjected to a second clean-up with AMPure XP beads. A Qubit 3.0 fluorimeter (ThermoFisher Scientific, RSA) was employed to quantify the indexed fragments using a high sensitivity double-stranded assay kit (ThermoFisher Scientific, RSA). The quality and fragment size distribution of the libraries was assessed on an Agilent 2100 Bioanalyzer (Agilent Technologies, USA) generating a gel image (Supplementary Figure A2 and A3) and electropherogram for each sample (Supplementary Figure A4).

3.2.5. Sequence quality filtering and processing

Read quality was assessed using FastQC v0.11.5, available online (https://www.bioinformatics.babraham.ac.uk/projects/fastqc/). Reads below 200 bp and/or < Q25 were removed using PRINSEQ-lite v0.20.4 (Schmieder and Edwards, 2011). Forward and reverse reads were joined/paired using PEAR v0.9.11 (Zhang *et al.*, 2014). Chimeric sequences were identified in a reference-based manner and removed according to the QIIME pipeline (Caporaso *et al.*, 2010). The SILVA rRNA database project served as chimeric reference library for the bacterial and archaeal targeted reads, whereas the UNITE reference dataset was used for the ITS targeted reads (Quast *et al.*, 2012; Nilsson *et al.*, 2015). Operational taxonomic units (OTUs) were clustered at the 97 % threshold level against a reference sequence collection (open-reference OTU picking) via QIIME. Reads that did not correspond to the reference collection were clustered *de novo*. Bacterial and archaeal targeted reads were clustered against the SILVA ribosomal RNA (rRNA) reference database utilising USEARCH (Edgar, 2010; Quast *et al.*, 2012). Fungal (ITS) targeted reads were clustered against the UNITE QIIME release sequence collection (UNITE Community, 2019).

The processed gene sequence data was further analysed using RStudio (R Core Team, 2017; RStudio Team, 2020). The following R packages were used during the processing of the data: Phyloseq (McMurdie and Holmes, 2013), Tidyverse (Wickham *et al*., 2019), Vegan (Oksanen *et al.*, 2015), Reshape2 (Wickham, 2007), RColorBrewer (Neuwirth, 2014), Biomformat (McMurdie and Paulson, 2020) and ggplot2 (Wickham, 2016). A detailed script has been provided in Supplementary data B.

3.3. Results and Discussion

3.3.1. Quality control and data hygiene

During the evaluation of DNA sequencing quality, knowledge about the accuracy of sequences is extremely valuable (Richterich, 1998). FastQC, a quality control tool for raw sequence data, presents sequence quality scores in a simple visual manner, enabling quick evaluation of read distribution and the mean quality score across all reads. Figure 3.1 indicates the average Q score (y-axis) and base position within the targeted area (x-axis) for the domains (Archaea and Bacteria) and kingdom (Fungi) pre- and post-filtering.

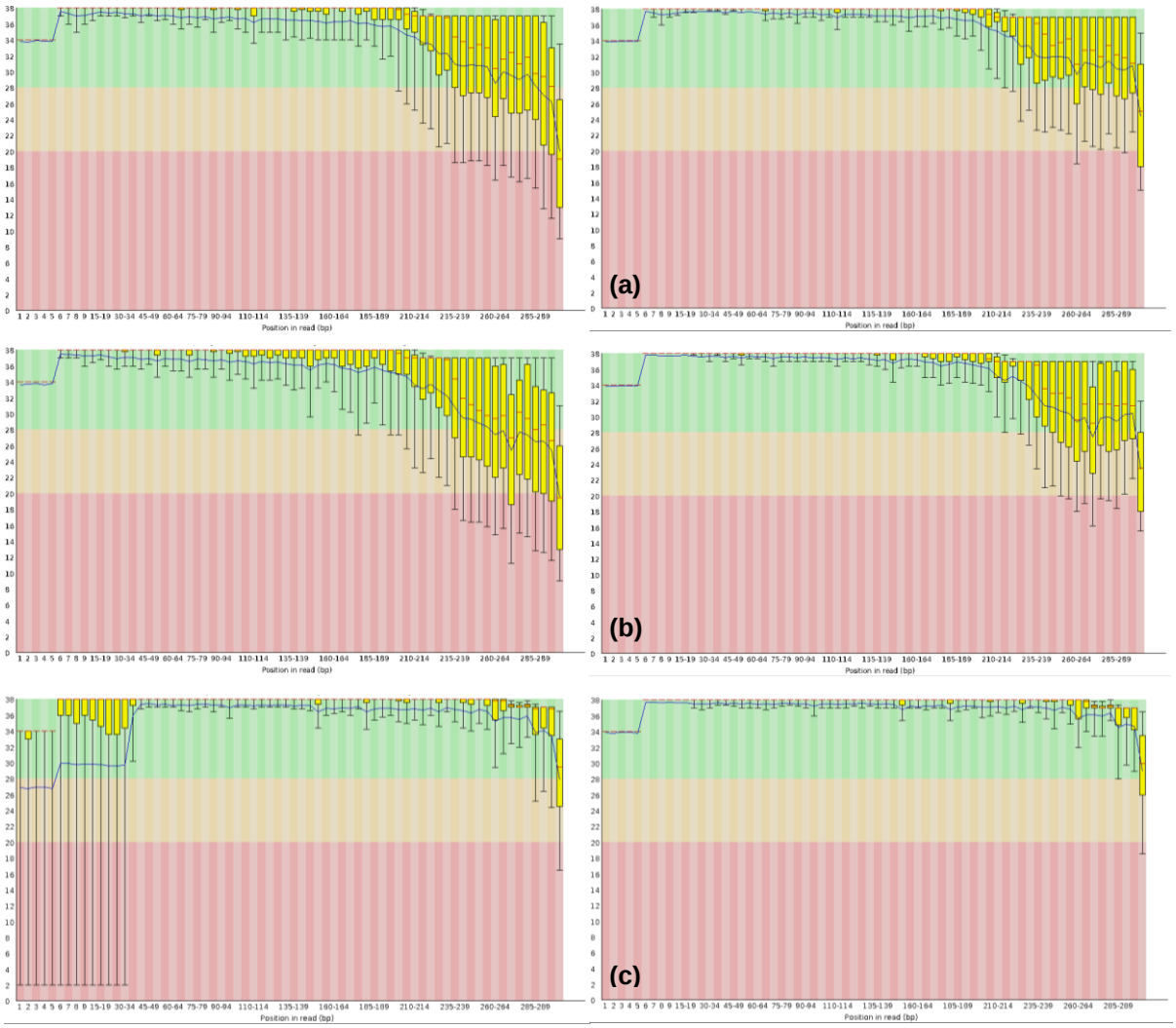

Figure 3.1. Per base quality scores. *Graphics indicate the quality scores of representative individuals from the sample group pre- (left column) and post-filtering (right column) with Prinseq-lite. Samples targeting the Bacteria (a) are arranged in the first row, Archaea (b) in the second row and Fungi (c) in the last row. The green region represents q-scores ranging from Q28 to Q38. Orange represents q-scores ranging between Q20 and Q28.*

Ewing and Green (1998) developed a program, PHRED, that calculates a quality score (*q*) for each base, which is linked logarithmically to the error probability (*p*).

$$q = -10 \times log_{10}(p) \quad (1)$$

Equation 1 describes the quality value (*q*) (Phred quality score) assigned to a base-call, where *p* is the estimated error probability for that base-call. This means that a base-call with a 1/1000 probability of being incorrect will have a quality score (Q score) of 30 (Ewing and Green, 1998). Therefore, a Q score of 30 (Q30) means that the base call accuracy (probability of correct base call) is 99.9 % and that nearly all reads will be perfect, containing zero errors and ambiguities. The fragments sequenced during this study had a mean length of 600 - 750 bp. Quality scores as shown in Figure 3.1 indicate that most of the reads have a mean quality score above Q20. Therefore, the sequences were trimmed to an average Q score of > Q25. Thus ensuring that the probability of a correct base call is between 99 - 99.9 %, resulting in a high-quality data set, which enables more accurate taxonomic distinction.

After quality control measures and the removal of chimeras, the average bacterial sequence count was 84 604 reads, representing an average of 1 703 bacterial OTUs. The average archaeal sequence count was 29 750 reads, representing on average 233 archaeal OTUs and the average fungal sequence count was 120 936 reads, representing an average of 2 609 OTUs. In order to mitigate challenges in data interpretation, data is often normalised prior to downstream analysis (Weiss et al., 2017). Normalisation refers to the process of eliminating artifactual biases within a dataset by transforming the data, with the aim of enabling accurate comparison of statistics from varying measurements. Rarefy is used as a means to normalise microbiome counts from libraries of different sizes by resampling the OTU table to ensure that all samples have the same library size (total sequence count). This method was employed on the current data set as recommended by Weiss and co-workers (2017). The samples were rarefied to 56 219 bacterial sequences, 7 000 archaeal sequences and 2 658 fungal reads. This step ensures that variations observed between different samples is statistically relevant and not the result of differing library sizes.

3.3.2. Microbial diversity

Mammals categorised as herbivores typically display the highest microbial gut diversity and have developed specialised anatomical structures that act as anaerobic fermentation chambers, establishing a niche for plant-degrading microbes (Shapira, 2016; Nishida and Ochman, 2018). Foregut fermenters or ruminants (e.g., cattle, sheep) benefit from protein and energy released during microbial fermentation in a pre-gastric chamber called the rumen (Flint *et al.*, 2008; Ilmberger *et al.*, 2014).

Hindgut fermenters (e.g., horses, elephants, rhinos) utilise the colon or caecum, an enlargement of the gut downstream of the stomach, for the main fermentation process (Flint *et al.*, 2008; Ley, Lozupone, *et al.*, 2008). The anaerobic chambers employed by these mammals may differ in their anatomical structure and chemical composition, but resemble each other in microbial composition (Shapira, 2016). The microbial communities found within the rumen are responsible for the rapid bioconversion of lignocellulose in nature and as such have received considerable interest in terms of biotechnological potential and the production of energy from renewable plant biomass (Flint *et al.*, 2008; Morrison *et al.*, 2009; Weimer, Russell and Muck, 2009). Alternatively, the microbial communities found within hindgut fermenters could also prove industrially relevant, yet these communities are often overshadowed and less well explored (Ilmberger *et al.*, 2014). Improved insight into one of Africa's prominent hindgut fermenters, the African elephant, could prove beneficial to South Africa's (SA) developing biogas industry.

Anaerobic digestion is split into four stages: hydrolysis, acidogenesis, acetogenesis and methanogenesis (Wikandari and Taherzadeh, 2019). The process is highly dependent on the interactions between the diverse communities of microorganisms that facilitate each of the four stages (Meegoda *et al.*, 2018). In order for anaerobic digestion to proceed, certain groups of microorganisms are therefore essential. Figure 3.2 is a simple summary of the anaerobic digestion process, indicating the flow of intermediates produced during one stage of the process to the next, yielding methane as the main product.

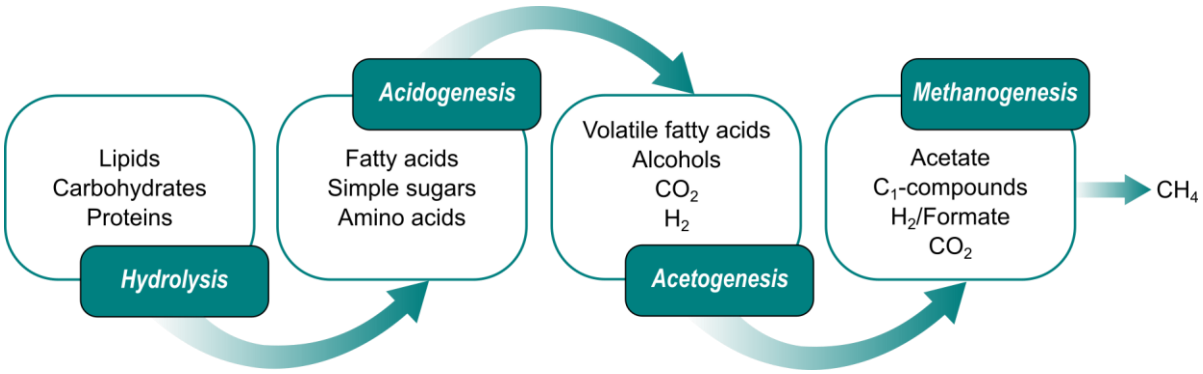

Figure 3.2. Summary of the anaerobic digestion process. *Anaerobic digestion is divided into four successive stages defined by the primary catabolic reactions that occur in that phase namely hydrolysis, acidogenesis, acetogenesis and methanogenesis. The products produced during one phase are sequentially utilised during the next phase to ultimately yield methane.*

3.3.2.1. Bacterial diversity

The four major bacterial phyla found in the faeces of the African elephant were Planctomycetes (55 - 61.4 % relative abundance), Bacteroidetes (9.9 - 12.6 %), Firmicutes (13.6 - 21.5 %) and Synergistetes (3.6 - 8.3 %). These four phyla were present in varying degrees in all seven sampled individuals with, Planctomycetes, representing the dominant phylum (Figure 3.3).

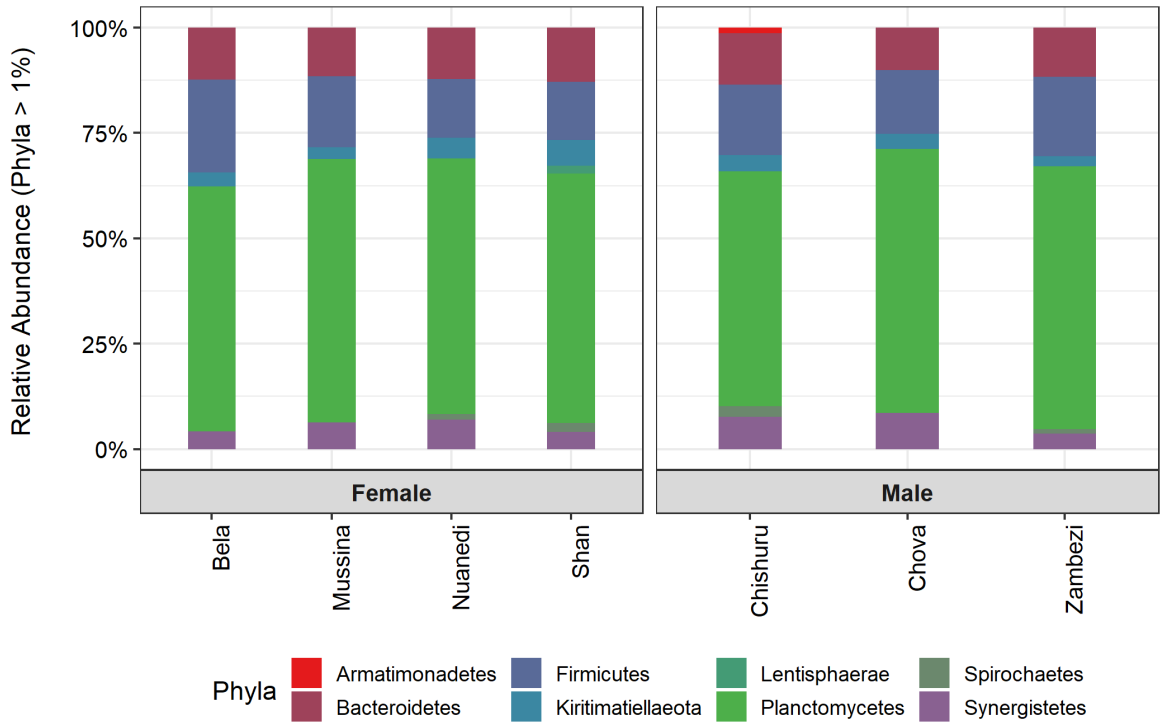

Figure 3.3. Relative abundance (> 1 %) of bacterial phyla. *Phyla diversity was determined from the amplified 16S rRNA sequences using the Silva reference database. The diversity is split into female and male members of the sample group, however little variance is noted between the various individuals.*

The remaining bacterial diversity was made up of five phyla: Armatimonadetes with an abundance of 1.3 % in the sample obtained from Chisuru, Kiritimatiellaeota (2.4 - 6.1 %) present in all samples, Lentisphaerae (1.7 %) from Shan's sample and Spirochaetes (1 - 2.5 %) present in four of the seven samples. From the data, it may seem that some phyla are unique to certain individuals. However they are represented at an abundance < 1 % in some individuals and as such are not displayed in the bar plots. These differences in abundance are however not significant in terms of age, sex or maturity. Studies have shown that mammalian species, like the elephant, harbour distinct gut microbiomes with a high level of host- and species specificity (Nishida and Ochman, 2018; Youngblut *et al.*, 2019).

A study of over 100 mammalian species identified four phyla, namely Actinobacteria, Bacteroidetes, Firmicutes and Proteobacteria as the core mammalian microbiome (Nishida and Ochman, 2018). Other less abundant (< 10 %) phyla commonly found in the gastrointestinal microbiome of mammals include, among other, Verrucomicrobia, Fusobacteria, Spirochaetes, Fibrobacteres and Planctomycetes (Ley, Hamady, *et al.*, 2008; Youngblut *et al.*, 2019). The microbial groups observed during the current study corresponded to the core mammalian microbiome, as well as the gut microbiome of other large herbivores (Youngblut *et al.*, 2019). However, the relative abundance of the observed microbial groups deviate from those observed in other studies. Literature presents an even larger proportion of Firmicutes and Bacteroidetes than that observed currently, representing up to 80 % of the overall relative abundance (Morrison *et al.*, 2009; Nishida and Ochman, 2018). Actinobacteria and Proteobacteria are underrepresented at < 1 % within the current dataset and the Plactomycetes are overrepresented, as this group generally represents one of the minor phyla within the mammalian microbiome (Ley, Hamady, *et al.*, 2008). However, multiple factors may influence taxonomic assignment, including DNA extraction methods, the sequencing platform, primer binding, the selected amplification site and the complexity of the isolated community (D'Amore *et al.*, 2016).

A meta-analysis of 16S rRNA gene sequences available on public databases has produced a collective overview of the microbial diversity found in anaerobic digesters (Nelson, Morrison and Yu, 2011). During this analysis, four major phyla were identified (Chloroflexi, Proteobacteria, Firmicutes and Bacteroidetes) that represent over 60 % of the total diversity, including twenty-four minor phyla of which only Planctomycetes and Actinobacteria represented approximately 1 % of the total diversity. The phyla Proteobacteria, Firmicutes and Bacteroidetes are likely ubiquitous in nearly all anaerobic digesters as multiple studies have identified sequences from these phyla within anaerobic digesters (Toerien and Hattingh, 1969; Werner *et al.*, 2011; Lee *et al.*, 2012). Furthermore, several species of these phyla are known to participate in one or more of the phases of anaerobic digestion (Li *et al.*, 2013; Ortseifen *et al.*, 2016). The Firmicutes and Bacteroidetes are likely involved in the breakdown of polysaccharides (hydrolysis) and the subsequent fermentation (acidogenesis) of the produced monomers (Vanwonterghem *et al.*, 2014). The abundance of these two phyla within the elephant microbiome supports anaerobic digestion, although the presence of a particular phylum does not guarantee that the species that perform these functions will be present. On the other hand, the low abundance of Proteobacteria (< 1 %) in the elephant faecal samples, may suggest the need for enrichment of the phylum prior to using the manure as seeding material in an anaerobic digester system.

During the first phase of anaerobic digestion, hydrolytic bacteria secrete extracellular enzymes (e.g., lipase, protease, amylase) that convert macromolecules to their constituent monomers (Weiland, 2010; Meegoda *et al.*, 2018; Laiq Ur Rehman *et al.*, 2019) (Figure 3.4). This phase does not require specialised microorganisms (*Chapter 1, Figure 1.3*) and as such, a vast number of microbial groups can facilitate this phase (Campanaro *et al.*, 2016). Nevertheless, some substrates, like lignocellulose contain recalcitrant materials (lignin, cellulose and hemicellulose) that pose a particular challenge to biodegradation and as such can remain inaccessible to microbes lacking the required enzymes to degrade these compounds (Mao *et al.*, 2015; Meegoda *et al.*, 2018). The products of hydrolysis are absorbed through the cellular membranes of acidogenic microorganisms, where they act as substrates for acidogenesis/fermentation, the second phase of anaerobic digestion (Meegoda *et al.*, 2018).

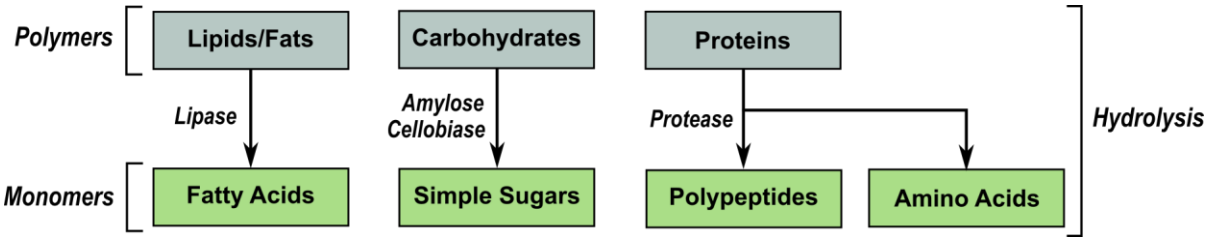

Figure 3.4. Summary of hydrolysis, the first phase of anaerobic digestion. *Summary of the substrates, products and main enzymes involved during hydrolysis, the first non-methanogenic phase of anaerobic digestion. Polymers such as lipids, carbohydrates and proteins are digested by hydrolytic enzymes to their constituent monomers, fatty acids, simple sugars and amino acids. These products are utilised in the subsequent phases of anaerobic digestion. Adapted from Mao et al., 2015.*

Figure 3.5 displays the most abundant bacterial genera detected. At the genus level, bacterial communities were dominated by the p-1088-a5 gut group (50.5 - 62.5 %). This group consists of uncultured Planctomycetes isolated from environmental samples like the rumen, bioreactors, municipal wastewater treatment plants, the mammalian gut and the wastewater treatment plant of an alcohol manufacturing plant (Chouari *et al.*, 2003; Tajima *et al.*, 2007; Yang *et al.*, 2007; Ley, Hamady, *et al.*, 2008; Garcia *et al.*, 2011; Shinkai *et al.*, 2014; Konda *et al.*, 2019). The Planctomycetes are an unusual group of bacteria, long believed to possess unique eukaryote-like morphological and biochemical features, including intracellular compartmentalisation and an absence of peptidoglycan within their cell walls (Fuerst, 1995; Ward, 2010; Fuerst and Sagulenko, 2011). Even though recent advancements have altered these views regarding their cell biology, comprehensive understanding of their functional- and metabolic roles within the environment remains limited (Jeske *et al.*, 2015; Boedeker *et al.*,

2017; Dedysh and Ivanova, 2019). This is mainly due to the challenge of obtaining pure cultures, restricting Planctomycete characterisation (Lage and Bondoso, 2012). However, the genomes of these bacteria have been shown to encode a wide variety of carbohydrate-active enzymes (CAZymes) and metatranscriptomic analysis has indicated versatile hydrolytic capabilities (Ivanova *et al.*, 2017; Dedysh and Ivanova, 2019). Carbohydrate-active enzymes refer to a diverse selection of enzymes that build or breakdown complex carbohydrates and glycosylated compounds (glycoconjugates) (Cantarel *et al.*, 2009). Genomic data also indicates that they encode genes for the expression of endo-β-xylanases, an industrially relevant group of glycoside hydrolases (Naumoff, Ivanova and Dedysh, 2014). These enzymes are responsible for the digestion of heteroxylans, a structural group of polysaccharides that compose the basis of hemicellulose in higher plant cell walls (Naumoff, Ivanova and Dedysh, 2014; Saini *et al.*, 2015). These properties indicate that Planctomycetes likely play a role during the hydrolysis of complex plant polysaccharides (e.g., xylan, starch, cellulose), an ability crucial to the initial stages of anaerobic digestion and lignocellulose digestion (Dedysh and Ivanova, 2019).

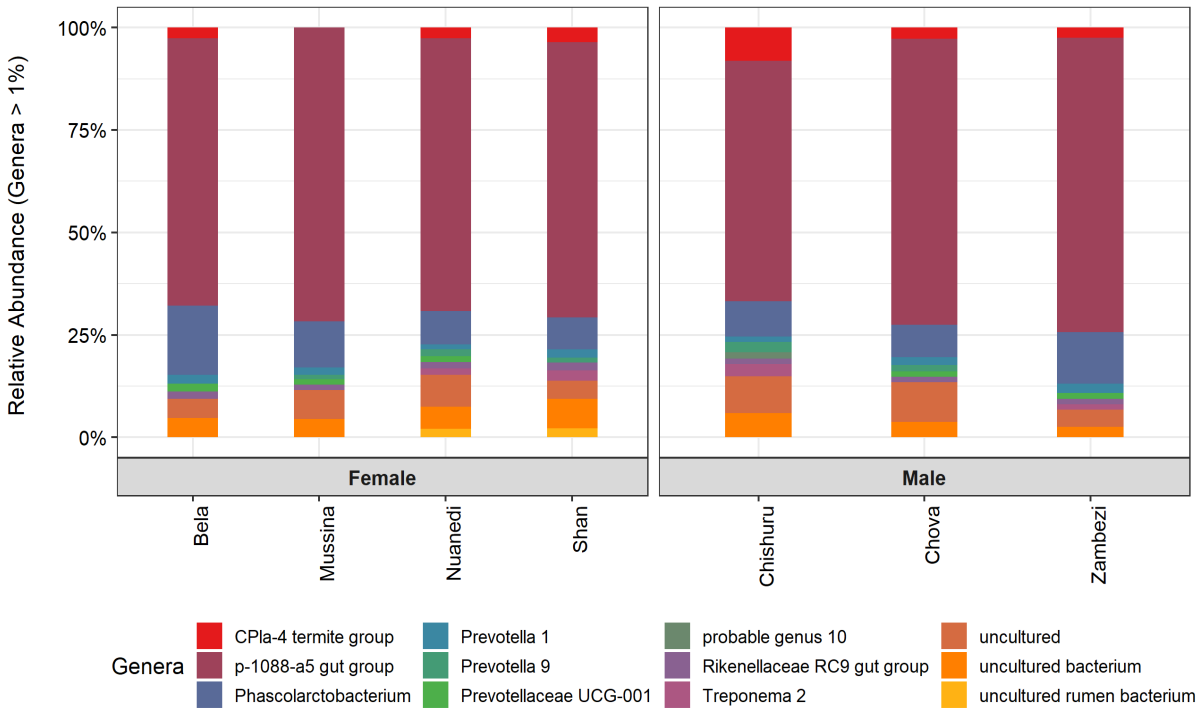

Figure 3.5. Relative abundance (> 1 %) of bacterial genera. *Bacterial genera diversity as determined from the amplified 16S rRNA targeted sequences using the SILVA reference database.*

The genus *Phascolarctobacterium* (6.6 - 14.9 %) of the phylum, Firmicutes, represented another abundant bacterial group. Species of this genus produce short-chain fatty acids and specifically have the ability to ferment succinate to propionate (Engels *et al.*, 2016; Wu *et al.*, 2017). This process is essential during acidogenesis and contributes to the production of volatile fatty acids (VFAs) (Pore *et al.*, 2016). Volatile fatty acids, produced from the products of hydrolysis, refer to a class of organic acids that include acetic-, propionic- and butyric acid (Mussatto, Dragone and Roberto, 2006). The other products produced during acidogenesis include hydrogen (H_2), carbon dioxide (CO_2) and alcohols like ethanol (Anukam *et al.*, 2019). The production of acetate during acidogenic fermentation means that a portion of the original substrate has been converted to a suitable substrate for acetoclastic methanogenesis. However, most VFAs remain inaccessible to methanogens and must first undergo acetogenesis, a process which converts these VFAs and other intermediates to acetate, producing H_2 as by-product (Weiland, 2010; Meegoda *et al.*, 2018). Figure 3.6 illustrates the process of acetogenesis. Genera of the phyla, Firmicutes and Bacteroidetes (e.g., *Prevotella*), likely facilitate acetogenesis via multiple fermentative pathways, producing the final precursors for methanogenesis. Lastly, the presence of the CPla-4 termite group (2.2 - 6.9 %) (Figure 3.5) could be attributed to the sequencing methodology. Next-generation sequencing (NGS) technology amplifies all available DNA within a sample, whether from live or dead cells. As the elephants ingest large volumes of woody material, termite DNA is to be expected. However, its presence may also point to common protein sequences, associated with the deconstruction of woody biomass.

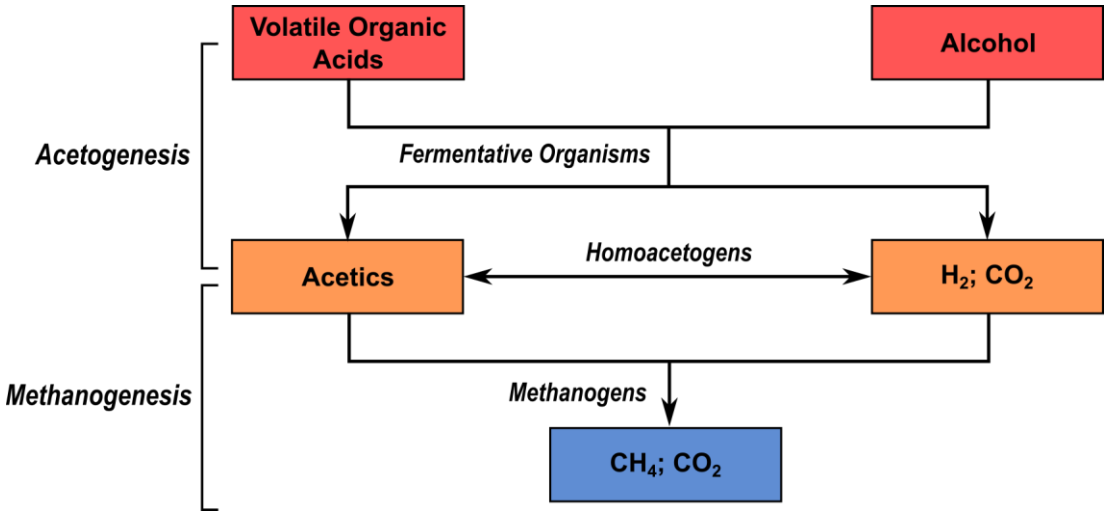

Figure 3.6. Summary of the methanogenic phase of anaerobic digestion. *Substrates, products and main organisms involved during acetogenesis and how these flow into methanogenesis, the final step of the anaerobic digestion process. Volatile organic acids and alcohol produced during acidogenesis are converted to hydrogen, carbon dioxide and acetate. These products are ultimately converted to methane and carbon dioxide (biogas).*

3.3.2.2. Archaeal diversity

The limiting factor and final phase of anaerobic digestion is the production of methane by methanogenic microorganisms, a group of slow growing, obligate anaerobic Archaea (Leclerc, Delgènes and Godon, 2004). Nearly all of the archaeal-targeted sequences (> 99 %), from this study, were clustered into the phylum, Euryarchaeota (Figure 3.7). Thus far, all methanogens belong to the phylum Euryarchaeota, however the phylum also contains non-methanogenic Archaea (Enzmann *et al.*, 2018). To date, methanogens are grouped into seven archaeal orders, which differ in their biochemical catabolic approaches and ecological niches (Lang *et al.*, 2015).

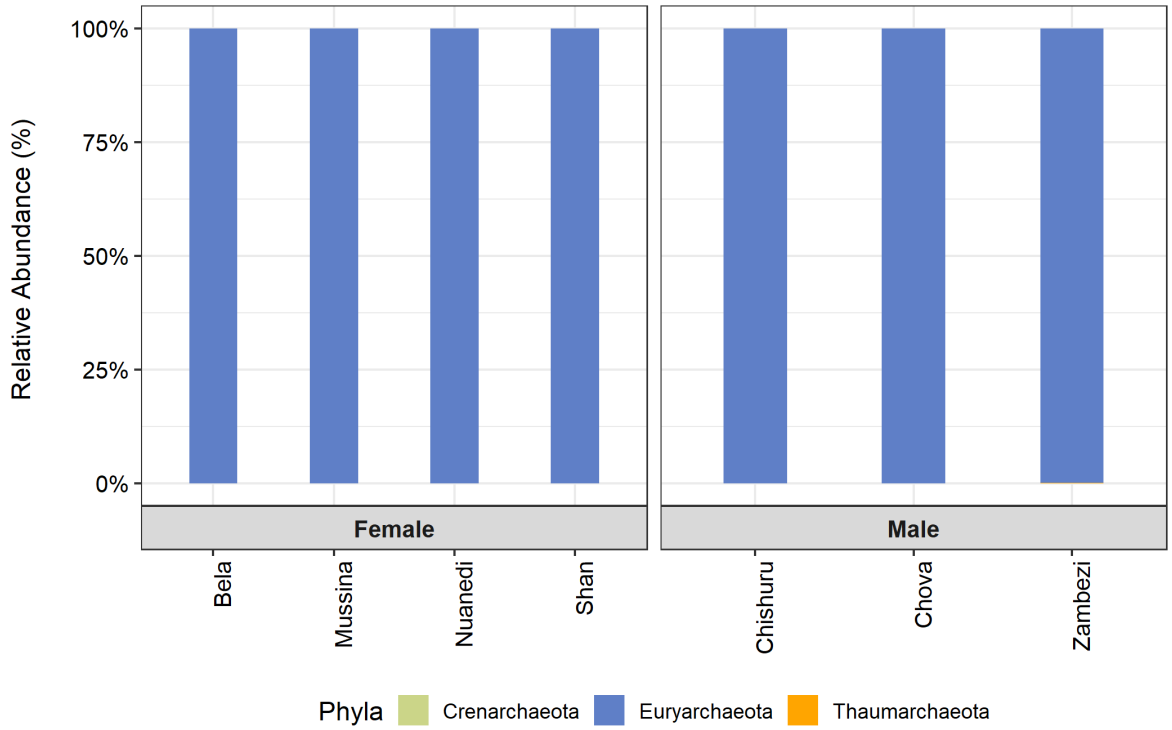

Figure 3.7. Relative abundance (%) of archaeal phyla. *Archaeal phyla diversity as determined using the SILVA reference database. Diversity was dominated by the Euryarchaeota. The phyla Crenarchaeota and Thaumarchaeota were only detected at an abundance level below 1 %.*

Methanogens make use of a small selection of substrates, which can be converted to methane via three pathways, the hydrogenotrophic-, methylotrophic- and acetoclastic pathway (Ferry, 2011; Guan *et al.*, 2014). All three these pathways ultimately converge on the reduction of methyl-CoM to methane (CH_4) (Meegoda *et al.*, 2018). The pathways harbour a similar central pathway for CO_2 reduction, but differ in the energy conservation method at the last methanogenic step (Richards *et al.*, 2016). Figure 3.8 illustrates these three pathways with their substrates and subsequent product, methane.

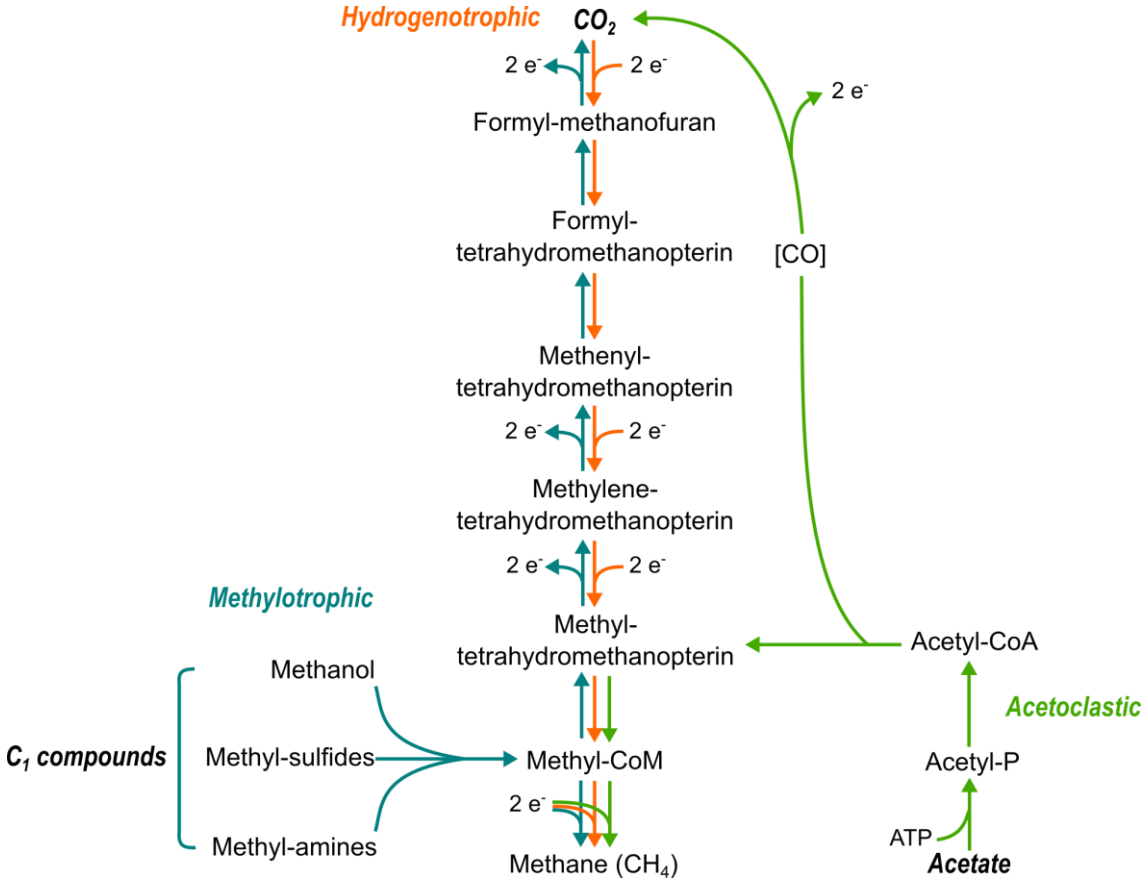

Figure 3.8. Summary of the three methanogenesis pathways. *In orange, the hydrogenotrophic pathway, which is the reduction of carbon dioxide (CO_2) to methane using electrons derived by the oxidation of hydrogen (H_2). In green, the acetoclastic pathway, the conversion of acetate to methane and lastly in blue, the methylotrophic pathway, the oxidation and reduction of C_1 compounds like methanol to produce methane. Adapted from Galagan et al., 2002.*

The archaeal genus level diversity, for this study, is outlined in Figure 3.9. The community was dominated by two genera: *Methanobrevibacter* (7.1 – 49 %) from the order, Methanobacteriales and *Methanocorpusculum* (45.9 - 73.1 %) from the order, Methanomicrobiales. Species of the orders Methanobacteriales and Methanomicrobiales are obligate hydrogenotrophic methanogens and use H_2 and CO_2 as substrate (Welte and Deppenmeier, 2014; Lang *et al.*, 2015). During the hydrogenotrophic pathway (Figure 3.8; orange arrows), CO_2 is reduced in a step-wise manner by utilising electrons obtained from the oxidation of H_2, produced during acetogenesis (Richards *et al.*, 2016). Most hydrogenotrophic methanogens are also capable of oxidising formate, which is converted to H_2 and CO_2 before being used for methane production (Welte and Deppenmeier, 2014).

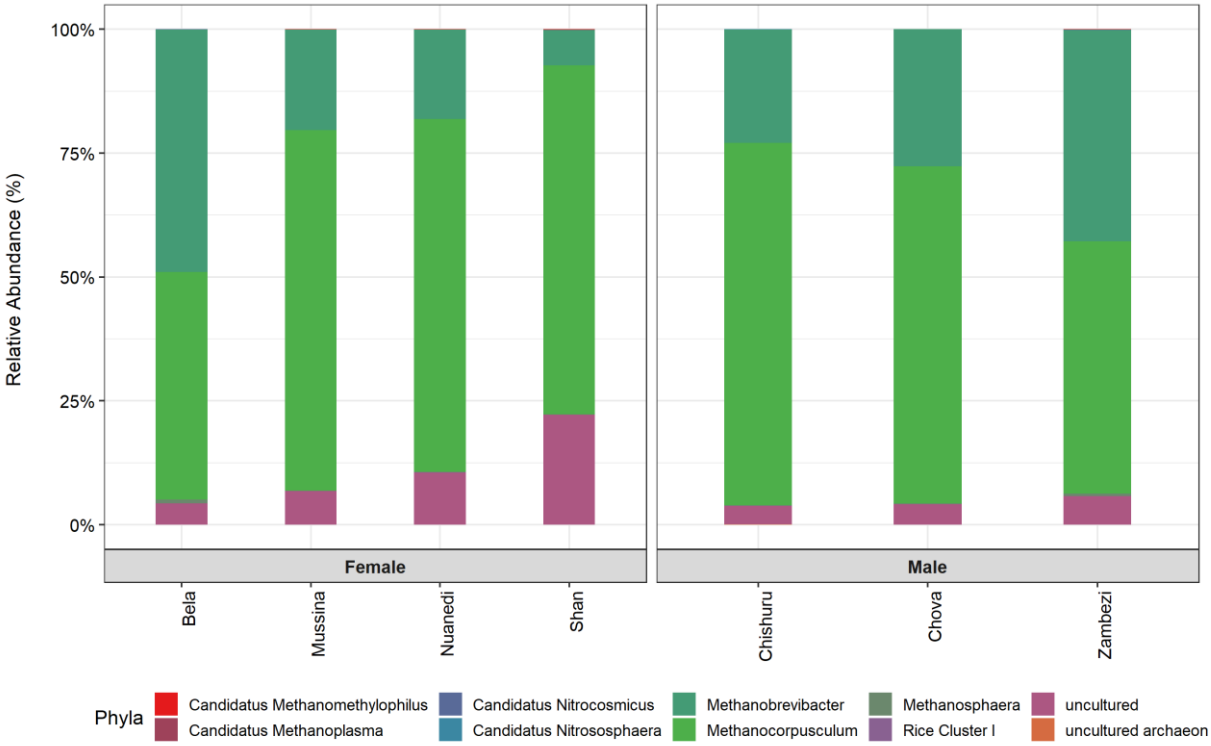

Figure 3.9. Relative abundance (%) of archaeal genera. *Archaeal genera diversity as determined utilising the SILVA reference database. The diversity was dominated by two groups, Methanobrevibacter and Methanocorpusculum.*

The remaining diversity was assigned to the genus, *Methanosphaera,* from the order Methanobacteriales and a cluster of uncultured belonging to the order Methanomassiliicoccales. Methanomassiliicoccales, originally known as Methanoplasmatales, is the seventh order of methanogens. Members of this order are restricted to growth on methanol and methylamines, however they lack cytochromes and are unable to oxidise these intermediates to CO_2 (central CO_2 reduction pathway) (Söllinger *et al.*, 2016). Methanogens lacking cytochromes employ a truncated version of the methylotrophic pathway (Figure 3.8, blue arrows), where one molecule of a C_1 compound (e.g., methanol) is oxidised to produce electrons for the subsequent reduction of three additional molecules, ultimately producing methane (Galagan *et al.*, 2002). These methanogens lack the genes encoding the C_1 pathway for the reduction of CO_2 to methyl-CoM, but possess the complete gene set for the utilisation of methanol and methylamines, making them obligately H_2-dependent (Lang *et al.*, 2015). Methylotrophic methanogens with cytochromes oxidise methyl groups to CO_2 using a membrane-bound electron transport chain and follow the central pathway of CO_2 reduction (Vanwonterghem *et al.*, 2016).

Only members of the Methanosarcinales employ the acetoclastic pathway for energy conservation (Vanwonterghem *et al.*, 2016). The acetoclastic pathway (Figure 3.8; green arrows) involves splitting acetate into a methyl group and enzyme-bound CO group, where the CO is oxidised to produce electrons for the reduction of the methyl group to methane (Galagan *et al.*, 2002). Studies into the diversity of methane producing archaea in anaerobic digesters present contradictory results concerning the main metabolic pathway used. Generally, the acetoclastic methanogens are regarded as the dominant methane producers within anaerobic digesters, however some studies have noted a larger population of hydrogenotrophic methanogens (Nettmann *et al.*, 2010; Zhu *et al.*, 2011). In ruminants, the acetic acid produced during fermentation is reabsorbed to act as an energy source for the host. This facilitates a larger abundance of hydrogenotrophic methanogens within the gastrointestinal tract (Thauer *et al.*, 2008). Considering the similarities between the microbial communities found within ruminants and hindgut fermenters, it is possible to speculate that this same trend will prevail in hindgut fermenters.

3.3.2.3. Fungal diversity

The fungal diversity of the African elephant faecal matter was dominated by the phylum, Ascomycota, with a relative abundance greater than 85 % in all samples except one (Chisuru). The remaining abundance consisted of the phyla, Basidiomycota (1.9 - 7.6 %) and an unidentified phylum (3 - 11.4 %). Figure 3.10 indicates the relative abundance > 1 % of the fungal phyla present in the African elephant faeces.

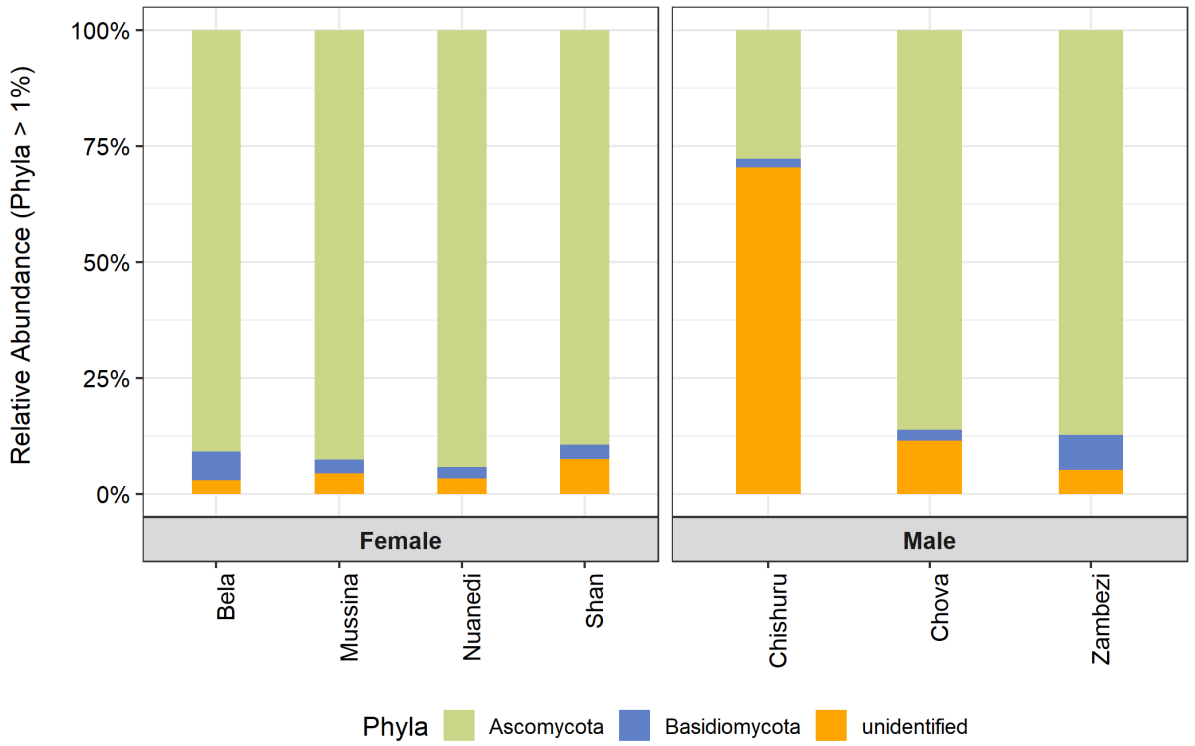

Figure 3.10. Relative abundance (> 1 %) of fungal phyla. *Phyla diversity of fungal targeted sequences as determined using the UNITE reference database. Diversity was dominated by the Ascomycota, followed by the Basidiomycota and a section of unidentified fungi.*

Fungi are known for their lignocellulolytic capabilities and as such are of great interest in the bio-energy industry, even if they do not partake or contribute to anaerobic digestion directly (Malherbe and Cloete, 2002). The components of lignocellulose (cellulose, hemicellulose and lignin) are intermeshed and chemically bonded together by non-covalent forces, covalent cross linkages, hydrogen bonding and van der Waals forces resulting in a recalcitrant matrix resistant to degradation (Sindhu, Binod and Pandey, 2016; Yarkwan and Anyanwu, 2016). The hydrolysis of lignocellulosics to fermentable sugars is enhanced through the use of biological-, physical-, chemical- and physio-chemical pre-treatment strategies (Saritha and Arora, 2012; Karimi and Taherzadeh, 2016).

However, these methods are considered harsh and cost/energy intensive, whereas biological pre-treatment is mild and environmentally friendly (Rabemanolontsoa and Saka, 2016). Biological pre-treatment generally employs white-rot fungi, soft-rot fungi, actinomycetes and bacteria that produce lignin-degrading enzymes (Saritha and Arora, 2012). To date, all known white- and brown-rot wood-decaying fungi are basidiomycetes of the phylum Basidiomycota (Vaz *et al.*, 2017). White-rot fungi can degrade all of the structural components of lignocellulose (Morgenstern, Klopman and Hibbett, 2008). Brown-rot fungi degrade the carbohydrate components, cellulose and hemicellulose, but lignin digestion is limited (Worrall, Anagnost and Zabel, 1997). Soft-rot fungi of the phylum, Ascomycota, create cavities to selectively degrade cellulose and hemicellulose within the central layer of the secondary plant cell wall (Schwarze, 2007). However, their capacity to modify lignin is extremely limited. The overall dominance of the Ascomycota and low abundance of Basidiomycota in the elephant microbiome could point to alternative strategies of lignocellulose digestion. Mechanical grinding with large molar-like teeth (physical pre-treatment) prior to ingestion combined with even a small number of lignin-degrading fungi could enhance access to the digestible parts of lignin-rich material (Bayané and Guiot, 2011). However, another option is that elephants ingest larger quantities of food to counteract the effect of only partially digesting lignocellulosic materials. Both these options would necessitate alternative measures like enrichment of white- and brown-rot Basidiomycota to effectively utilise this microbial community for biological pre-treatment.

Traditional methods of describing new fungal species requires a fair amount of time and dedication, however at the current rate it could take up to 4 000 years to describe all the species of fungi (Yahr, Schoch and Dentinger, 2016). In the interim, high-throughput barcoding to analyse community structures, especially from environmental samples, is resulting in increasing numbers of unidentified fungal ITS sequences and OTUs. In 2008 and 2009, the number of environmental clusters deposited in GenBank exceeded the number of species described based on specimens (Hibbett *et al.*, 2011). This large influx could affect classification during diversity studies resulting in large proportions of sequences without proper taxonomic annotations as observed by Buée and co-workers (2009). These observations could also account for the notable proportion of unidentified fungal sequences present in the African elephant faecal matter. This ever growing collection of unnamed OTUs and environmental clusters has increased miscommunication about the true fungal diversity of certain communities and highlights the need for a classification system based on environmental samples (Hibbett *et al.*, 2011). Figure 3.11 displays the genera level diversity of the fungal-targeted sequences.

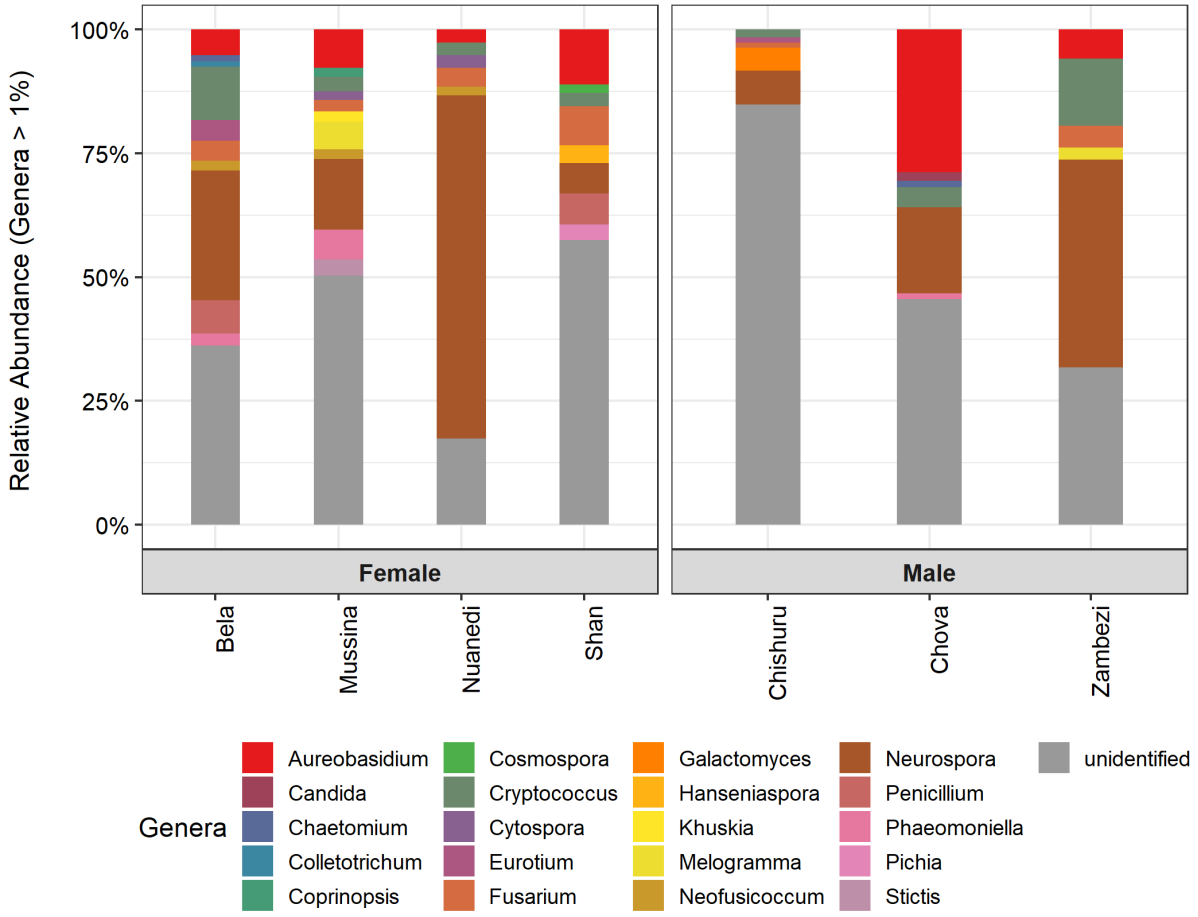

Figure 3.11. Relative abundance (> 1 %) of fungal genera. *Fungal genera level diversity as determined using the UNITE reference database. Genera level diversity varies considerably between individual members of the sample group, however no apparent correlation exists based on specimen sex.*

In contrast to the bacterial and archaeal diversity, the fungal diversity differed notably between the seven elephant samples. However, one genus, the *Neurospora* (5.5 – 67 %) presented as the dominant group in a number of samples (Figure 3.11). Filamentous fungi are among the most efficient decomposers of plant biomass and are the main source of commercial enzymes used to degrade lignocellulose (Glass *et al.*, 2013). The model filamentous fungi, *Neurospora crassa*, among other filamentous ascomycete fungi, are notorious for their cellulolytic capabilities and produce a number of enzymes capable of cellulose deconstruction (Znameroski and Glass, 2013). However, enzymatic hydrolysis is greatly affected by the accessibility of these cellulolytic enzymes to cellulose. Pre-treatment therefore aims to improve the accessibility of cellulose by removing the barriers halting hydrolysis (Bhutto *et al.*, 2017).

Cellulose microfibrils (glucan chains composed of repeating β-(1-4) D-glucose units) are encased within lignin and hemicellulose (*Chapter 1, Figure 1.5*) (Bhutto *et al.*, 2017). Lignin acts as a physical barrier and inhibitor by binding to cellulolytic enzymes, whereas hemicellulose reduces the mean pore size of substrates, reducing the accessibility of hydrolytic enzymes to cellulose (Esteghlalian *et al.,* 2000; Saritha and Arora*,* 2012). The removal of either xylan, a component of hemicellulose, or lignin enhances the rate of enzymatic saccharification (Glass *et al.*, 2013). Yet, glucan chain accessibility is directly affected by xylan removal, which could prove more advantageous to enhance enzymatic hydrolysis (Sindhu, Binod and Pandey, 2016). Oleaginous yeasts can be found within the genus, *Cryptococcus,* from the phylum Basidiomycota. This genus represents 1.5 - 11.9 % of the total fungal diversity from the elephant faecal matter (Figure 3.11). Apart from their cellulolytic capabilities, a study by Jiménez and co-workers (1991) also showed the ability to produce xylanase, an enzyme utilised for the degradation of xylan (Štursová *et al.*, 2012). This genus could therefore aid the hydrolysis of cellulose and hemicellulose from lignocellulosic biomass. Furthermore, species of this genus have been shown to co-ferment mixed sugar solutions (Hu *et al.,* 2011). Generally when presented with multiple sugars like glucose and xylose, the sugars are metabolised sequentially, first glucose then xylose. Yet, this method of metabolism causes extended cultivation periods and results in the inefficient utilisation of the substrates, decreasing the total process productivity (Yu *et al.*, 2014). Co-fermentation could circumvent this and enhance fermentation during anaerobic digestion, specifically following depolymerisation of sugars from recalcitrant biomass.

Species from the genus, *Aureobasidium*, have been compared to that of *Aspergillus*, a group of versatile, biotechnologically significant yeasts (Chi *et al.*, 2009). Species of *Aspergillus* are known to produce extracellular enzymes targeted toward the deconstruction of cellulose, hemicellulose and pectin, through the production of enzymes like cellulase, amylase and xylanase (Gawande and Kamat, 1999; Chi *et al.*, 2009; Cleveland *et al.*, 2009; Kausar *et al.*, 2010; Gostinčar *et al.*, 2014). These enzymes all contribute to the digestion of complex plant biomass. The remaining genus of interest, regarding lignocellulose breakdown, is *Fusarium* (1.1 - 7 %) from the phylum, Ascomycota. Several strains of *Fusarium* spp. have lignin degrading capabilities (Sutherland, Pometto III and Crawford, 1983). Strains from the species *F. solani* are characterised as soft-rot fungi (Sutherland, Pometto III and Crawford, 1983; Norris 1980). However, a study by Sutherland and co-workers (1983) confirmed that a variety of *Fusarium* strains are also capable of degrading lignin, although at a slower rate than that commonly seen in white- and brown-rot fungi.

3.3.3. Potential functional capabilities of microbial community

Functional annotation of prokaryotic taxa (FAPROTAX) is a manually assembled database used to map prokaryotic taxa to metabolic or other ecologically relevant functions based on the available literature (Louca, Parfrey and Doebeli, 2016). This database can be used to infer the potential functional capabilities of a prokaryotic community based on peer-reviewed literature of cultured representatives.

3.3.3.1. Putative bacterial functional capabilities

Each phase of anaerobic digestion is facilitated by a variety of microorganisms that work in conjunction to breakdown and utilise various compounds, thus covering numerous functional fields (Peng *et al.*, 2018). The bacterial functional diversity of the African elephant was dominated by two groups: the chemoheterotrophs (39 - 45.2 %) and fermenters (38.7 - 45.2 %) (Figure 3.12). Heterotrophs are dependent on the consumption of organic carbon compounds, including sugars and organic acids, for a source of carbon and energy (Parker, 2001; Liang, Sarkany and Cui, 2009). Saprotrophs or decomposers, which deconstruct large molecules from decaying organic material into smaller absorbable units, also fit this category. Fermentation is loosely described as the catabolism of organic compounds without the consumption of oxygen (Madigan *et al.*, 2012). To maintain redox balance during fermentation, a vast number of fermentation products are generated dependent on the original substrate. As such, the representatives within this group of the FAPROTAX database span various fermentative pathways, including butyric acid-, propionic acid-, ethanol- and lactic acid fermentation to name just a few. A full list of the microbes included within this category and cited literature can be found within the database available on the Louca lab website (http://www.loucalab.com).

Hydrolysis and acidogenesis/fermentation are characterised by a diverse range of functional groups, including chemolithotrophic-, chemoorganotrophic-, phototrophic-, proteolytic-, cellulolytic- and denitrifying microorganisms (Toerien and Hattingh, 1969; Nelson, Morrison and Yu, 2011). Chemoorganotrophs or microbes that conserve energy from organic chemicals are by definition heterotrophs (Madigan *et al.*, 2012). Therefore, the dominant bacterial functional groups, chemoheterotrophs and fermenters, are ideally suited to these initial phases of anaerobic digestion. The described functional roles enable the breakdown of complex polymers and subsequent fermentation of the hydrolysed products. These findings support the taxonomic diversity and functional capabilities inferred according to bacterial diversity already discussed.

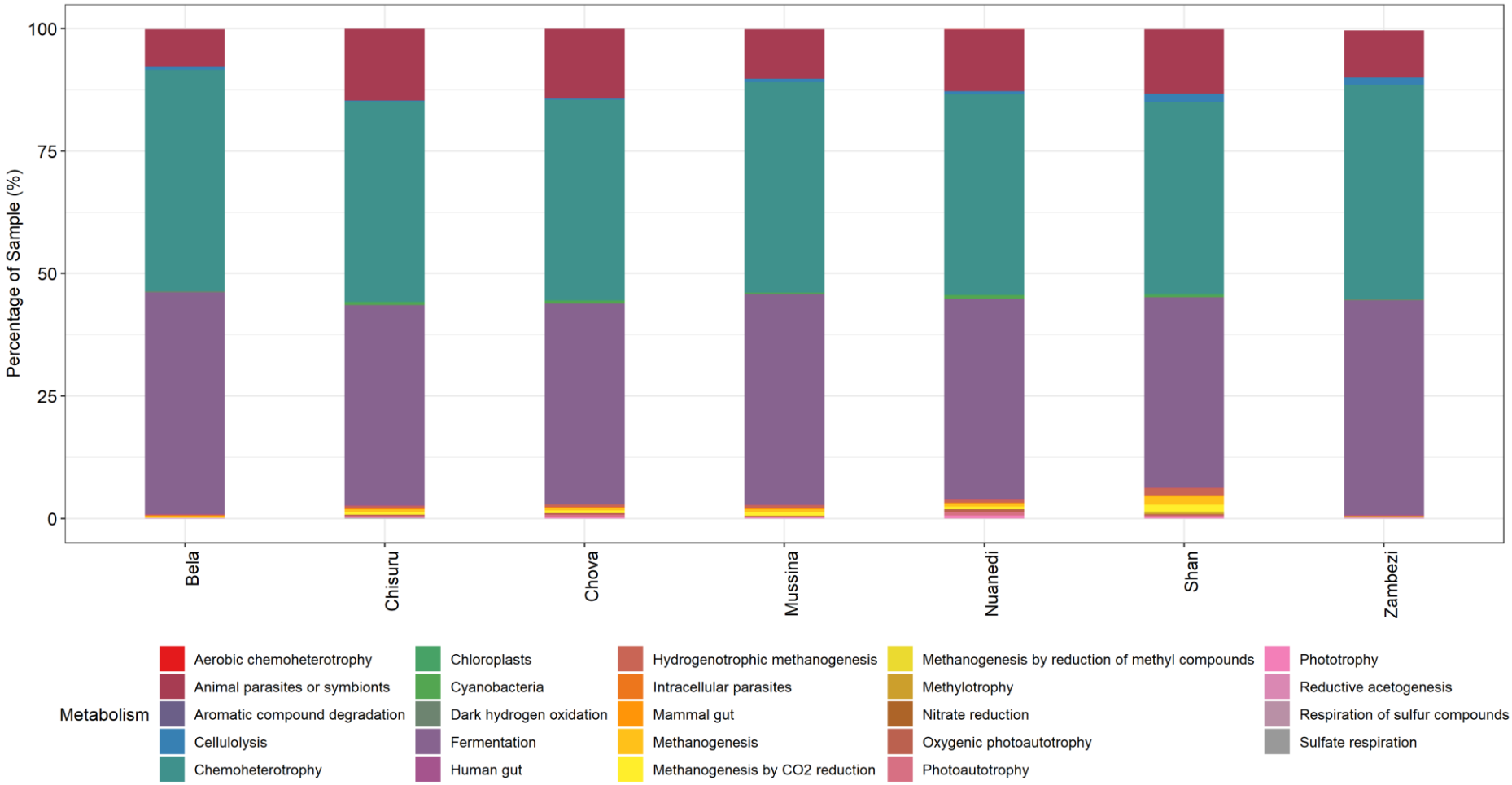

Figure 3.12. Potential functional diversity of bacterial targeted sequences. *The putative functional diversity as determined using the FAPROTAX database. The dominating groups include chemoheterotrophs and fermenters. Functionality amongst members of the sample group indicate a large degree of similarity.*

Minor bacterial functional roles of interest, detected in the African elephant faecal matter, include cellulolysis and reductive acetogenesis. From the bar plot it may seem that only a small group of acetogenic microorganisms (0.1 - 0.2 %) were detected, however this is not necessarily the case. Due to the manner in which the FAPROTAX database has been assembled (descriptions based on literature), some acetogens may also fall into the fermentation category. The FAPROTAX database is non-exhaustive, this means that some organisms known to perform specific functions may be missing or only partially included. In this case, some species are capable of acetogenesis and fermentation, however they may only have been annotated as acetogens, but not as fermenters or *vice versa*, because the second function was only shown in a subsequent publication (Misoph and Drake, 1996; Louca lab).

The last function of interest is cellulolysis, detected at 0.1 - 1.8 %. Although the FAPROTAX database is predominantly for prokaryotic microorganisms, the list of cellulolytic organisms does include examples of both bacteria and some fungi, even if to a much lesser extent. This group is of particular interest for the pre-treatment of cellulosic biomass in the bio-energy industry. A full list of the organisms on the FAPROTAX database and cited literature can be found within the database available on the Louca lab website (http://www.loucalab.com). Yet, similar to the acetogens, microbes capable of cellulolysis are also included within the fermentation category under cellulose fermentation. The fermentation category further includes the fermentation of xylan and pectin, components of hemicellulose. Due to the diversity within this functional category, it encompasses functional roles applicable to multiple phases of anaerobic digestion. These results also support the assumptions drawn from the bacterial diversity. Nonetheless, although the potential to facilitate the first three stages of anaerobic digestion does seem plausible, the deciding factor will remain the production of methane gas. The potential functional capabilities of the archaeal-targeted sequences were therefore also investigated via the FAPROTAX database (Figure 3.13).

3.3.3.2. Putative archaeal functional capabilities

The archaeal functional diversity was dominated by three groups: hydrogenotrophic methanogenesis (24.8 - 28.9 %), methanogenesis (24.8 - 28.9 %) and methanogenesis by CO_2 reduction (21.6 - 27.4 %) (Figure 3.13). These three categories together encompass representatives capable of facilitating all three methanogenesis pathways, with the hydrogenotrophic methanogenesis pathway representing the dominant pathway for methane anabolism within the current dataset.

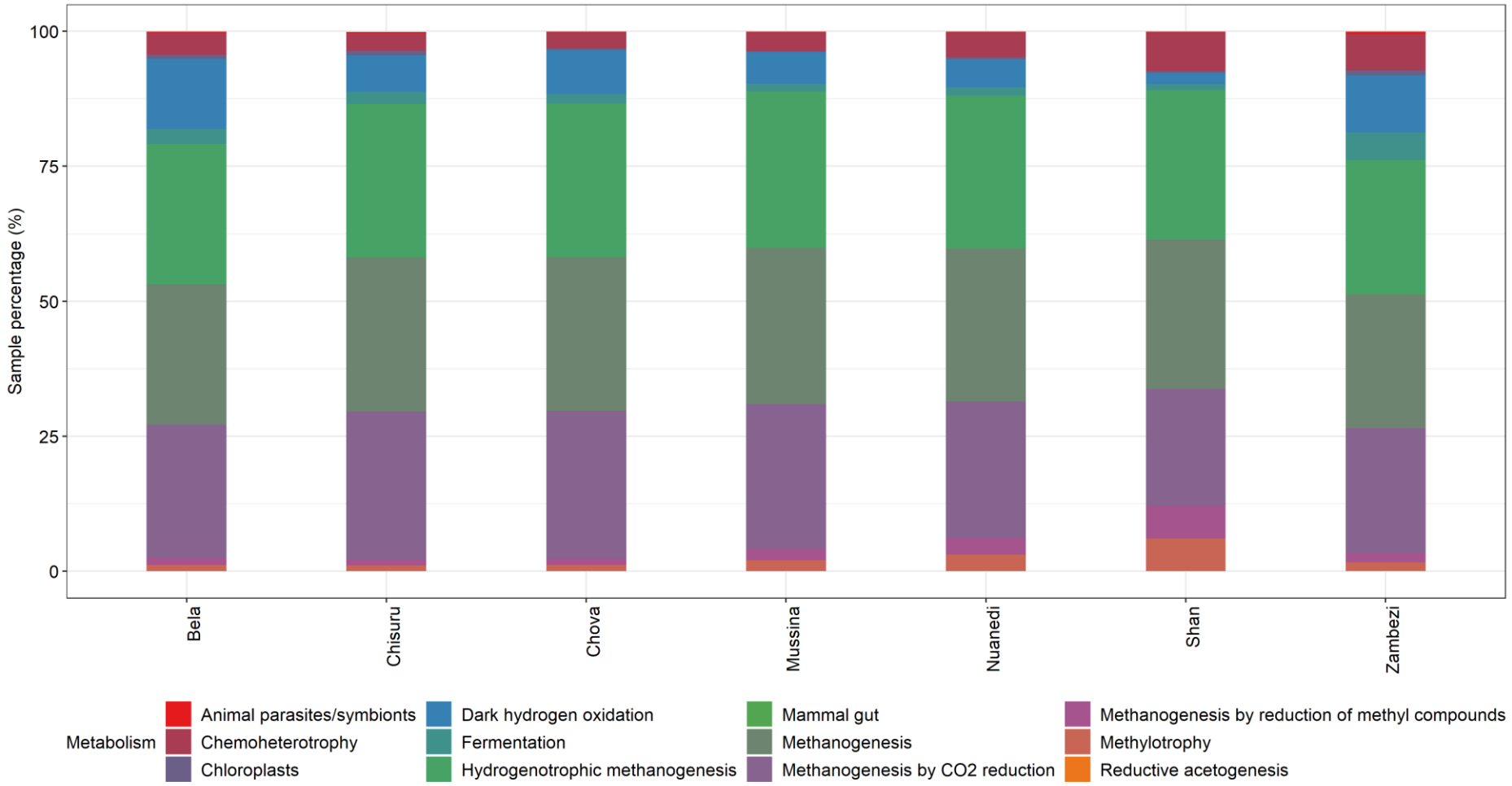

Figure 3.13. Potential functional capabilities of archaeal targeted sequences. *Potential archaeal functional capabilities as inferred from the FAPROTAX database indicates dominance of methanogenic archaea, including multiple pathways of methane generation.*

The categories, hydrogenotrophic methanogenesis and methanogenesis by CO_2 reduction, describe a similar pathway, but consist of different microbial representatives. Some methylotrophic methanogens also employ the central pathway for CO_2 reduction, similar to hydrogenotrophic methanogens and as such were grouped into methanogenesis by CO_2 reduction. The category, methanogenesis, includes representatives of all three methanogenesis pathways. Yet, the database only provides an overall percentage for a category rather than indicating a hit for a specific representative of the category. Considering the taxonomic data, this group likely got hits for methylotrophic- and hydrogenotrophic methanogens, rather than acetoclastic methanogens. Minor functional categories of interest were methanogenesis by reduction of methyl compounds (1.1 - 6.1 %) and methylotrophy (1.1 - 6.07 %). Methanogenesis by reduction of methyl compounds refers to the methylotrophic pathway of methane production. Methyltrophs are not to be confused with methanogens, where methanogens produce methane, methylotrophs consume C_1-compounds, like methane, for growth (Chistoserdova and Kalyuzhnaya, 2018). The abundance of hydrogenotrophic- and methylotrophic methanogens detected via the FAPROTAX database corresponded to the general abundance of genera associated with these functions present in the taxonomic data.

The FAPROTAX database is an extremely versatile and useful tool, however it does come with its own limitations and as such, the results obtained from it are predictions rather than a confirmation of functional capabilities. FAPROTAX extrapolates knowledge from a subset of well-studied and characterised organisms, whether cultures or isolates, to affiliate taxa with functional groups. As such certain assumptions have to be made, one such assumption is that if all the cultured members (at time of publication cited) of a taxon are capable of performing a certain function then all members of that taxon (cultured and non-cultured) should be able to perform said function. As technology advances and more organisms are cultured, some of these assumptions may prove false (Louca lab). Lastly since FAPROTAX was developed to map prokaryotic taxa, the same method could not be employed for the fungal-targeted sequences and as such, the fungal functional capabilities are based upon the genera already discussed.

3.4. Conclusions

Biogas is a versatile renewable energy source that can replace fossil-based fuels on a number of levels, thereby decreasing greenhouse gas emissions, reducing pollution and preventing the depletion of limited fossil fuel deposits. Lignocellulosic biomass presents an abundant renewable source of digestion fuel for the bio-energy industry. However, due to its recalcitrant nature, costly pre-treatment is required to convert lignocellulose to digestible sugars. Biological pre-treatment could offer the solution, although optimisation is required to improve the biogas yield from these lignocellulosic materials.

The gut microbiome refers to the totality of microorganisms residing within the gastrointestinal tract, including, Viruses, Archaea, Bacteria, Fungi and Protozoa. This study focused on three groups: Archaea, Bacteria and Fungi. Bacterial communities were dominated by the phyla, Firmicutes, Bacteroidetes and Planctomycetes. All three these phyla contain species capable of facilitating anaerobic digestion. The archaeal diversity was dominated by the Euryarchaeota, the phylum to which all methanogenic microorganisms are confined. Furthermore, multiple known methanogenic genera including *Methanobrevibacter* and *Methanocorpusculum* were detected within the African elephant faecal matter. Fungi are of particular interest for their lignocellulolytic capabilities. Lignin degrading fungi were not observed, but a large proportion of potentially cellulolytic fungi were identified within the sample matter. Most of these cellulolytic fungi are also capable of degrading hemicellulose, specifically the component xylan. Reduction of this polysaccharide greatly increases cellulase accessibility to cellulose, which in turn affects the efficiency of cellulose utilisation and subsequent hydrolysis rate.

The FAPROTAX database served as a means to predict the potential functional capabilities of the microbial community. The functional groups identified correspond to the different stages of the anaerobic digestion process, including multiple methanogenesis pathways. In terms of lignocellulosic degradation, the diversity was more indicative of a community able to degrade cellulose and hemicellulose, but likely not lignin. However, this will be impossible to state definitively without further experimentation and verification. The current absence of a certain group cannot be used to definitely state that that group is missing within the African elephant gastrointestinal microbiome. Various factors including, human error, quality control parameters and test group size could contribute to the exclusion of certain groups. Furthermore, the test group utilised for this study consists of only seven mammals kept in captivity and nutritionally supplemented. They may offer insight into the African elephant gastrointestinal microbiome, but may also not be representative of all African elephants.

Finally, an important note to remember is that the approach followed here used DNA, whether that DNA is from a live or dead cell. Therefore, the presence of a certain group of microorganisms does not mean that that group plays an active role within the community. In spite of these limitations, the currently observed microbial diversity does suggest that the African elephant gastrointestinal community is capable of facilitating the anaerobic digestion of plant biomass, even though lignin degradation seems unlikely considering the current information available. Yet, the efficiency of these microbial communities to improve biogas production remains to be determined.

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CHAPTER 4

RECONSTRUCTION OF BIOGAS METABOLIC PATHWAYS FROM THE AFRICAN ELEPHANT (*Loxodonta africana*) GUT MICROBIOME

4. Introduction

The world was built on the presumption of plentiful and inexpensive natural resources (Hassan, Williams and Jaiswal, 2018). However, the overutilisation of fossil fuel stores and environmental concerns surrounding the use of such energy sources has fuelled the shift towards renewable energy sources (Dogaris, Mamma and Kekos, 2013; Hassan, Williams and Jaiswal, 2018; Zhang *et al.*, 2019). This shift towards a bio-economy encourages the reuse and recovery of resources, rather than prolonged use of natural non-renewable sources (Förster and Gescher, 2014). The production of second generation biofuels is of particular interest since, contrary to first generation biofuels, this technology uses raw materials not suitable for the production of food products (Dogaris, Mamma and Kekos, 2013). The anaerobic digestion of organic material not only facilitates the production of a renewable green energy source (methane-gas), but also enables the reuse of recovered biomass (Zheng *et al.*, 2014; Divya, Gopinath and Merlin Christy, 2015). Nearly any organic biomass is suitable for biogas production, including reclaimed organic waste like animal manure, crop residues and municipal waste (Amon *et al.*, 2007; Momayez, Karimi and Taherzadeh, 2019). Anaerobic digestion involves an intricate community of microorganisms that yield biogas in four distinct steps: hydrolysis, acidogenesis, acetogenesis and methanogenesis (Weiland, 2010; Zheng *et al.*, 2014). The process necessitates a microbial population with specific functional capabilities to facilitate each of these steps.

As our understanding of anaerobic digestion increases, so does the interest in alternative waste streams such as lignocellulosic biomass, which consists of cellulose, hemicellulose and lignin (Brautaset and Ellingsen, 2011). Lignocellulose is a by-product, continually produced in large quantities, by numerous industrial sectors and as such is a promising source of inexpensive, easily accessible organic biomass (Lange, 2007). A process like anaerobic digestion is necessary to convert the carbon-rich lignocellulose to usable energy (Jingura and Kamusoko, 2017). However, the components of lignocellulose yield a recalcitrant matrix, resistant to digestion (Mussatto and Teixeira, 2010). Pre-treatment offers a solution to this issue, however pre-treatment methods are often not feasible on an industrial scale due to cost and time constraints (Agbor *et al.*, 2011). Hindgut fermenters, like elephants, have adapted to a diet rich in complex plant carbohydrates, like cellulose, by lengthening the gut retention time to better accommodate bacterial fermentation (Ley *et al.*, 2008). At its core, anaerobic digestion of organic biomass is a series of fermentation reactions by a complex microbial community of Bacteria, Archaea and Eukaryota (Agler *et al.*, 2011). As such, the African elephant could potentially host a microbial community capable of methane production and lignocellulose digestion.

Lignocellulose treatment with a community of microorganisms capable of lignocellulose utilisation and biogas production could circumvent some of the aforementioned pitfalls by combining pre-treatment and energy conversion.

Shotgun sequencing, the methodology used in this study, involves the random fragmentation of large DNA segments into numerous smaller pieces (Dunham, 2005). These smaller fragments are randomly sequenced and the reads (sequences) reassembled to enable the reconstruction of the original non-fragmented DNA sequence. This method yields extremely large datasets that are difficult to assemble and annotate manually. Therefore, an open-source metagenomics analysis server (MG-RAST), which uses peer-reviewed, curated databases to infer the phylogenetic and functional properties of a metagenome was employed during this study (Meyer *et al.*, 2008). *Chapter 3* illustrated that the microbial communities within the African elephant gut microbiome potentially support anaerobic digestion. This was mainly inferred using taxonomic information and the functional annotation of prokaryotic taxa (FAPROTAX) tool (Louca, Parfrey and Doebeli, 2016). Yet, these predictions are limited by cultured representatives. Therefore, this chapter aims to determine, using culture-independent metagenomics, whether or not the gut microbiome possesses the functional capability to facilitate anaerobic digestion.

Chapter aims

- To identify and reconstruct the key metabolic pathways involved during the anaerobic digestion of organic biomass.
- To identify key genes products/enzymes essential to anaerobic digestion and lignocellulose digestion.
- To evaluate the potential of the elephant gastrointestinal microbiome to facilitate anaerobic digestion of organic material and lignocellulose digestion.

4.1. Materials and Methods

4.1.1. Sample collection and preservation

Sample collection and preservation was performed as previously described (*Chapter 3*, *Section 3.2.2*).

4.1.2. Genomic DNA extraction and sample preparation

Genomic DNA was extracted and visualised according to the methods described in *Chapter 3* (*Section 3.2.3*). The DNA concentration and purity were confirmed on the NanoDrop One^{C} spectrophotometer (Thermo Scientific, USA). Genomic DNA from each sample was diluted to 100 ng/µL, after which an equal volume of each sample was combined into a single sample containing roughly 1 200 ng DNA (± 21 ng/µL). This composite sample was shipped to Mr. DNA (Texas, USA) for shotgun metagenomic sequencing on an Illumina NovaSeq 6 000 (2 x 150 base pairs (bp), 30 million paired-end reads).

4.1.3. Data processing

The joined pair-end reads, along with the required metadata, were submitted to Metagenomic Rapid Annotations using Subsystems Technology v4.0.3 (MG-RAST) (Meyer *et al.*, 2008). Figure 4.1 outlines the automated pipeline for quality control and annotation offered by MG-RAST, as discussed in the sections to follow.

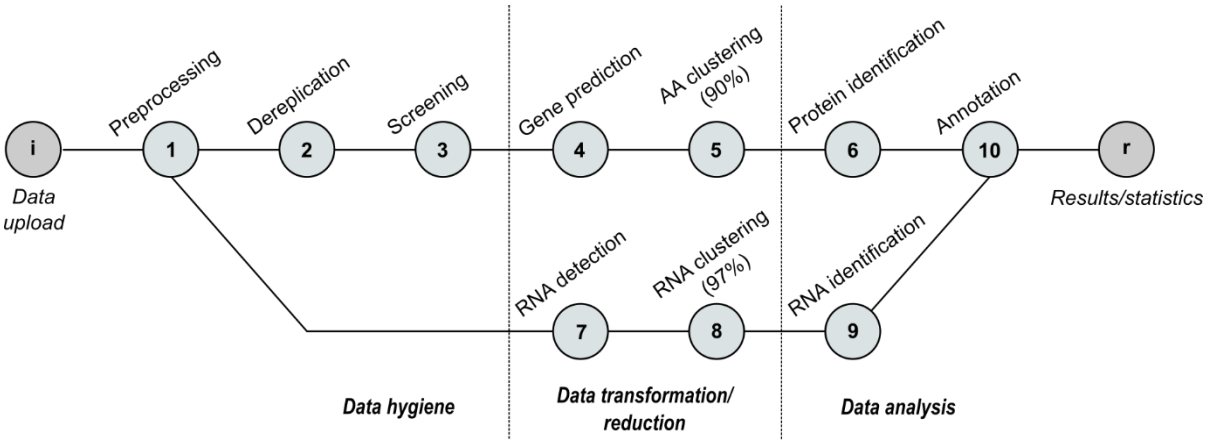


Figure 4.1. Outline of MG-RAST data processing pipeline. *Data processing is divided into three phases: data hygiene or quality evaluation, data transformation or reduction and finally analysis, which yields an annotated dataset set from which phylogeny and functionality is inferred. Image adapted from Tang et al., 2013 and Wilke et al., 2015.*

4.1.3.1. Sequence quality control

Sequencing adapters were detected and removed using Skewer (Jiang *et al.*, 2014), after which the reads were trimmed according to sequence length and quality parameters. Sequences with < 100 bp and/or < Q15 were removed. DRISEE was used to identify and remove sequencing artifacts before moving on to the next stage of the analysis pipeline (Keegan *et al.*, 2012). Lastly, reads were screened using Bowtie 2 (Langmead and Salzberg, 2012) to remove any potential host DNA contamination (set to default: H. sapiens. NCBI v36).

4.1.3.2. Feature extraction and annotation

Ribosomal ribonucleic acid (rRNA) feature identification (rRNA genecalling) was performed using SortMeRNA (Kopylova, Noé and Touzet, 2012). Genes were assigned based on sequence similarity (70 % identity) to ribosomal sequences from a reduced version of the custom M5RNA database (combining RDP, SILVA and GreenGenes) (DeSantis *et al.*, 2006; Quast *et al.*, 2012; Cole *et al.*, 2014). Sequence clustering was performed on a 97 % identity similarity. This yields clusters with at least 70 % identity to ribosomal sequences and 97 % sequence similarity. Putative protein coding regions within the sequences were predicted using FragGeneScan (prokaryotic gene calling algorithm), followed by the clustering of predicted proteins at 90 % identity (Rho, Tang and Ye, 2010). BLAT was used to calculate similarities for representatives from each cluster (Kent, 2002). The remaining pipeline is devoted to annotating and indexing the protein and RNA similarities and abundance, which is used to compile numerous statistics regarding the overall data. Further phylogenetic and functional analysis was performed using available databases on MG-RAST as described below.

4.1.3.3. Taxonomic and functional annotation

Sequence taxonomy was inferred using the Ribosomal Database Project (RDP) and the National Center for Biotechnology Information (NCBI) reference sequence database (RefSeq) (Cole *et al.*, 2014; Tatusova *et al.*, 2014). Functionality was inferred based on the KEGG ORTHOLOGY (KO) database and SEED subsystems (Kanehisa, 1997; Overbeek, 2005).

4.1.3.4. Metabolic pathway reconstruction

Biochemical pathways were reconstructed that illustrate the production of key intermediates, during hydrolysis, acidogenesis, acetogenesis and methanogenesis as determined by Cai and co-workers (2016). Pathways containing reaction steps and enzymes to produce the aforementioned intermediates were reconstructed with reference to the KEGG, SEED and BRENDA databases (Jeske *et al.*, 2019).

4.2. Results and Discussion

Targeted sequencing (16S rRNA) (*Chapter 3*, *Section 3.3.2*) indicated that prokaryotic diversity across animals appeared to be very similar. Furthermore, no significant correlation (supplementary Table C1) between microbial composition and age, gender or maturity of the individual elephants were found. Therefore, gDNA from all seven elephants was combined into a single sample to obtain a metagenomic overview in order to assess the functional potential present in the entire community, rather than properties of a single individual. However, the presented data may not be representative of all African elephants as the sample group is fairly small (7), in constant contact with humans and nutritionally supplemented.

The elephant test group regularly interacts with tourists and undergoes obedience training, up to 4 hours a day, depending on the number of tourist interactions. During training and/or interactions, the elephants are rewarded with 12 % grazer game cubes (Reg. no. V19237 Act 36/1947, EPOL Feeds, RSA). The remainder of the day, the elephants graze freely on natural vegetation (savannah, *Combretum* woodlands and *Acacia* thickets). Their diet is also supplemented with lucerne, oats hay and bana grass (*Pennisetum americanum* and *P. purpureum* hybrid). Genomic DNA was extracted from the same samples throughout the study to limit microbiome fluctuations that may be introduced due to the seasonal availability of food and subsequent modifications to diet composition (Nishida and Ochman, 2018).

4.2.1. Dataset statistical overview

The original dataset consisted of 75 928 131 paired-end sequences with a mean sequence length of 163 ± 39 bp. Following completion of the quality control (QC) pipeline, the dataset contained 57 890 798 paired-end sequences with an average length of 166 ± 40 bp. As indicated by Figure 4.2, of the sequences obtained, 23.77 % failed QC (mainly replicated sequences), 73.06 % (55 477 679 sequences) have a predicted feature and 3.18 % (2 413 119 sequences) remain unknown.

Of the sequences with predicted features, 56.85 % were described as proteins with unknown functions, 42.58 % were annotated proteins with known functions and 0.57 % rRNA (Figure 4.2). Matching reads/sequences to a collection/database of gene sequences is a commonly enlisted high-throughput method of identifying coding regions within metagenomic data (Mande, Mohammed and Ghosh, 2012; Sharpton, 2014). Nonetheless, this method may create the illusion of novel gene sequences due to the underrepresentation of certain genomes within the chosen database (Sharpton, 2014).

A large fraction of unknown proteins, as seen in this dataset, may represent unique gene sequences or may just be the result of under representation of similar communities within the chosen database. As databases expand and grow, annotation improves, allowing greater distinction between unknown and known proteins.

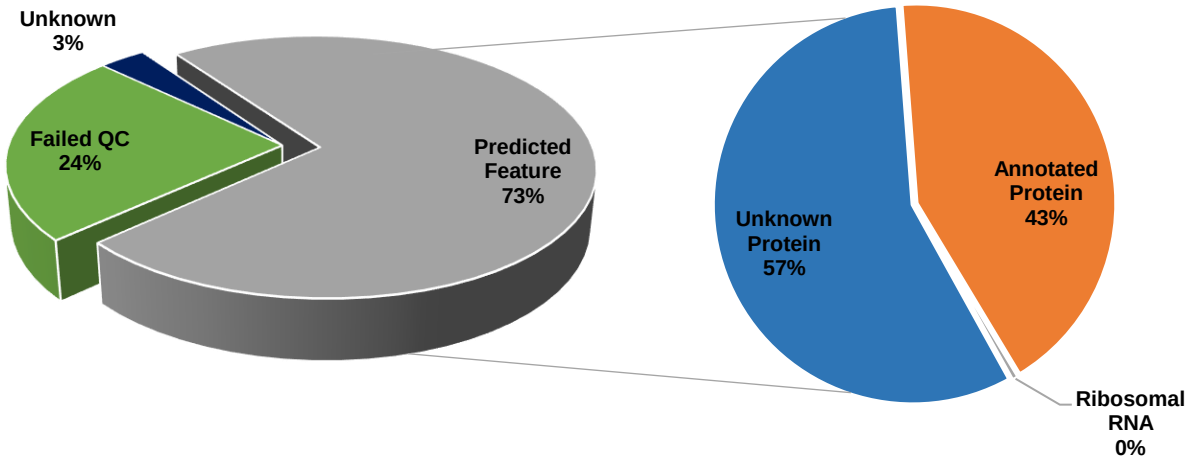


Figure 4.2. Pie chart illustrating data quality and feature prediction. *The original metagenomic dataset comprised over 75 million sequences, of which 23.77 % failed the initial quality control measures and 73.06 % represent predicted protein features. From the predicted protein features, coding regions were identified according to a reference based method against the M5RNA database (SILVA, GreenGenes, RDP) as per the MG-RAST pipeline. Original images generated via MG-RAST pipeline available in supplementary material, Figure C1 and C2.*

4.2.1.1. Sequencing adequacy

Rarefaction curves visually display sequencing effectiveness by plotting the number of reads against the number of species/operational taxonomic units (OTUs) (Sun *et al.*, 2015; Zhang *et al.*, 2019). The rarefaction curve for this dataset (Supplementary Figure C3) approaches a saturation plateau, indicating that a reasonable number of individuals were sampled and that more intensive sampling would likely only yield a few additional species.

4.2.2. Taxonomic composition

The following data represents the shotgun metagenome derived taxonomic composition found in the faeces of the African elephant as per annotation against the M5NR database (Meyer *et al.*, 2008). Sequences with a 97 % identity similarity were clustered together, as this is considered the same species on an rRNA level (Stackebrandt and Goebel, 1994). The three major microbial Kingdoms were Bacteria at 97.81 %, followed by the Eukaryota and Archaea at just over and under 1 % respectively (Figure 4.3). The RDP database was able to assign 574 (0.2 %) sequences up to a taxonomic rank of genus. The RDP is a curated database consisting of aligned and annotated small subunit rRNA gene sequences for Bacteria and Archaea, as well as fungal large subunit rRNA sequences (Cole *et al.*, 2014). Whereas with RefSeq, 29 423 294 (51 %) sequences were assigned to the taxonomic rank, genus. The RefSeq initiative is responsible for the maintenance and curation of a publicly available database containing curated genomic transcript- and protein sequence records. The initiative ensures that data submitted to the International Sequence Database Collaboration (INSDC) is pitted against computational and manual curation to yield a set of stable, non-redundant reference sequences (Tatusova *et al.*, 2014). Treu *et al.* (2016) noted during previous metagenomic analysis of the biogas microbiome that the vast majority of sequences were only assigned to high taxonomic levels. A possible indication that the biogas microbiome consists largely of uncharacterised species (Treu *et al.*, 2016).

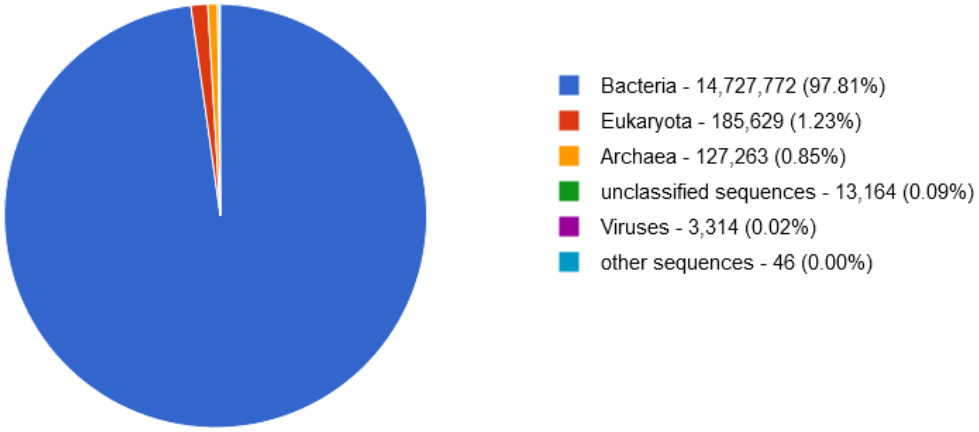


Figure 4.3. Community domain diversity distribution. *The overall taxonomic distribution of the dataset as determined using a contigLCA algorithm as per the MG-RAST pipeline. This algorithm assigns a single consensus taxonomic entity for all the features associated with every individual sequence.*

The two major bacterial phyla detected were Bacteroidetes (50.5 %) and Firmicutes (36 %) (Figure 4.4, a). Proteobacteria (3 %), Actinobacteria (2 %), Fibrobacteres (1.5 %) and Lentisphaerae (1 %) occupy the remaining diversity (Figure 4.4). Multiple studies of the biogas microbiome suggest that members of the Bacteroidetes, Firmicutes and Proteobacteria are intricately involved during the process of biogas production (Klocke *et al.*, 2007; Zverlov *et al.*, 2010; Campanaro *et al.*, 2016; Maus *et al.*, 2016). However, the abundance of some of these groups may vary from one study to another. These variations are attributed to the difference in composition of substrates treated within each digester (Kampmann *et al.*, 2012; De Francisci *et al.*, 2015).

Dominant orders (Figure 4.4, b) include the Bacteroidales of the phylum Bacteroidetes at 51.5 % and the Clostridiales of the phylum Firmicutes at 28.6 %. Members of the order Bacteroidales specialise in polysaccharide (e.g. cellulose, starch) utilisation (Comstock, 2009; De Francisci *et al.*, 2015). The two major bacterial genera (Figure 4.4, c) were the *Prevotella* (29 %) and *Bacteroides* (19 %), from the order, Bacteroidales. Another prominent bacterial genus was *Clostridium* (5 %), from the phylum, Firmicutes. Some *Clostridium* species are highly effective at degrading crystalline (natural) cellulose, associated with plant biomass (Zverlov *et al.*, 2010). These species may also degrade hemicellulose and are commonly isolated from anaerobic digesters (Zverlov *et al.*, 2010; Kampmann *et al.*, 2012; Pore *et al.*, 2015).

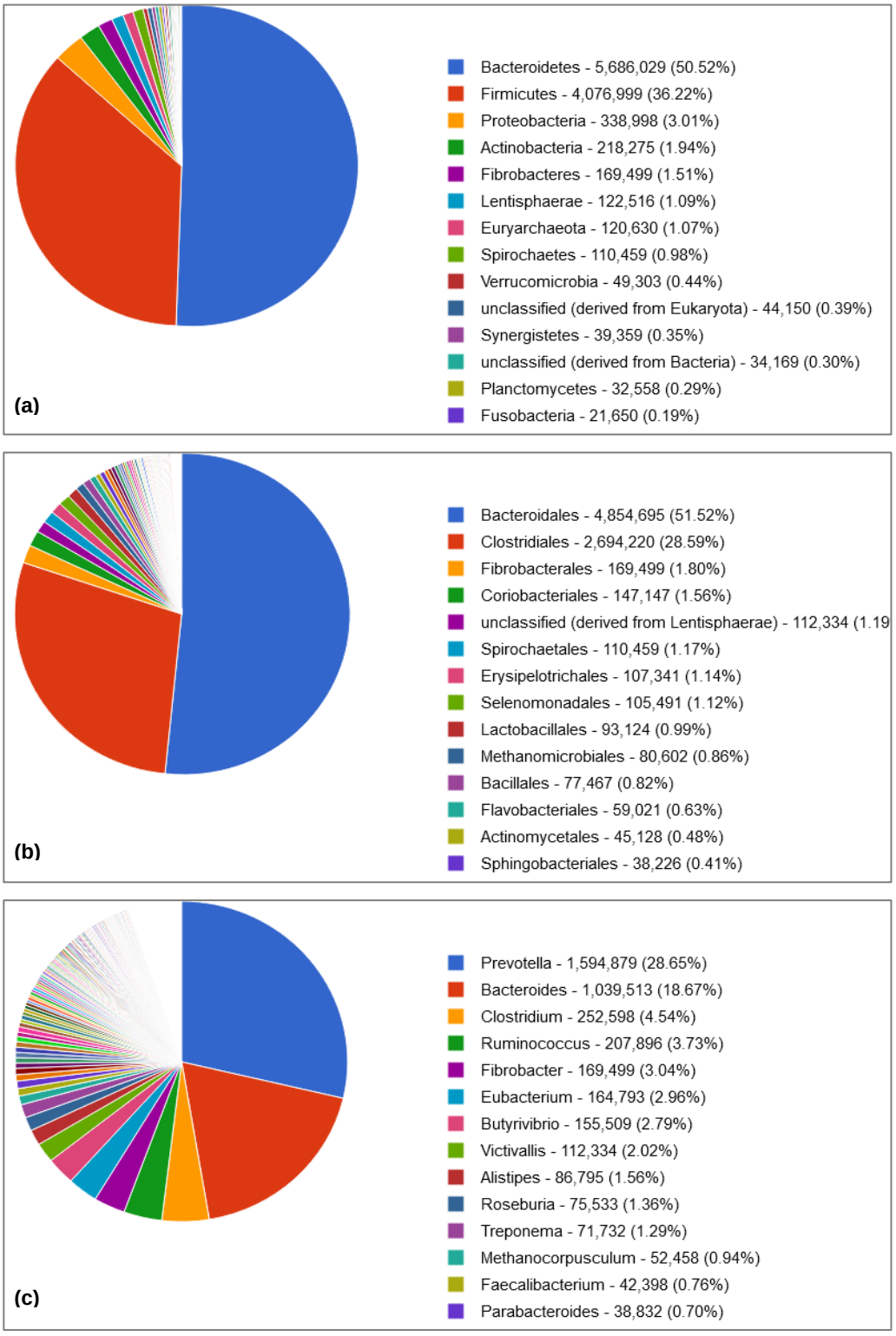


Figure 4.4. Community taxonomic assignment. (a) *Phylum-level taxonomic distribution of dataset as determined using a contigLCA algorithm. This algorithm assigns a single consensus taxonomic entity for all the features associated with every individual sequence.* (b) *Order level taxonomic distribution of dataset as per contigLCA algorithm.* (c) *Genus level taxonomic distribution of dataset as per contigLCA algorithm. Images generated via MG-RAST pipeline.*

All five major archaeal phyla were represented within the dataset, however, combined Nanoarchaeota, Korarchaeota, Thaumarchaeota and Crenarchaeota represent < 1 % (2 000 reads) of the archaeal phyla composition. The phylum, Euryarchaeota, which includes all currently known methanogens, represents 1.07 % (120 630 reads) of the archaeal phyla (Figure 4.4, a) (Cai *et al.*, 2016). The dominant archaeal order is the methanogenic Methanomicrobiales (0.86 %) (Figure 4.4, b). Members of this order are largely associated with hydrogenotrophic methanogenesis (reduction of carbon dioxide (CO_2) in the presence of hydrogen (H_2)) (Pyzik *et al.*, 2018). The only dominant archaeal genus (Figure 4.4, c) was *Methanocorpusculum* (0.94 %). However, when focusing on the genera classification according to the RefSeq database (Figure 4.5), other well-known methanogenic genera were present. These genera include *Methanobrevibacter*, *Methanococcus*, *Methanosarcina* and *Methanosphaera*. Members of these genera commonly utilise the hydrogenotrophic pathway of methane production, however some are also known methyltrophic methanogens (Cai *et al.*, 2016). *Methanosarcina* is currently considered the only methanogen capable of utilising all three methanogenesis pathways (Pyzik *et al.*, 2018).

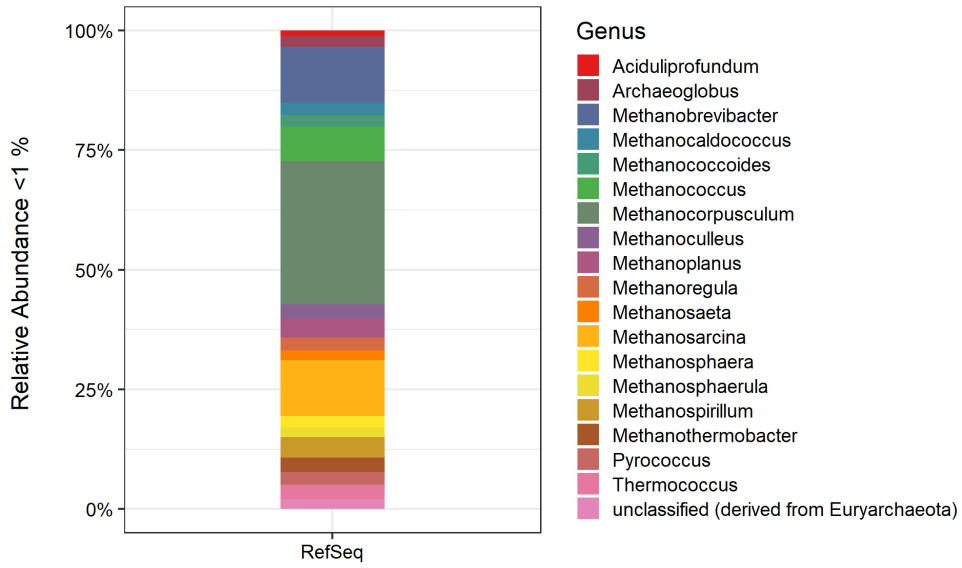
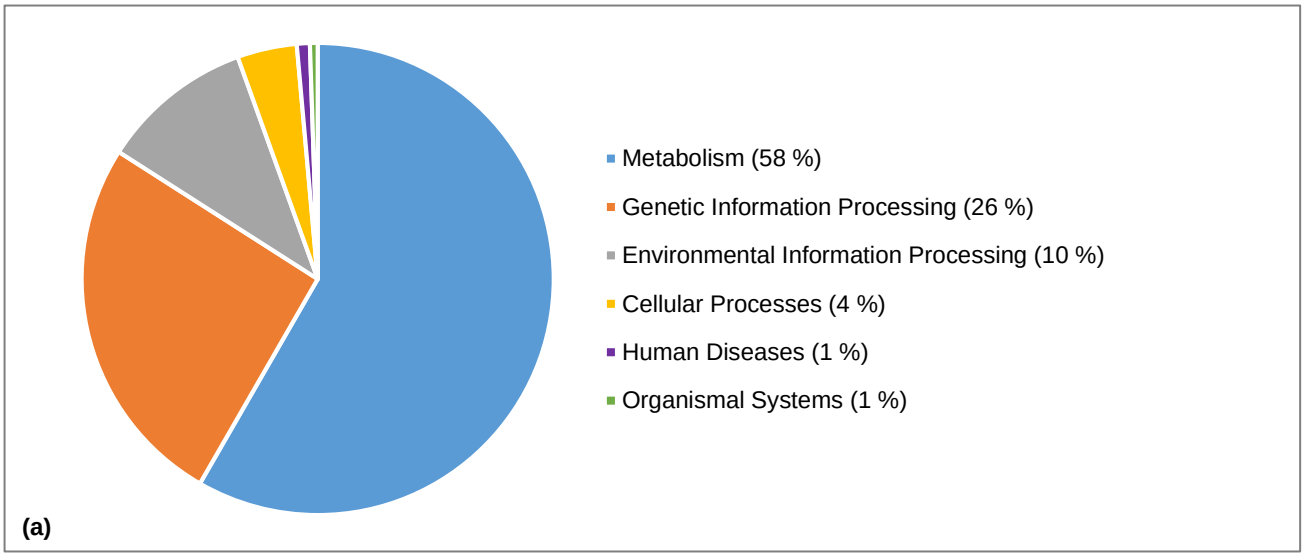


Figure 4.5. RefSeq Archaeal genera assignment. *Archaeal genera with an abundance > 1 % as assigned based on the RefSeq database. Data obtained from MG-RAST, image generated via Rstudio v1.1.463 (R Core Team, 2017; RStudio Team, 2020).*

4.2.3. Functional composition

Reads were annotated via a search against the KO and SEED subsystems databases to yield a metabolic function profile for the metagenomic data. KEGG is used to predict protein interaction networks and associated cellular functions by matching genes in the genome to gene products in KEGG pathways (Kanehisa, 2002). Genes encoding functions from metabolism-related KO categories were dominant, representing over 50 % of the reads (Figure 4.6, a). Within this KO category, prevalent categories were carbohydrate metabolism, amino acid metabolism, nucleotide metabolism, lipid metabolism, energy metabolism and biosynthesis of secondary metabolites. A similar distribution of genes to these metabolic categories have been noted in other metagenomic studies of anaerobic digesters and may indicate a metabolism suited to anaerobic digestion (Li *et al.*, 2013; Zhang *et al.*, 2017).

A subsystem is a set of functional roles (protein families) that together encompass a specific biological process (Overbeek, 2005). The SEED subsystems database houses protein families from complete genomes arranged into five hierarchy levels. This hierarchy contains homologous genes (share the same function), used to infer gene function and metabolic pathways from metagenomic data (Overbeek, 2005; Overbeek *et al.*, 2014). Dominant functional SEED subsystem categories (Figure 4.6, b) of level 1 (broadest level) were carbohydrate metabolism (16 %), clustering-based subsystems (15 %), protein metabolism (10 %) and amino acids and derivatives (9 %). Prevalent subsystems within the carbohydrate metabolism category were central carbohydrate metabolism, saccharide metabolism, one-carbon metabolism, fermentation, CO_2 fixation and sugar alcohol metabolism. Similar to the KO categories, these dominant subsystems were noted in other anaerobic digesters and mainly involve the digestion of organic matter (Cai *et al.*, 2016).



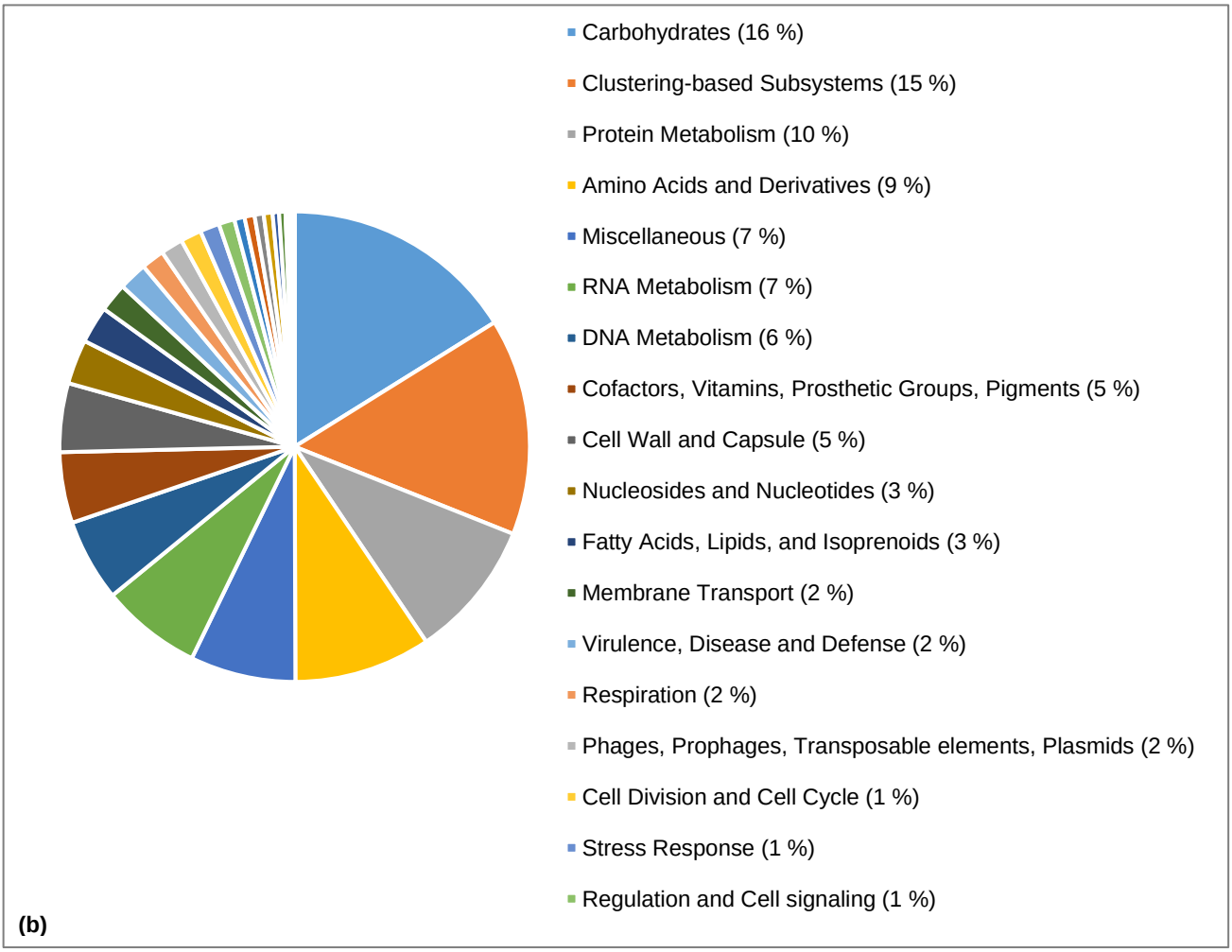


Figure 4.6. Distribution of functional categories at the highest level supported. (a) *Percentage (wedge) of reads with predicted protein functions annotated to a category set out by the KEGG ORTHOLOGY database.* (b) *Predicted protein functions annotated to a particular category as set out by the SEED subsystems database. Categories with a percentage <1 % are not displayed in the legend. Image adapted from MG-RAST pipeline. See supplementary Figure C4 and C5 for original images generated via MG-RAST pipeline.*

4.2.4. Reconstruction of functional pathways

Figure 4.7 illustrates the major steps of anaerobic digestion and the key intermediates/products (a-h) produced during these steps. The genera listed beside each intermediate represent the top four genera within the dataset capable of facilitating the final reaction toward the production of each intermediate.

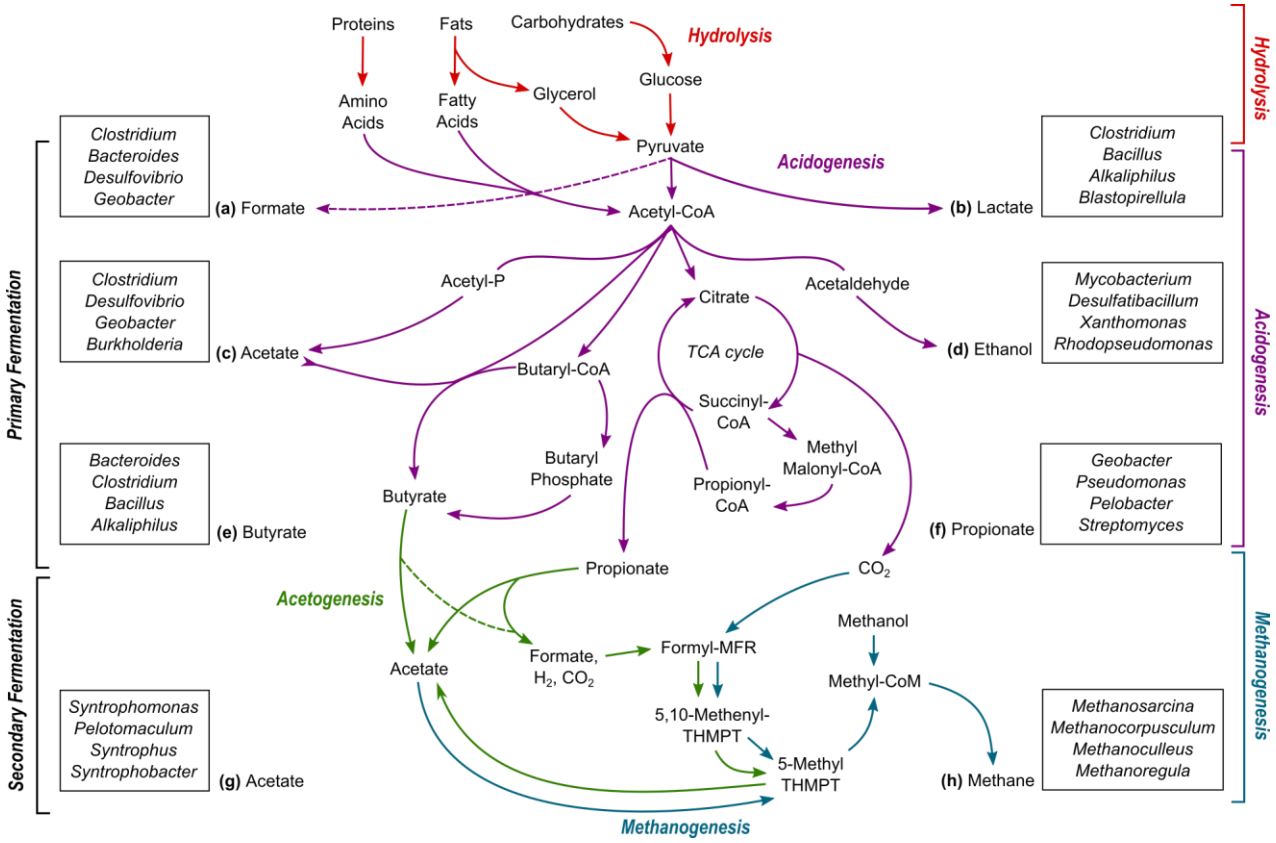


Figure 4.7. Summary of the major steps of anaerobic digestion. *Anaerobic digestion consists of four main phases: hydrolysis, acidogenesis, acetogenesis and methanogenesis, producing eight key intermediates/products (a-h), essential to the process. Genera listed beside key intermediates represent the top four genera capable of facilitating the reactions that produce each intermediate. Adapted from Cai et al. 2016.*

Metabolic pathways are not closed systems, substances and intermediates interact, moving from one pathway to the next. Just as multiple reactions may yield a single compound, multiple biological pathways produce similar compounds. Generalist microorganisms (e.g., *Bacillus subtilis*) rely on multiple alternative pathways to improve substrate utilisation and improve resilience to gene loss (Mahadevan and Lovley, 2008). In the same manner, numerous metabolic pathways could yield the eight key intermediates (Figure 4.7), essential to the progression of anaerobic digestion.

As anaerobic digestion progresses, the metabolic processes involved become more specialised, continually requiring more specialised microorganisms (Campanaro *et al.*, 2016). This general trend of increased specialisation resembles that of a funnel, ending in the highly specialised methanogens, a select group of Archaea. The complex microbial community facilitating biogas production is divided into three main functional groups, primary fermenters, anaerobic oxidising bacteria and methanogenic archaea (Toerien and Hattingh, 1969). Initially, the primary fermenters hydrolyse complex polymers (e.g., lipids and proteins) to their constituent monomers (e.g., amino acids) (Kampmann *et al.*, 2012; De Francisci *et al.*, 2015). The monomers produced via these pathways are converted to alcohols, short-chain fatty acids, organic acids, H_2 and CO_2. Bacteria oxidise these reduced products to acetate, H_2, formate and CO_2. Lastly, highly specialised acetoclastic methanogens convert acetate to methane, whereas the hydrogenotrophic methanogens reduce CO_2 to methane using H_2.

To illustrate the progression of anaerobic digestion and the production of the eight key intermediates select metabolic pathways were reconstructed centered on carbon metabolism, fermentation and methanogenesis. The reconstructed pathways were based on the MG-RAST KEGG pathway plugin and existing KEGG pathway reference maps available at GenomeNet (https://www.genome.jp). The current absence of particular gene products/enzymes does not necessarily exclude these enzymes from the microbial community since numerous factors may influence the observed results including data quality and annotation.

4.2.4.1. Hydrolysis

Complex polymers like carbohydrates, proteins and lipids are hydrolysed (Meegoda *et al.*, 2018) via well-known metabolic pathways including glycolysis (hsa00010) and the citrate cycle (TCA cycle) (hsa00020). Enzymes crucial to glycolysis present in the metagenome were phosphotransferases (EC 2.7.1.-, EC 2.7.1.11), isomerases (EC 5.4.2.-, EC 5.3.1.9), aldolase (EC 4.1.2.13), oxidoreductases (EC 1.2.1-), phosphoglycerate kinase (EC 2.7.2.3) and pyruvate kinase (EC 2.7.1.40) (see Supplementary Figure C6 for full pathway map) (Kanehisa, 1997; Overbeek *et al.*, 2014). A complete gene set for the citrate cycle was found including select transferases (EC 2.3.3.-, EC 2.3.1.61), lyases (EC 4.2.1.-), isocitrate (NADP) dehydrogenase (EC 1.1.1.42), oxoglutarate dehydrogenase (EC 1.2.4.2), acetyl-CoA ligase (EC 6.2.1.-), succinate dehydrogenase (EC 1.3.5.1) and malate dehydrogenase (EC 1.1.1.37) (see Supplementary Figure C7 for full pathway map). These processes produce intermediates such as pyruvate, acetyl-CoA and oxaloacetate, which enter subsequent phases of anaerobic digestion (Agler *et al.*, 2011).

Due to the non-specialised nature of these metabolic pathways, various microorganisms were identified within the metagenome that participate in carbohydrate-, protein- and lipid

metabolism. The three major bacterial phyla were Firmicutes, Bacteroidetes and Proteobacteria (Figure 4.8). Genera from the phylum Bacteroidetes generally include metabolically flexible hydrolysing and fermenting microorganisms capable of degrading complex organic macromolecules (Zhang *et al.*, 2017). Members of the Firmicutes (e.g., *Clostridium*) play a crucial role throughout various phases of anaerobic digestion as members of this phylum facilitate acetogenesis and produce typical fermentation products like volatile fatty acids (VFAs) and alcohols (Krakat, Schmidt and Scherer, 2011). The Clostridiales (Firmicutes) are closely linked to the degradation of proteins and cellulose, whereas Bacilli, Gammaproteobacteria and Bacteroidetes are linked to the digestion of lipids and carbohydrates (Li *et al.*, 2013). Prevalent genera from these groups included *Clostridium*, *Bacillus*, *Eubacterium*, *Bacteroides*, *Prevotella*, *Escherichia* and *Geobacter*.

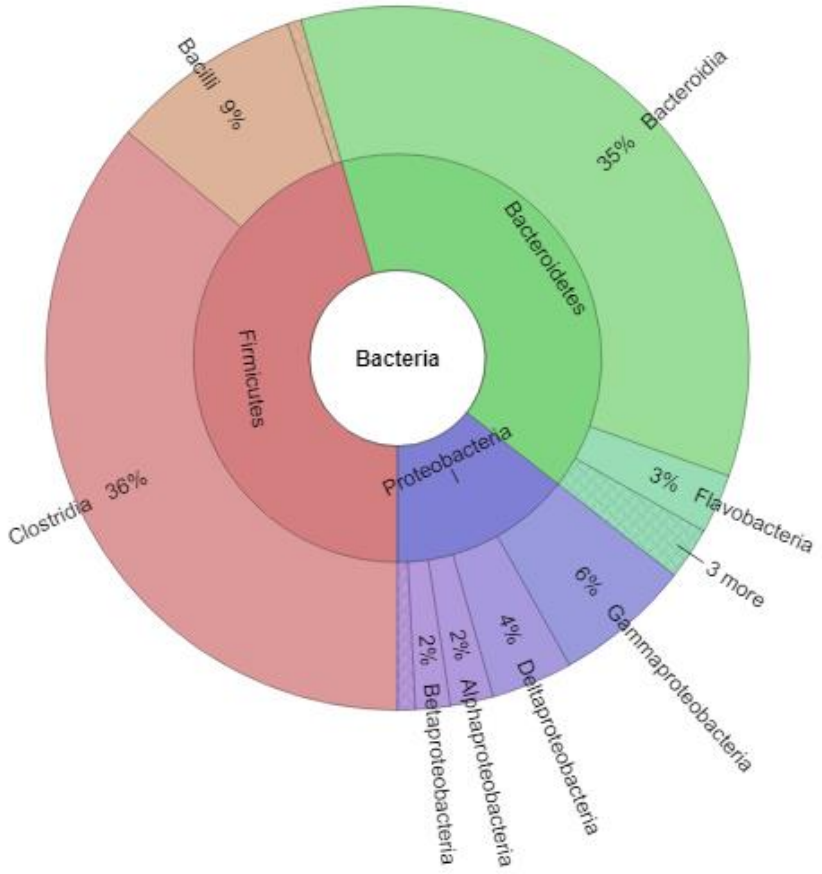


Figure 4.8. Major bacterial phyla responsible for hydrolysis. *The inner ring corresponds to the three major bacterial phyla, with the outer ring indicating the prevalent bacterial classes within each phylum.*

4.2.4.2. Acidogenesis: Primary fermentation

In the absence of oxygen or an alternative electron acceptor, microbes metabolise carbon sources by fermentation, producing energy from the partial oxidation of glucose and other carbon sources (Ciani, Comitini and Mannazzu, 2008; Ward, 2015). The fermentation pathways of acidogenesis yield a number of products, including organic acids (e.g., acetate, butyrate, propionate, lactate) and alcohols (e.g., butanol, ethanol) (Wirth *et al.*, 2012). As an essential intermediate metabolite of multiple fermentation pathways, pyruvate marks the transition from hydrolysis to acidogenesis (Agler *et al.*, 2011). Figure 4.9 illustrates the role of pyruvate in fermentation pathways such as mixed acid-, alcoholic-, lactic acid-, formic acid-, butyric acid- and propionic acid fermentation.

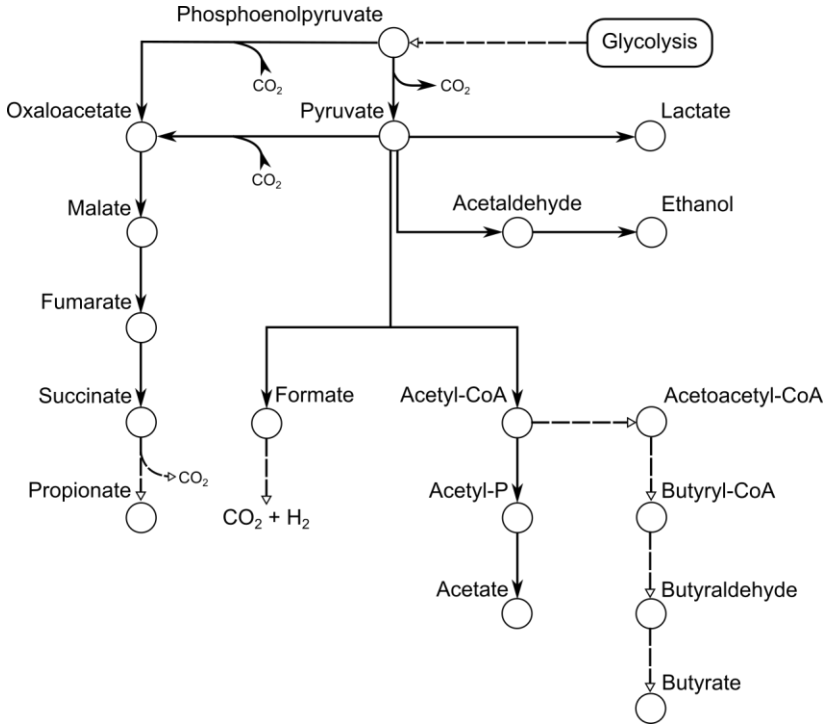


Figure 4.9. Fermentation pathways by way of pyruvate. *Pyruvate is an intermediary metabolite of various fermentation pathways, including mixed acid-, lactic acid-, formic acid-, butyric acid- and propionic acid fermentation. Solid lines indicate the common pathway for mixed acid fermentation within Escherichia coli and dotted lines illustrate alternative fermentative pathways utilised by other microorganisms.*

The pyruvate metabolism pathway was selected for detailed reconstruction due to its role in the aforementioned fermentation reactions. However, due to the complexity of the process, the pathway was split into two sections, A and B. Part A (Figure 4.10) of pyruvate metabolism illustrates the production of lactate (lactic acid in solution). Part B (Figure 4.11) shows the production of the intermediates, formate, acetate and ethanol.

Figure 4.10 illustrates detected pathways involved in lactate generation, including lactic acid fermentation and the methylglyoxal pathway. Lactic acid fermentation describes the conversion of glucose via pyruvate to lactate and/or ethanol, acetate and CO_2 in one of two pathways (Yang *et al.*, 2010). Homolactic fermentation yields lactic acid as the sole fermentation product (Steinkraus, 1983, Madigan *et al.*, 2012). During heterolactic fermentation, lactic acid remains the principle metabolite, but a significant quantity of ethanol, CO_2 and acetate (to a lesser degree) is produced (De Moss, Bard and Gunsalus, 1951). However, the final reaction of lactic acid fermentation is the interconversion of pyruvate and lactate by the enzyme lactate dehydrogenase (EC 1.1.1.-, EC 1.1.2.-) (Figure 4.10, a and b).

The difference between homo- and heterolactic fermentation approaches can be tied to the presence or absence of the glycolytic enzyme aldolase (EC 4.1.2.13) (Madigan *et al.*, 2012). Homofermentative lactic acid bacteria (e.g., *Enterococcus*, *Streptococcus*, some *Lactobacillus*) utilise aldolase via the standard glycolytic pathway to yield lactate (Ciani, Comitini and Mannazzu, 2008, Madigan *et al*., 2012). In contrast, heterofermentative lactic acid bacteria (e.g., *Weissella*, some *Lactobacillus*) lack aldolase and therefore employ an alternative pathway (pentose phosphoketolase pathway) (Ciani, Comitini and Mannazzu, 2008; Liu *et al.*, 2012). The final reaction of this pathway involves the conversion of pentose phosphate to glyceraldehyde-3-phosphate (triose phosphate) and acetyl phosphate by the key enzyme, phosphoketolase (EC 4.1.2.9) (Spector, 2009). The presence of phosphoketolase and lactate dehydrogenase confirms homo- and heterofermentative capabilities within the elephant gut microbiome. However, the lactate isomer (L-lactate or D-lactate) produced may be influenced by the lactic acid bacteria present in the microbiome (González-Vara *et al.*, 1996). *Lactobacillus* (Firmicutes), a genus capable of yielding both lactate isomers was detected as the dominant lactic acid bacterium within this dataset. This genus also includes both homo- and heterofermentative lactic acid bacteria (Yang *et al.*, 2010).

Gene products of the methylglyoxyl pathway, yielding both isomers of lactate, were detected in the African elephant gut microbiome. This pathway provides an alternative catabolic pathway for triose phosphates produced during glycolysis and enables the release of phosphate from glycolytic metabolites during phosphate starvation (Hopper and Cooper, 1972). Glycerone phosphate (a.k.a., dihydroxyacetone phosphate) generated during glycolysis is converted to methylglyoxyl by methylglyoxal synthase (EC 4.2.3.3) (Figure 4.10). However, physiologically produced methylglyxoyl is a toxic, growth inhibitory metabolite (Chakraborty, Karmakar and Chakravortty, 2014). Organisms therefore convert this metabolite to lactate via lactaldehyde and ultimately pyruvate as a means of detoxification. Gene products involved in this pathway were detected from the genus *Clostridia* and members of the class Deltaproteobacteria and Gammaproteobacteria (e.g., *Escherichia*).

The enzyme fructose-6-phosphate phosphoketolase (EC 4.1.2.22) was detected in the datatset (not visually indicated). This enzyme is key to an alternative heterolactic fermentation pathway known as the bifid or fructose-6-phosphate shunt unique to members of enteric *Bifidobacteria* (Actinobacteria) (Ward, 2015). This pathway enables the conversion of glucose to lactate and acetate. Acetate and CO_2 resulting from heterolactic fermentation (Figure 4.11) can be directly utilised by acetoclastic and hydrogenotrophic methanogens respectively (Section 4.2.4.5) (Pyzik *et al.*, 2018).

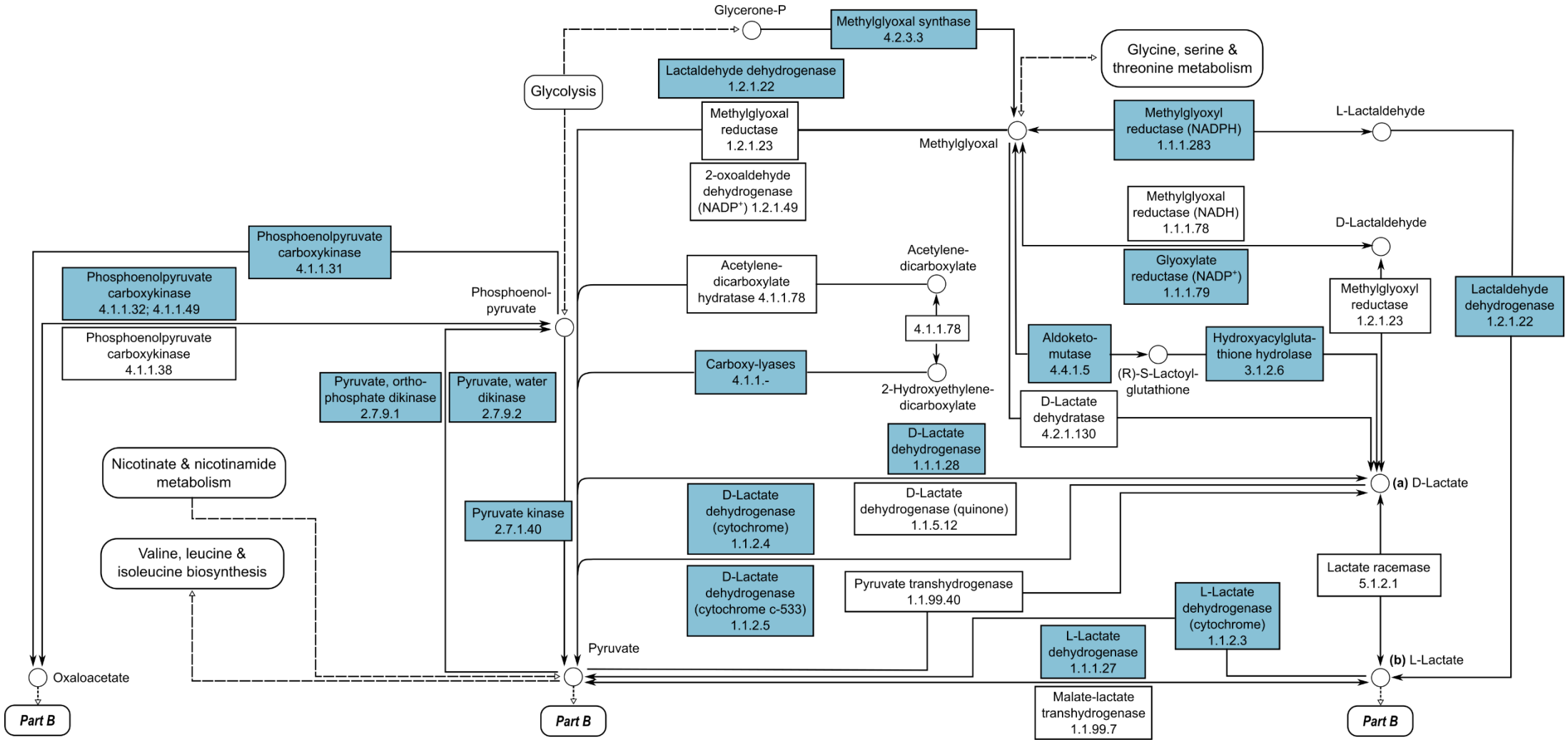


Figure 4.10. Pyruvate metabolism (hsa00620) (Part A). *The production of D-Lactate (a) and L-Lactate (b) from pyruvate produced during hydrolysis of carbohydrates and other complex polymers. Rectangular boxes represent the gene products that catalyse each biochemical reaction. An enzyme name and EC number identifies each gene product, with coloured blocks indicating gene products present within the analysed metagenome. Circles indicate other molecules such as the substrates or products of a particular reaction.*

Alcoholic/ethanol fermentation is a well-recognised, simple fermentative pathway that involves the conversion of sugars such as glucose to ethanol and CO_2 (Figure 4.11, c) (Fenchel, King and Blackburn, 2012). Traditionally pyruvate is decarboxylated to acetaldehyde, catalysed by the enzyme pyruvate carboxylase (EC 6.4.1.1); followed by the reduction of acetaldehyde to ethanol, catalysed by alcohol dehydrogenase (EC 1.1.1.1) (Ciani, Comitini and Mannazzu, 2008; Spector, 2009; Ward, 2015). Although the enzymes for this route were detected in the metagenome, the reaction is not displayed in Figure 4.11. Alternatively, pyruvate is converted to acetaldehyde via multiple redox (reduction/oxidation) reactions before the final reduction to ethanol (Figure 4.11).

Alcoholic fermentation is generally associated with yeast (e.g., *Saccharomyces cerevisiae*), but some bacterial groups do produce ethanol as a fermentation by-product (Spector, 2009; Mussatto and Teixeira, 2010). The yeast genera *Saccharomyces* and *Neurospora* were linked to alcoholic fermentation within this dataset, however, at a relatively low abundance (< 100 reads). Members of *Neurospora,* such as the model organism, *Neurospora crassa*, has been shown to convert sugar and cellulose polymers to ethanol (Dogaris, Mamma and Kekos, 2013). Bacterial genera associated with alcoholic fermentation included *Clostridium* and *Zymomonas* (< 100 reads). *Zymomonas mobilis* is an essential part of bacterial alcoholic fermentation and has been utilised for industrial ethanol production, however the use of yeast remains preferential (Ciani, Comitini and Mannazzu, 2008). The CO_2 produced during alcoholic fermentation is converted to methane in the presence of H_2 by hydrogenotrophic methanogens (Section 4.2.4.5) (Costa and Leigh, 2014). Alternatively, the incomplete oxidation of ethanol by acetic acid bacteria produces acetate and water (H_2O) as end-products (Schuchmann and Müller, 2016). Acetic acid bacteria include genera of the Alphaproteobacteria (e.g., *Acetobacter*, *Gluconobacter*) (Yamada and Yukphan, 2008). Similarly, the elongation of carboxylates (conjugate base of carboxylic acid) with ethanol produces acetate and H_2 (Agler *et al.*, 2011). As with lactate, the resulting acetate is used by acetoclastic methanogens.

Mixed acid fermentation refers to the use of two or more different pathways in the terminal stages of fermentation (Ward, 2015). This type of fermentation is commonly associated with the Enterobacteriaceae (e.g., *Escherichia*, *Enterobacter* and *Salmonella*) and some species of anaerobic fungi (e.g*., Neocallimastix*) (Lowe, Theodorou and Trinci, 1987; Ciani, Comitini and Mannazzu, 2008; Spector, 2009). These microbes ferment glucose to a mixture of formate, lactate, acetate, succinate and ethanol (Figure 4.9, 4.10 and 4.11) (Fenchel, King and Blackburn, 2012). Acetate and one carbon (C_1) molecules like formate or methanol serve as substrates for methyltrophic methanogenesis (Section 4.2.4.5) (Li *et al.*, 2013; Costa and Leigh, 2014; Vanwonterghem *et al.*, 2016).

Alternatively, formate is an essential reagent of the Wood-Ljungdahl pathway (Section 4.2.4.4), utilised by acetogenic bacteria for CO_2 fixation and energy conservation (Schuchmann and Müller, 2016). Succinate produced during mixed acid fermentation (Figure 4.12) enters the propanoate metabolism pathway where it is converted to propionate, a substrate of acetogenesis and the next phase of anaerobic digestion, via secondary fermentation (Fenchel, King and Blackburn, 2012). The enzymes pyruvate formate-lyase (EC 2.3.1.54) (Figure 4.11) and lactate dehydrogenase (Figure 4.10) control entry to mixed acid fermentation. These enzymes were detected within the dataset supporting mixed acid fermentation within the African elephant gut. Genera detected involved in mixed acid fermentation include *Enterobacter*, *Escherichia*, *Salmonella*, *Klebsiella*, *Desulfatibacillum* and *Desulfovibrio*. Additionally, Clostridial species carry out mixed acid fermentation producing butyrate as one of the end-products (Fenchel, King and Blackburn, 2012; Ward, 2015). Butyrate serves as a substrate for acetogenesis, the next phase of anaerobic digestion.

Genes encoding enzymes that catalyse reactions which yield acetate are abundant within this metagenome. As illustrated in the above sections, multiple pathways were detected that enable the conversion of other fermentation intermediates to acetate (secondary fermentation). Alternatively, acetate is also synthesised from the direct conversion of pyruvate (Figure 4.11, b). Pyruvate undergoes a redox reaction catalysed by pyruvate oxidase (EC 1.2.3.3) to yield acetyl-phosphate (acetyl-P) coupled to the release of CO_2. The phosphate group is cleaved by acylphosphatase (EC 3.6.1.7), yielding acetate (Figure 4.12). Pyruvate is also directly converted to acetate by pyruvate dehydrogenase (EC 2.1.5.1) or indirectly via acetaldehyde, catabolysed by the enzyme aldehyde dehydrogenase (EC 1.2.1.-) (Figure 4.11). Acetate is utilised directly by the acetoclastic methanogens (detailed discussion of this pathway in Section 4.2.4.5). Genera detected in the metagenome that facilitate such reactions include *Clostridium* (Firmicutes) and members of the Proteobacteria, *Geobacter*, *Burkholderia* and *Desulfovibrio*.

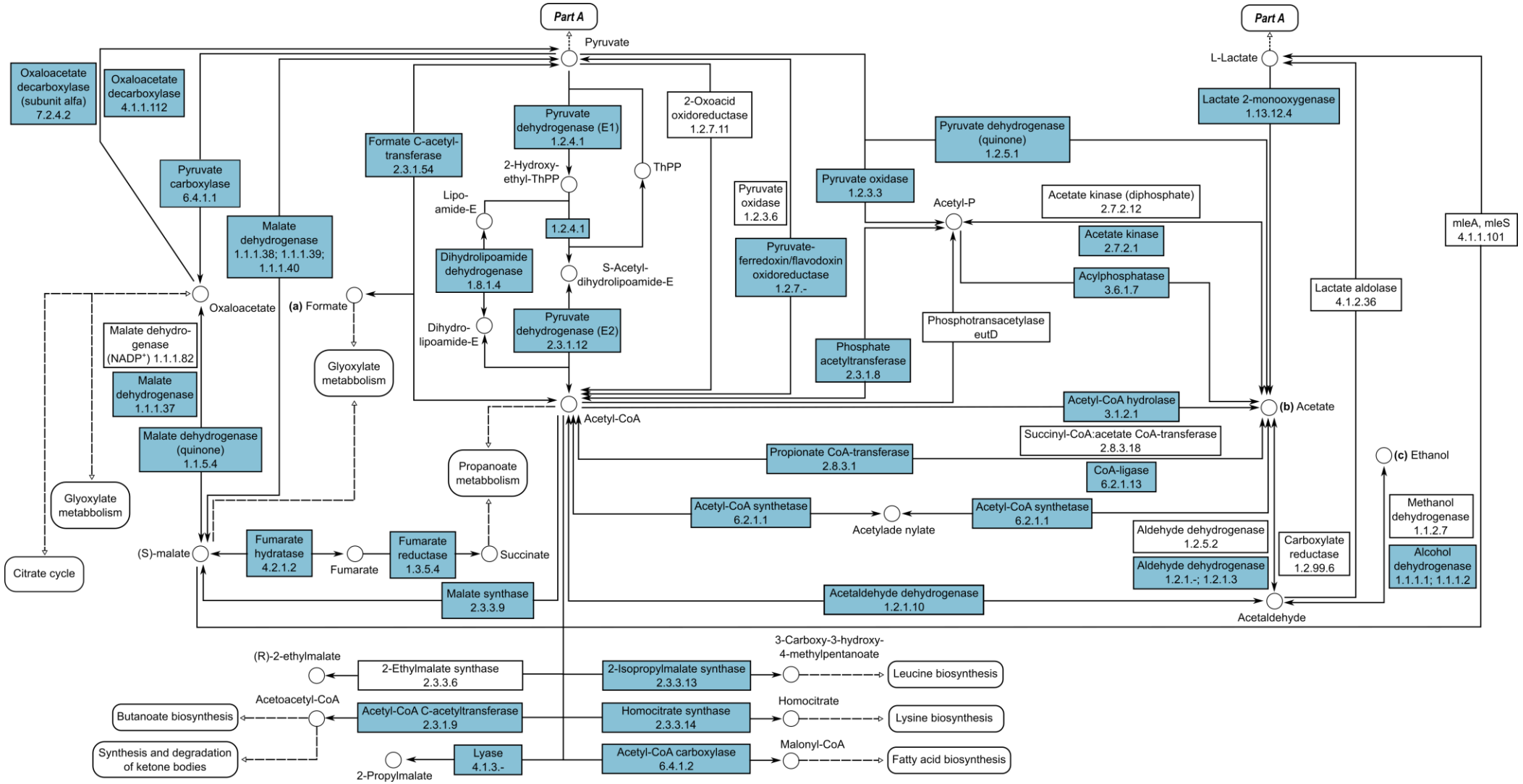


Figure 4.11. Pyruvate metabolism (hsa00620) (Part B). *(a) Conversion of pyruvate and coenzyme A (CoA) to formate and acetyl-CoA. (b) Acetate production from multiple sources including lactate, acetyl-CoA and pyruvate. (c) Alcoholic fermentation producing ethanol and carbon dioxide.*

In the presence of protein-rich substrates, some “proteolytic” *Clostridium* species can ferment amino acids (Ciani, Comitini and Mannazzu, 2008). These organisms are often associated with the degradation of dead organisms in nature (Madigan *et al.*, 2012). Such fermentation reactions yield short chain VFAs (e.g., acetic-, propionic- and butyric acid) through the reductive deanimation of aliphatic amino acids or coupled deanimation via Stickland fermentation (Nisman, 1954; Merlin Christy, Gopinath and Divya, 2014). Stickland fermentation describes redox reactions between pairs of amino acids, where one amino acid serves as the electron donor and the other serves as the electron acceptor (Ciani, Comitini and Mannazzu, 2008; Merlin Christy, Gopinath and Divya, 2014). The major products of Stickland fermentation include ammonia (NH_3), CO_2, acetic acid, short chain fatty acids ($C_{1\text{-}5}$) and H_2 (Nisman, 1954; Ciani, Comitini and Mannazzu, 2008). Alcohols and phenols represent some of the minor products of Stickland fermentation. The role of these metabolites in anaerobic digestion have been outlined under mixed acid fermentation and alcoholic fermentation.

Butyrate (a.k.a., butanoate), a key precursor of acetogenesis, is a short chain fatty acid produced by bacterial fermentation of carbohydrates and fibre (Leschelle et al., 2000). Several obligate anaerobic bacteria belonging to genera such as *Clostridium*, *Fusobacterium*, *Eubacterium* and *Butyrivibrio* produce butyrate as a major product of pyruvate metabolism (Spector, 2009). In addition to butyrate, varying quantities of acetate, acetone, ethanol, CO_2, H_2, butanol, isopropanol and 2,3-butanediol is produced by some butyric acid fermenters. Butyric acid fermenters employ glycolysis to oxidise saccharides, and occasionally pectin and amylose to pyruvate (Ciani, Comitini and Mannazzu, 2008). Pyruvate is subsequently oxidised to acetyl-CoA by the pyruvate ferredoxin oxidoreductase (EC 1.2.7.1) enzyme system (Figure 4.12), coupled to the release of CO_2 and H_2. Acetoacetyl-CoA generated from acetyl-CoA is reduced in a stepwise manner to butanoyl-CoA by way of crotonoyl-CoA, catalysed by enzymes such as acetyl-CoA C-acetyltransferase (EC 2.3.1.9), enoyl-CoA hydratase (EC 4.2.1.17) and butyryl-CoA dehydrogenase (EC 1.3.8.1) among others (Figure 4.12). Butanoyl-CoA is directly converted to butyrate by the enzyme acetate CoA-transferase (EC 2.8.3.8) or indirectly via butanoyl-phosphate, catalysed by the enzymes phosphate butyryltransferase (EC 2.3.1.19) and butyrate kinase (EC 2.7.2.7) (Figure 4.12). The genus *Clostridium* (Firmicutes) was detected as the dominant group responsible for butyrate production within this metagenome dataset. Members of *Alkaliphilus* (Firmicutes), *Bacillus* (Firmicutes), *Fusobacterium* (Fusobacteria) and *Butyrivibrio* (Firmicutes) were also linked to butanoate metabolism, however at notably lower abundance levels than *Clostridium*.

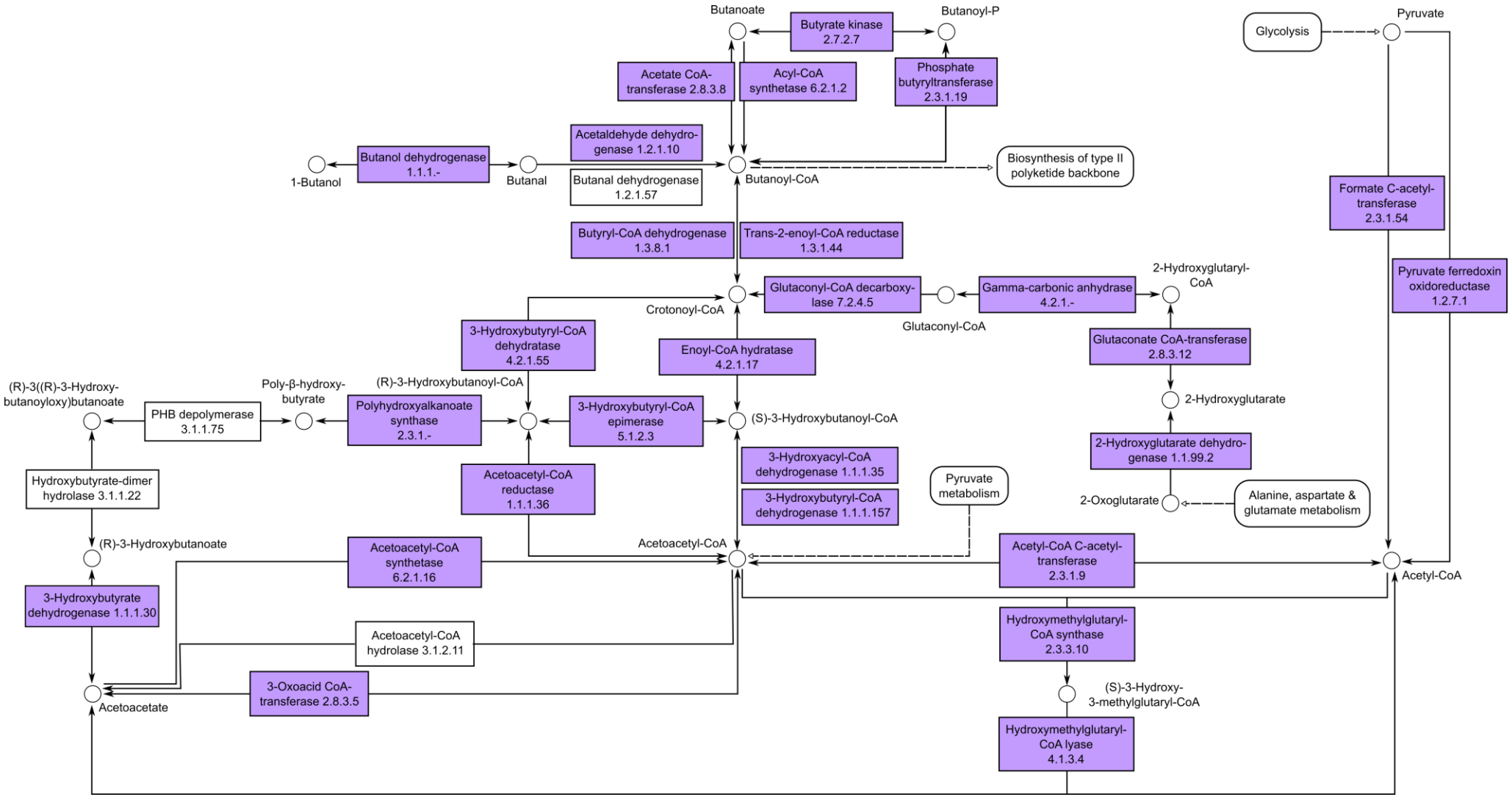


Figure 4.12. Butanoate metabolism reconstructed KEGG pathway (hsa00650). *Complete reconstructed pathway of butanoate production and metabolism via multiple metabolic reactions. Coloured blocks represent gene products coding for enzymes within the metagenome that catalyse the indicated reaction.*

4.2.4.3. Acidogenesis: Secondary fermentation

In the simplest terms, secondary fermentation involves the fermentation of metabolites yielded during previous fermentative reactions. Propionic acid fermenters (e.g., *Propionibacterium*, *Bacteroides*, *Clostridium*) produce propionic acid (a.k.a., propanoate), acetic acid and CO_2 as the major products of either glucose, lactate or glycerol fermentation (Figure 4.13) (Stams *et al.*, 1998; Spector, 2009). Lactate, the fermentation product of lactic acid bacteria, is considered the main substrate of propionic acid fermentation in natural habitats, as the microbes involved in these pathways are often found in close proximity (Madigan *et al.,* 2012).

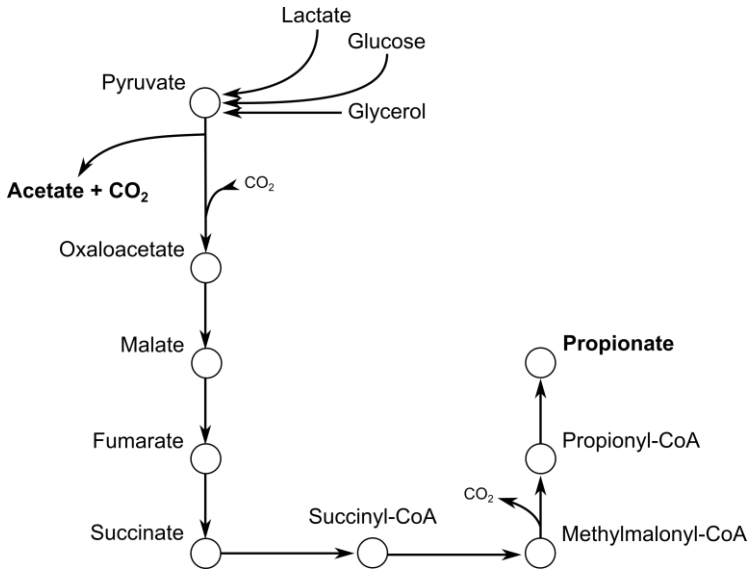


Figure 4.13. Propionic acid fermentation from lactate and glucose. *Simplified schematic showing the major intermediates of propionic acid fermentation. Major products of this pathway are indicated in bold. Adapted from (Ciani, Comitini and Mannazzu, 2008, Madigan et al., 2012)*.

Compounds produced during glycolysis, the citrate cycle and pyruvate metabolism participate in propanoate metabolism (Figure 4.14). The dehydration of lactate to lactoyl-CoA and subsequently to acryloyl-CoA catalysed by propionate CoA-transferase (EC 2.8.3.1) and lactoyl-CoA dehydratase (EC 4.2.1.54) respectively (Figure 4.14, b) is facilitated by select species of *Clostridium* (e.g., *C. propionicum*) and *Bacteroides* (e.g., *B. ruminicola*) (Spector, 2009). Acryloyl-CoA is further reduced to propanoyl-CoA and ultimately propanoate. All enzymes required for the conversion of lactate to propanoate were not detected within the dataset, however this does not confirm their absence from the microbial community, but might suggest the need for enrichment prior to biogas production.

Following pyruvate metabolism, succinate (Figure 4.14; c) enters propanoate metabolism and is converted to propanoyl-CoA via one of two pathways, the methylcitrate cycle or through the conversion of succinyl-CoA to propanoyl-CoA. Three enzymes specific to the methylcitrate cycle were detected in the dataset, methylisocitrate lyase (EC 4.1.3.30), methylcitrate dehydratase (EC 4.2.1.79) and methylcitrate synthase (EC 2.3.3.5) (Upton and McKinney, 2007). The methylcitrate pathway was originally considered unique to fungi, however recent years have linked multiple bacteria to this pathway including *Bacillus subtilis* and members of the enterobacteria (e.g., *Escherichia coli*, *Salmonella enterica* and *Campylobacter* spp.) (Dolan *et al.*, 2018). Alternatively, succinate is converted to succinyl-CoA by succinyl-CoA synthetase (EC 6.2.1.4/5) and subsequently to methylmalonyl-CoA, catalysed by methylmalonyl-CoA mutase (EC 5.4.99.2). Methylmalonyl-CoA is ultimately converted to propionate (Figure 4.15, d) via propanoyl-CoA by enzymes that include methylmalonyl-CoA carboxytransferase (EC 2.1.3.1) and acetyl-CoA synthetase (EC 6.2.1.1).

Acetyl-CoA (Figure 4.14, a) produced during pyruvate metabolism is converted to propanoyl-CoA via malonyl-CoA or malonate semialdehyde, catalysed by enzymes such as methylmalonyl-CoA carboxytransferase (EC 2.1.3.1) and malonate semialdehyde dehydrogenase (EC 1.2.1.18). These reactions are the first in a long series of reactions that terminate in the final conversion of propanoyl-CoA to propanoate (Figure 4.14, d) by multiple enzymes that include acetyl-CoA synthetase (EC 6.2.1.1), propionyl-CoA synthetase (EC 6.2.1.17), propionate CoA-transferase (EC 2.8.3.1) and acetate-CoA ligase (EC 6.2.1.13).

Glycerol enters the glycolytic pathway as glycerone-P, from where it is converted to glyceraldehyde-3-phosphate (Campbell and Farrell, 2013). From there, glycerol is converted to propionate via the same reactions as lactate and glycerol. However, glycerone-P can also enter propanoate metabolism without proceeding through glycolysis (Figure 4.14). Cleavage of the phosphate from glycerone-P by the enzyme methylglyoxal synthase (EC 4.2.3.3) yields methylglyoxal and phosphate. This methylglyoxal is converted to propanal via a series of reactions that employ enzymes like methylglyoxal reductase (EC 1.1.1.283), glycerol dehydrogenase (EC 1.1.1.6), lactaldehyde dehydrogenase (EC 1.1.1.77) and propanediol dehydratase (EC 4.2.1.28). However, the final enzyme required for the conversion of propanal to propanoyl-CoA and ultimately propionate, propionaldehyde dehydrogenase (EC 1.2.1.87), was not detected within the current dataset.

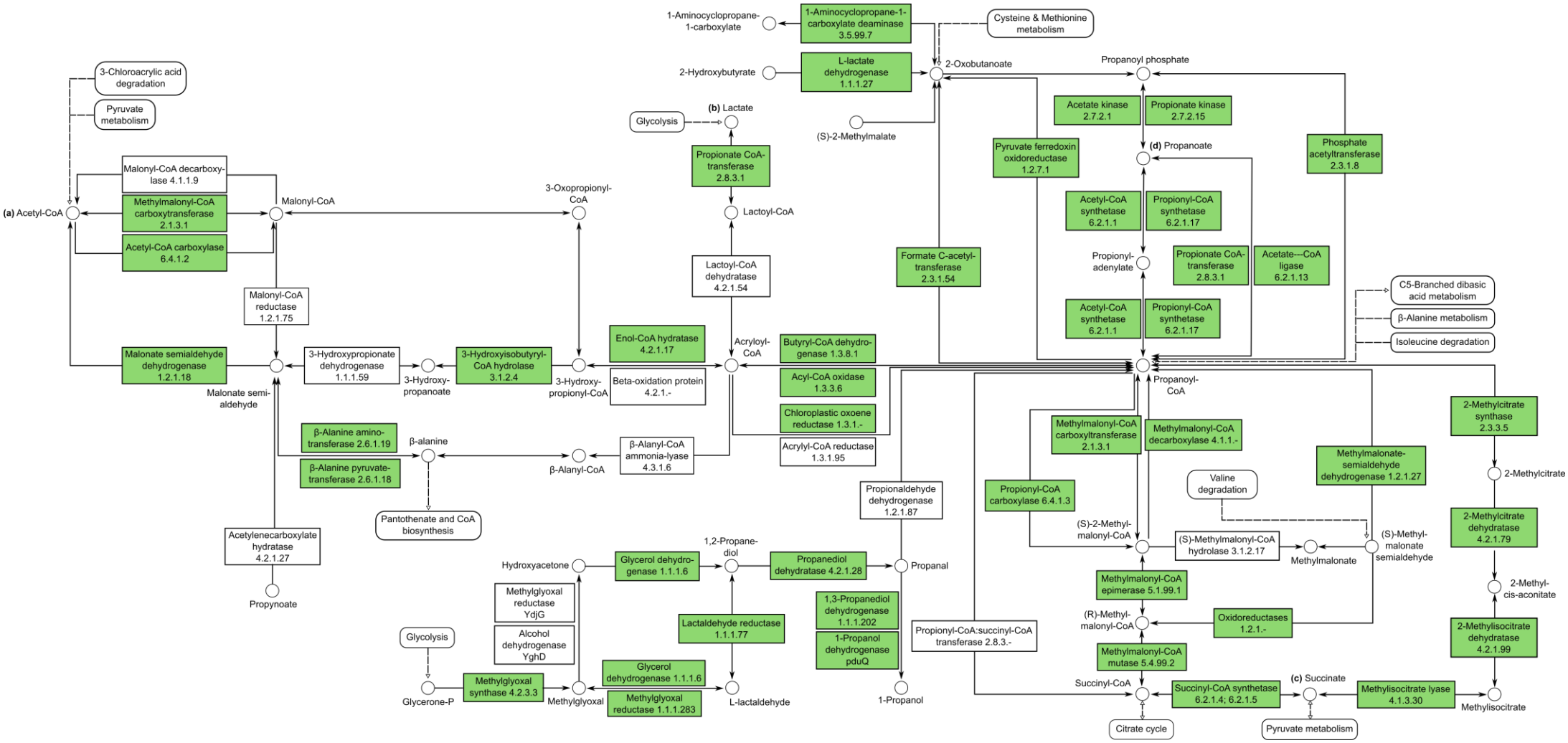


Figure 4.14. Propanoate metabolism reconstructed KEGG pathway (hsa00760). *Complete reconstructed pathway of propanoate production and metabolism via multiple metabolic reactions from compounds including acetyl-coenzyme A (acetyl-CoA), lactate and propionoyl-CoA. Coloured blocks represent gene products coding for enzymes within the metagenome that catalyse the indicated reactions*.

4.2.4.4. Acetogenesis: The role of propionate and butyrate

Acetogens describe obligately anaerobic bacteria that employ the Wood-Ljungdahl pathway as their main mechanism for energy conservation (Ragsdale and Pierce, 2008; Schuchmann and Müller, 2016). During this process, formate is converted to acetyl-CoA and ultimately acetate in the presence of two molecules of CO_2 (Figure 4.15) (Schuchmann and Müller, 2016). On the other hand, under low pH conditions, formate is converted to CO_2 and H_2 via the enzyme formate hydrogen lyase (Ward, 2015). However, this enzyme was not detected within the current dataset.

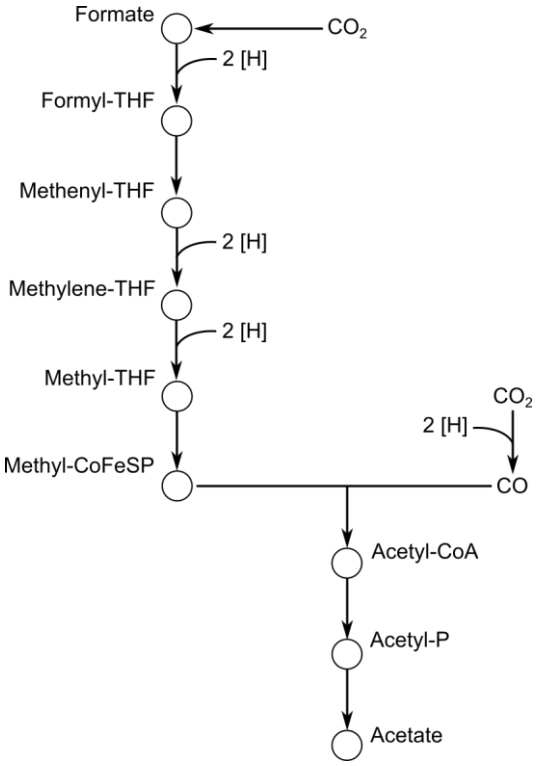


Figure 4.15. The Wood-Ljungdahl pathway employed by acetogenic bacteria. *[H] represents a redox equivalent (e.g., H_2) (electron + proton). Tetrahydrofolate (THF). Corinoid iron-sulfur protein (CoFeSP). Acetogenic bacteria employ this pathway for energy conservation, however not all acetogens produce acetate as a final end product and rather employ this pathway for acetyl-CoA production. Adapted from Schuchmann and Müller, 2016.*

The conversion of short-chain fatty acids like propionate and butyrate to intermediates such as acetate, formate, H_2 and CO_2 under standard conditions (substrate and product concentration of 1 M, P_{H2} of 1 atm, temperature at 298 K) is an endergonic (energy consuming) reaction (Müller *et al.*, 2010). However, under anoxic conditions, this process is energetically feasible, as methanogens consume these intermediates, thereby maintaining low levels of H_2, formate and acetate. The coupling of metabolic H_2 production and consumption is the basis of syntrophic “interspecies hydrogen transfer” (Fenchel, King and Blackburn, 2012). In natural

microbial communities, acetogens and hydrogenotrophic methanogens may compete for CO_2 and H_2, while acetogens form syntrophic relationships with acetoclastic methanogens (Ragsdale and Pierce, 2008). The final steps of anaerobic digestion are therefore closely linked due to syntrophic relationships between acetogenic- and methanogenic microbes (Müller *et al.*, 2010; Chistoserdova and Kalyuzhnaya, 2018). In natural habitats, acetogens often resort to other metabolic pathways due to competition with hydrogenotrophs. As such, these microbes have a highly diverse metabolic repertoire that comprises biodegradation products of natural polymers such as cellulose, lignin, alcohols and organic acids like propionic- and butyric acid (Ragsdale, 2008; Ragsdale and Pierce, 2008). The oxidation of propionate and butyrate to acetate is catalysed by an array of enzymes summarised in Table 4.1. All listed enzymes were detected within the African elephant faecal matter.

Table 4.1. *Selected enzymes necessary for the oxidation of propionate and butyrate to acetate (acetogenesis). Adapted from Sikora et al., 2019.*

Propionate oxidation to acetate	
$Propionate + 3H_2O \rightarrow Acetate + HCO_3 + H^+ + 3H_2$	
Enzymes	**EC number**
Pyruvate carboxylase	6.4.1.1
Malate dehydrogenase	1.1.1.37
Fumarate hydratase	4.2.1.2
Fumarate reductase	1.3.5.4
Succinate dehydrogenase	1.3.5.1
Succinyl-CoA synthetase	6.2.1.4
Methylmalonyl-CoA mutase	5.4.99.2
Methylmalonyl-CoA epimerase	5.4.99.1
Methylmalonyl-CoA decarboxylase	4.1.1.41
Propionate-CoA transferase	2.8.3.1
Butyrate oxidation to acetate	
$Butyrate + 2H_2O \rightarrow 2Acetate + 2H^+ 2H_2$	
Butyrate-acetoacetate CoA-transferase	2.8.3.9
Butyryl-CoA dehydrogenase	1.3.8.1
Crotonase-3-OH-butyryl-CoA dehydratase	4.2.1.55
Acetyl-CoA acetyltransferase	2.3.1.9
Hydroxybutyryl-CoA dehydrogenase	1.1.1.157
Phosphotransacetylase	2.3.1.8
Acetate kinase	2.7.2.1

Acetogens are versatile microbes and as such these microbes are linked to various different phyla including multiple members of the order Clostridia from the phylum Firmicutes (Schuchmann and Müller, 2016). The final step of the Wood-Ljungdahl pathway, acetyl-P to acetate, is catalysed by the enzyme acetate kinase (EC 2.7.2.1). Genera within the dataset linked to the gene product of this enzyme include *Syntrophomonas* and *Pelotomaculum* from the order Clostridia. *Syntrophobacter* and *Syntrophus* of the class Deltaproteobacteria were also linked to the acetate kinase gene product.

4.2.4.5. Methanogenesis

Since methane production is essential to biogas formation, the final step of anaerobic digestion, methanogenesis, remains the determining factor. Methanogens compose a group of diverse, highly specialised Archaea belonging to the phylum Euryarchaeota (Thauer *et al.*, 2008; Costa and Leigh, 2014). These highly specialised microorganisms are metabolically restricted to the use of a limited number of substrates, including formate, acetate, H_2, CO_2 and methyl containing compounds to produce methane (CH_4) (Thauer *et al.*, 2008). Methane is produced via one of three metabolic pathways under anoxic conditions, the hydrogenotrophic-, acetoclastic- and methylotrophic methanogenesis pathways. Regardless of the methanogenesis pathway utilised, the last common reaction involves the reduction of methyl-CoM to methane, catalysed by methyl-CoM reductase (EC 2.8.4.1) (Pyzik *et al.*, 2018).

Figure 4.16 outlines the aforementioned methanogenesis pathways with orange rectangles representing gene products/enzymes common to all three pathways. The genetic coding to facilitate all three methanogenesis pathways was detected within the African elephant faecal matter, however the abundance of genes for each pathway differed considerably. Genes encoding enzymes of the hydrogenotrophic pathway were dominant, with genes of the acetoclastic methanogenesis pathway poorly represented by a limited number of reads. The sample source and subsequent energy constraints could offer one explanation for these observations. Methanogen communities from natural sources (e.g., rumen, manure) show a higher abundance of hydrogenotrophic- and methyltrophic methanogens than communities from artificial reactors, which favour acetoclastic methanogens (Alvarado *et al.*, 2014; Pyzik *et al.*, 2018). This is likely because the hosts (e.g., ruminants) reabsorb metabolically produced acetic acid within the intestinal tract to use as a source of energy (Thauer *et al.*, 2008). Therefore, restricting methanogen growth within the intestinal tract mainly to substrates like H_2, CO_2 and formate, favouring hydrogenotrophic- and methyltrophic methanogens.

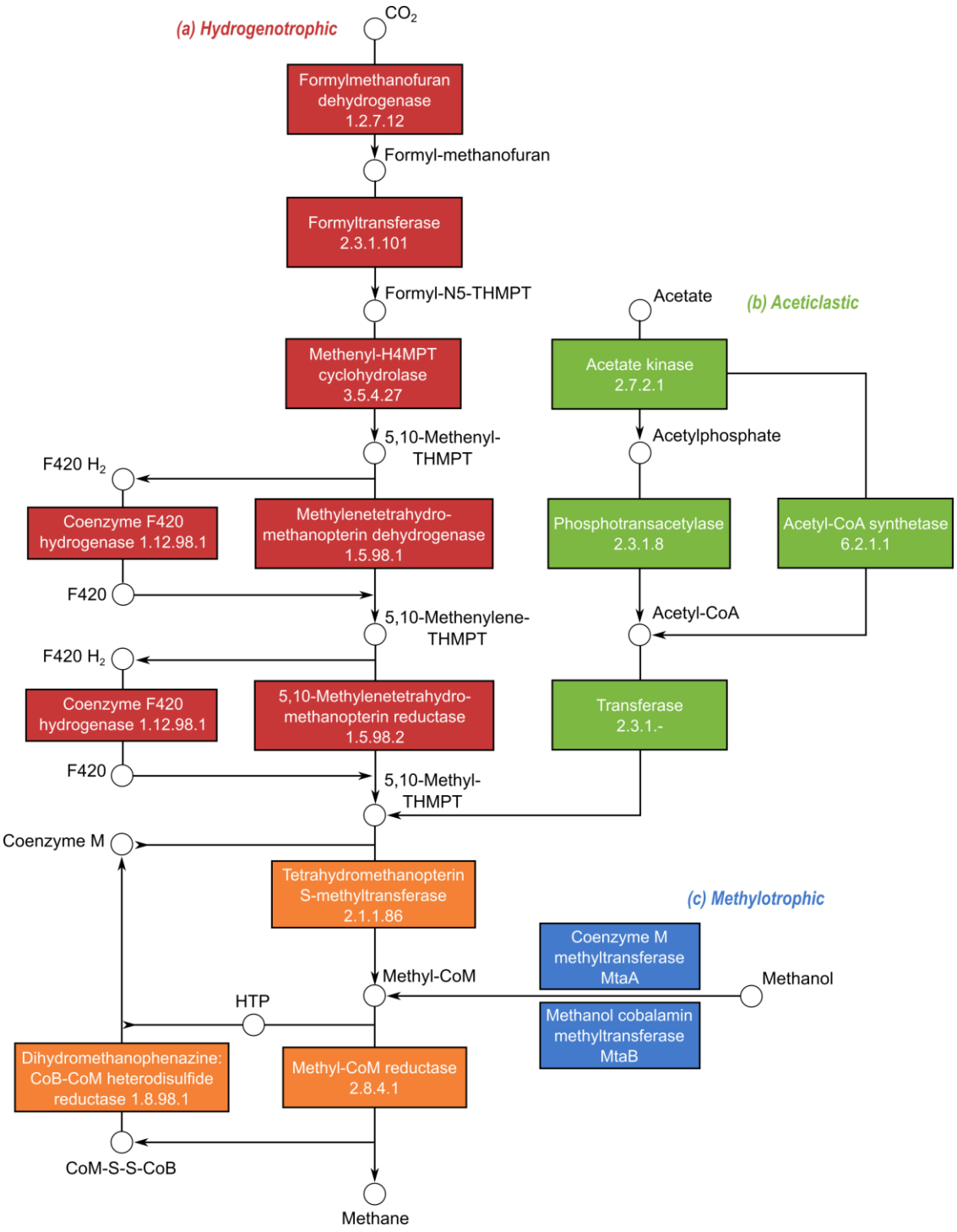


Figure 4.16. Methanogenesis pathways. *Methane production proceeds via one of three pathways: the reduction of carbon dioxide (CO_2) in the presence of hydrogen (H_2) or the hydrogenotrophic pathway (red rectangles). Methane production from acetate or the acetpclastic pathway (green rectangles) and finally the methyltrophic pathway (blue rectangles), involving the conversion of C_1 compounds like methanol and formate to methane. Coloured rectangles represent gene products present in the metagenome essential to the indicated biochemical reactions. Orange rectangles represent gene products/enzymes common to all three methanogenesis pathways. THMPT - Tetrahydromethanopterin*

Hydrogenotrophic methanogens (Figure 4.16, a) use H_2 or formate as an electron source for the reduction of CO_2 to methane (Richards *et al.*, 2016). Like acetogens, hydrogenotrophic methanogens employ the Wood-Ljungdahl pathway for CO_2 fixation, however methanogens conserve energy by converting H_2 and CO_2 to methane (Ragsdale and Pierce, 2008; Borrel, Adam and Gribaldo, 2016). Carbon dioxide is reduced to a formyl group covalently linked to the methanofuran (MF) carrier molecule (Blaut, 1994; Costa and Leigh, 2014). The formyl group is then transferred via formyltransferase (EC 2.3.1.101) to the next carrier molecule, tetrahydromethanopterin (THMPT). Cyclisation catalysed by the enzyme methenyl-H4MPT cyclohydrolase (EC 3.5.4.27) following dehydration yields methenyl-THMPT. Subsequent reduction reactions catalysed by methylenetetrahydromethanopterin dehydrogenase (EC 1.5.98.1) and 5,10-methylenetetrahydromethanopterin reductase (EC 1.5.98.2) convert methenyl to methylene and finally to a methyl group. The third carrier molecule is the sulfhydryl-containing coenzyme M (CoM). Lastly, the methyl group is converted to methane by the oxidation of CoM via methyl-CoM reductase together with another sulfhydryl- containing coenzyme, coenzyme B (CoB). Alternatively, hydrogenotrophic methanogens can form syntrophic relationships with acetate-oxidising bacteria (Barret *et al.*, 2013). These acetate-oxidising bacteria convert acetate to H_2 and CO_2, which is then converted to methane by the hydrogenotrophic methanogens.

Archaea within this dataset linked to hydrogenotrophic methanogenesis all belong to the class Methanomicrobia. The dominant genera were *Methanocorpusculum*, *Methanoregula and Methanoculleus* of the order Methanomicrobiales. To date, *Methanocorpusculum* is the only genus belonging to the family Methanocorpusculaceae, created in 1989 to host mesophilic methanogens isolated from different anaerobic wastewater treatment plants (Tabatabaei *et al.*, 2010; Oren, 2014). All species of *Methanocorpusculum* and *Methanoculleus* grow on H_2/CO_2 or formate, however select species also reduce primary- (e.g., ethanol) (*Methanoculleus*) and secondary alcohols (e.g., 2-propanol) (Zellner and Winter, 1987; Maestrojuan *et al.*, 1990; Oren, 2014; Manzoor *et al.*, 2016). *Methanoregula* hosts strains of acidophilic and mesophilic hydrogenotrophic methanogens growing exclusively on H_2/CO_2 and formate (Bräuer *et al.*, 2011; Pyzik *et al.*, 2018). *Methanosarcina* of the order Methanosarcinales was also linked to this pathway, but at a lower abundance than the members of the Methanomicrobiales. Members of *Methanosarcina* are metabolically extremely versatile and to date considered the only methanogens capable of utilising all three methanogenesis pathways (Galagan *et al.*, 2002; Pyzik *et al.*, 2018).

Acetoclastic methanogens produce methane and CO_2 from the conversion of acetate (Pore *et al.*, 2016). These methanogens apply the Wood-Ljungdahl pathway in the oxidative direction (reverse) (Figure 4.16, b) to split acetate into a methyl group and a CO group (Galagan *et al.*, 2002; Ragsdale and Pierce, 2008). Initially, acetate is phosphorylated via acetate kinase (EC 2.7.2.1) to yield acetyl-phosphate (Welte and Deppenmeier, 2014; Pyzik *et al.*, 2018). Next, the acetyl group is transferred to the carrier molecule coenzyme A (CoA) by the enzyme phosphotransacetylase (EC 2.3.1.8). Alternatively, acetate is converted directly to acetyl-CoA via acetyl-CoA synthetase (EC 6.2.1.1). Acetyl-CoA is then split into a methyl group and a CO group. The CO group is subsequently oxidised to provide electrons for the reduction of the methyl group to methane (Galagan *et al.*, 2002). The methyl group is transferred to the carrier molecule THMPT by transferase (EC 2.3.1.-) before conversion to methyl-CoM and finally methane via methyl-CoM reductase (EC 2.8.4.1). Thus far, only two methanogenic genera *Methanosarcina* and *Methanothrix* (formerly *Methanosaeta*), both of the order Methanosarcinales can grow on acetate as the sole carbon and energy source (Stams, Teusink and Sousa, 2019). Acetate kinase, acetyl-CoA synthetase and methyl-CoM reductase were used as indicators of the acetoclastic methanogenesis pathway within the dataset (Pyzik *et al.*, 2018). Both *Methanosarcina* and *Methanothrix* were detected within the African elephant gut microbiome. The genera *Metahanobrevibacter* and *Methanocorpusculum* were also linked to these gene products, however as hydrogenotrophic methanogens, these microbes only utilise acetate as a carbon source for cell synthesis and not methane production (Jetten, Stams and Zehnder, 1990; Siegert *et al.*, 2014).

Methylotrophic methanogens (Figure 4.16, c) employ methanol or methyl containing compounds like methylamines and methylsulfides to yield methane (Nettmann *et al.*, 2010). These substrates enter methanogenesis as methyl-S-CoM (Costa and Leigh, 2014). Methyl-S-CoM undergoes reduction to yield methane by utilising electrons from either H_2 or from the oxidation of a second methyl-S-CoM to CO_2 (Galagan *et al.*, 2002; Costa and Leigh, 2014). This method of methane production makes use of a single class of enzymes, methyltransferases, including methylamine-specific methyltransferase (MtaA) and methanol-specific methyltransferase (MtaB) (Pyzik *et al.*, 2018). These methyltransferases serve as highly specific indicators of methyltrophic methanogenesis. *Methanosarcina* and *Methanosphaera* were detected as the dominant methylotrophic methanogens. *Methanosphaera* is distinguished from other members of the mostly hydrogenotrophic order, Methanobacteriales, by its ability to grow exclusively by reducing methanol to methane using H_2 as the electron donor (Bonin and Boone, 2006).

Due to the complete dominance of hydrogenotrophic methanogens within the African elephant gut microbiome, a transition period may be necessary to aid methane production. A transition period could allow the methanogenic community to adapt to an artificial bioreactor setting if this community were to be used for methane production.

4.2.4.6. Lignocellulose metabolism

Lignocellulose refers to a renewable, non-edible source of biomass and may include food waste residues, agricultural waste or industrial waste (Hassan, Williams and Jaiswal, 2018). Composed mainly from cellulose, hemicellulose and lignin, lignocellulose is bound in a complex, recalcitrant hierarchical structure (Figure 4.17). Despite the high aromatic carbon content of lignocellulose, converting it to usable products presents a challenge (Brink *et al.*, 2019). Cellulose is sheathed in hemicellulosic polysaccharides (mannans and xylans) linked by covalent and hydrogen bonds resulting in a structure resistant to chemical and biological hydrolysis (Malherbe and Cloete, 2002). Lignin acts as a physical barrier, intricately associated with hemicellulose to further shield hemicellulose and cellulose from direct enzymatic hydrolysis (Figure 4.17).

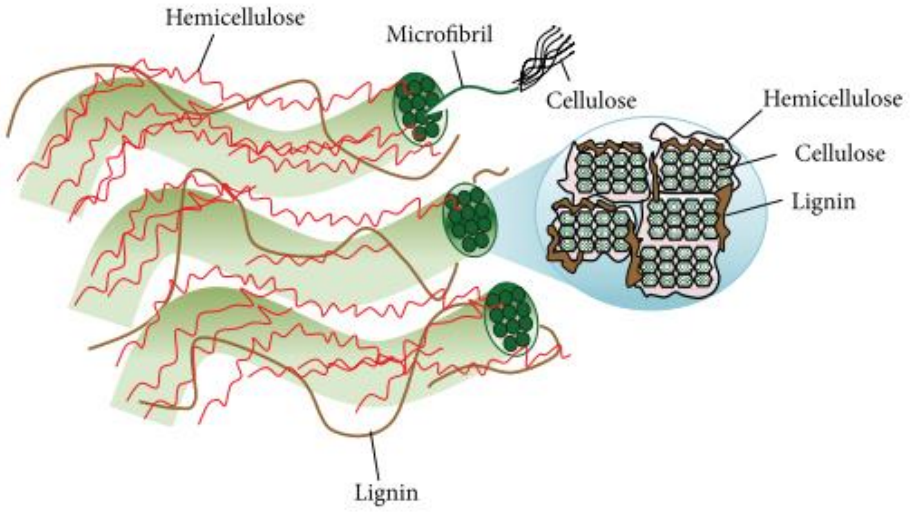


Figure 4.17. Plant cell wall structure. *Structural organisation of lignocellulose within plant cell walls. Enlarged microfibril cross-section shows strands of cellulose enveloped in a matrix of hemicellulose and lignin. Obtained from Lee, Hamid and Zain, 2014.*

Pre-treatment is necessary to liberate the fermentable sugars derived from cellulose and hemicellulose components prior to fermentation of the carbon rich components (Mussatto and Teixeira, 2010; Agbor *et al.*, 2011). Effective utilisation of lignocellulosic biomass requires at least the separation or complete conversion of all lignocellulose components (Philbrook, Alissandratos and Easton, 2013; Teeravivattanakit *et al.*, 2017).

Biological pre-treatment, using purified enzymes or microbial consortia, has potential as a treatment method for lignocellulosic materials, however this process is often time-consuming and costly (Philbrook, Alissandratos and Easton, 2013). Figure 4.18 illustrates the enzyme classes required for the breakdown of the components of lignocellulose.

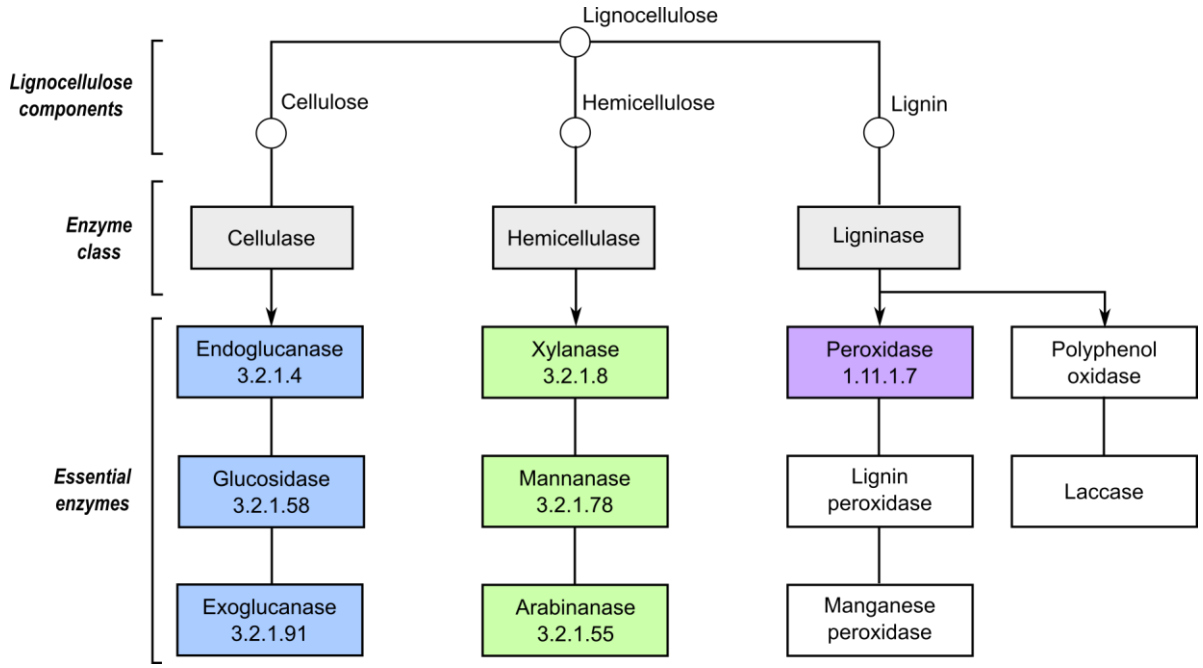


Figure 4.18. Enzymes required for the efficient degradation of lignocellulose. *Lignocellulose is composed of three components, cellulose, hemicellulose and lignin. These compounds form a recalcitrant matrix, resistant to degradation. Rectangles represent enzyme classes necessary for the digestion of each component with coloured rectangles representing gene products present in the analysed metagenome.*

Cellulases (Figure 4.18) target the polysaccharide components of cellulose either via complex extra-cellular enzyme systems or free cellulase, which aim to hydrolyse lignocellulosic substrates by penetrating the substrate structure (Ward, 2015; Rabemanolontsoa and Saka, 2016; Siqueira *et al.*, 2017). Endoglucanases target regions of low crystallinity, producing free chain ends, which is further degraded by the removal of cellobiose units by exoglucanase (Mussatto and Teixeira, 2010). Hemicellulases (Figure 4.18) target linkages between the components of lignocellulose, specifically aimed at polysaccharides, like xylans, mannans and less abundant sources like arabinases (Sindhu, Binod and Pandey, 2016). Peroxidases (lignin and manganese peroxidase) are a major component of the lignolytic enzyme system targeting phenolic and non-phenolic lignin units (Saloheimo *et al.*, 2002). Laccases coupled with peroxidases enable the complete degradation of lignin by catalysing the oxidation of phenolic units and compounds to radicals.

Members of the Kingdom Fungi (Eukaryota), are of particular interest to the bio-energy industry due to their ability to degrade lignocellulosics (Malherbe and Cloete, 2002). However, due to low read abundance, linking specific genera of fungi to specific degradation pathways presented a challenge. The phyla Ascomycota and Basidiomycota represent dominant Eukaryotic phyla within the African elephant gut microbiome. Even though these groups were not linked to a specific metabolic pathway, the role of these groups in lignocellulose degradation is clearly set out in *Chapter 3* (*Section 3.3.2.3*). Some bacterial genera were linked to the enzymes presented in Figure 4.18. Yet, even though bacteria are capable of metabolising the individual components of lignocellulose, catabolising lignocellulose as a whole is unlikely as bacteria often require additional pre-treatment to effectively catabolise the individual components (Mussatto and Teixeira, 2010). *Lactobacillus* species can convert cellulose to lactate via lactic acid fermentation. Whereas members of *Clostridia* and *Bacillus* were linked to hemicellulose bioconversion. *Clostridium* and *Bacteroides* species were also linked to cellulose conversion (Spector, 2009; Zverlov *et al.*, 2010).

The enzymatic hydrolysis of cellulose is strongly influenced by the access of cellulases to cellulose (Sindhu, Binod and Pandey, 2016; Siqueira *et al.*, 2017). Lignin and xylan removal both improve hydrolysis rates, however xylan removal is more favourable than lignin removal as it directly affects glucan chain (polysaccharide derived from D-glucose) accessibility (Sindhu, Binod and Pandey, 2016; Teeravivattanakit *et al.*, 2017; Shimizu *et al.*, 2020). Improved access to glucan chains enables proper utilisation of cellulose by exo- (EC 3.2.1.91) and endoglucanase enzymes (EC 3.2.1.4). This method of degradation could offer an alternative access point to the fermentable sugars derived from cellulose and hemicellulose degradation. The genus, *Gibberella*, of the Ascomycota was linked to xylananse (EC 3.2.1.8), an enzyme involved in xylan digestion. The bacterial genera *Bacteroides* and *Clostridium* were also linked to xylan removal.

Enzymes linked to the degradation of lignin were not detected within the current sample. This does not exclude lignin degradation from the equation, as these sequences may have been lost during data handling due to a low abundance or poor sequence quality. Furthermore, lignin degrading enzymes are often linked to fungal species (Malherbe and Cloete, 2002). Although, Eukaryota are represented within the M5RNA database utilised by MG-RAST, representatives of this domain are far fewer than those of the Bacteria and Archaea, limiting proper identification and annotation (Meyer *et al.*, 2008). Furthermore, of the reads assigned to a taxonomic rank, less than 2 % represent Eukaryota. These factors may contribute to an inaccurate representation of lignocellulose degrading microbes and enzymes.

4.3. Conclusions

Anaerobic digestion offers a viable, industrially relevant pathway for the production of clean energy and value added products (e.g., natural fertiliser). Knowledge about the mechanisms and microbes involved during anaerobic digestion in nature (e.g., ruminants, hindgut fermenters) could improve our ability to produce methane gas from available waste streams such as lignocellulose. The reconstruction of key metabolic pathways involved during anaerobic digestion indicated that the microbial community present in the African elephant gut microbiome possesses the metabolic repertoire to produce methane gas (biogas). This was attributed to the presence of genes coding for enzymes involved in each phase of anaerobic digestion (hydrolysis, acidogenesis, acetogenesis), including all three methanogenesis pathways. Genera employing hydrogenotrophic methanogenesis (e.g., *Methanocorpusculum, Methanoculleus*) were the dominant methanogens within the microbiome. The genus *Methanosarcina*, which employs all three methanogenesis pathways also contributed considerably to the methanogen diversity. The difference in abundance of genes for specific methanogenic pathways may be influenced by the sample source (elephant manure) or point to a preference for specific methanogenesis pathways, possibly due to metabolic constraints within the elephant gut.

The recalcitrant structure of lignocellulose necessitates pre-treatment to facilitate proper access of hydrolytic enzymes. Pre-treatment steps hinder the industrial implementation of biogas produced from lignocellulose. A microbial community that could facilitate biogas production and lignocellulose digestion would alleviate these hindrances. Targeted sequencing of the elephant mycobiome (*Chapter 3*) indicated the presence of lignocellulolytic fungal genera. However, metagenome analysis only identified enzymes involved in cellulose and hemicellulose digestion. Yet, only a small fragment of the data is attributed to Eukaryota (< 2 %), possibly due to limited representatives within reference databases. Nonetheless, xylan removal may offer an alternative method to access the polysaccharide components of lignocellulose. Genes associated with xylanase, an enzyme linked to xylan removal, were detected in the African elephant gut and linked to fungal and bacterial representative genera. Therefore, elephant manure could serve as anaerobic digestion feedstock with the aim of producing methane. However, more data is required to definitively state whether this community could be used to simultaneously treat lignocellulosic biomass.

4.4. References

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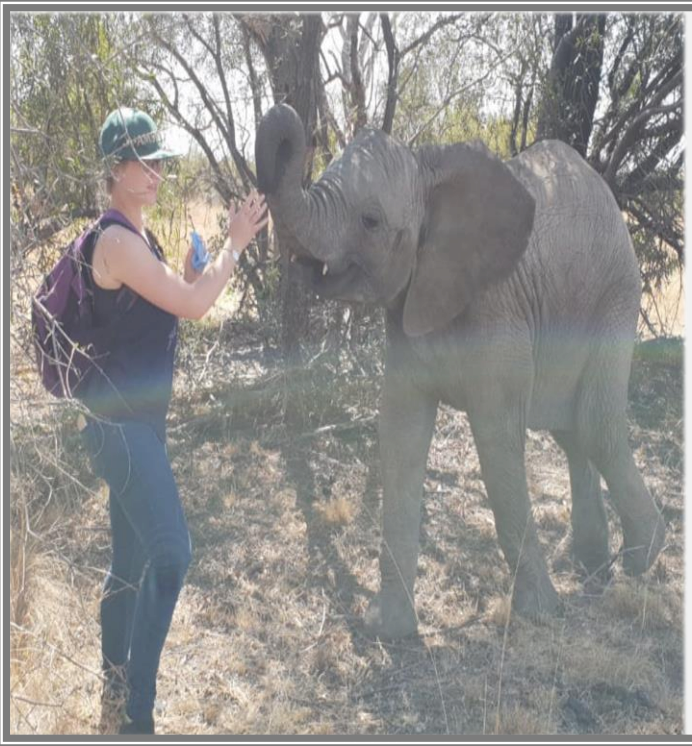

CHAPTER 5

GENERAL CONCLUSIONS

AND

SUMMARY

5. General conclusions and summary

5.1. General conclusions

Biogas is a renewable, green energy source, which could be used to mitigate dependence on traditional fossil fuels and decrease greenhouse gas emissions, especially in a developing country such as South Africa. Foregut- and hindgut fermenting herbivorous mammals employ biochemical reactions, similar to those utilised during the production of biogas, to extract energy from their plant-based diets. These mammals are dependent on the microorganisms housed within their gastrointestinal tracts to facilitate these biochemical reactions.

In this study, the hindgut fermenting African bush elephant's gastrointestinal microbiota was characterised using culture independent methods (high throughput targeted sequencing and metagenomics). Taxonomic diversity analysis showed that the four major bacterial phyla in the African elephant faeces were Planctomycetes (55 - 61.4 %), Bacteroidetes (9.9 - 12.6 %), Firmicutes (13.6 - 21.5 %) and Synergistetes (3.6 - 8.3 %). The Archaeal diversity was dominated by the methanogenic Euryarchaeota (> 99 %). Fungal diversity was dominated by the phyla Ascomycota (> 85 %) and Basidiomycota (< 10 %). Metabolic pathway reconstruction indicated that the gastrointestinal microbiota of the African elephant has the potential to perform anaerobic digestion and that therefore its microbiome could facilitate the production of biogas. The African bush elephant derives energy from a wide variety of complex plant materials and woody vegetation (e.g., tree bark, -roots, fruit), rich in lignocellulose, an attractive and abundant substrate for biogas generation. Genes that code for enzymes capable of digesting the carbohydrate components of lignocellulose (e.g., glucosidases, xylanases) were also detected. An indication that these mega herbivores may possess a gastrointestinal microbiome adapted to extracting energy from lignocellulose-rich compounds that could prove beneficial for the production of biogas from lignocellulosic plant material.

According to our knowledge, this is the only study that has characterised the African elephant gastrointestinal microbiota in such depth and determined its possible application in the bio-energy industry. However, further experimentation is required to determine to what extent this microbial community could digest lignocellulose and the quantity of biogas that could be produced by utilising this community.

5.2. Summary

In summary, this is the first study that has characterised the African elephant gastrointestinal microbiota with the specific aim of evaluating its potential application for the simultaneous degradation of lignocellulose and production of biogas. The results obtained show that these large hindgut fermenters and their gastrointestinal microbiomes have adapted to extracting energy from food that offers little nutritional value. This diet has provided the microbiome with the capacity for cellulose and hemicellulose digestion, two out of the three components of lignocellulose. However, in the current dataset we were unable to identify enzymes capable of lignin digestion. This does not conclusively exclude the ability of the gastrointestinal microbiome to degrade native lignocellulose and points out that further experimentation is required to determine the true capacity of this microbial community to degrade all the components of lignocellulose. The metagenomic results revealed complete metabolic pathways to facilitate each phase of anaerobic digestion. Thus, indicating a community with the metabolic capacity to degrade at least some lignocellulose components and to facilitate the production of biogas.

Key words: African bush elephant, anaerobic digestion, biogas, gastrointestinal microbiome, lignocellulose, *Loxodonta africana*

Supplementary data A

Table A1. *NanoDrop quantification and purity indication of extracted gDNA. The A260/A280 ratio is an indicator of protein or RNA contamination and should ideally be within 1.8 to 1.9. The A260/A230 ratio may indicate the presence of other contaminants, a ratio of around 1.8 indicates pure DNA.*

Sample_ID	[DNA] (ng/µL)	A260/A280	A260/A230
Bela	87	1.84	1.89
Zambezi	114.3	1.72	1.05
Mussina	69.8	1.84	2.05
Shan	65.6	1.73	1.14
Nuanedi	81.6	1.79	1.27
Chova	153.2	1.57	0.62
Chisuru	56.5	1.86	1.8

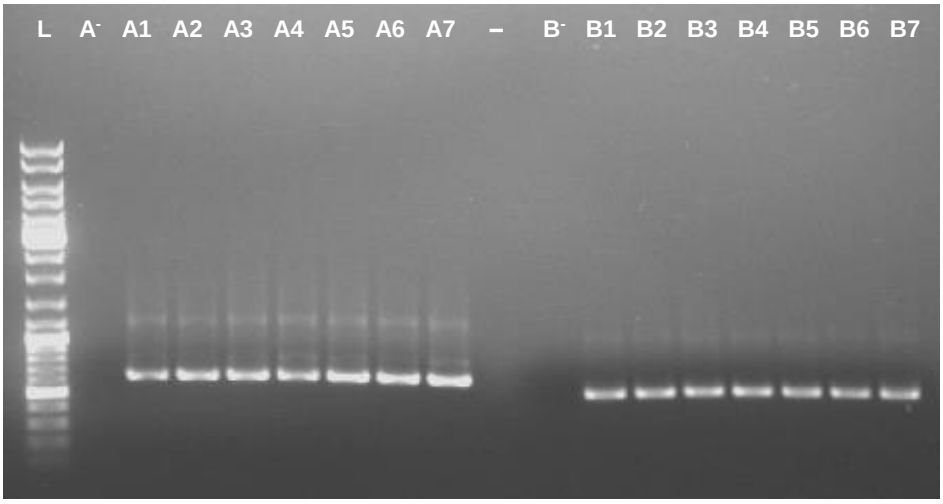

Figure A1. Electrophoresis gel image of amplified 16S rRNA targeting Bacteria and Archaea. *Lane L – DNA ladder; A- - negative control (Archaea); A1 – Bela; A2 – Mussina; A3 – Nuanedi; A4 – Zambezi; A5 – Shan; A6 – Chova; A7 – Chisuru. Lane B- - negative control (Bacteria); B1 – Bela; B2 – Mussina; A3 – Nuanedi; A4 – Zambezi; A5 – Shan; A6 – Chova; A7 – Chisuru.*

Table A2. *DNA concentrations of indexed libraries determined by Qubit 3.0 High sensitivity double stranded DNA (dsDNA) assay.*

Targeted domain	Sample ID	Concentration (ng/µL)
Bacteria	Bela	55.0
	Mussina	53.0
	Nuanedi	51.0
	Zambezi	57.0
	Shan	Out of range*
	Chova	53.0
	Chisuru	52.0
Archaea	Bela	Out of range*
	Mussina	48.7
	Nuanedi	Out of range*
	Zambezi	45.4
	Shan	50.0
	Chova	58.0
	Chisuru	Out of range*
Eukarya (Fungi)	Bela	18.3
	Mussina	20.2
	Nuanedi	18.9
	Zambezi	21.8
	Shan	23.0
	Chova	12.2
	Chisuru	15.0

*Sample concentration out of Qubit detection range (> 60.0 ng/µl)

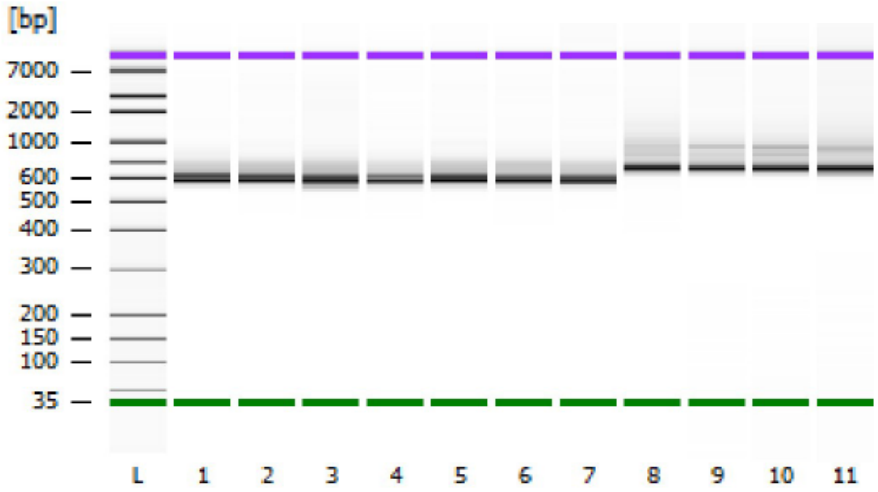

Figure A2. Gel image from Agilent 2100 Bioanalyzer for Bacteria and Archaea. *Lane L – Ladder; lane 1 – Bela (bacteria); lane 2 – Mussina (bacteria); lane 3 – Nuanedi (bacteria); lane 4 – Zambezi (bacteria); lane 5 – Shan (bacteria); lane 6 – Chova (bacteria); lane 7 – Chisuru (bacteria); lane 8 – Bela (archaea); lane 9 – Mussina (archaea); lane 10 – Nuanedi (archaea); lane 11 – Zambezi (archaea).*

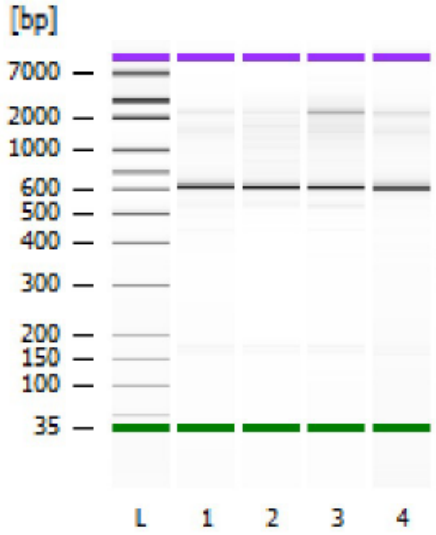

Figure A3. Gel image from Agilent 2100 Bioanalyzer for fungi. *Lane L – Ladder, lane 1 – Bela (Fungal ITS), lane 2 – Mussina (Fungal ITS), lane 3 – Nuanedi (Fungal ITS), lane 4 – Zambezi (Fungal ITS).*

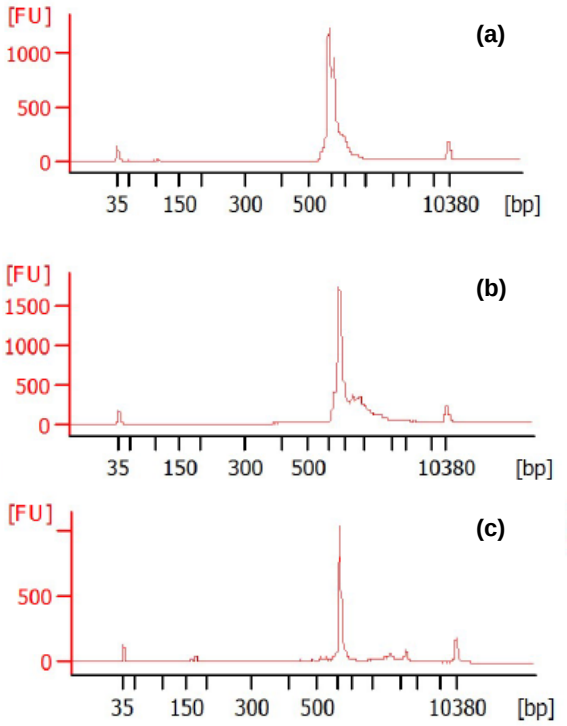

Figure A4. Electropherogram produced by Bioanalyzer. *Electropherogram produced to validate libraries and establish the average amplicon size. A – Bacteria (average size 630 bp); B – Archaea (average size 728 bp); C – Fungi (average size 624 bp).*

Supplementary data B

##Packages utilised

>library(Phyloseq)

>library(Tidyverse)

>library(Reshape2)

>library(vegan)

>library(RColorBrewer)

>library(dplyr)

>library(Biomformat)

set.seed(425)

##Import mapping file

#First column = sample_ID

>Object_1_name<-read.table("file_name.txt", header=T, row.names=1, sep ='\t')

#Convert Object_1 into phyloseq format

>Variable_1_name<-sample_data(Object_1)

#Creates new variable with phyloseq format of Object_1

##Parse Taxonomy Silva

#Specifically for Archaeal and Bacterial sequences

>source("Parse_Taxonomy_Silva.R")

##Load OTU table

#For Archaea and Bacteria

>otufile="file_name.biom"

>biomfile=import_biom(otufile, parseFunction = parse_taxonomy_silva_128)

#For fungal sequences

>Fungi_otufile="file_name.biom"

>Fungi_biomfile=import_biom(Fungi_otufile, parseFunction = parse_taxonomy_greengenes)

```
##Merge the three objects in phyloseq
>Merged_object_name=merge_phyloseq(biomfile,Variable_1)

##Rename columns
#For Archaea and Bacteria
>colnames(tax_table(Merged_object)) = c("Kingdom", "Phylum", "Class", "Order", "Family", "Genus", "Species")

#For fungal sequences
>colnames(tax_table(F_merged_object)) = c("Kingdom", "Phylum", "Class", "Order", "Family", "Genus", "Species","Rank8", "Rank9", "Rank10", "Rank11", "Rank12", "Rank13", "Rank14")

##Extract taxonomy and otu tables from a phyloseq object
#Parameters remain the same for all 3 sequence types unless otherwise stated

>otus<-as(otu_table(Merged_object),"matrix")
>taxonomy<-as(tax_table(Merged_object),"matrix")

##For bacterial sequences
#Filter out NA and Archaea, plus mitochondria and chloroplast
>Merged_object<Merged_object%>%
        subset_taxa(Kingdom=="Bacteria"&
        Family!="mitochondria"&
        Class!="Chloroplast")

##For archaeal sequences
#Filter out NA and Bacteria, plus mitochondria and chloroplast
>A_merged_object<-A_merged_object%>%
        subset_taxa(Kingdom=="Archaea"&
        Family!="mitochondria"&
        Class!="Chloroplast")
```

```
##For fungal sequences
#Filter out NA, Bacteria and Archaea
>F_merged_object<-F_merged_object%>%
        subset_taxa(Kingdom=="Fungi")

##Remove doubletons
#Parameters remain the same for all 3 types of sequences
>Filtered_object_name <-prune_taxa(taxa_sums(Merged_object) > 2,
        Merged_object)

##Rarefy
#Parameters remain the same for all 3 sequence types
>Rarefy_object_name <-rarefy_even_depth(Filtered_object,
        rngseed=FALSE)

##Stacked barplots
#Phylum level
>prune_phylum_name <- Rarefy_object %>%
        tax_glom(taxrank="Phylum") %>%
        transform_sample_counts(function(x) {x*100/sum(x)}) %>%
        psmelt() %>%
        filter(Abundance > 1) %>%
        arrange(Phylum)

>phylum_colors <- c("#CBD588", "#5F7FC7", "orange","#DA5724", "#508578", "#CD9BCD",
        "#AD6F3B", "#673770","#D14285", "#652926", "#C84248",
        "#8569D5", "#5E738F","#D1A33D", "#8A7C64", "#599861")

>prune_phylum$Phylum <- factor(prune_phylum$Phylum,
        levels = unique(prune_phylum$Phylum))
```

```
>ggplot(prune_phylum, aes(x=Description, y=Abundance, fill=Phylum)) +
        geom_bar(stat="identity", position="fill", width=0.4) +
        facet_wrap("Mapfile_parameter (e.g., sex)", nrow=1, scales="free_x",strip.position
        ="bottom")+
        scale_fill_manual(values=phylum_colors) +
        theme_bw() +
        theme(strip.text.x = element_text(colour = "black"))+
        scale_y_continuous(labels=scales::percent)+
        theme(text = element_text(size=15))+
        theme(axis.title.x = element_blank()) +
        theme(axis.title.x = element_blank(), axis.text.x = element_text(angle=90, hjust=1,
        colour = "black"), panel.background = element_rect(fill = 'white', colour = 'white')) +
        theme(axis.text.y = element_text(colour = "black")) +
        guides(fill = guide_legend(reverse = FALSE, keywidth = 1, keyheight = 1)) +
        ylab("Relative Abundance (Phyla > 1%) \n") +
        ggtitle("Bacterial Composition") +
        labs(fill = “Phyla”) +
        theme(legend.position = “bottom”) +
        theme(strip.text = element_text(face = “bold”, size = 10, lineheight = 5.0))

#Save/export bar plot
>ggsave("Phyla_bac.png", plot_name)

##Rarefaction curves
>source("rarefaction.R")
>p <- ggrare(Merged_object, step = 1000, color = "Description", label = NULL, se = FALSE) +
        theme_bw()
>plot <- p + scale_color_manual(values = getPalette_Samples(colourCount_Samples)) +
        theme_bw()

#Save/export rarefaction curve
>ggsave("Rarefaction_bac.png", p, width = 15, height = 10)
```

##Permanova

#Calculate Bray-Curtis distance matrix

>Matrix <- phyloseq::distance(Rarefy_object, method = "bray")

#Import metadata (text file) as object

#Convert metadata into phyloseq format

>Sampledata <- sample_data(metadata_object_name)

#Adonis test

>adonic(Matrix ~ metadata_category*, data = metadata_object_name)

*Category examples for this study include maturity, sex and ages (< 10, < 20 and < 30)

Supplementary data C

Table C1. *Statistical values obtained through dissimilatory testing to establish whether the differences noted in microbial diversity among the test group were statistically relevant. A p-value of < 0.05 would indicate statistical significance.*

	Age		Maturity		Sex	
Microbial Group	R^2-value	*p*-value	R^2-value	*p*-value	R^2-value	*p*-value
Archaea	0.26	0.55	0.40	0.09	0.15	0.45
Bacteria	0.33	0.39	0.07	1	0.12	0.71
Fungi	0.29	0.86	0.16	0.52	0.17	0.39

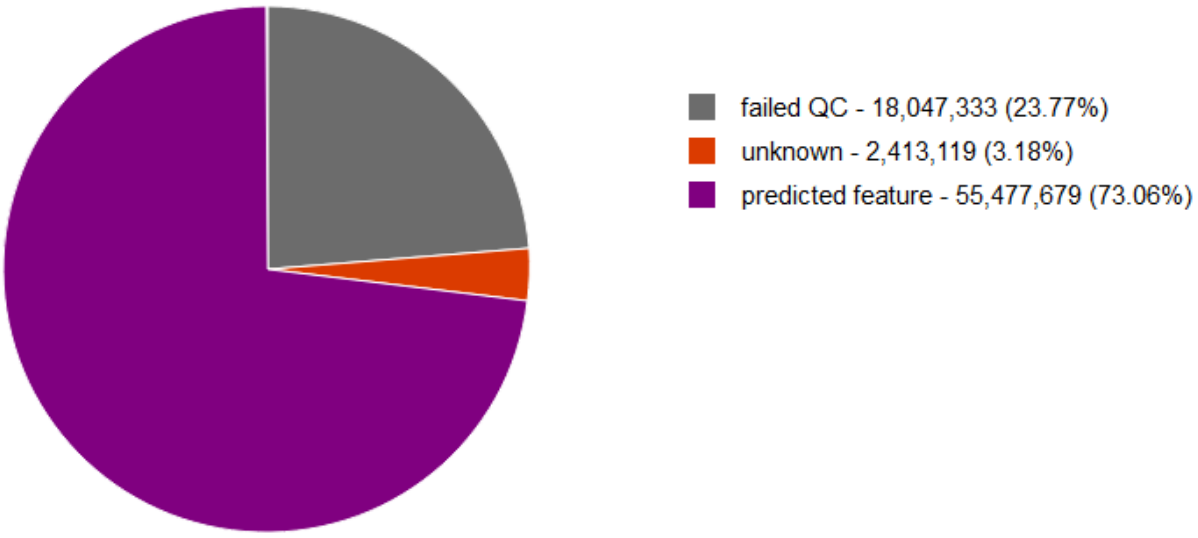

Figure C1. Pie chart illustrating sequence breakdown. *Of the original metagenomic dataset, 23.77% of the sequences failed the initial quality control measures, 73.06% indicate predicted protein features and 3.18 % remain unknown. Image generated via MG-RAST pipeline.*

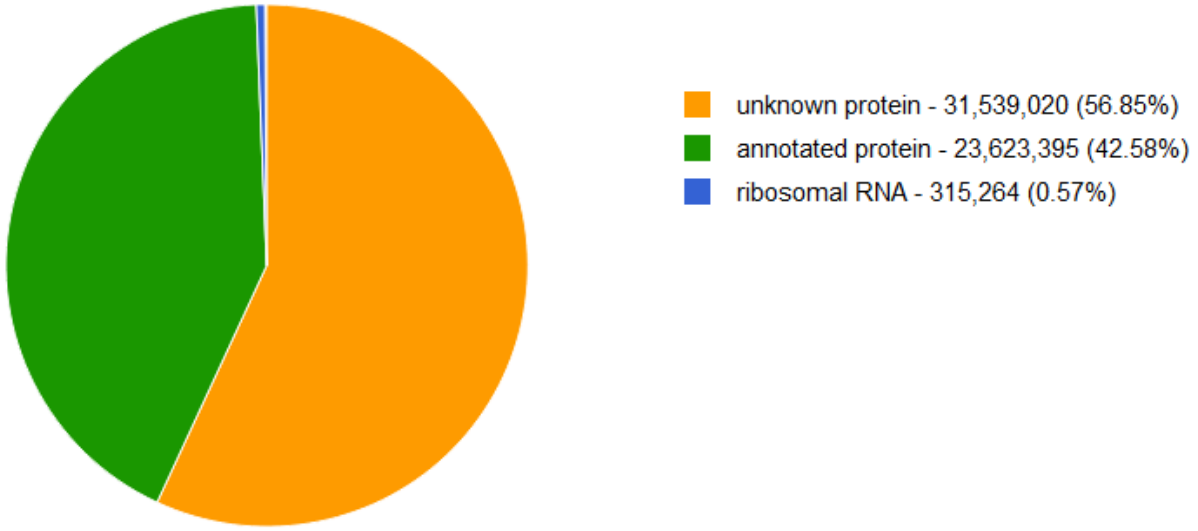

Figure C2. Chart illustrating predicted features. *Coding regions were identified according to a reference based method against the M5RNA database (SILVA, GreenGenes, RDP). Image generated via MG-RAST pipeline.*

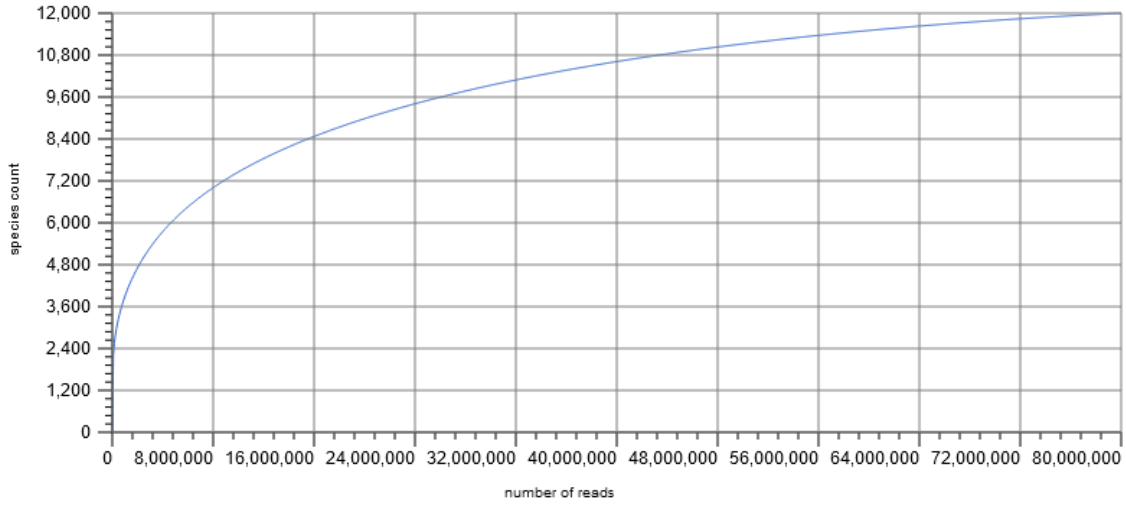

Figure C3. Data rarefaction curve. *The general trend of the curve, which slants to the right and approaches a saturation plateau indicates proper coverage of the species diversity. Additional reads would likely not greatly affect the currently observed diversity. Image generated via MG-RAST pipeline.*

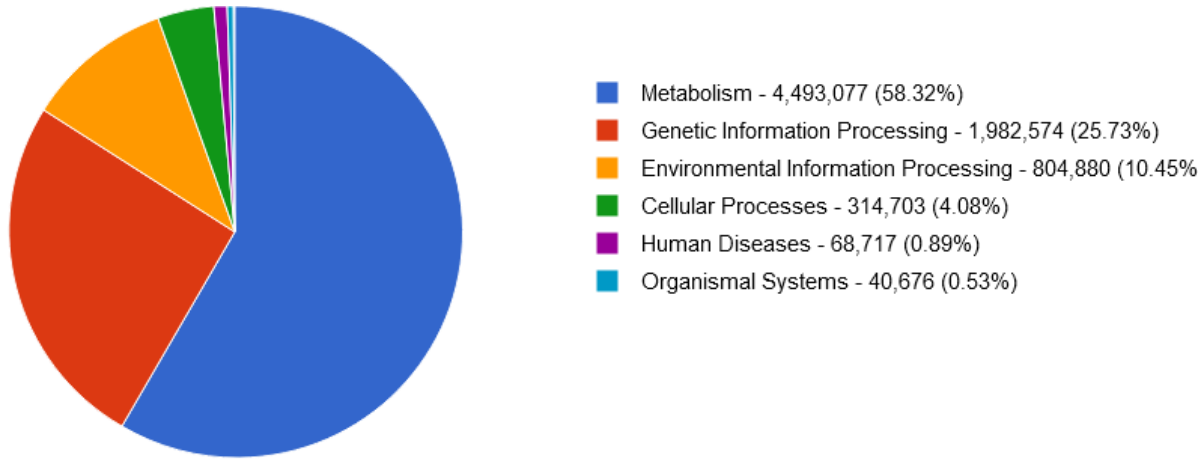

Figure C4. Distribution of functional categories at the highest level supported by the hierarchy of the KO database. *Each wedge or slice represents the percentage of reads with predicted protein functions annotated to a category determined by the KO database. Image generated via MG-RAST pipeline.*

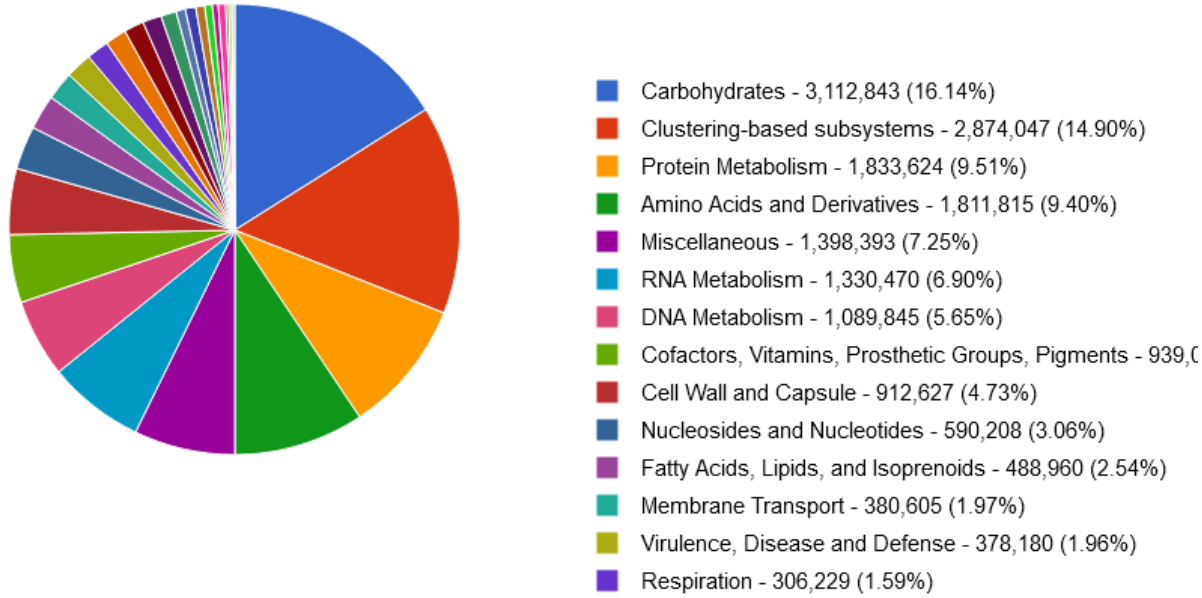

Figure C5. Distribution of functional categories at the highest level supported by the hierarchy of the SEED subsystems database. *Each wedge or slice represents the percentage of reads with predicted protein functions annotated to a category determined by the SEED database. Image generated via MG-RAST pipeline.*

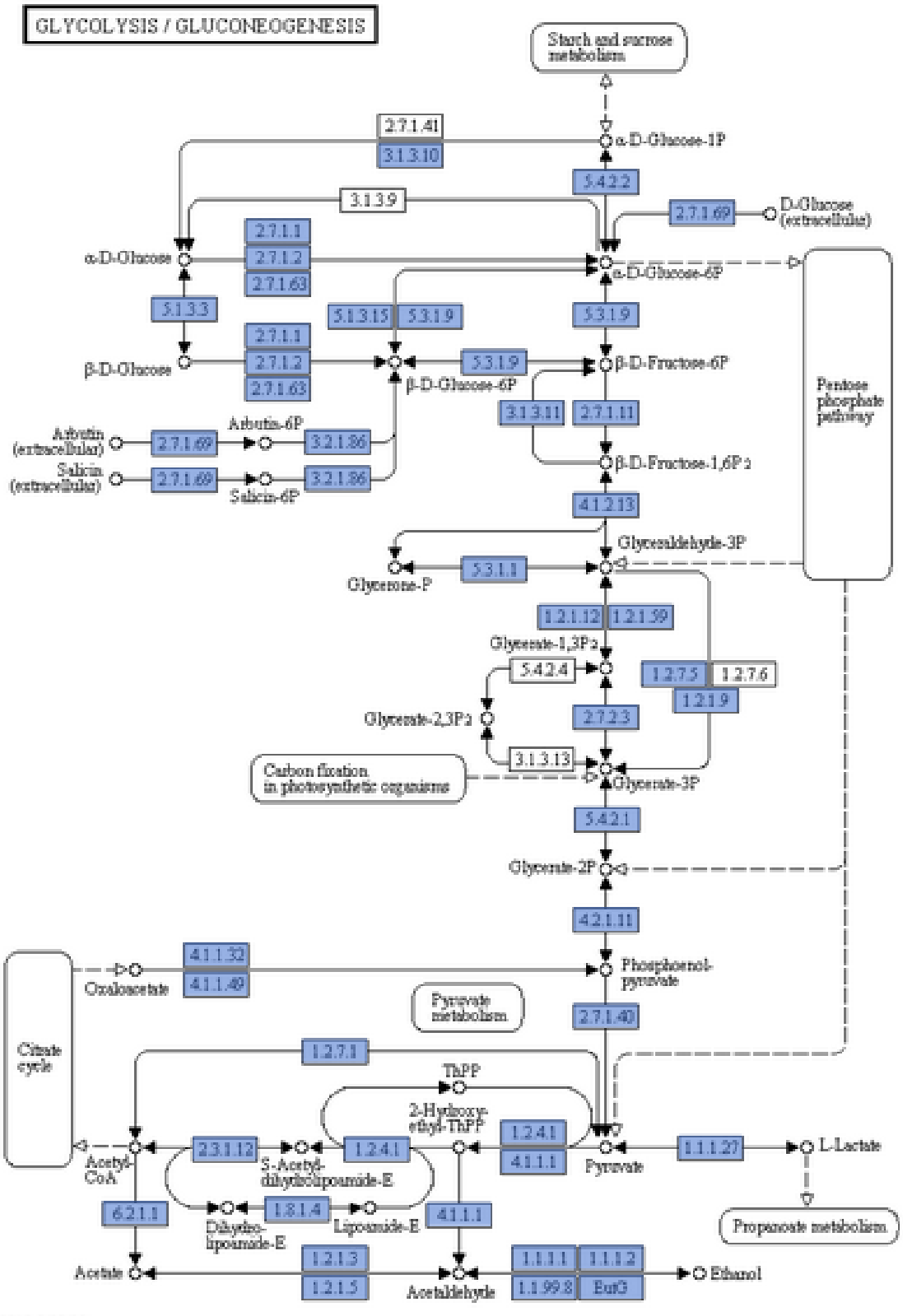

Figure C6. Reconstructed biochemical glycolysis/gluconeogenesis (ksa00010) KEGG pathway. *Numbered rectangles represent gene products/enzymes necessary to catalise reactions within the pathway. Blocks coloured blue are present within the analysed metagenome. Image obtained from MG-RAST KEGG plugin.*

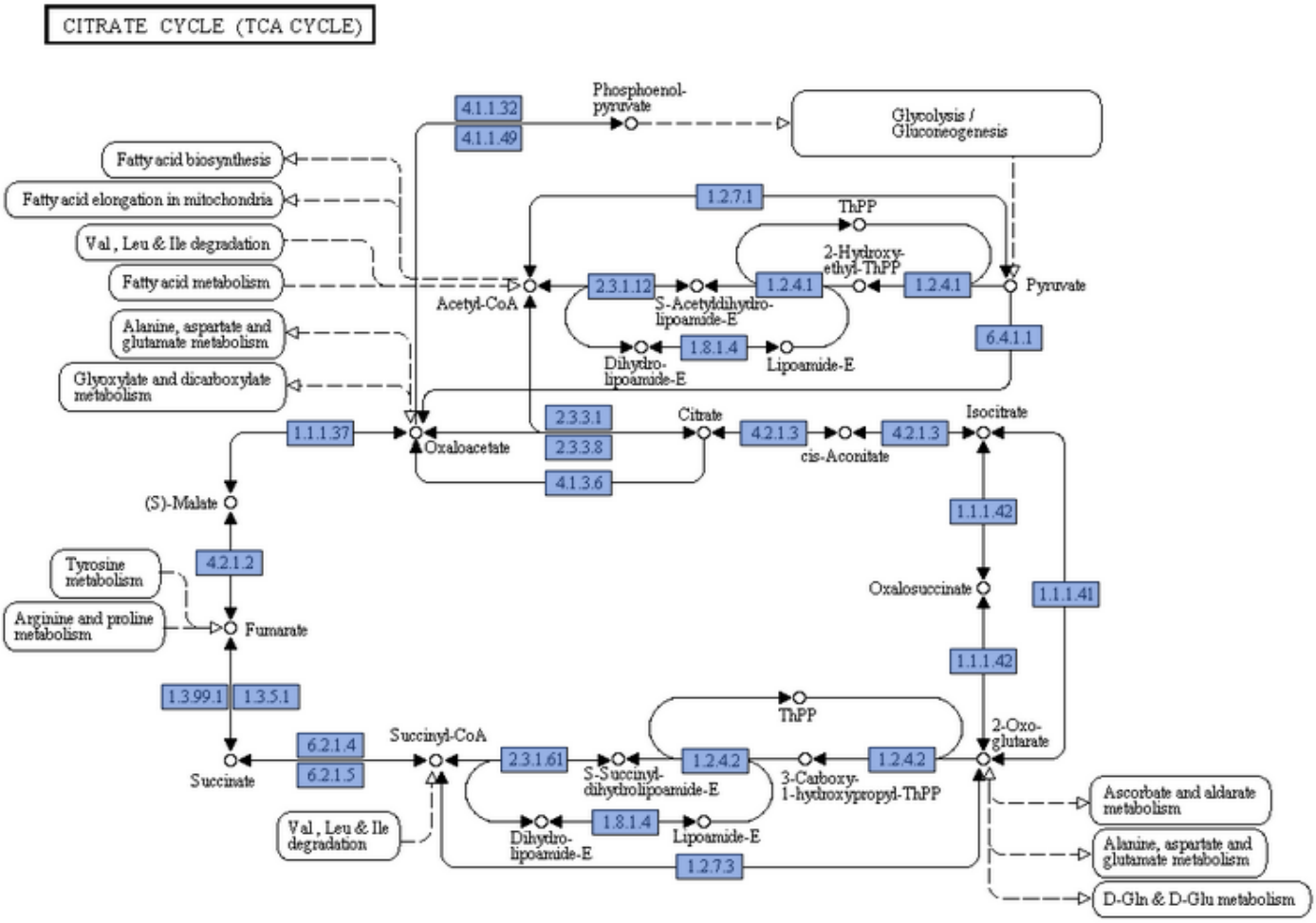

Figure C7. Reconstructed citrate cycle (TCA cycle) (ksa00020) KEGG pathway. *Numbered rectangles represent gene products/enzymes necessary to catalise reactions within the pathway. Blocks coloured blue are present within the analysed metagenome. Image obtained from MG-RAST KEGG plugin.*