ECOLOGICAL GUILD OF MICROBES THAT DRIVE PRODUCTION OF BIOGAS FROM MULTIPLE FEEDSTOCK

by

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DEDICATION

This dissertation is dedicated to my beloved mother Josephine Tshilidzi Nemaguvhuni, aunt Asnath Nemaguvhuni and my late father Phophi Alfred Mukhuba for being good parents throughout my educational career.

DECLARATION

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I declare that the above dissertation/thesis is my own work and that all the sources that I have used or quoted have been indicated and acknowledged by means of complete references.

_____ _____

SIGNATURE DATE

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"The fear of the Lord is the beginning of knowledge, but fools despise wisdom and instruction''.
Proverbs 1:7

RESEARCH OUTPUT

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- **Mukhuba M*,** Roopnarain A, Adeleke R and Moeletsi M**.** Agricultural Research Council (ARC), Professional and Development Programme annual Conference. The effects of microbial composition in the production of biogas from multiple feedstock.17-19th October 2015. Pretoria, South Africa.
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- **Mukhuba M*,** Roopnarain A, Adeleke R and Moeletsi M**.** Annual National Renewable and Sustainable Energy Post Graduate symposium (REPS 2016). Potential use of microorganisms isolated from digestate (slurry) for the promotion of plant growth. University of Fort Hare, 05-06 September 2016.
- **Mukhuba M*,** Roopnarain A, Adeleke R and Moeletsi M**.** Agricultural Research Council (ARC), Professional and Development Programme annual Conference. Potential use of microorganisms isolated from digestate (slurry) for the promotion of plant growth, 24-25th October 2016.Pretoria, South Africa.
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2. Publications

- **Mukhuba, M.,** Roopnarain, A., Adeleke, R., Moeletsi, M. and Makofane, R., 2018. Comparative assessment of bio-fertiliser quality of cow dung and anaerobic digestion effluent. *Cogent Food & Agriculture*, *4*(1), p.1435019 (Published)**.**

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ABSTRACT

Anaerobic digestion (AD) is becoming a widely adopted technology for conversion of organic waste and nutrient-rich fertiliser production due to its cost-effectiveness and sustainability. In this study, a batch experiment was conducted using five different types of food waste and cow dung (CD). No significant difference was observed among the four substrates that produced the highest methane ($P<0.05$). Based on the batch experiment results, two substrates were selected for semi-continuous digestion and the highest methane yield (67%) was obtained from co-digestion (CO). PCR-DGGE results revealed higher bacterial and archaeal diversity indices in CO as compared to mono-digestion of CD and mixed food waste. The high-throughput sequence analyses revealed that the Operational Taxonomic Units (OTUs) belonging to the phyla *Bacteroidetes*, followed by *Firmicutes*, *Actinobacteria* and *Proteobacteria*, were dominant in all treatments. The enhanced methane production in CO could be attributed to the neutral pH and partial shift of archaea from *Methanosaeta* to *Methanosarcina*. The digestate and fresh CD were screened for plant growth promoting bacteria (PGPB), nutrient and heavy metal content. The dung contained higher concentrations of heavy metals ($P<0.05$) and potential pathogens in comparison to the digestate. The use of digestate may, therefore, enhance soil fertility with minimal negative environmental effects.

Key words: Anaerobic digestion, cow dung, plant growth promoting bacteria, PCR-DGGE, heavy metals, co-digestion, digestate, food waste.

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LIST OF ABBREVIATIONS

AD	Anaerobic digestion
ADL	Acid detergent lignin
ADF	Acid detergent fibre
CD	Cow dung
CH_4	Methane
CO	Co-digestion
CO_2	Carbon dioxide
CSTR	continuous stirred tank reactor
DEA	Department of environmental affairs
DGGE	Denaturing gradient gel electrophoresis
FISH	fluorescent in situ hybridisation
FVWs	fruit and vegetable wastes
GHG	Greenhouse gas
GW	Gigawatts
HRT	Hydraulic retention time
K	kelvin
KPa	Kilopascal
m^3	Cubic metre
MFW	Mixed food waste
mg	milligram
Min	Minutes
NDF	Neutral detergent fibre
^oC	Degree Celsius
PCR	Polymerase chain reaction
QIIME	Quantitative insights into microbial ecology
RFLP	Restriction fragment length polymorphism
SA	South Africa
SABIA	South African Biogas Association
Sec	Seconds
SSCP	single strand conformation polymorphism
TGGE	Temperature gradient gel electrophoresis

TS	Total solids
VS	Volatile solids
W/V	Weight per volume
WTE	Waste-to-energy

CHAPTER 1: GENERAL INTRODUCTION

1.1 Background

South Africa (SA) relies mostly on non-renewable sources such as natural gas, oil and coal to meet its energy demand (Figure 1.1), with coal providing 72% of the energy (Mwakasonda, 2007; BP, 2014). The state-owned company Eskom generates about 95% of its electricity from coal (Eskom, 2013). The South African Department of Environmental Affairs (2013) reported that the energy sector is the main contributor of greenhouse gas (GHG) emissions, with more than 70% emitted in the year 2000 and 78% in 2010. SA is therefore one of the countries with the highest GHG emissions in the world (Department of Environmental Affairs, 2013). This is an indication that SA is over-reliant on non-renewable energy.The use of non-renewable energy resources is a global challenge because of the negative effect of GHG emissions on the environment (Thassitou and Arvanitoyannis, 2001; Franke-Whittle *et al*., 2014; Roopnarain and Adeleke, 2017).

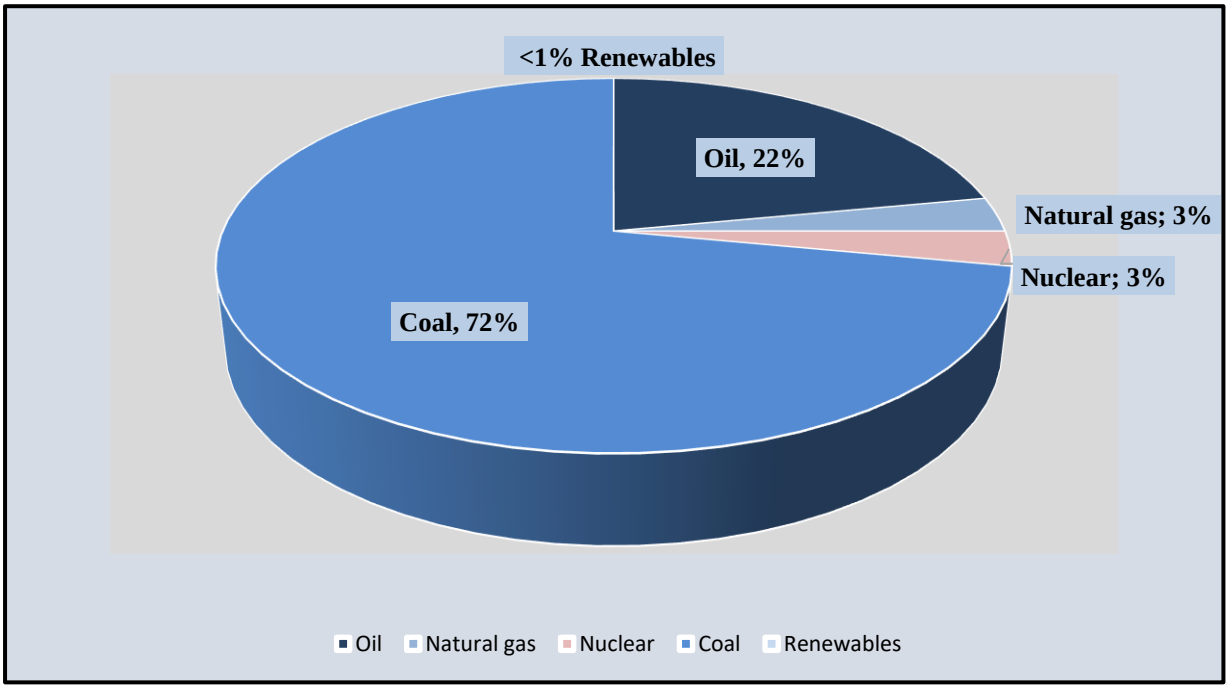


Figure 1.1: Total energy consumption in SA 2013 (BP, 2014)

Furthermore, SA is also facing the challenge of uncontrolled waste production. In 2011, it was reported that SA generated 108 million tons of waste, 59 million tons of which were municipal waste, more than 10% was categorised as organic waste and about 35% classified as non-recyclable (Department of Environmental Affairs, 2012). Poor management of such waste, especially animal manure and agricultural and municipal waste, is a major cause of environmental pollution (Pardo *et al*., 2017). Moreover, more than 89% of waste produced in SA is landfilled (Department of Environmental Affairs, 2012). Disposal of solid waste in landfills is still a popular method of waste management and the decomposition of landfill waste leads to the release of GHGs (AGAMA 2009; Van Rooy *et al*., 2013). Another environmental risk of landfill sites is the production of leachate that carries contaminants such as heavy metals, posing the danger of ground water contamination (Shin *et al*., 2001; Curry and Pillay, 2012). Furthermore, owing to a growing population that requires land for residential, agricultural and industrial development landfilling is no longer an option as there is limited space.

Environmental pollution, increases in energy prices and waste management are challenges that triggered significant interest in waste-to-energy (WTE) technologies (Seadi *et al*., 2013; Franke-Whittle *et al*., 2014). These technologies include thermo-chemical processes (gasification, combustion and pyrolysis) and biological processes that consist of anaerobic digestion (AD) and fermentation (Gumisiriza *et al*., 2017). Anaerobic digestion, especially treating substrates that contain a high moisture content like food waste (fruit and vegetable waste), is ideal and eco-friendly compared to thermo-chemical processes (Sitorus *et al*., 2013; Gumisiriza *et al*., 2017).

Biogas is the key product from AD process. The AD process takes place in an oxygen-free environment. This process employs a consortium of microorganisms such as methanogens to break down organic matter to produce biogas and nutrient-rich fertiliser called slurry or digestate (Asikong Bassey *et al*., 2013; Roopnarain and Adeleke, 2017). The AD process occurs in four stages: hydrolysis, acidogenesis, acetogenesis and methanogenesis (Yu *et al*., 2014) at different temperature ranges. The commonly used temperature ranges are the mesophilic (20 ℃ -40 ℃) and the thermophilic temperature range (50 ℃ -65 ℃) (Al Seadi et al., 2008).

Biogas is a combustible gas that consists of about 60% methane (CH_4) and 39% carbon dioxide (CO_2) with trace amounts of water vapour, hydrogen, sulphide and ammonia (Sitorus and Panjaitan, 2013). Biogas is used as a source of energy for cooking, generating electricity and heating in households (Kumar, 2012; Kigozi *et al*., 2013).

There are a vast number of feedstocks that can be used for biogas production, including animal waste, food waste, crop residues, industrial waste and sewage sludge (Appels *et al*., 2011). However, AD is currently not used to its full potential because of a number of shortcomings, such as controlling operational conditions, improved biodigester designs and, most important, maintaining a favourable environment for microorganisms. The microbial communities involved in each stage of the AD process play an important role in converting organic wastes (Franke-Whittle *et al*., 2014). In spite of the increasing global acceptance of AD technology, intensive research on microbial diversity is still needed (Franke-Whittle *et al*., 2014).

1.2 Aim of the study

The aim of the study is to investigate microbial drivers of biogas production when FW and CD are used as feedstock.

1.3 Hypotheses

- There is increased microbial diversity when FW and CD are co-digested compared to mono-digestion of either FW or CD
- There is a link between microbial diversity, choice of substrate and biogas yield

1.4 Specific objectives

- To conduct batch mesophilic AD experiments using different types of FW and CD for biogas production
- To determine the quantity of biogas production from mono and co-digestion of CD and FW using semi-continuous digestion
- To determine the diversity and abundance of microbes involved in the AD process and relate to the biogas production

- To determine the fertilizer potential of anaerobic digestate

1.5 Study rationale

Renewable-energy use is increasing significantly because of the escalating energy crisis, efforts to mitigate the environmental impact of conventional fuels and waste disposal challenges (Mao *et al*., 2015), and more research is being done into alternative biomass for energy production through, for instance, AD. The conversion of biomass to biogas is dependent on the interaction of microorganisms in the digester (Akuzawa *et al*., 2011; Franke-Whittle *et al*., 2014). Many of the research studies that have been conducted focused on the performance of the AD digester, biogas production and the quality of the digestate (Weiland, 2010; Alfa *et al*., 2014; Roopnarain and Adeleke, 2017). However, AD is a microbe-driven process and therefore the microbiology of the process should be regarded as the most important aspect of the AD process (Roopnarain and Adeleke, 2017).

Studies conducted by Lee *et al*. (2009), Pycke *et al.* (2011) and Franke-Whittle *et al.* (2014) reported that although biogas technology is now receiving more attention, information about the microbial diversity of the actual process is insufficient. Weiland (2010) reports that little is known about the interaction of microorganisms that govern the overall process in the digester, with only an insignificant percentage of archaea and bacteria isolated. Therefore, in order to optimise conditions in the biogas digester, knowledge of the ecology and functions of the microbial communities involved in each step of the AD process is required for better control of the biological processes.

CHAPTER 2: LITERATURE REVIEW

2.1 Introduction

Energy is one of the basic needs of today's society. The demand for energy is increasing every day because of rapid global economic and population growth. In SA, inadequate energy supply remains a challenge and the country has experienced frequent load shedding. Energy generation is mainly from non-renewable resources (Roopnarain and Adeleke, 2017). A non-renewable resource is defined as a resource that cannot be replaced once consumed, for example natural gas, coal and oil, while renewable energy is naturally replenished; examples include biomass, solar energy, water and wind (De Vries *et al*., 2007).

The majority of people in developing countries, including SA, do not have easy access to fossil-derived energy because of poverty. They depend on traditional biomass such as firewood and the combustion of dried manure for cooking and lighting, especially in rural areas (Gashaw, 2016). A study conducted by the International Energy Agency (2009) estimated that more than two billion people worldwide use traditional biomass as the most accessible form of energy for cooking, while more than one billion people do not have access to electricity. Biomass can be used directly or indirectly as a source of energy (Muvhiiwa *et al*., 2016). Direct burning of traditional biomass like firewood contributes to GHG emissions and produces harmful fumes that can result in health-related problems (Adelekan and Adelekan, 2004) such as mild respiratory illness and lung cancer (Ezzati, 2005).

Energy poverty mostly affects women and young girls, who often have to for walk long distances to collect firewood (Clancy *et al*., 2002). Overcoming energy poverty is linked to achieving one of the Millennium Development Goals, namely, making sustainable energy accessible (World Future Council, 2010). Sustainable and cost-effective ways of producing renewable energy from biomass have therefore gained interest (Ahmad *et al*., 2016). Biomass as a renewable energy source consists of all forms of organic material derived from plants, including land-based vegetation and trees, different waste materials such as municipal solid waste (MSW), sewage and animal manure, forestry and agricultural residues, and industrial

waste (Sriram and Shahidehpour, 2005; Rao *et al*., 2010). Such biomass generally consists of carbon, hydrogen, oxygen and small amounts of sulphur (Rao *et al*., 2010). When compared to other renewable energy resources, biomass is cheap (Schröder *et al*., 2008). Biomass can be used indirectly by converting it into various bioenergy forms such as bioethanol, biodiesel and biogas (Saxena *et al*., 2009). These forms of bioenergy have received much attention throughout the world. The production of biogas from biomass using the AD process is more advantageous to the rural population in Africa than other forms of bioenergy production (Weiland, 2010; Okudoh, 2014).This is due to an abundance of substrates available for the AD process and suitable climatic conditions for biogas digester operation.

2.2 History of AD

Anaerobic digestion of organic waste is one of the oldest technologies (Chen *et al*., 2016). This technology has been used since the 10th century B.C in Assyria, where it was used for heating up bath water (He, 2010; Bond and Templeton, 2011). Jan Baptista Van Helmont first experimented on the release of CH_4 from decaying organic matter in 1630 (Khoiyangbam *et al*., 2011). The year 1808, the production of CH_4 using cattle manure was demonstrated by Sir Humphry Davy in AD process (Lusk, 1997). Anaerobic digesters were built in Bombay (India) and England in 1859 (Bond and Templeton, 2011).

In 1921, Guorui Luo built an 8 m^3 hydraulic biogas tank fed with household waste and used the energy for cooking and lighting. In 1929, he founded a company, named Zhonghua Guorui Gas Lamp, in Shantou and in 1931 the company extended its operations to Shanghai to popularise biogas technology (He, 2010). Later, in the 1950s, the use of AD slowed down because fossil fuels became increasingly available at relatively low prices. Anaerobic digestion as an energy source again attracted attention as an alternative to fossil fuels in early 1970s owing to the rising prices of non-renewable resources (Ni and Nyns, 1996). In Africa, the AD technology gained a foothold in the early 1950s in countries such as Kenya (Amigun *et al*., 2012). In SA, Mark Fry built the first digester in 1957, producing biogas from pig manure (Amigun and Von Blottnitz, 2010).

2.3 Overview of biogas in South Africa, Africa and the world

2.3.1 Biogas in South Africa

South Africa is a developing country with many industries. It is situated on the southern part of the African continent (Mwakasonda, 2007) and has a population of about 54 million in 2015 (Statistics S.A, 2015). It is estimated that up to 300 000 homes in SA own more than one head of cattle without electricity (Brown, 2006). A study conducted by Brown (2006) estimated that more than 40 % of schools in SA have no electricity.

No fewer than 200 anaerobic digesters have been installed in SA (Tiepelt, 2013). Reasons for the slow spread of biogas technology include a lack of information about the technology, lack of a trained labour force, high capital expenditure on setting up commercial plants, and generally inadequate and unreliable government support policies (Agama Energy Pty, 2008). However, biogas technology in SA is gaining popularity because of environmental challenges, load shedding and escalating non-renewable energy costs.

Several companies have been playing a role in the installation of biogas digesters in SA and these include AGAMA Energy, Finishes of Nature, Nova biogas, TPA Biogas and Africa Green Energy. Furthermore, the National Energy Regulator of SA has registered about 38 biogas plants since 2011. These plants are located mainly in rural areas of the Gauteng, Free State, KwaZulu-Natal, Limpopo and Western Cape provinces of SA (NERSA, 2013). In addition, through establishment of the plants about 1700 jobs have been created. The largest biogas plant that has been built thus far in SA is the Bronkhorstspruit plant that has been developed by Bio2Watt (waste-to-energy company) which generates about 4.4 MW of electricity (Bio2Watt, 2014).

Another interesting biogas plant is the 8 m^3 community scale bio-digester built in Giyani (Mpfuneko CS's rural domestic biogas) in the Limpopo province. This was established in 2007 by Jotte van Ierland in collaboration with some members of the Gawula village. The plant uses sewage and CD as the major substrate for AD (Roopnarain and Adeleke, 2017). The South African Biogas Association (SABIA) stated that biogas potential in SA has a market potential

of 10 billion and can generate about 2 gigawatt (GW) of electricity as well as thousands of jobs (Da Silva, 2013).

2.3.2 Biogas in Africa

The second biggest continent after Asia is Africa, on which more than 10 % of the world's population lives (International Energy Agency, 2014). In Africa, access to energy resources is still a huge challenge (Mshandete and Parawira, 2009). Most of the sub-Saharan African countries rely mainly on biomass combustion to generate energy (International Energy Agency, 2014). Overpopulation has led to overexploitation of traditionally available biomass and to an increased need for arable land for food production (Mulinda *et al*., 2013). Furthermore, collecting traditional biomass such as firewood in forests is risky and always requires hard labour (Figure 2.1), so energy generation by means of AD of waste biomass ought to be a viable alternative.

Figure 2.1: African women collecting fire wood from the forest (http://newsofthesouth.com/wp-content/uploads/2015/04/four-women-carrying-firewood.jpg) [Accessed 14/2/2017]

Africa has the potential for installing biogas digesters, but the adoption rate of the technology is still low (Amigun and Von Blottnitz, 2002). Biogas digesters have to date been installed in several sub-Saharan countries, such as Burkina Faso, Ethiopia, Kenya, Tanzania, Uganda and Cameroon, as shown in Table 2.1 (Pandey *et al.*, 2007; Amigun and Von Blottnitz, 2007). However, very few of these digesters are working properly because of poor maintenance, inexperienced labour and limited knowledge of biogas technology (Parawira, 2009).

Table 2.1: Number of biogas digesters installed in Africa

Country	programme took off in	No. of biogas plant installed in 2008	No. of biogas installed in 2009	cumulative number of biogas plants installed up to 2009
Rwanda	2007	120	213	434
Ethiopia	2008	98	30	128
Kenya	2008	-	3	3
Tanzania	2008	3	103	106
Uganda	2008	-	40	40
Burkina Faso	2009	-	1	1
Cameroon	2009	-	23	23

2.3.3 Biogas in the world

A large number of operational biogas plants are found in Europe (Al Seadi *et al*., 2008). Countries such as Germany, Austria, Denmark and Sweden have the largest number of modern biogas plants (Al Seadi *et al*., 2008). Four GW plant of total biogas power has been installed in Germany (Wagner, 2015), which contributes about 3% of the total electricity required in the country (Fachverban, 2011).

In other parts of the world such as China and India, AD technology is mostly used to generate energy on a smaller scale (Curry and Pillay, 2012). Approximately four million household bio-digesters have been installed in India and this created about 85 000 jobs (IRENA, 2015). In China, about 100 000 large-scale bio-digesters and 43 million rural household bio-digesters were operational in 2014 (IRENA, 2015). Biogas digesters have also been installed in the United States and Canada, Argentina, Peru, Brazil, Chile and Mexico (Comparetti *et al*., 2013).

2.4 The microbiology of AD

Microbial communities consisting of bacteria and methanogenic archaea play vital role in biogas production (Town *et al*., 2014). Their metabolic activities and diversity are influenced by the composition of the feedstock, environmental factors and the operating mode of the digester (Yu *et al*., 2014). The entire AD process can be divided into four stages based on the microbial composition and activities. Each step in AD is accomplished by the guild of microorganisms. Ecological guild refers to the group of species that exploit the same resources, or who exploit different resources in related ways (Simberloff and Dayan, 1991). The microbes are strongly linked to drive four stages of AD including hydrolysis, acidogenesis, acetogenesis and methanogenesis as shown in Figure 2.2 (Ritari *et al*., 2012; Yu *et al*., 2014).

2.4.1 Hydrolysis

This is the initial stage of the AD process. In this stage, fermentative bacteria convert the macromolecules such as proteins, carbohydrates and fats into soluble molecules such as sugars, amino acids and fatty acids (Ralph and Dong, 2010). The breakdown of these molecules is achieved with the aid of different extracellular enzymes secreted by the microorganisms (Ali Shah *et al*., 2014). Examples of extracellular enzymes used for macromolecule breakdown

include proteinases, cellulases, hemicellulases, amylases, lipases and pectinases (Weiland, 2010; Ali Shah *et al*., 2014). Bacteria that are active in hydrolysis include the genera *Bacteriodes, Clostridium, Peptostreptococcus, Acetivibrio, Streptococcus* and *Enterobacterium* (Ali Shah *et al*., 2014).

The hydraulic retention time, which is the average time that the substrate is retained in the digester, is determined by the type or nature of the substrate. Hydrolysis is generally the rate limiting stage of AD, that is, the time required for the breakdown of the substrate (Kondusamy and Kalamdhad, 2014). This depends on the nature of the feedstock. The degradation process of feedstock rich in cellulose, protein and fats requires more time for the hydrolysis step than feedstock rich in carbohydrates (Weiland, 2010; Ali Shah *et al*., 2014). However, if hydrolysis occurs too rapidly, acid accumulates and the pH of the process decreases, which inhibits methanogens (Leung and Wang, 2016).

2.4.2 Acidogenesis

Acidogenesis is the second stage of the AD process and microorganisms are most active during this stage. During acidogenesis, bacteria convert the products formed by hydrolysis to form volatile fatty acids (propionic acid, acetic acid and butyric acid) and some gases (CO_2, hydrogen and ammonia) (Kondusamy and Kalamdhad, 2014). Facultative and obligate anaerobic bacteria are present during this stage (Kothari *et al*., 2014). Acid-phase bacteria belonging to the facultative anaerobes utilize the oxygen that may be introduced into the digester during feeding.

Facultative anaerobes are microorganisms that can survive in the presence or absence of oxygen. This action is very important in creating favourable conditions for the obligate anaerobes such as *Pseudomonas, Bacillus, Clostridium, Micrococcus and Flavobacterium* (Ali Shah *et al*., 2014). These microorganisms are able to withstand low pH, high temperatures and a high organic loading rate (Ahring *et al*., 2001).

2.4.3 Acetogenesis

In the third stage, acetogenic bacteria - also known as acid-forming bacteria such as *Syntrophomonas* and *Syntrophobacter* convert long chain fatty acids, volatile fatty acids and alcohols into simple organic acids, CO_2 and hydrogen (Ingrid *et al*., 2014). The acetogenic bacteria grow faster than the methanogens and the interaction of the two groups of bacteria is important for the performance of the anaerobic digester (Amani *et al*., 2010). *Methanobacterium suboxydans* plays a role in the decomposition of pentanoic acid to propionic acid and *Methanobacterium propionicum* convert propionic acid to acetic acid (Ali Shah *et al*., 2014). At this stage, about 25% of the acetate and 11% of the hydrogen are produced (Schink, 1997).

2.4.4 Methanogenesis

The last stage of AD is methanogenesis. In this stage, acetate and hydrogen are converted to CH_4 and CO_2 by methanogens (Gashaw and Teshita, 2014). The methanogens are the dominant species and are strict anaerobes. The end product can be formed in two ways, either by means of cleavage of acetic acid molecules to generate CO_2 and CH_4 (equation 1) or by the reduction of CO_2 with hydrogen to form CH_4 and water (equation 2) (Ostrem and Themelis, 2004).

It has been observed that up to 70% of CH_4 is produced from acetate by acetotrophic methanogenesis (equation 1) and 30% is generated from hydrogen or CO_2 through hydrogenotrophic methanogenesis (equation 2) (Čater *et al*., 2013; Jain *et al*., 2015). Acetotrophic methanogens are obligate anaerobes, some are *Methanosaeta* species and *Methanosarcina* species (Čater *et al*., 2013). The microorganisms that are most responsible for the production of CH_4 via the hydrogenotrophic pathway belong to the orders *Methanobacteriales, Methanococcales, Methanomicrobiales, Methanopyrales* and *Methanocellales* (Demirel and Scherer, 2008; Kröber *et al*., 2009, Čater *et al*., 2013).

$$4CH_3COOH \longrightarrow 4CH_4 + 4CO_2 \quad \textbf{(1)}$$

$$CO_2 + 4H_2 \longrightarrow CH_4 + 2H_2O \quad \textbf{(2)}$$

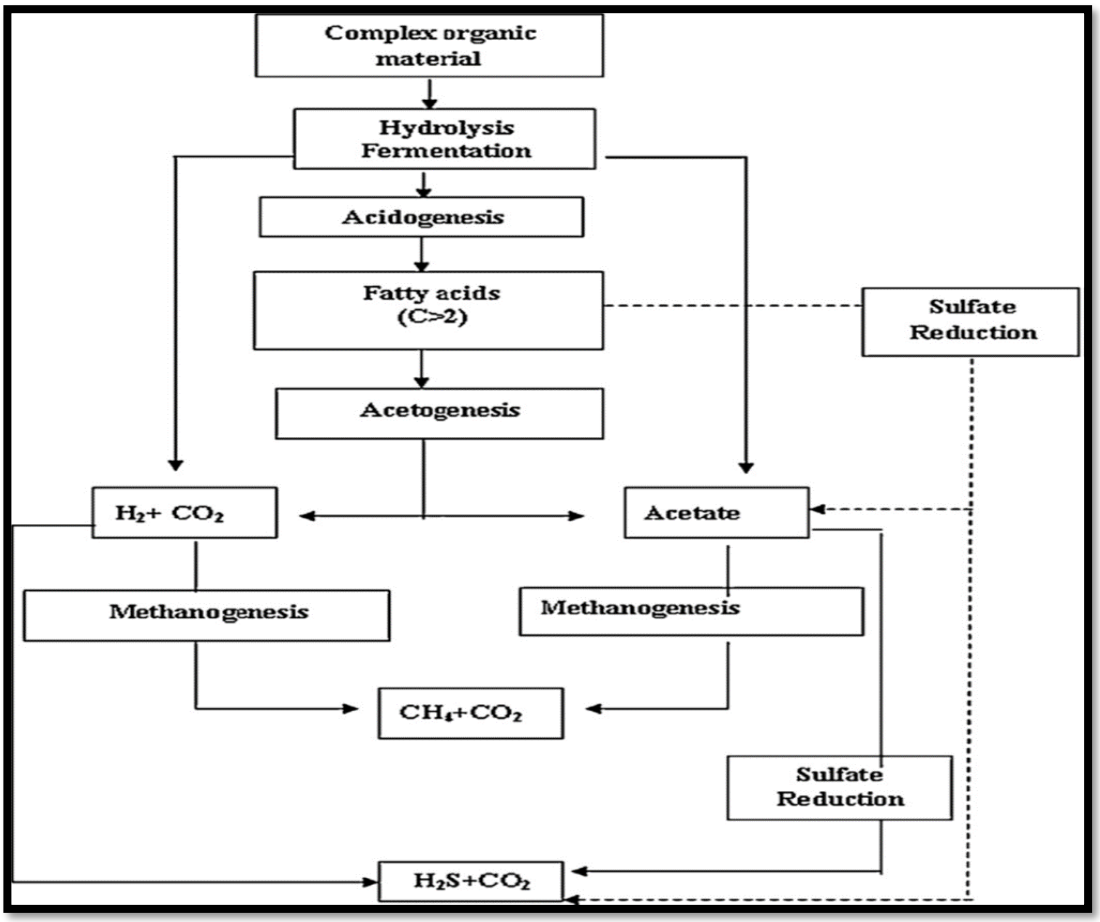


Figure 2.2: Schematic diagram of AD (Kondusamy and Kalamdhad, 2014)

2.5 Microbial identification

Identifying the species in a sample is crucial in microbiology research. Sequencing is a technique used for the identification of various microorganisms in samples collected from the digester (Čater *et al*., 2013). Sequencing technology is used worldwide (Caporaso *et al*., 2012) and is particularly useful in identifying microorganisms that are difficult to culture. First-generation sequencing known as the Sanger technique and next generation sequencing (NGS) techniques have been developed for the identification of microbes (Wirth *et al*., 2012). The species identification process starts with extracting DNA, purifying the extracted DNA, quantification and sequencing (Caporaso *et al*., 2010). For data analyses, bioinformatics software, such as Mothur and quantitative insights into microbial ecology (QIIME), is used (Caporaso *et al*., 2010). Sequences are further grouped into Operational Taxonomic Units (OTUs) in order to classify groups of related species (Caporaso *et al*., 2010). The alpha and beta diversity can be computed from the generated OTUs using such software. It is also

important to mention that nucleotide sequences are submitted to the NCBI (BLAST) for the purpose of identifying microorganisms.

2.6 Techniques for identifying microbial communities

Methodological approaches are required for the detailed analysis of the microbial community involved in the AD process. Culture-dependent and culture-independent techniques have been widely used to identify microbial populations involved in the AD process (Manyi-Loh *et al*., 2013). Culture-dependent techniques involve the characterisation and isolation of microorganisms using growth media such as Nutrient agar. Its limitation is that less than 1% of microbes can be cultured (Manyi-Loh *et al*., 2013). These techniques are laborious and time-consuming.

Molecular techniques, particularly those based on the 16S rRNA, have been studied as an alternative (Rastogi and Sani, 2011; Joyce, 2016). Techniques such as cloning, denaturing gradient gel electrophoresis (DGGE), fluorescence *in situ* hybridisation (FISH), single-strand conformation polymorphism (SSCP) and temperature gradient gel electrophoresis (TGGE) have been used to determine the community structure of bacteria and archaea (Ye et *al*., 2007; Čater *et al*., 2013). Most of the molecular techniques require that DNA be extracted by means of DNA extraction kits or any non-kit-based extraction methods, followed by PCR (polymerase chain reaction) (Čater *et al*., 2013). Molecular techniques to study microbial communities, such as PCR-DGGE, are fast fingerprinting techniques that can differentiate between closely related species (Ezeokoli *et al*., 2016).

DGGE is a common method used to define the phylogenetic relationship between microbial communities. It is based on the mobility of denatured DNA fragments of the same size but with a different nucleic acid sequence that generates a banding pattern that represents the microbial diversity in the sample (LaPara *et al*., 2000). It is also used to study the changes in community structure (Ali Shah *et al*., 2014). Separation of the bands in DGGE is based on the electrophoresis of the PCR amplified DNA gene fragments in a polyacrylamide gel with a low to high denaturant (urea and formamide) gradient (Muyzer *et al*., 1993). Temperature gradient gel electrophoresis (TGGE) works in the same way as DGGE, but the difference is that in TGGE

the gradient is obtained from the temperature instead of the denaturant. The study conducted by Carballa *et al.* (2011) used these two molecular techniques for the identification of mesophilic and thermophilic microbial communities in the AD process.

2.7 Important operating parameters in the AD Process

Anaerobic digestion is a complex process that involves different types of microorganisms with various environmental requirements (Leung and Wang, 2016). According to Khalid *et al*. (2011), microorganisms do not perform the same function under different conditions and in different environments. It is therefore important to monitor the operating conditions of AD. The biogas yield and the growth of anaerobic microorganisms in the digester are influenced by many abiotic and biotic factors. These factors include pH, C/N ratio, microbial composition, nutrient content, temperature, hydraulic retention time, moisture content, design of the digester, concentration of feedstock and many more (Behera *et al*., 2010; Jeong *et al*., 2010; Khalid *et al*., 2011; Comparetti *et al*., 2013). These parameters are explained below.

2.7.1 pH and alkalinity

The acid concentration in aqueous systems is expressed by the pH value. pH may indicate the activity of anaerobic bacteria, especially the methanogens, because they are sensitive to low pH values in the digester: their growth can be inhibited by acidic conditions with a pH value less than pH 6.5 (Chen *et al*., 2010). The amount of alkalinity in the digester represents the buffering capacity (Manyi-Loh *et al*., 2013). The pH in a biogas digester is directly dependent on the retention time (Chen *et al*., 2010). The AD process normally occurs at neutral pH and is dependent on the organic loading rate and the buffering capacity of the substrates (Manyi-Loh *et al*., 2013).

Livestock manure such as CD has a high buffering capacity (Molinuevo-Salces *et al*., 2010). Each group of microorganisms in AD survives in a different pH range. The optimal pH for methanogens is between 6.5 and 8.2 (Lee *et al*., 2009). The study conducted by Kim *et al*. (2003) shows that hydrolysis and acidogenesis occur optimally between pH 5.5 and 6.5. However, the best pH for AD has been suggested to be between 6.8 and 7.2 (Ward *et al*., 2008).

2.7.2 Temperature

Anaerobic digestion has occurs in three different temperature ranges, namely the psychrophilic, mesophilic and thermophilic ranges (Al Seadi *et al*., 2008). The most commonly used temperature ranges that provide optimum digestion conditions for the production of CH_4 are the mesophilic and thermophilic ranges. The mesophilic range is between 20 ℃ and 40 ℃ and the thermophilic temperature range is between 50 ℃ and 65 ℃ (Al Seadi *et al*., 2008). It has been observed that operating the digester at thermophilic temperatures reduces the retention time (Ostrem and Themelis, 2004). However, the diversity of microorganisms at thermophilic temperatures is low as microbes are very sensitive to fluctuations of temperatures (Weiland, 2010). Acidification can also occur during thermophilic AD, which inhibits biogas production. Other disadvantages include low-quality effluent, larger energy requirements and poor methanogenesis (Mao *et al*., 2015).

A study conducted by Pycke *et al.* (2011) reports that more diverse bacterial and archaeal species were found in a digester operating at mesophilic temperatures. The operation of digesters at mesophilic temperatures is more stable and does not require a lot of energy (Ward *et al*., 2008; Khalid *et al*., 2011). The disadvantages of mesophilic AD are poor degradability and nutrient imbalance. Methanogenic bacteria have been isolated from an AD digester operating at mesophilic temperature in the range 30-45℃ range.

Examples of microorganisms associated with the mesophilic temperature range include the genera *Methanobacterium Methanobrevibacter, Methanosphaera, Methanolobus* and *Methanococcus*. Others include *Methanosarcina, Methanocorpusculum, Methanoculleus, Methanoplanus, Methanospirillum, Methanococcoides, Methanolobus* and *Methanohalophilus.* However, species such as *Methanohalophilus, Methanohalobium* and *Methanosarcina* have been identified from an AD digester operating at the thermophilic temperature range (Gerardi, 2003).

It has been suggested that temperature changes should be less than 0.6 ℃ to maintain process stability. In addition, it should not exceed 1 ℃ in a day because that can lead to process failure

(Appels *et al*., 2011). Moreover, recent studies reveal that the rate of solubilisation was lower in thermophilic digestion that in mesophilic digestion (Komemoto *et al*., 2009). Therefore, the optimal conditions for AD would be operating at mesophilic temperature consisting of two-phase digestion process (Mao *et al*., 2015). Another study conducted by Lattieff (2016), suggested the use of AD under mesophilic conditions was the best when fruit wastes were used.

2.7.3 Carbon to Nitrogen Ratio (C/N)

The relationship between the amount of carbon and nitrogen present in organic material is represented by the C/N ratio (Adelekan and Bamgboye, 2009). The C/N ratio is used as an indicator of nutrient deficiency in the AD process (Hartmann, 2002; Kondusamy and Kalamdhad, 2014). For the AD process to function optimally, it is important to maintain the composition of the feedstock within a required C/N range (Gashaw, 2016). The perfect C/N ratio for AD is between 20:1 and 30:1 (Adelekan and Bamgboye, 2009). The study conducted by Kondusamy and Kalamdhad (2014) states that bacteria in AD utilise carbon 25 to 35 times more than nitrogen. Methanogenic bacteria and archaea use nitrogen to meet their protein requirements (Kayhanian and Rich, 1995; Khalid *et al*., 2011) and carbon as a source of energy.

Unbalanced nutrient composition of substrates results in low biogas production. Furthermore, microbial populations increase slowly and the retention time is longer if the percentage of nitrogen is low (Leung and Wang, 2016). Nitrogen levels exceeding the limit can also be problematic as they can cause ammonia inhibition (Kondusamy and Kalamdhad, 2014). To overcome this problem, substrate with low carbon can be co-digested with substrates with high nitrogen to balance the C/N ratio (Gashaw, 2016). An increase in C/N ratio represent a rapid intake of nitrogen by methanogenic archaea and results in low biogas production (Adelekan and Bamgboye, 2009; Kumar, 2012). The optimum biogas production with fruits and vegetable waste occurs at a C/N ratio of between 20 and 25 according to Khalid *et al*. (2011).

The highest CH_4 production, for instance, was achieved with the co-digestion of wheat straw and animal and chicken manure at a C/N ratio of 27.2 (Wang *et al*., 2012). A similar study conducted by Karthikeyan and Visvanathan (2012) reports a successful AD process with a C/N ratio of 27. However, recent research reports the success of AD at low C/N ratios when composting green waste and FW (Kumar *et al*., 2010). Zhang *et al*. (2013) co-digested FW with

cow dung and found that the optimum C/N range was 15.8. Therefore, the C/N ratio depends on the feedstock (Zhang *et al*., 2014).

2.7.4 Degradability of feedstock

The organic dry matter is used to characterise the organic waste and to estimate the biogas yield that can be produced (Leung and Wang, 2016). The reduction of VS in the AD process shows substrate degradability (Leung and Wang, 2016). Feedstock that has less than 60% of VS produces less biogas, while more biogas is produced when the VS is above 70% (Vögeli *et al*., 2014). The VS contains fats, protein and carbohydrates. Studies conducted by Gunaseelan (2004) and Deublein and Steinhauser (2011) report that market wastes have DM and VS between 8% and 20% and 75% and 90% respectively.

2.7.5 Hydraulic retention time and organic loading rate

Hydraulic retention time (HRT) is the average time the substrate remains inside the digester (Al Seadi *et al*., 2008; Kothari *et al*., 2014). The loading rate is the amount of raw material per unit volume of digester capacity fed into the digester per day and it can be expressed in g COD/L, g TS/L and g VS/L (Manyi-Loh *et al*., 2013). The organic loading rate plays a vital role in AD because it determines the gas that can be produced in the biodigester (Kothari *et al*., 2014). The chemical oxygen demand (COD) and the biological oxygen demand (BOD) are used to measure the rate or level of decomposition of organic material during the AD process (Ray[1] *et al*., 2013). The retention time varies according to substrate types, environmental conditions and temperature (Ostrem and Themelis, 2004).

The retention time in the mesophilic temperature range varies from 10 to 40 days (Kothari *et al*., 2014). Overloading the reactor leads to low biogas production (Ray[1] *et al*., 2013) because of the accumulation of inhibitory substances, such as fatty acids, in the reactor (FAO, 1996). In countries with colder climates, the HRT may be up to 100 days, whereas the values lie between 30 and 50 days in warmer climates. There is a linear relationship between retention time and the digester temperature. Higher temperature ranges result in lower retention times (FAO, 1996).

2.7.6 Moisture content

Moisture content play a very important role in metabolic processes during AD (Gashaw, 2016). Excess moisture in the substrate leads to a decrease in biogas production because of low total solids (TS). On the other hand, substrates with insufficient moisture leads to an accumulation of acids that inhibit the AD microbes and therefore reduces biogas production (Kumar, 2012). The highest CH_4 production has been reported to take place with a 60-80% moisture content (Bouallagui *et al*., 2003; Gashaw, 2016).

2.7.7 Nutrient content

Microbes require nutrients for their growth and functioning. Anaerobic microbes require macronutrients such as carbon, nitrogen and phosphorus and micronutrients such as iron, nickel and cobalt are required (Al Seadi *et al*., 2008; Gashaw and Teshita, 2014). If energy crops are used for biogas production, trace elements such as nickel, iron, molybdenum, selenium, tungsten and iron can be added to enhance the growth of the microorganisms (Abdoun and Weiland 2009). All methanogenic bacteria require nickel for the synthesis of the cell components responsible for CH_4 production (Weiland, 2010).

2.7.8 Volatile fatty acids

Volatile fatty acids (VFA) are intermediate compounds that are produced during AD of organic waste. Example of VFAs are acetate, propionate, butyrate and lactate (Al Seadi, 2001; Leung and Wang, 2016). Methanogenic bacteria utilise these VFAs and convert them to biogas. The accumulation of VFAs in high concentrations as a result of overloading can prevent the growth of methanogens, which can lead to process failure (Gerardi, 2003; Palacio-Barco *et al*., 2010; Zhang *et al*., 2015; Leung and Wang, 2016). The accumulation of VFAs lowers the pH in the AD system. Previous research has shown that propionic to acetic acid concentrations exceeding 0.8 g/L are disadvantageous to the AD system. Therefore, the propionic acid to acetic ratio is one of the parameters that can be used to determine process stability (Buyukkamaci and Filibeli, 2004).

2.7.9 Ammonia

In AD, the end-product ammonia is formed during the degradation of substrates rich in protein and nitrogen. Inorganic nitrogen exists in two forms, namelys ammonium (NH_4^+) and free ammonia (NH_3) (Rajagopal *et al*., 2013; Zhang *et al*., 2014). Ammonia is used as a source of nutrients for microbes, but if its concentration exceed the threshold required by the microbes it causes problems (Kim and Oh, 2011). A low ammonia concentration is the result of substrates with a nitrogen deficiency (Rajagopal *et al*., 2013). Rajagopal *et al*. (2013), confirms that ammonia is inhibitory at high concentrations (Rajagopal *et al*., 2013). However, a study conducted by Wang *et al*. (2012) shows that ammonia could increase the buffering capacity of the process because it neutralises the VFAs formed in the process.

Researchers reported that the acetoclastic methanogens that produce CH_4 and CO_2 from acetate are more sensitive to ammonia inhibition than hydrogenotrophic bacteria (Chen *et al*., 2008; Walker *et al*., 2011). Therefore, whenever there is ammonia inhibition, the CH_4 production stops. Microbes such as *Methanosaeta concilii* and *Methanosarcina barkeri* have been identified as being more sensitive to high ammonia production (Sprott and Patel, 1986). Whenever the temperature rises and the pH increases, the concentration of free ammonia increases as well (Rajagopal *et al*., 2013).

2.7.10 ***Mixing***

Mixing in the digester reduces the particle size, increases the contact between the microorganisms and the substrate which helps microbes to obtain nutrients (Monnet, 2003). In addition, mixing in an anaerobic digester keeps the solids in suspension. The mode of mixing in the digester and the mixing intensity have a direct effect on CH_4 production (Lindmark *et al*., 2014). Thoroughly mixing throughout the AD process can reduce the formation of layers inside the digester. However, gentle homogenisation is advised to avoid disruption of microbial activities as microorganisms are sensitive to mixing intensity (Monnet, 2003; Deublein and Steinhauser, 2008).

2.8 Overview of the feedstock for biogas production

Millions of tons of different types of organic wastes are generated each year. Such wastes include animal manure, wastewater sludge, FW, agricultural residues and municipal solid wastes (Al Seadi *et al*., 2008; Ali Shah *et al*., 2014). Waste composition and characterisation is one of the most important steps in AD technology. Knowing the composition of the substrate in the digester is needed to calculating the amount of biogas that can be produced as well as the amount of energy the feedstock contains (Curry and Pillay, 2012). The FW composition differs from place to place and seasonally (Leung and Wang, 2016). The feedstock used for AD should be degradable and should contain nutrients (Kothari *et al*., 2013) such as carbohydrates, fats, cellulose, hemicellulose and proteins (Weiland, 2010).

According to Leungand and Wang (2016), food rich in lipids can produce a CH_4 yield of 69%, proteins 68% and carbohydrates 50%. The AD process of carbohydrates occurs fast but has low biogas production. Therefore, the yield of biogas produced depends on the feedstock type and retention time (Weiland, 2010). Woody materials are not ideal for AD as they have a high lignin content that is difficult for bacteria to break down.

2.8.1 Livestock manure

Livestock manure is the largest agricultural waste category consisting mainly of dairy and pig manure. Improper disposal of livestock waste such as cattle manure can result in health problems, causing unpleasant odours, and pathogen contamination (Abubakar *et al*., 2012). Historically, manure has been a valuable source of nutrients for crop cultivation. However, the intensification of modern livestock production has caused an extreme manure surplus. Livestock dense areas pose a risk of nutrient losses such as leaching of nitrogen (N) and phosphorus (P) into waterways and emissions into the atmosphere in the form of polluting gases (Oenema *et al*., 2005).

Biogas production from CD, chicken droppings and pig manure using AD has often been reported in recent years (Table 2.2). According to Mata-Alvarez *et al* (2000), CD is a good substrate for AD and it is widely available. It also contains a naturally occurring mixture of microorganisms responsible for AD. In addition, it is an important resource to improve soil fertility because of its high content of micro and macro- nutrients for crop growth, which makes

it a good bio-fertiliser (Ibrahim *et al*., 2016). The study conducted by El-Mashad and Zhang (2010) found that the CH_4 yield of screened and unscreened manure after 30 days were about 302 and 241 L/kg VS respectively. Cow dung and pig manure were reported to contain a total solid content in the of 3-12% range. Chicken manure contains 10-30% dry matter (Jain *et al*., 2015). The volatile fatty acid concentration is higher in pig slurry than in CD (Jain *et al*., 2015). Usually, the volatile fatty acid concentration of animal slurry does not cause inhibition, but it causes fast degradation of organic molecules (Jain *et al*., 2015).

2.8.2 Food waste

Food waste, is a global challenge, costs SA about R61 billion a year (Oliveira, 2013). Food waste account for a large portion of the municipal solid waste in both developed and developing countries (Zhang *et al*., 2014). Leftovers from restaurants and cafeterias, food lost in the manufacturing process, uneaten food (Zhang *et al*., 2007) and any food that is no longer fit for human consumption are considered as FW. Most of these losses occur during handling, distribution, packaging and storage (Van Schie, 2013). Unfortunately, the quick deterioration of FW causes environmental pollution and bad smells (Hartman and Ahring, 2006).

It has been reported that the GHG emission from the food supply chain is between 2.8 and 4.14 tons of CO_2 equivalent per ton of food (Oliveira, 2013). The AD process treating FW is becoming more popular because of the high moisture content and organic matter contained in this waste stream, which is needed for microbial growth (Zhang *et al*., 2011). The composition of FW plays an important role in determining the bio-methane potential. A study conducted by Sebola *et al* (2014) states that kitchen waste has a higher moisture content and can be easily degraded. Food waste yielded 0.50 m^3 CH4/kg VS during continuous digestion trials (Lin *et al*., 2011).

2.8.3 Fruit and vegetable waste

Fruit and vegetable waste (FVW) form a very important class of residues because they are produced in large quantities in wholesale markets (Scano *et al*., 2014). The fruit industry produces many different types of fruit (citrus, tropical and deciduous fruit) in different seasons. These fruit contain low TS, high volatile solids (VS) and is easily degraded in the AD process. However, hydrolysis occurs within a very short period and can lead to acidification of the

process, which inhibits the growth of methanogens (Ward *et al*., 2008). The fruit and vegetables can be obtained from canning industries, the frozen vegetable industry, fruit pulping and tomato juice industries (Thassitou and Arvanitoyannis, 2001).

Different studies reported on mono-digestion of vegetable and fruit waste as feedstock for biogas production. A study conducted by Bouallagui *et al*. (2005) reveals that fruits and vegetable waste is a very highly degradable substrate with about 70-95% organic matter that can be converted into biogas. In 2004, Gunaseelan studied the biochemical CH_4 potential from fruits which ranged from 0.18 to 0.73 g/VS and vegetables from 0.19 to 0.41 g/VS as substrates for biogas production. Lin *et al*. (2011) investigated the CH_4 production of fruit waste during continuous digestion at 35℃ which produced 0.30 m^3 CH_4/kg VS. Fruits and vegetable wastes are quickly degraded especially when they are damaged or extremely ripe. The average bio-methane potential of FW is around 0.367m^3 CH4/kg VS (Curry and Pillay, 2012). Previous studies using FVW as feedstock for AD are shown in Table 2.2.

Table 2.2: Biogas production using different substrates (Deublein and Steinhauser, 2008; Rajendran *et al*., 2012).

Category	Substrate	DM%	Ash %	TDN%	Biogas yield (m^3 kg^{-1} VS)
Manure	Pig	20-25	NA	NA	0.27-0.45
	Buffalo	14	NA	NA	NA
	Poultry	89	33	38	0.3-0.8
	Horse	28	NA	NA	0.4-0.6
	Cow dung	38	14	92	0.6-0.8
Food waste	Whey	94	10	82	NA
	Fruit waste (apples)	17	2	70	NA
	Vegetable waste	20	NA	NA	0.4
	Kitchen /restaurant waste	27/13	013/8	NA	0.506-0.65(CH_4)
	Left overs food	14-18	NA	NA	0.2-0.5
	Egg waste	25	NA	NA	0.97-0.98
	cereals	85-90	NA	NA	0.4-0.9
Agricultural residues	rice straw	91	13	40	0.55-0.62
	maize straw	85	NA	NA	0.4-1.0
	coffee pulp	28	8	NA	0.300-0.450
	Corn stalk	80	7	54	0.350-0.480
	Wheat straw	91	13	40	0.55-0.62

*DM (dry matter), TDN (total digestible nutrients; NA (not available), VS (volatile solids), TS (total solids).

2.9 Co-digestion

Co-digestion is the breaking down of more than one substrate or waste type in the same digester and is considered profitable in terms of CH_4 production (Iyagba *et al*., 2009; Khalid *et al*., 2011; Gashaw and Teshita, 2014). The studies conducted by Sebola *et al.* (2015) and Wang *et al* (2014) found that CO of animal manure with any substrate with high a C/N ratio can increase the yield of the biogas (Gashaw and Teshita, 2014). Co-digestion is also advantageous as it eliminates or reduces challenges associated with bacterial inhibition during the AD process (Sebola *et al*, 2015). Gashaw and Teshita (2014) showed that there is a nutrient balance when FW and animal manure are co-digested that positively influences microbial growth and functioning.

Co-digestion of dairy cow manure, municipal waste and cotton gin waste has resulted in the CH_4 production of 87 m^3/ton of dry waste (Macias-Corral *et al*., 2008). Callaghan *et al*. (2002) studied the CO of cattle slurries with FVW and chicken manure in a continuous stirred tank reactor (CSTR) with an improved CH_4 yield from 0. 23 to 0. 45 m^3 CH_4/kg VS added. Li *et al*. (2009) obtained a 44% CH_4 increase when co-digesting CD with FW.

2.10 Anaerobic digesters

A biogas digester is an airtight and waterproof vessel that can be cube-shaped or cylindrical (Renwick *et al*., 2007). It should be designed to maximise or enhance production of CH_4 in a short retention time (Ward *et al*., 2008). The quantity, characterisation and availability of the feedstock should be taken into consideration when designing and operating the bio-digester (Sebola *et al*., 2014). There are different types of bio-digesters that operate in different modes but the most commonly used ones are batch reactors, one stage continuously fed and two stage continuously fed reactors (Khalid *et al*., 2011).

In a batch reactor, the substrate is added to the digester, left for a certain period of time and emptied afterwards (Weiland, 2006; Ward *et al*., 2008). Batch reactors are advantageous as the AD process occurs fast, they have low maintenance requirements and they are not expensive (Kothari *et al*., 2014). There are three types of the batch systems, namely the single stage system, sequential system and the upflow anaerobic sludge blanket (UASB) reactor (Monnet,

2003). The single stage system involves re-circulating of the leachate at the top of the same reactor. The sequential system comprises of two or more reactors. The leachate form the first reactor is re-circulated to the second reactor where the final stage occurs (Monnet, 2003). The UASB reactor is almost the same as the two-stage batch system. Batch processes are simple to operate, cheap and give results that are more robust. The disadvantage is that it requires a long retention time (Okudoh *et al*., 2014).

In a one-stage continuously fed reactor, all the AD stages occur in the same reactor (Ward *et al*., 2008; Khalid *et al*., 2011; Kondusamy and Kalamdhad, 2014). The substrate is continuously fed into the reactor and an equal amount of what is fed into inside the reactor is removed as effluent (Kothari *et al*., 2014). The disadvantage of this reactor is that the effluent might be mixed with less digested material (Kothari *et al*., 2014). The growth rate of fermentative bacteria is faster than acetogenic and methanogenic bacteria. As the pH falls, acids accumulate and this leads to a collapse of the process (Manyi-Loh *et al*., 2013). The third type is two stage continuously fed bioreactor. In this type of reactor, all the AD stages (hydrolysis, acidogenesis, acetogenesis and methanogenesis) are separated (Ward *et al*., 2008). Common designs for continuous biogas digesters for domestic or rural use include the fixed dome, floating drum and bag type digesters (Cheng and Liu, 2002).

2.11 Substrate pre-treatment

Pre-treatment of a substrate involves removing inorganic material and providing small size particles for efficient digestion. Pre-treatment also involves the addition of chemicals. Pre-treatment is a necessary step to increase biogas yield (Jain *et al*., 2015). The AD of lignocellulosic material is challenging as it is not easily biodegradable. If the particles size of the substrate are big, it is not easy to create a surface area for the microorganism and the digestion process may take time. Therefore, chemical, physical and biological pre-treatment methods can be applied to accelerate the rate of the AD process especially the hydrolysis step (Andriani *et al*., 2014). In European countries, substrates such as municipal solid waste (MSW) and FW have to be pasteurized or sterilized prior to AD. The various pre-treatment methods have their advantages and disadvantages, depending on the type of substrate used (Ariunbaatar *et al*., 2014).

2.12 Digestate

The sustainability of AD depends on the use of all the products of the AD process (Alburquerque *et al*., 2012). Except the biogas, the solid residue also known as the digestate or slurry can be used as a fertiliser provided the pathogens have reduced to acceptable level (Cioabla *et al*., 2012). The digestate or slurry produced is an excellent fertiliser, because it is rich in phosphorus, potassium and nitrogen (Teodorita *et al*., 2008; Islam *et al*, 2010; Zhang *et al*, 2016). Moreover; it provides macronutrients that may be inadequate in the soils. Therefore, it can be used as a source of plant nutrients. Mineral nitrogen contained in the digestate is readily available to plants in the form of ammonium (Makádi *et al*., 2012). The direct use of digestate as a fertiliser or soil amendment in agriculture is currently considered inexpensive (Alburquerque *et al*., 2012) in comparison with chemical fertilisers. However, the digestate might contain pathogens and heavy metals, depending on the type of substrate used in and the operational conditions of the AD process. The study conducted by Sahlström (2003) found that AD can destroy harmful pathogens such an *Escherichia coli* during digestion especially at high temperatures ensuring that the slurry is free of pathogens.

Conventional agriculture, which has been practiced for a very long time to meet the food demands of the rapidly growing global human population, depends to a large degree on chemical fertilisers, and the frequent or continuous use of chemical fertilisers has led to declining of soil fertility, environmental pollution and heavy-metal pollution (Zhu *et al*., 2012). It has been shown that a large portion of inorganic phosphates applied to the soil as chemical fertiliser is rapidly immobilised after application and becomes unavailable to the plants (Yadav and Dadarwal, 1997; Rengel and Marschner 2005). Microorganisms contained in the digestate can be utilised in agriculture as inoculants for biological nitrogen fixation, phosphate solubilisation and for their ability to produce plant growth regulators or phytohormones such as indole acetic acid (indole-3-acetic acid, IAA) to improve crop quality and yields (Higa, 1994). Microorganisms which have been isolated from the AD the digestate include *Pseudomonas, Klebsiella, Salmonella, Penicillin, Shigella, Bacteriodes, Aspergillus* and *Bacillus* (Owamah *et al*., 2015). *Azotobacter, Azospirillum, Enterobacter, Xanthomonas, Serratia. Klebsiella* and *Clostridium* species are nitrogen fixers, while *Bacillus* and *Pseudomonas* species are phosphate solubilisers (Alfa *et al*., 2014).

CHAPTER 3: METHODS AND MATERIALS

3.1 Ethical clearance

Ethical clearance for this study has been granted by the University of South Africa Research Ethics Review Committee.

3.2 Study area

Food waste amples were collected from the Tshwane Market in Pretoria (Site A: latitude: -25.713525 ^{o}S, longitude of 28.131973 ^{o}E) and cow dung in the Free State province (Site B: Latitude -28.3652 ^{o}S, longitude 28.87208 ^{o}E) (Figure 3.1). The Tshwane Market was selected because of the continuous production of organic waste such as fruit and vegetables spoiled during packaging, transportation and distribution. The Free State province was selected because of the abundant availability of animal waste and biogas digesters installed for smallholder farmers for energy production.

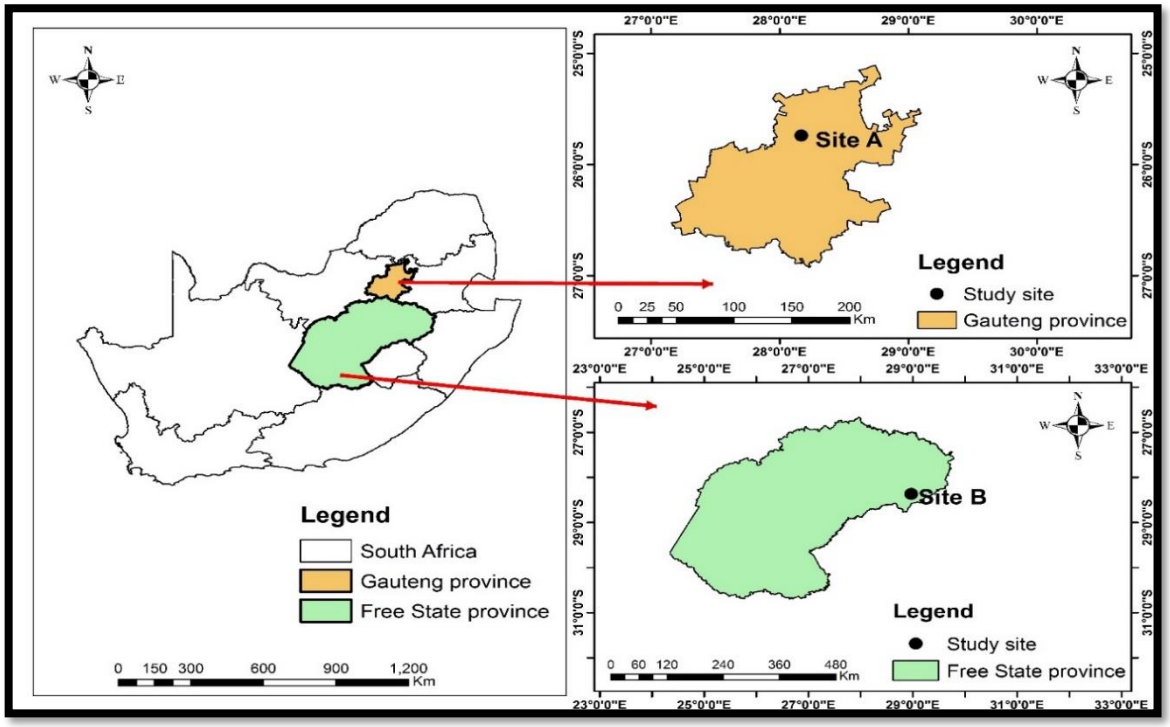


Figure 3.1: Study area map showing the location where samples were collected.

3.3 Sample collection

The inoculum was collected from a working pre-fabricated household digester operating at ambient temperature, installed and monitored by the Agricultural Research Council (ARC) on a small scale farm situated in Maluti-a-Phofung (Free State). The bio-digester was fed every second day with 20 kg of fresh CD diluted in water in a ratio of 1:1. The biogas produced was used for cooking purposes. Fresh CD was also collected from the farm in Maluti-a-Phofung. Food waste was collected in Tshwane Market. The FW included potato peels, mixed vegetables (spinach, baby marrow and cabbage), mixed fruit (lemon, orange, watermelon and mango) and tomatoes.

All samples were collected once. The samples were collected by hand, wearing gloves, and placed on ice inside a cooler box. All samples were stored at a temperature of -4 ℃. Impurities such as plastic, glass and steel were removed from CD and FW within 24 hours of sampling. Prior to the batch AD experiments, the FW was homogenised using a blender (Bouallagui *et al*., 2005), pasteurised at 70℃ for 1 hour (Kirchmayr *et al*, 2003) and stored at -20℃ until use.

3.4 Analysis of physico-chemical parameters

The physico-chemical parameters analysed were the pH, total solids (TS), VS and moisture content. For compositional analysis such as the lignin, protein and cellulose samples were sent to the Agricultural Research Council-IRENE laboratory. The hemicellulose, cellulose and lignin content was detected using traditional methods (Robertson and Van Soest, 1981). All FW and CD were analysed prior to the initiation of batch experiments. The pH was measured using a pH meter (AD 1030).

3.4.1 Determination of dry matter (total solids)

Dry matter (DM) content was determined in an oven at 105℃ for 24 h by drying a known weight of the sample (W_s) and measuring the weight after drying (W_{DM}), as described by Poulsen *et al.* (2011). The percentage of DM was calculated as follows:

$$DM\ (\%) = 100 \times W_{DM}/W_S \quad \textbf{(1)}$$

Where:

W_s= refers to the weight of the sample

3.4.2 Determination of volatile solids

Volatile solids were measured using the method described by Cioabla *et al*. (2012) with some modification. Dried samples were combusted at 550℃ for 24 h and the weight of the ash (W_{ash}) was measured afterwards. The VS was calculated as follows:

Volatile solid (%) = 100 × (W_{DM} - *Wash*)/ W_{DM} **(2)**

Where:

W_{DM}= weight after drying

W_{ash} = weight of the sample after drying at 550℃

3.4.3 Determination of moisture content

Samples were weighed in a dish and dried in an oven at 105℃ overnight (Cioabla *et al*., 2012). The weight of the dried sample plus the empty dish was noted and the percentage of moisture was measured using the following equation:

Moisture content (%) = (m_2-m_1) - (m_3-m_1) / (m_2-m_1) ×100 **(3)**

Where:

m_1= mass in grams of the empty dish,

m_2= mass in grams of sample before drying plus the empty dish,

m_3= mass in grams of sample after drying plus empty dish.

3.5 Biochemical methane potential test

The batch culture experiments were conducted in airtight 500 mL gas infusion bottles with rubber septa. The fresh inoculum was used as suggested by Angelidaki *et al*. (2009) from the bio-digester producing biogas. Fruit and vegetable waste is known to be acidic in nature. When using acidic substrates in AD, a high inoculum to substrate ratio (ISR) is recommended because it increases the rate of biogas production and improves the buffering capacity (Raposo *et al*., 2009). A 3:1 (inoculum: substrate) ratio was therefore chosen for batch analyses.

Each substrate without inoculum served as controls. All the experiments had a working volume of 250 mL. In all controls, 25 g of substrate was added (Table 3.1). For all experiments, 18.75 g of inoculum with 6.25 g of the substrate were mixed and water was added to a total volume of 250mL. The headspace in all batch bottles was flushed or purged with nitrogen gas for 5

minutes to remove excess oxygen inside the bottles. The bottles were incubated at 30℃ (mesophilic condition) in a shaker incubator at 90 rpm to ensure continuous homogenisation of the substrate and the inoculum throughout the experiment (Figure 3.2). The experiment was conducted for 21 days in triplicate.

Table 3.1: Treatment patterns for biochemical methane potential.

Substrates (3:1)	**Inoculum**	**Fruit**	**Veg**	**Tomato**	**Potato peels**	**Mixed FW**	**Cow dung**	**Controls**
1	25g	0	0	0	0	0	0	0
2	18.75g	6.25g	0	0	0	0	0	0
3	18.75g	0	6.25g	0	0	0	0	0
4	18.75g	0	0	6.25g	0	0	0	0
5	18.75g	0	0	0	6.25g	0	0	0
6	18.75g	0	0	0	0	6.25g	0	0
7	18.75g	0	0	0	0	0	6.25g	0
8	0	0	0	0	0	0	0	25g

*Controls refer to fruits, vegetables, tomato, potato peels, mixed food waste (MFW) without addition of inoculum

Figure 3.2: Experimental setup of batch AD cultures in a shaker incubator operating at 30℃

3.6 Biogas analysis

Biogas production was monitored by measuring the pressure build up in the headspace of the bottles. The measurements were taken every second day. The pressure (mbar) was measured using a digital manometer (MP 210 KIMO® THERMO-ANEMO-MANOMETERS). The daily biogas production was calculated using equation 4 (El-Mashad and Zhang, 2010).

$$V_{Biogas} = \frac{P.V_{head}.C}{R.T} \quad \textbf{(4)}$$

Where:

V_{Biogas}= daily biogas volume (L)

P= absolute pressure difference (mbar)

V_{head}= volume of the head space (L)

C=molar volume (22. 41 $Lmol^{-1}$)

R= universal gas constant (83. 14 L $mbarK^{-1}mol^{-1}$)

Temperature =absolute temperature (K)

The biogas composition was monitored using gas chromatography (GC) model 8610C (SRI, USA) equipped with thermal conductivity detector (TCD) with helium as a carrier gas. After measuring the pressure, 2 mL of biogas sample was injected into the GC for compositional analysis. Gases measured included CH_4 and CO_2. The CH_4 production was recorded as a percentage and converted into volume of CH_4 in (L CH_4) to calculate the accumulated CH_4 production (Nurliyana *et al*, 2015). The CH_4 yield was thereafter converted to volume of CH_4 produced relative to the quantity of the organic matter added. The CH_4 yield was calculated using equation 5, as previously used by Nurliyana *et al.* (2015).

$$\text{Methane yield } (\text{L}CH_4/\text{gVS}) = \frac{\text{Cumulative volume of CH4 } (\text{L}CH_4)}{\text{Mass of the original VS content (gVS)}} \quad \textbf{(5)}$$

3.8 Semi-continuous digestion

Based on the results of the batch experiments, two substrates that produced the highest CH_4 yield were chosen for semi-continuous culture trials. The semi-continuous digestion experiments were conducted in 20 L continuous stirred tank reactors with a working volume of 14 L. Three runs were carried out (two mono digestions and one co-digestion). For mono-digestion, cow dung and MFVW were used individually and for co-digestion, MFVW and cow dung were digested together (1:3). The digesters were operated for a period of 40 days at a constant temperature of 30 ^{o}C ±1 (Figure 3.3). Freshly prepared substrate mixture (1400 ml) was added to each reactor every second day and an equivalent volume was removed before each addition to maintain a constant volume inside the reactor. The leachate samples were used for microbiology analysis and the pH, VS, DM and ash content were measured. The VS, DM and moisture contents were analysed as set out in sections 3.4.1, 3.4.2 and 3.4.3 respectively. The biogas production was monitored every second day using the automated gas counter and 2 mL samples were analysed into the GC for gas composition analysis.

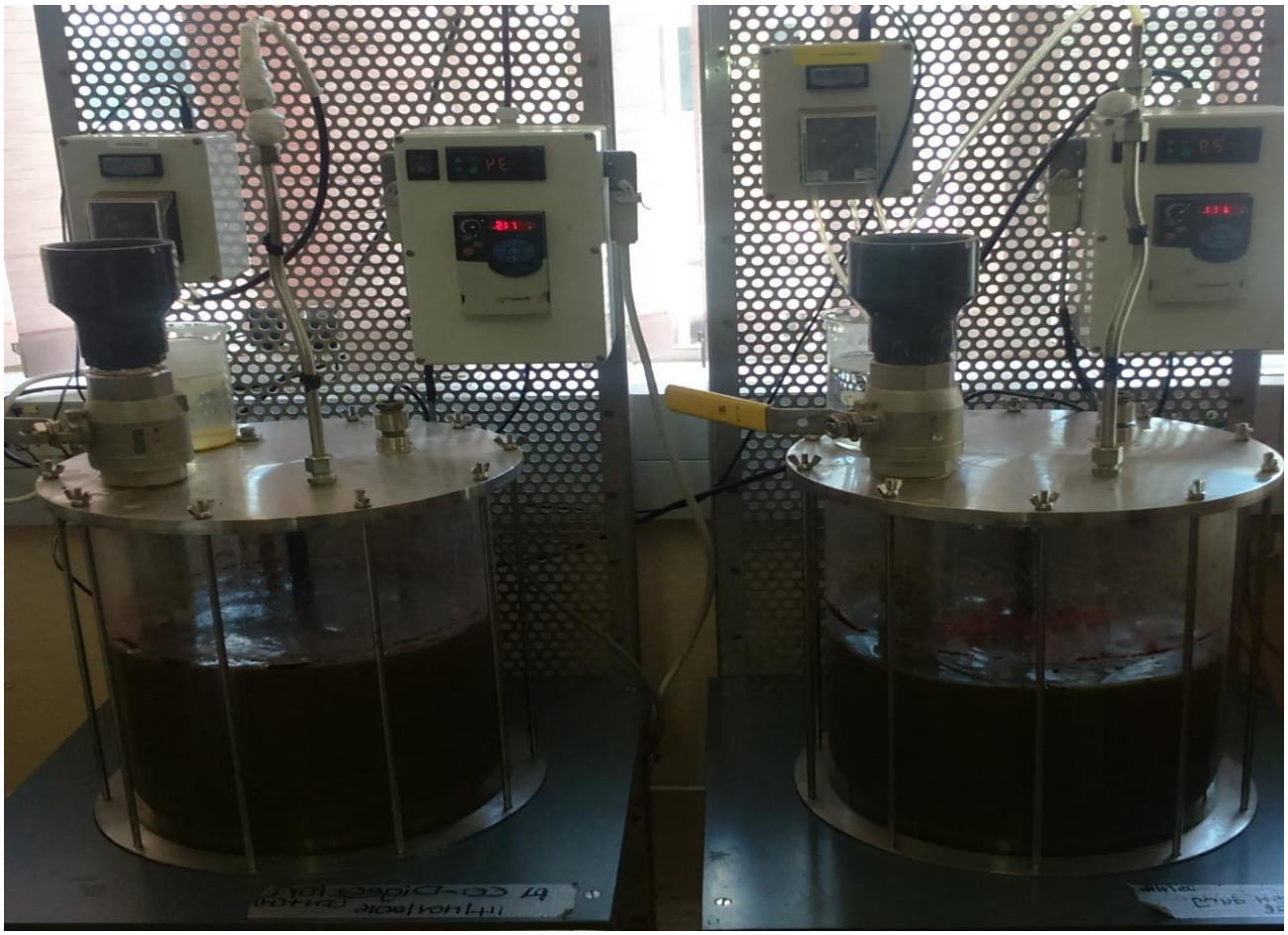


Figure 3.3: Semi-continuous AD trials in 20 L continuous stirred tank reactor (CSTR).

3.9 Culture independent method (molecular techniques)

3.9.1 DNA extraction

Prior to DNA extraction, the samples collected from the semi-continuous digestion run were centrifuged at 11 200rpm for 10 minutes. The supernatant was then removed using a pipette. Genomic DNA was extracted using the Powersoil DNA extraction kit (MO BIO Laboratories) recommended by Roopnarain *et al*. (2017).The DNA was extracted according to the manufacturer's instructions. The resulting DNA extract was stored at -20 ℃ until further processing.

3.9.2 DNA quantification

A Qubit fluorimeter (Invitrogen, USA) was used to measure the quantity of DNA extracted using the Qubit® dsDNA HS Assay Kit as per manufacturer's instructions.

3.9.3 Polymerase chain reaction (PCR)

The V3-V4 region of the 16S rRNA genes were amplified using universal bacterial primers 341F and 907R (Muyzer *et al*., 1993) and archaeal primers 0357F and 915aR (Table 3.2) as suggested by Watanabe *et al* (2004). The PCR was performed in a T100^{TM} thermal cycler (Bio-Rad Laboratories). The PCR cycling conditions are listed in Table 3.3. Each PCR reaction contained: 0.2 µM of each primer set, 10 µL of 2x Phusion Flash PCR Master Mix (Thermo Scientific), 50 ng of the template DNA and nuclease free water was added to make up the final volume of 20 µL. The integrity of the PCR product was evaluated by gel electrophoresis on a 1 % agarose gel (w/v) stained with ethidium bromide. The gel was viewed using the Bio-Rad Gel Doc^{TM} EZ imager (Bio-Rad Laboratories, Hercules, CA).

Table 3.2: Nucleotide sequences of primers used for molecular identification of bacteria and archaea targeting V3-V4 region

Primer	Nucleotide sequence (5'-3')	Annealing temperature (°C)	Reference
341F	CCTACGGAGGCAGCAG	50.9	(Myzer *et al*.,1993)
907R	CCGTCAATTCCTTTGAGTTT		
0357F	CCCTACGGGGCGCAGCAG	69	(Watanabe *et al*., 2004)
0915aR	GTGCTCCCCCGCCAATTCCT		

Table 3.3: PCR cycling conditions used for the amplification of DNA from bacteria and archaea with different annealing temperatures

Cycle steps	Temperature (℃)	Time (s)	Cycles
Initial denaturation	98	10	1
Denaturation	98	1	
Annealing	Listed (Table 3.2)	5	30
Extension	72	15	
Final extension	72	15	1

3.9.4 DGGE

DGGE was performed using the protocol of Muyzer *et al*. (1993) with a Dcode^{TM} Universal Mutation Detection System (Bio-Rad Laboratories, SA). A 100% denaturing solution that contained 40% formamide (vol/vol) and 7 M urea was prepared. Touchdown PCR was performed for bacteria using 341F-GC and 907R primers. The annealing temperature used decreased from 65ºC to 55ºC in 20 cycles. For archaea, a nested PCR was conducted using 0357F and 0915aR primers (Table 3.2). The obtained PCR product was then re-amplified using 0357F-GC and 0691R primers (Table 3.4). The annealing temperature was 57 ºC (Ikenaga *et al*., 2004; Watanabe et *al*., 2004). The sequence-specific separation of the PCR fragments was obtained in 8% (wt/vol) polyacrylamide (40% acrylamide-N, bisacrylamide, 37.5:1[wt/vol]) using denaturing gradient ranges of 60-40% for bacterial samples and 60-25% for archaeal samples. A stacking gel containing 8% (wt/vol) polyacrylamide was applied onto the denaturing gel. A volume of 18 µL PCR samples with 5µL of the loading dye was loaded onto the stacking gel. Electrophoresis was performed at 200 V for 15 minutes, then at 100 V for 16 hours. The 0.5 X TAE buffer was maintained at 60°C throughout the run. Gels were stained with GelRed for 45 minutes, it was then viewed and photographed using a UV transilluminator (Bio-Rad Gel DocTM EZ imager [Bio-Rad Laboratories, Hercules, CA]).

Table 3.4: Nucleotide sequences of PCR-DGGE primers used.

Primer	Nucleotide sequence (5'-3')	Reference
0691R	GGATTACARGATTTCAC	(Watanabe *et al*., 2004)
0357F-GC	CGCCCGCCGCGCGCGGCGGGCGGGGCGGGGGCA CGGGGGGCCCTACGGGGCGCAGCAG	
341F-GC	CGCCCGGGGCGCGCCCCGGGCGGGGCGGGGGCA CGGGGGGCCTACGGGAGGCAGCAG	(Muyzer *et al*.,1993)
907R	CCGTCAATTCCTTTGAGTTT	

3.9.5 Analysis of DGGE profiles

DGGE band detection and quantification of band intensity were performed using Image Lab software (Bio-Rad). Equations 6 and 7 were used to calculate the Shannon-Wiener (*H*') as suggested by (Xu *et al*., 2013) and Simpsons indices (Magurran, 1988).

$$H' = -\sum_{i=1}^{s} pilnpi \quad \textbf{(6)}$$

$$D = \sum_{i=1}^{i=n} {p_i}^2 \quad \textbf{(7)}$$

where pi is the proportion of the community composed of species i (intensity of band i/the total intensity of all bands in the lane) and S is the total number of bands in each sample lane. To ensure that the Simpsons index increases with increasing diversity, 1/D was used instead of the original formula.

3.9.6 PCR amplification for illumina Next Generation Sequencing (NGS)

PCR amplification of the 16S rRNA gene hypervariable V3-V4 region was performed with 341F and 805R primers (Table 3.5). Overhangs were attached to the 5' end of the forward and reverse primers (Illumina Inc., Calfornia, USA). PCR was performed in a T100 ™ thermal cycler (Bio-Rad laboratories). The amplification mix contained 12.5 ng template DNA, 5 µM of forward and reverse primers, 12.5 µL of 2x KAPA HiFi HotStart ReadyMix (KAPA

Biosystems, Massachusetts, USA) and nuclease-free water to make up a final volume of 25 µL. Samples were initially denatured at 95 ℃ for 3 min, 30 cycles denaturation at 95 ℃ for 30 min, annealing followed at 55 ℃ for 30s and a final extension of 30s at 72 ºC. The positive and negative controls were used to check primer or DNA contamination. PCR products were run on a 1% agarose gel. Sequencing was performed at the Agricultural Research Council-biotechnology platform.

Table 3.5: Nucleotide sequences of primers used for Next generation sequencing targeting V3-V4 region

Primer	Nucleotide sequence (5'-3')	Annealing temperature (℃)	References
341F	CCTACGGAGGCAGCAG	55	(Muyzer *et al*.,1993)
805R	GACTACHVGGGTATCTAATCC		

3.9.7 Data analysis for NGS sequencing

The received 16S sequencing data was analysed using the bioinformatics pipeline QIIME (Ziganshina *et al*., 2017). The quality of the sequence reads were checked using FastQC software. Merging of the reverse and forward sequences was accomplished using PANDAseq. The sequences were clustered into operational taxonomic units (OTU) with "pick_open.reference_otus.py" script using Mothur software (Caporaso *et al*., 2010). The alpha diversity (Chao analyses, coverage, evenness value, Shannon and Simpson index) were calculated using R to characterise the microbial diversity of the biogas digester samples (Claesson *et al*., 2012).

3.10 Culture dependent methods

For this objective, fresh CD used as feedstock for the digester and the digestate from the digester outlet were collected in triplicate in sterile plastic bags. Samples were collected from a working pre-fabricated, fixed dome household anaerobic digester situated in Qwa-Qwa, Free State province in SA (Latitude -28, 3652 °S, longitude 28, 87208 °E).

3.10.1 Analytical methods

The influent and effluent samples were analysed before and after for the pH, VS, DM and ash content as set out in (sections 3.4.1, 3.4.2 and 3.4.3). For detection of heavy metals and nutrient content (N, P and K), samples of fresh CD and digestate were transported to the Agricultural Research Council-Institute for Soil Climate and Water (ARC-ISCW) analytical laboratory in Pretoria. The dried and ground samples were digested by HNO_3 to determine heavy metal concentrations. A 1.0g sample was digested after adding 21mL of acid. The analyses were conducted using inductively coupled plasma mass spectrophotometry (ICP-MS).

3.10.2 Isolation of bacteria

One gram of dung and digestate was transferred to sterile distilled water and serially diluted up to 10^{-8}. For each dilution, 100 µL was spread on nutrient agar and the plates were incubated at 37°C for 24 hours. Individual bacterial colonies were selected on the basis of their distinct morphological characteristics and sub culturing was conducted until pure colonies were obtained. Pure isolates were preserved in glycerol (-80 °C freezer).

3.11 Assessment of soil fertility attributes

3.11.1 Screening for phosphate solubilisation bacteria

The phosphate solubilisation ability of the bacterial isolates was investigated using the National Botanical Research Institute's phosphate (NBRIP) growth medium (Nautiyal, 1999; Adeleke *et al*., 2015). The medium contained per litre; 10g of glucose, 5 g of insoluble $Ca_3(PO_4)_2$ as a source of phosphate, 5 g $MgCl_2$, 0.25 g $MgSO_4$, 0.2 g KCl, 0.1 g $(NH_4)_2SO_4$ and 15 g agar. The pH of the medium was adjusted to 7. 0. Ten micro liter of 48 hour culture was transferred to the wells created on the NBRIP medium. The halo zones and colony diameters were measured after 7 days of incubation at 37 ^oC. Formation of visible halo zones around the microbial colonies on NBRIP media indicates the phosphate solubilisation ability of the microorganisms. The ability of bacteria to solubilise insoluble phosphate was described by the solubilisation index (SI) (equation 6) (Bhawalkar, 1996).

$$SI = \frac{Colony\ diameter + halo\ zone\ diameter}{\text{Colony diameter}} \quad \textbf{(8)}$$

3.11.2 Nitrogen fixation

The isolates were grown on nitrogen free (Burk's) medium. The medium contained in a litre; 0.2 g $MgSO_4$, 0.80 g K_2HPO_4, 0.2 g KH_2PO_4, 0.130 g $CaSO_4$, 0.00145 g $FeCl_3$, 0.000253 g Na_2MoO_4, 20 g Sucrose and 15 g Agar. Plates were incubated at 30°C for 2- 3 days. The isolates that were able to grow after incubation indicated the potential to fix nitrogen, which is advantageous for plant growth (Bello-Akinosho *et al*., 2016).

3.11.3 Indole acetic assay (IAA)

The detection of IAA production was carried out as described by Bric *et al*. (1991) with some modifications. Isolates were cultured in LB broth supplemented with L-Tryptophan for 24 hours. Fully grown cultures were centrifuged at 10 000 rpm for 10 min at 4 °C. One mL of the supernatant was mixed with 2 mL of Salkowski reagent and incubated in the dark for 30 minutes at room temperature. A colour change of the reagent from yellow to pink indicated indole production.

3.11.4 16S rDNA gene amplification using colony PCR

Pure isolates were subjected to colony PCR (Obi *et al*., 2016). Pure colonies were used as a template DNA. The 16S rRNA was amplified using universal bacterial primers (907R and 341F).The cycling conditions are indicated in Table 3.3.

3.12 Phylogenetic assignment, alignment and clustering of 16S rRNA gene fragments

The PCR products were sent for Sanger sequencing at the Central Analytical Facilities (CAF) at the Stellenbosch University. Sequences were inspected and edited using BioEdit Sequence Alignment Editor (http://www.mbio.ncsu.edu/bioedit/bioedit). For the preliminary identification of isolates, the National Centre for Biotechnology Information (NCBI) gene bank was used, applying the basic alignment search tool (BLAST) (http://www.ezbiocloud.net/eztaxon). Mothur software was used to cluster sequences into OTUs at a sequence similarity of 97% (Bello-Akinosho *et al*., 2016). A phylogenetic tree was constructed with a neighbour-joining tree using the maximum composite likelihood model and 1 000 bootstrap replications in MEGA7.

3.13 Statistical analyses

The significant differences between the CH_4 yield and the physico-chemical parameters measured were evaluated by two-way ANOVA using XLSTAT version 2015. Student's t-LSD (least significant difference) was calculated at a 5% significance level to compare the means of significant source effects. The correlation to determine the association between variables was performed using SAS version 9.4 (Statistical Analysis System Institute, 1999).

CHAPTER 4: RESULTS

4.1 Anaerobic digestion in batch experiment

4.1.1 Compositional analyses

Compositional analysis of raw substrates was conducted prior to batch culture experiments. Substrates were characterised individually and a mixture of all FW in equal amounts was characterised. The results of the physico-chemical and biochemical characteristics of the substrates used in the current study are shown in Table 4.1. Potato peels had the highest carbohydrate content (calculated based on the dry matter). The VS ranged between 80 and 95%, potato peels having the highest. The moisture content ranged between 78 and 95% with tomato waste having the highest moisture content followed by vegetable waste, fruits waste, MFW, potato peels and CD respectively.

Table 4.1: The initial chemical composition of substrates used in batch experiment. Neutral detergent fibre (NDF), Acid detergent fibre (ADF), Acid detergent lignin (ADL).

Analyses	MFW	Vegetables	Fruits	Tomato	Potato peels	CD	Inoculum
Dry matter %	8.10	6.00	6.36	4.61	14.25	21.25	8.95
VS (DM %)	87.5	80.00	90.92	87.18	93.13	86.29	5.51
Moisture %	91.9	94.00	93.64	95.39	85.75	78.75	91.05
Protein (DM %)	13.95	17.83	11.47	22.99	9.75	19.95	8.26
Fat (DM %)	6.41	13.83	4.24	4.98	2.52	11.81	2.45
Carbohydrates (DM %)	69.87	49.66	77.044	60.30	81.89	55.53	50.83
NDF (DM %)	28.64	29.66	27.67	33.18	18.03	46.02	35.97
ADF (DM %)	18.51	18.83	22.01	31.67	8.91	23.48	16.20
ADL (DM %)	4.44	1.83	4.40	8.67	4.14	5.76	1.34
Cellulose (DM %)	14.07	17.00	17.61	22.99	4.77	18.07	14.86
Hemicellulose (DM %)	10.12	10.83	5.66	1.51	9.12	22.54	34.63

*Cellulose =ADF-ADL

Hemicellulose=NDF-ADF

4.1.2 pH

The initial and final pH of substrates used in the batch experiments are presented in Figure 4.1. All the controls had a pH range of between 4 and 5 before and after AD, except for the inoculum which had a pH above 7. Potato peels and CD with inoculum had a high pH before AD but the pH decreased after AD. In the rest of the substrates with inoculum, the pH value increased after the AD process.

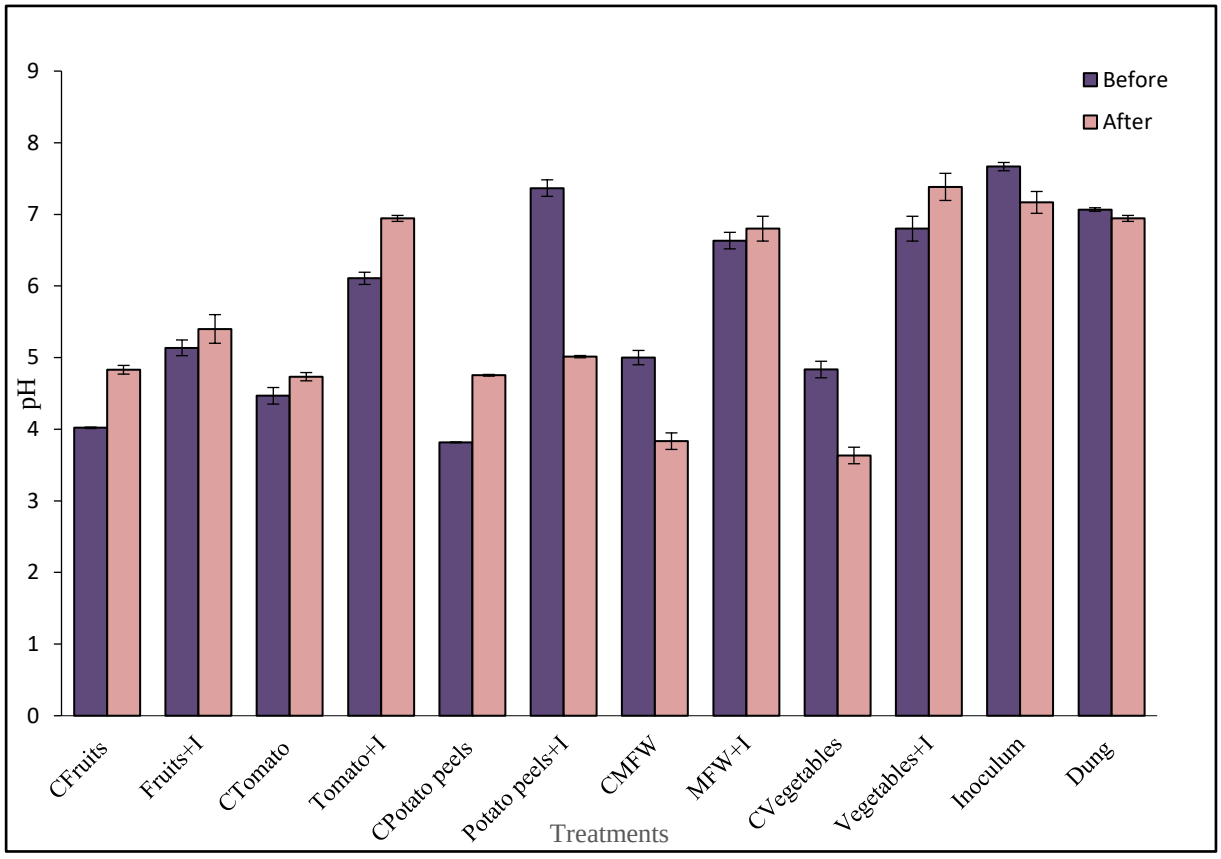


Figure 4.1: pH measurements before and after AD process (batch experiments). Error bars representing standard deviation (n=3). I=inoculum and MFW=mixed food waste, C=control (digestion with FW alone are specified as control).

4.1.3 Batch methane production

The cumulative CH_4 production during AD in the batch experiments is shown in Figure 4.2. All FW and CD produced CH_4 but with varying yield. No significant difference was observed in the CH_4 yield with CD, MFW, vegetables and tomatoes ($P>0.05$) at the end of the digestion process. However, the CH_4 yield obtained from fruits waste and potato peels were lower than of tomatoes, CD, MFW and vegetables ($P<0.05$). The fruit and potato peel wastes produced the lowest CH_4 ($P>0.05$). None of the FW controls produced CH_4 but only CO_2 was produced. The vegetables, tomatoes, MFW, CD and fruits had low C/N ratio ranging between 12-19, and potato peels had a C/N ratio of 27 (Table 4.2).

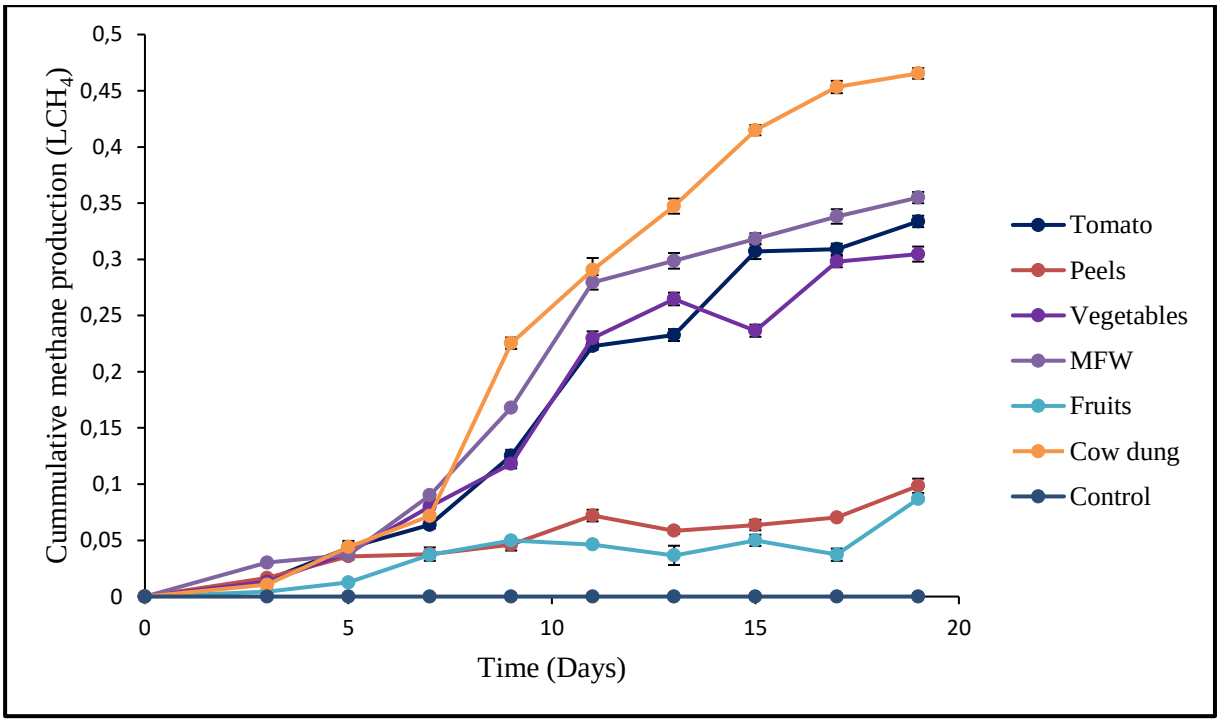


Figure 4.2: Cumulative methane production using different substrates. Error bars representing standard deviation (n=3). Digestion with FW alone are specified as control.

Table 4.2: Summary of the AD digestion parameters using FW and CD

Substrates	C	N	C/N	L CH_4/g VS (last day)
Tomato	41.49	3.39	12.22	0.25
Potato peels	40.18	1.46	27.56	0.05
Vegetables	36.81	2.73	13.50	0.23
MFW	205.6	12.11	16.97	0.24
Fruits	42.55	2.21	19.28	0.06
CD	44.64	2.31	19.31	0.21

4.2 Biogas production in semi-continuous digestion

The cumulative biogas yield for all semi-continuous experiments is shown in Figure 4.3. The highest yield was achieved with CO followed by CD and MFW respectively. The trends in treatments were the same when biogas yield increased with time.

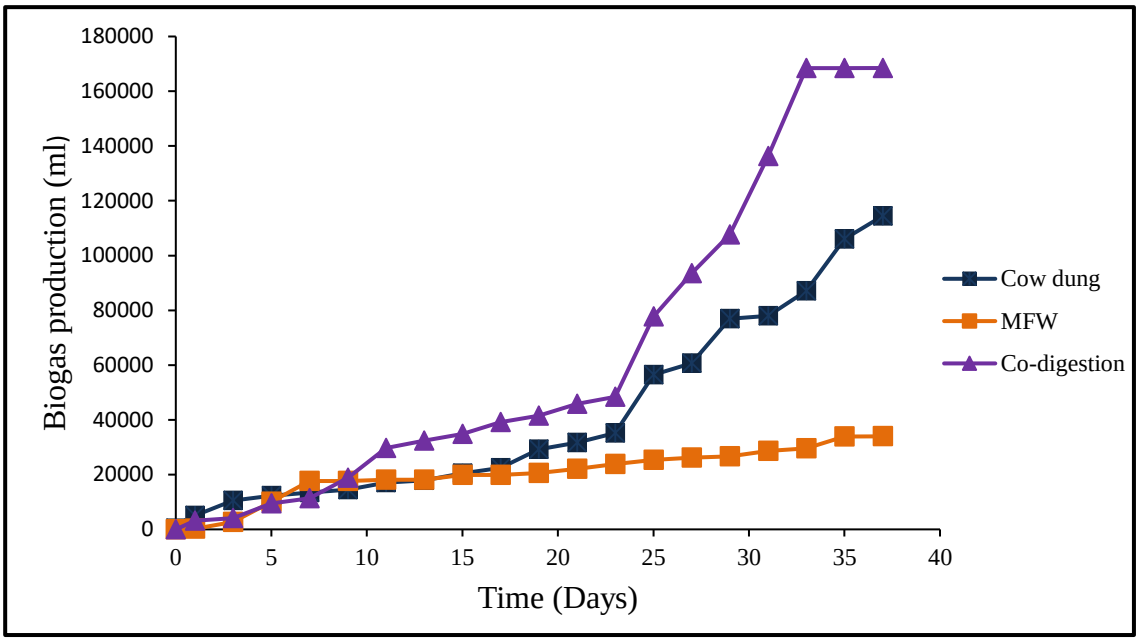


Figure 4.3: Influence of substrate type on biogas production in semi-continuous digestions

The pH was not controlled during the entire process but measured at different intervals. The initial pH for all experiments varied. Cow dung and MFW decreased at the beginning of the process while in CO, a drop in pH was observed on day 11 (Figure 4.4). The pH in CO and CD trials increased again and was almost within the range or stabilised during the entire digestion process. However, the pH of MFW decreased significantly and it was within the range of 4.3-4.9.

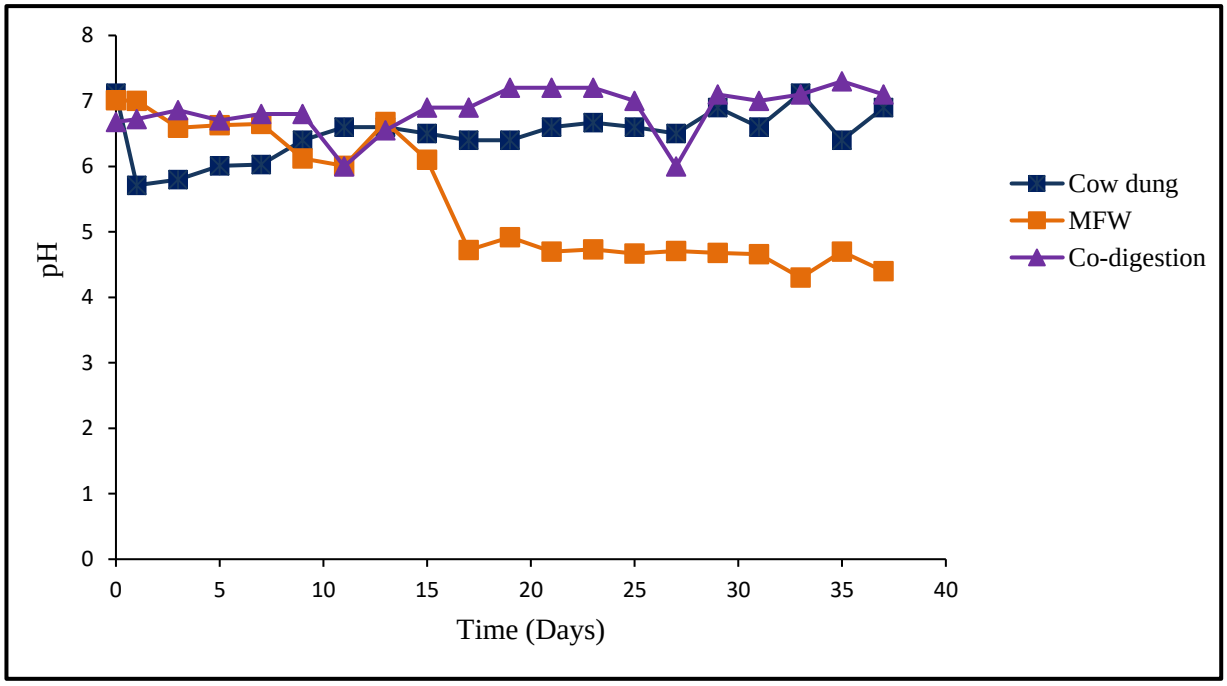


Figure 4.4: pH measured at different days for all substrates used.

The highest CH_4 production (67%) in total biogas produced was obtained from CO followed by CD (52%) and the lowest CH_4 was achieved from MFW at the end of the digestion process. Fluctuations in CH_4 production were recorded mostly in MFW after day 10 compared to other treatments. The CH_4 production decreased significantly and 18% was recorded at the end of the digestion process for MFW (Figure 4.5). The CO_2 content was higher at the beginning of the process but decreased with increasing CH_4 content in CD and CO.

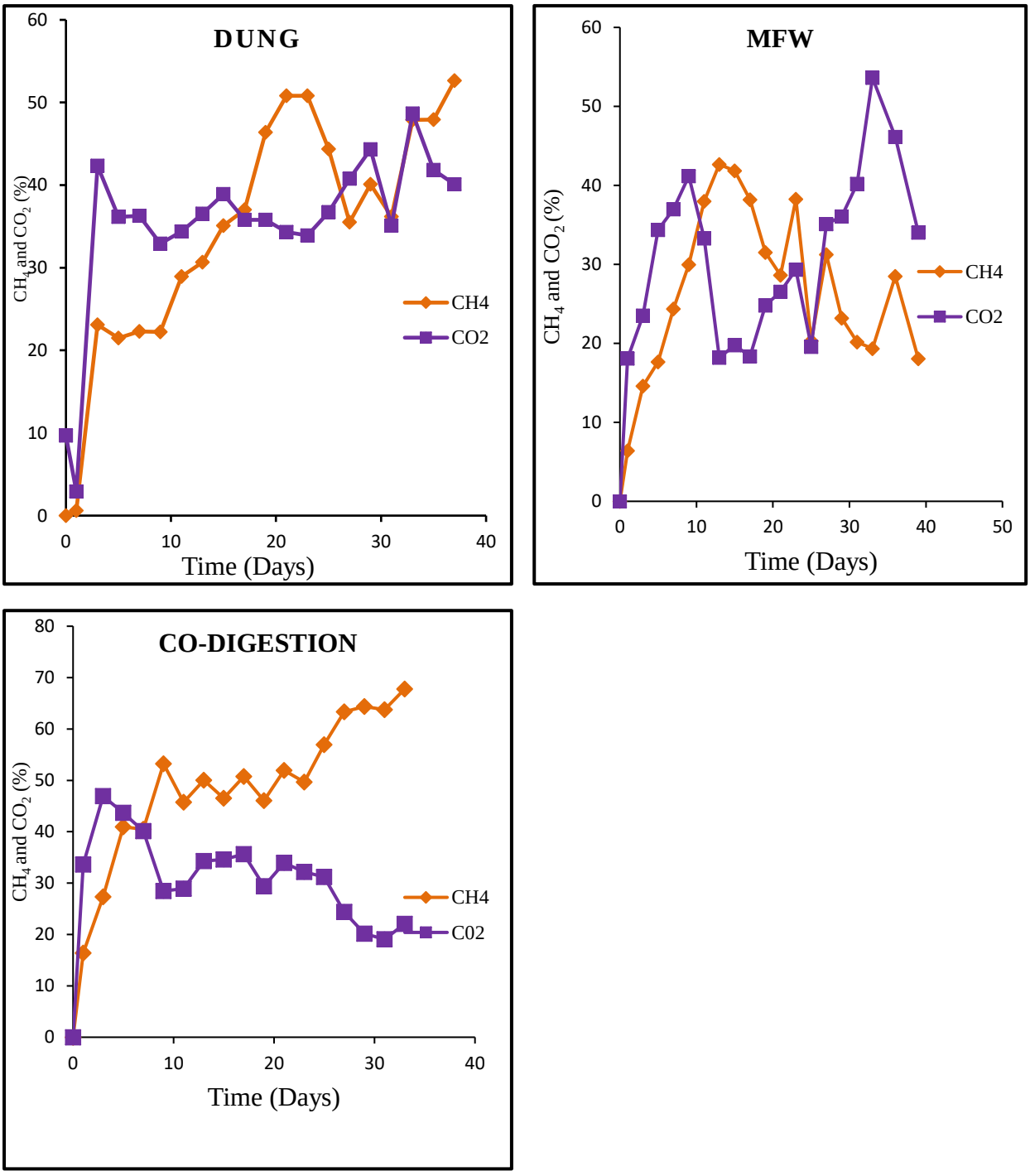
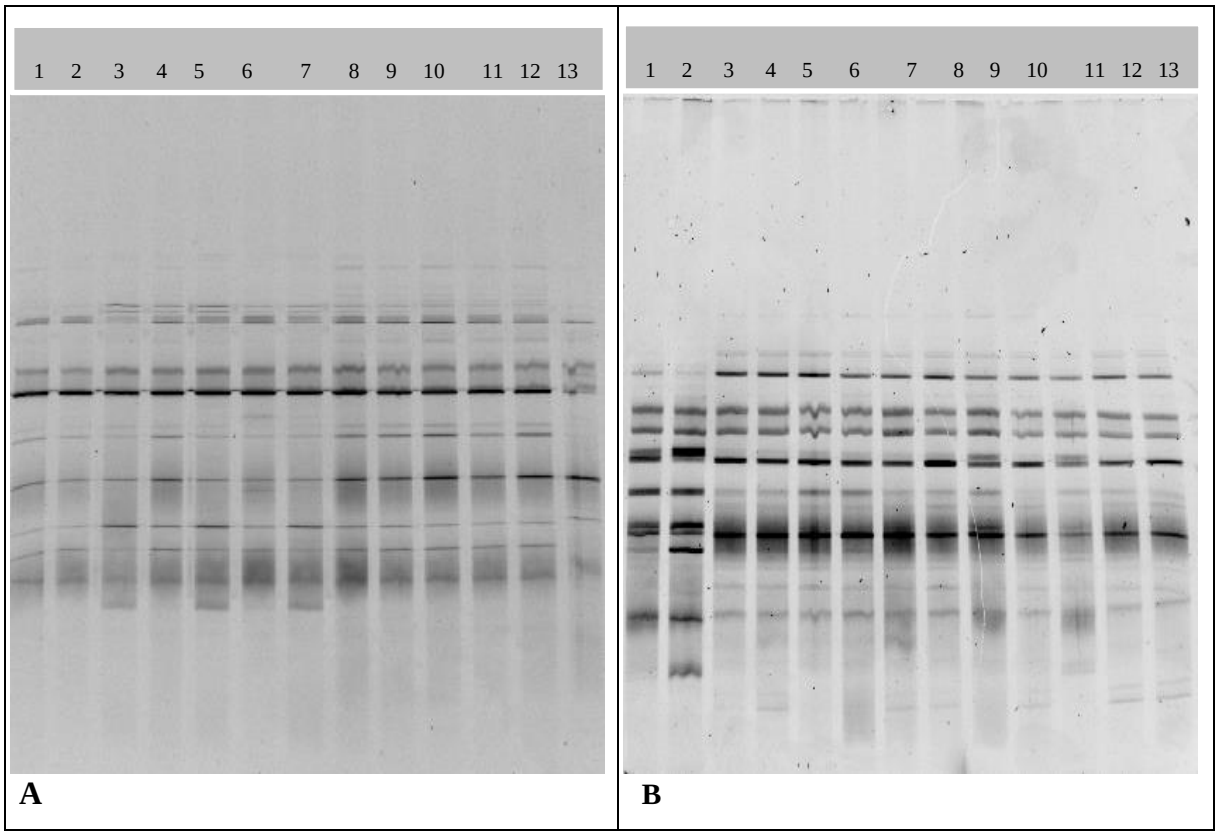


Figure 4.5: Cumulative methane production during AD digestion process.

4.3 Denaturing gradient gel electrophoresis (DGGE)

Denaturing gradient gel electrophoresis was performed to investigate the bacterial and archaeal community shifts during the AD process. The PCR-DGGE profiles showed highly diverse microbial assemblages in CO in comparison with MFW and CD. The DGGE pattern changed slightly as the digestion process progressed in all treatments. In the initial stage, the banding pattern was similar especially with archaea (Figures 4.6 &4.7). The intensity and quantity of bands in CO were also stronger compared to those of CD and MFW digested alone. The increased diversity of bacterial species with bright and faint bands resulted in a smear in the DGGE pattern from CD.

The Shannon-Weaner (*H'*) and inverse Simpson's indices (1/D) were used to estimate the bacterial and archaeal diversity (Table 4.3). The Shannon-Weaner index represents the species abundance and uniform distribution (Dilly *et al*., 2004). The *H*' and 1/D values fluctuated throughout the digestion process. The highest *H'* and 1/D were observed with the CO of substrates and the lowest with CD. The *H'* values in CO ranged from 2.12 to 2.84 in archaea and from 2.63 to 3.24 in bacteria. The *H*' and 1/D order were as follows: CO >MFW> CD (Table 4.3). Lower CH_4 production towards the end of the process was observed in MFW digestion with higher *H*' and 1/D values.



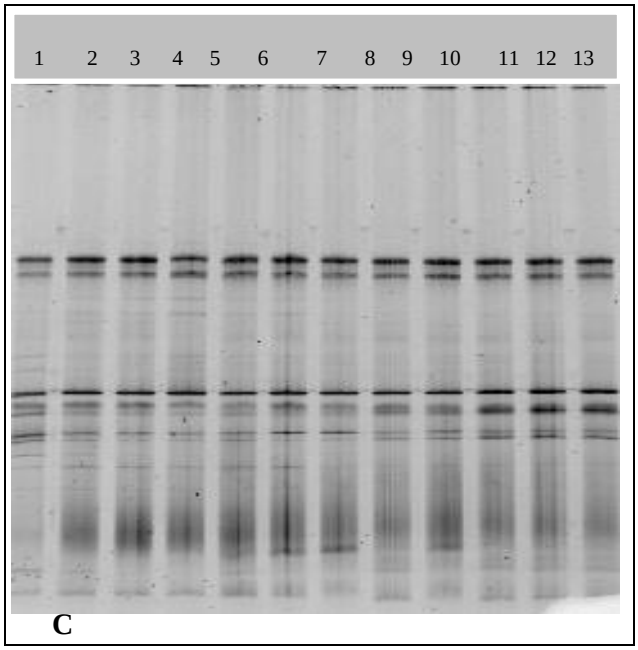
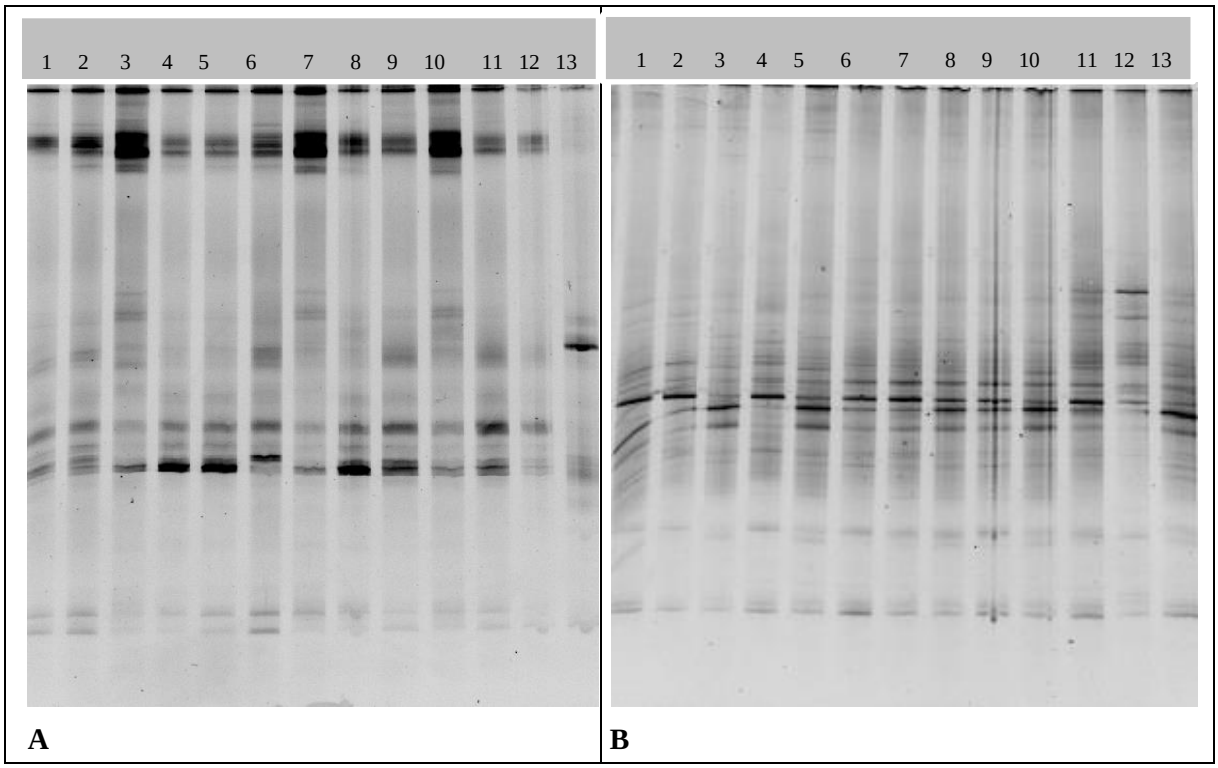


Figure 4.6: DGGE gel showing the dominant archaeal communities obtained from the digester. The bands were separated on 8% polyacrylamide gel with denaturing gradient of 60-25%. A-CD, B-MFW and C-CO. Lane 1-13, samples collected during semi-continuous AD of each treatment.



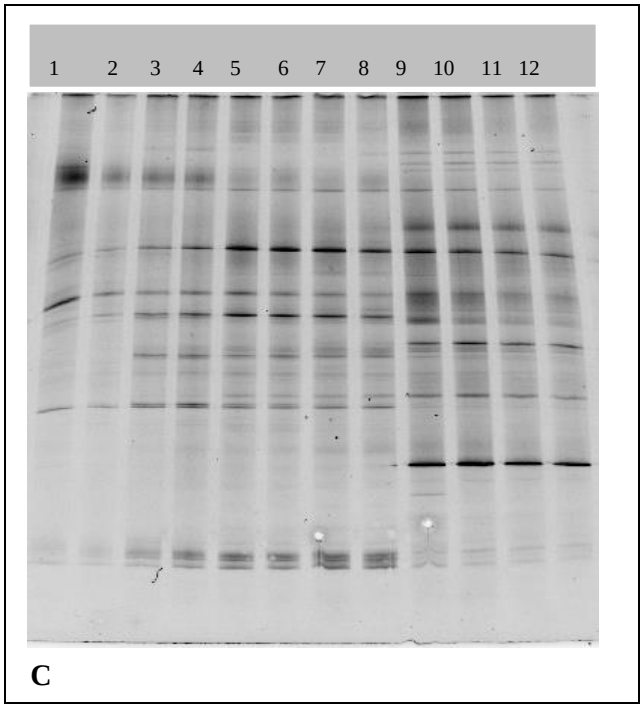


Figure 4.7: DGGE gel showing the dominant bacterial communities obtained from the digester. The bands were separated on 8% polyacrylamide gel with denaturing gradient of 60-40%. A-CD, B-MFW and C-CO. Lane 1-13, samples collected during semi-continuous AD of each treatment.

Table 4.3: Bacterial and Archaeal diversity indices obtained from CD (A), MFW (B) and CO (C)

A

Archaea	CD											
H’	1.96	2.08	2.22	2.16	2.32	2.28	4.15	2.07	2.11	2.19	2.18	2.34
1/D	4.80	5.37	5.71	6.01	6.42	6.69	4.96	5.66	6.01	6.61	6.69	7.28
Bacteria												
H’	2.07	2.39	2.29	1.69	2.26	2.38	2.21	2.25	2.07	2.36	2.20	2.02
1/D	4.82	6.14	6.19	4.3	5.83	6.6	6.17	7.28	4.87	8.00	6.48	3.87

B

Archaea	MFW											
H’	2.20	2.54	2.43	2.28	2.29	2.28	2.49	2.38	2.37	2.62	2.33	2.66
1/D	6.16	10.11	8.69	6.24	6.12	6.50	6.9	6.52	7.06	10.19	6.35	10.95
Bacteria												
H’	2.23	2.75	2.77	2.82	2.84	2.94	3.03	2.99	3.00	3.02	3.18	2.82
1/D	6.25	10.00	8.75	10.13	10.26	10.83	12.23	12.58	13.63	11.90	16.96	10.35

C

Archaea	CO											
H’	2.84	2.56	2.12	2.25	2.34	2.56	2.56	2.50	2.66	2.64	2.55	2.51
1/D	12.01	8.55	4.54	5.30	5.93	9.24	9.24	8.90	9.36	9.72	9.21	8.96
Bacteria												
H’	2.47	2.63	2.91	2.94	3.04	3.03	3.05	3.24	3.08	3.05	2.94	2.86
1/D	7.31	8.10	12.94	14.09	13.34	14.31	14.41	20.50	15.48	13.47	12.16	10.40

4.4 Next generation sequencing (NGS)

To investigate the relationship between the microbial diversity and biogas production, samples were taken when the CH_4 levels were low, intermediate and high from semi-continuous digester runs using CD, MFW and CO i.e. CD and MFW. The microbial community richness is indicated by the number of observed OTUs, Chao1, Shannon index and Simpson's index. The number of OTUs observed in each treatment is shown in Table 4.4. The highest number of OTUs was observed when the CH_4 production was intermediate in CD followed by MFW and lastly CO. The Chao1 richness estimator (Table 4.4) indicated that there was greater species richness when the CH_4 was in an intermediate phase in all reactors. In general, the microbial communities in all reactors were diverse.

The relative abundance and taxonomic distribution of microbial communities in each sample were analysed at phylum, class, order and genus level. Thirty-four phyla were identified including *Euryarchaeota, Miscellaneous Crenarchaeotic Group, Acidobacteria, Actinobacteria, Armatimonadetes, Atribacteria, Bacteroidetes, Candidate division WS6, Chlorobi, Chloroflexi, Cloacimonetes, Cyanobacteria* and *Deferribacteres.* Other identified phyla are *Deinococcus-Thermus, Elusimicrobia, Fibrobacteres, Firmicutes, Fusobacteria, Gemmatimonadetes, Hydrogenedentes, Latescibacteria, Lentisphaerae, Microgenomates, Nitrospirae, Parcubacteria, Planctomycetes, Proteobacteria, SHA-109, Saccharibacteria, Spirochaetae, Synergistetes, Tenericutes, Thermotogae* and *Verrucomicrobia.*

However, the phyla *Bacteroidetes* followed by *Firmicutes, Actinobacteria* and *Proteobacteria* were dominant or distributed in varying amounts in all treatments (Figure 4.8). *Firmicutes* were predominant in MFW when the CH_4 production was high, (MFW-H, 55%) followed by CD (CD-H, 50.3%). Lower abundance of the phylum *Actinobacteria* was observed in CO which range between (0.2-0.4%), MFW (0.05-7.13%) and highest in CD (0.2-15.91%). In the phyla *Firmicutes* and *Bacteroidetes*, the class *Bacteroidia* followed by *Clostridia* were present in abundance in all treatments. However, the classes *Negativicutes* and *Gammaproteobacteria* were dominant in MFW especially when the CH_4 production was in the intermediate and high phases, and least distributed in CO (Figure 4.9). The genus *Syntrophomonas* predominated in CO. (Figure 4.11). An enormous portion of unclassified and uncultured bacteria was obtained at the genus and order level. In archaea, two phyla *Eurychaeota* and *Crenarchaeota* were

detected in all samples. However, the phylum *Euryarchaeota,* which includes the methanogens that are responsible for CH_4 production was predominant in CO in comparison with other treatments. The relative abundance of archaeal communities at genus level is shown in Figure 4.12. *Methanobrevibacter*, *Methanosarcina,* and *Methanobacterium* in that order were present in large quantities. Other methanogens found were *Methanosphaera*, *Methanocorpusculum, Methanoculleus*, *Methanoregula*, *Methanospirillum*, *Methanosaeta*, *Candidatus Methanogranum*, *Candidatus Methanoplasma*, *Methanomassiliicoccus* and *uncultured archaeon. Methanobrevibacter* genera were present in large quantities in CD and MFW but less prevalent in CO. Comparably, the genus *Methanocarsina* was present in large quantities in CO compared to the other treatments. *Candidatus* and *Methanoplasma* were less abundant in all treatments. When the CH_4 production was low the methanogens were present in small quantities.

Table 4.4: Community richness and alpha diversity from bacterial and archaeal communities. Chao 1 estimates the richness (higher value means higher richness). The CH_4 production was categorised into three classes: Low (0-25%), Intermediate (26-40%) and high (41-80%).

Treatments	Categories	CH_4 (%)	Observed OTUs	chao1	Shannon index (H')	Simpson index (D)
CD	Low	23	2414	3042.51	5.91	9.04
	Intermediate	36	2889	3515.12	6.64	24.55
	High	50	2018	2686.99	6.39	33.32
	High	52	2110	2753.00	6.51	33.26
MFW	Low	15	1737	2389.63	6.20	23.66
	Intermediate	29	2673	3418.92	6.89	39.01
	High	41	1840	2529.37	5.93	27.40
	High	42	1973	2682.22	5.92	15.437
CO	Low	17	2316	3061.79	6.63	25.59
	Intermediate	27	2540	3425.78	5.91	10.51
	High	49	1703	2204.00	6.32	27.15
	High	67	1942	2533.22	6.40	25.97

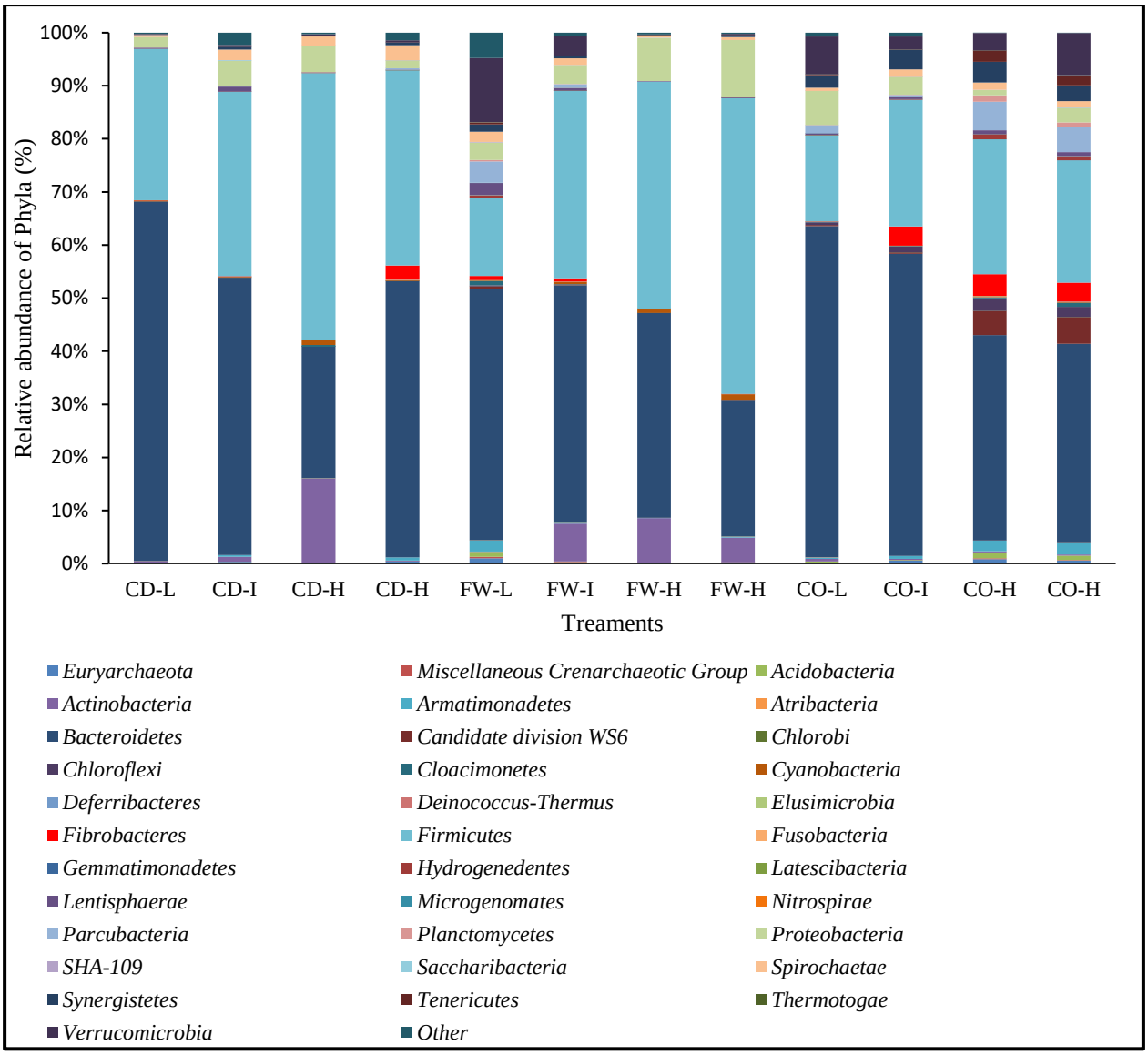


Figure 4.8: Relative abundance of the microbial communities of different treatments at phyla level (CD- cow dung, FW- mixed food waste, CO-codigestion of cow dung and mixed food waste).

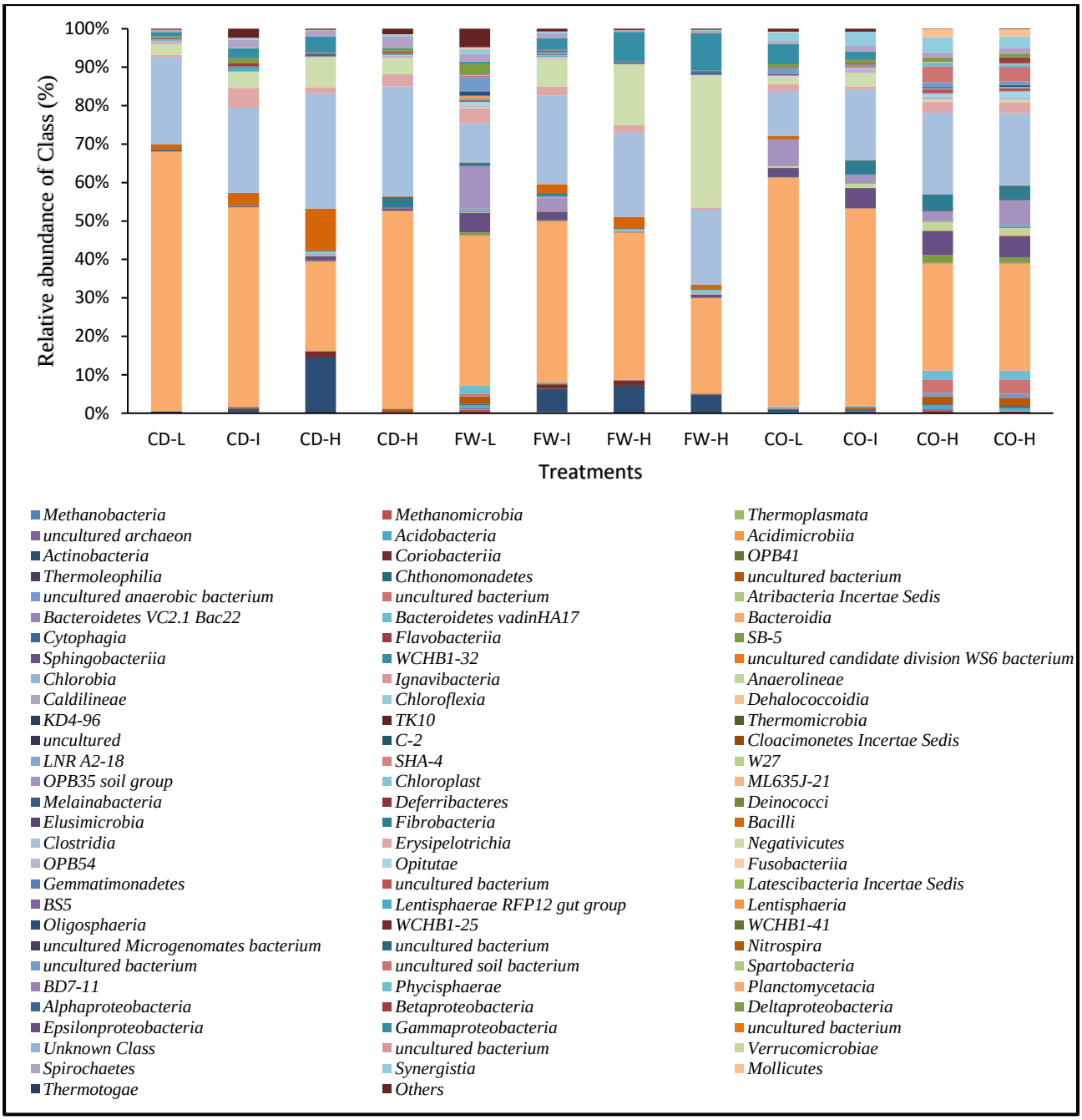


Figure 4.9: Relative abundance of the microbial community of different treatments at class taxa-level (CD- cow dung, FW- mixed food waste, CO-codigestion of cow dung and mixed food waste).

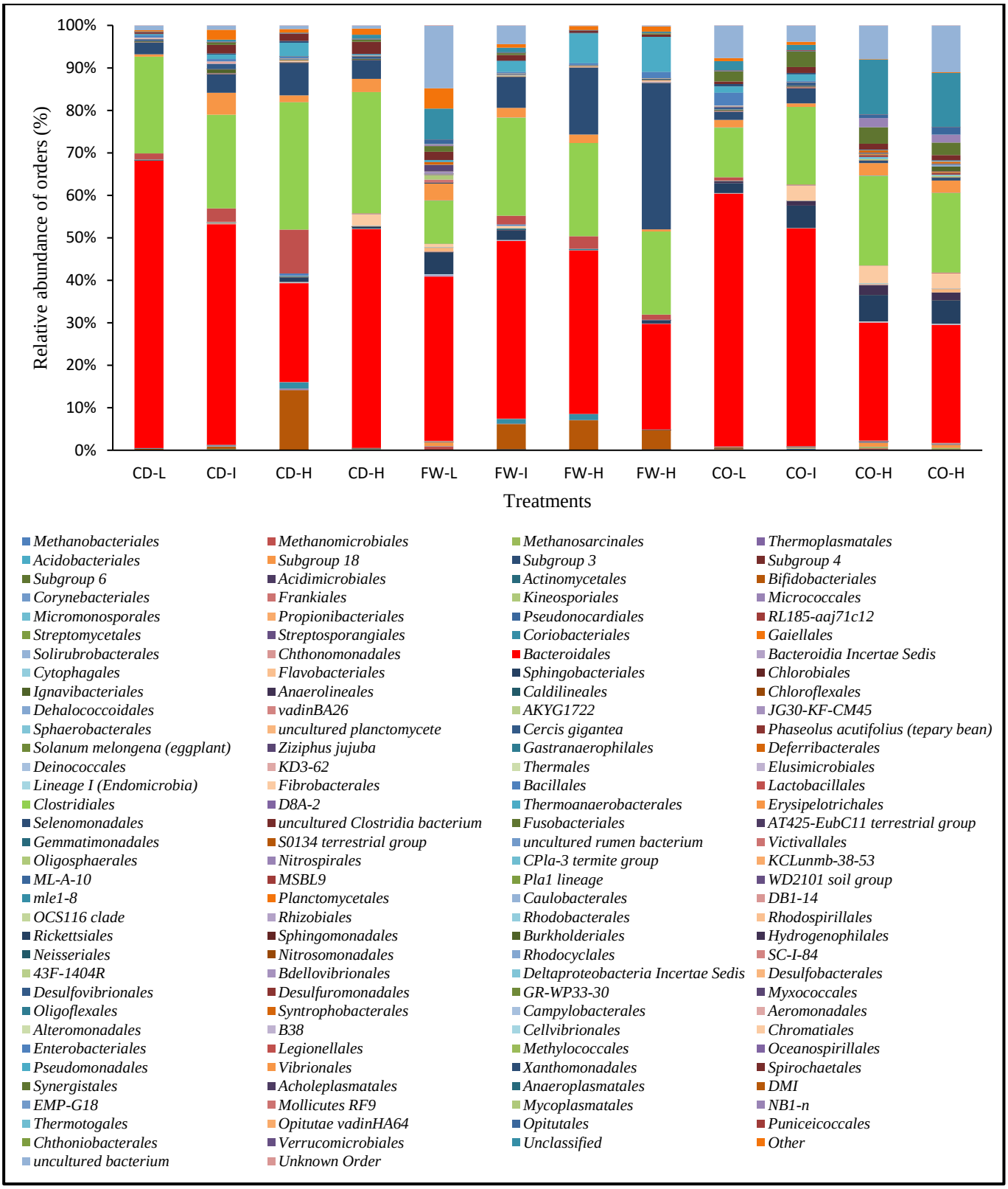


Figure 4.10: Relative abundance of the microbial community of different treatments at orders taxa (CD- cow dung, FW- mixed food waste, CO-codigestion of cow dung and mixed food waste).

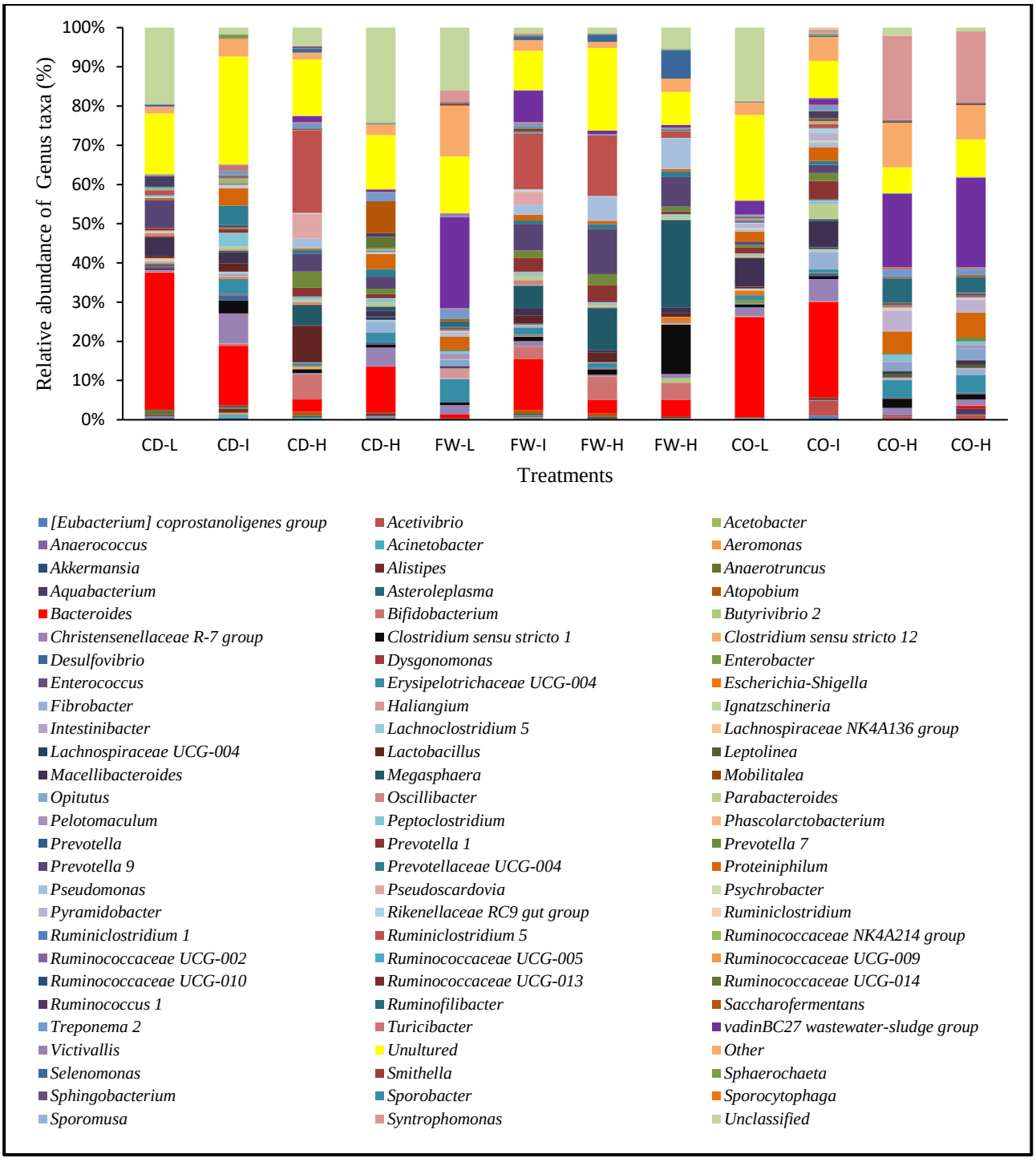


Figure 4.11: Relative abundance of the microbial community of different treatments at genus taxa. All sequences with less than 1% were excluded (CD- cow dung, FW- mixed food waste, CO-codigestion of cow dung and mixed food waste).

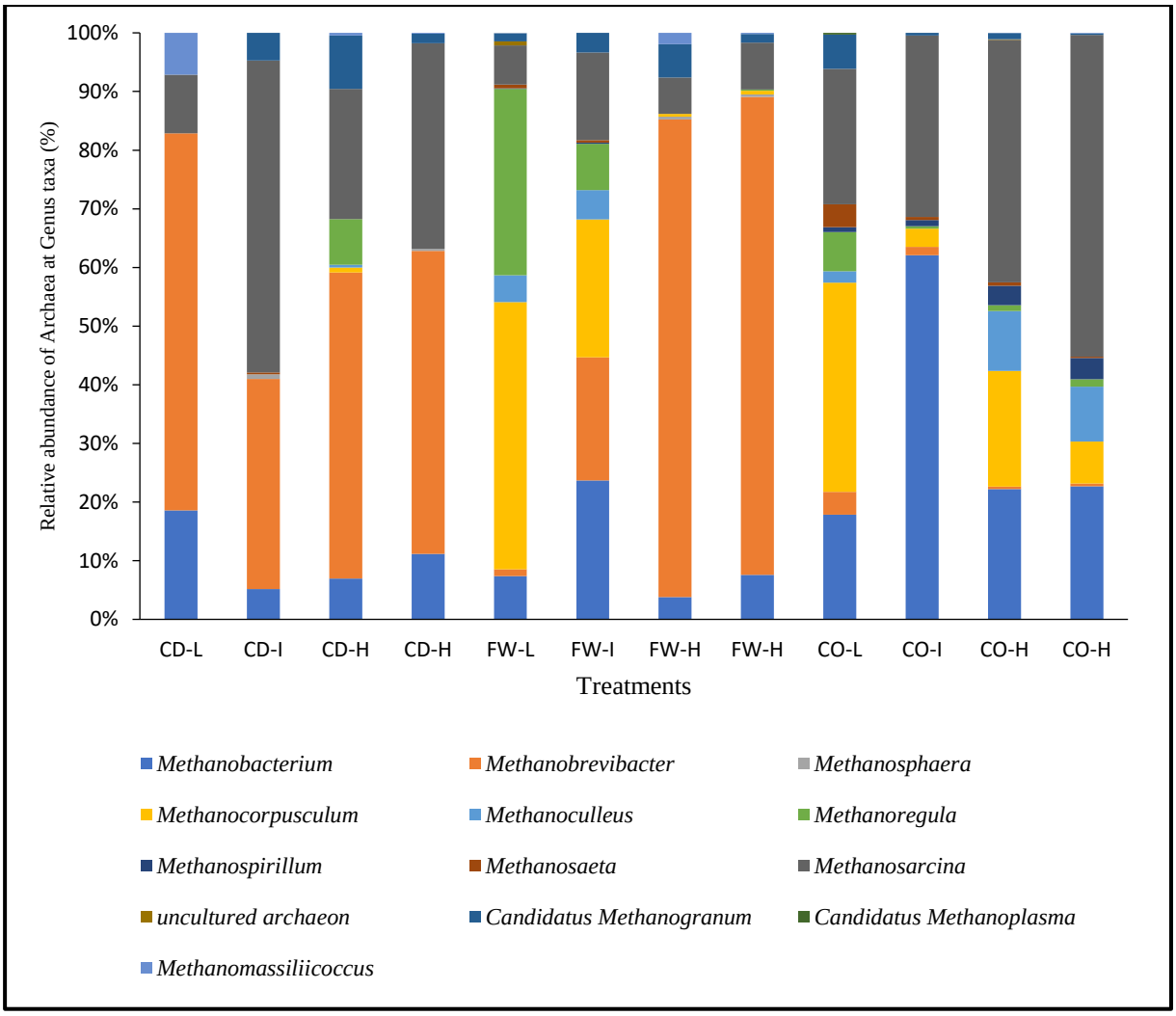


Figure 4.12: Relative abundance of Archaeal community of different treatments at genus level (CD- cow dung, FW- mixed food wastes, CO-codigestion of cow dung and mixed food waste).

4.5 Plant growth promotion experiments

4.5.1Physico-chemical parameters before and after AD

The physico-chemical characteristics of dung and digestate were compared (Table 4.5). The dry matter, potassium, phosphorus and VS decreased in the digestate ($P<0.05$). However, no significant variation was evident in the pH, nitrogen and carbon content of the dung and digestate ($P>0.05$). Furthermore, the C/N ratio was higher in the dung than in the digestate.

Table 4.5: The physico-chemical parameters of the digester feedstock before and after AD

Nutrient content	Dung	Digestate
pH (%)	7.4±0.15	7.73±0.05
DM (%)	21.78± 0.43	14.21± 0.22
VS (%)	80.98 ±0.67	63.98± 0.33
Nitrogen (ppm)	1.6±0.05	1.91±0.02
Potassium (ppm)	0.9±0.03	0.56±0.02
Phosphorus (ppm)	0.43±0.02	0.26±0.01
Carbon (%)	39.651±0.46	36.76±0.28
C/N	24.78	19.24

n=3; ± standard deviation

4.5.2 Heavy metal analysis

In SA, there is no legislation or guidelines for heavy metal content in digestate. Therefore, the fertiliser regulations of the Department of Agriculture, Forestry and Fisheries (DAFF, 2012) were used. The heavy metals in the dung and the digestate were below the threshold recommended by DAFF. The mean concentration of heavy metals before and after the AD process is shown in Figure 4.13. There was a significant decrease in the levels of Mn, Cu, Sr, Sn, and Ba after AD ($P<0.05$).

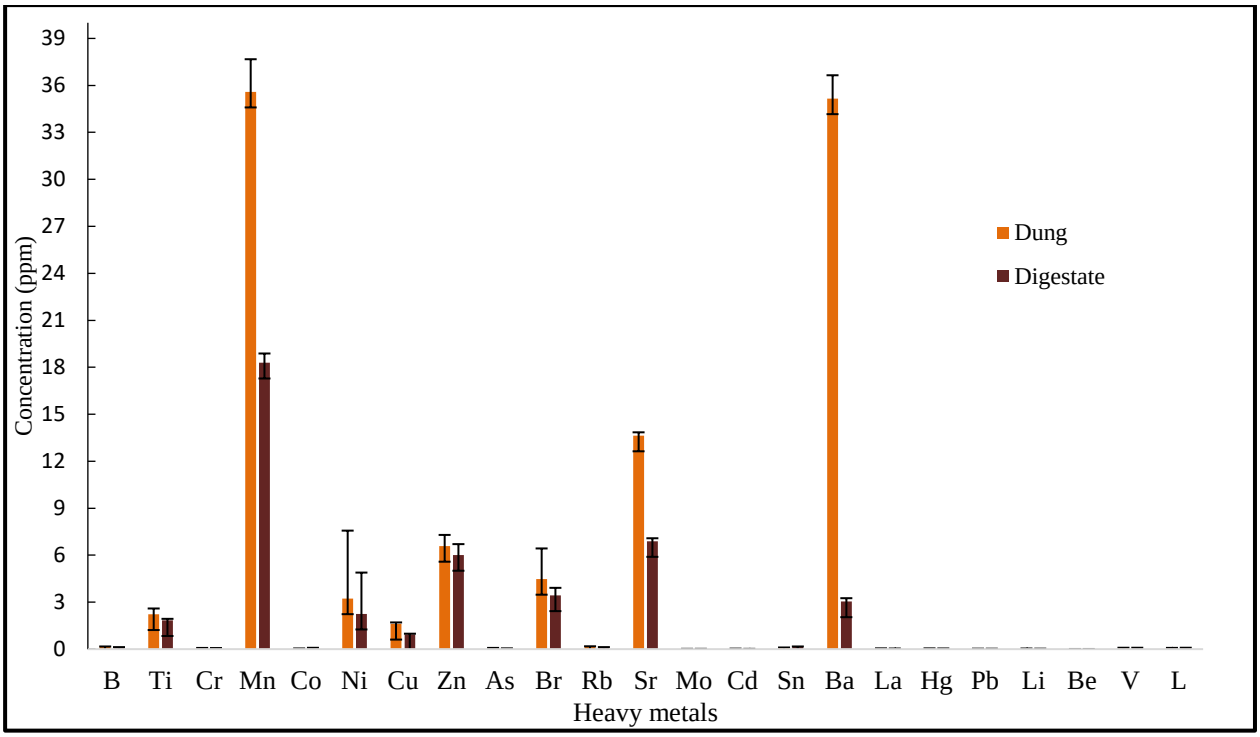


Figure 4.13: Heavy metal analyses from the dung and digestate: Error bars represent standard deviation (n=3)

4.5.3 Screening for phosphate solubilisation, IAA production and nitrogen fixation

Twenty-four bacterial isolates were obtained, 15 from the dung and 9 from the digestate. Selection of isolates was based on variations in the morphological characteristics of the bacterial colonies (Table 4.6). The isolates were screened for their ability to solubilise insoluble forms of phosphate. More isolates from the dung were able to solubilise phosphate than those obtained from the digestate (Figure 4.14). From the nitrogen fixation and IAA production assays, similar trends were observed in that more isolates from the dung were able to fix atmospheric nitrogen and produce IAA than the digestate isolates.

Table 4.6: Morphological characterisation of bacterial isolates

No. of isolates	Shape	Elevation	Margin	Colour
7	Round	Raised	Smooth	Off white
4	Round	Raised	Smooth	Whitish
9	Round	Raised	Smooth	Yellowish
2	Round	Raised	Smooth	Brownish
2	Round	Flat	smooth	yellowish

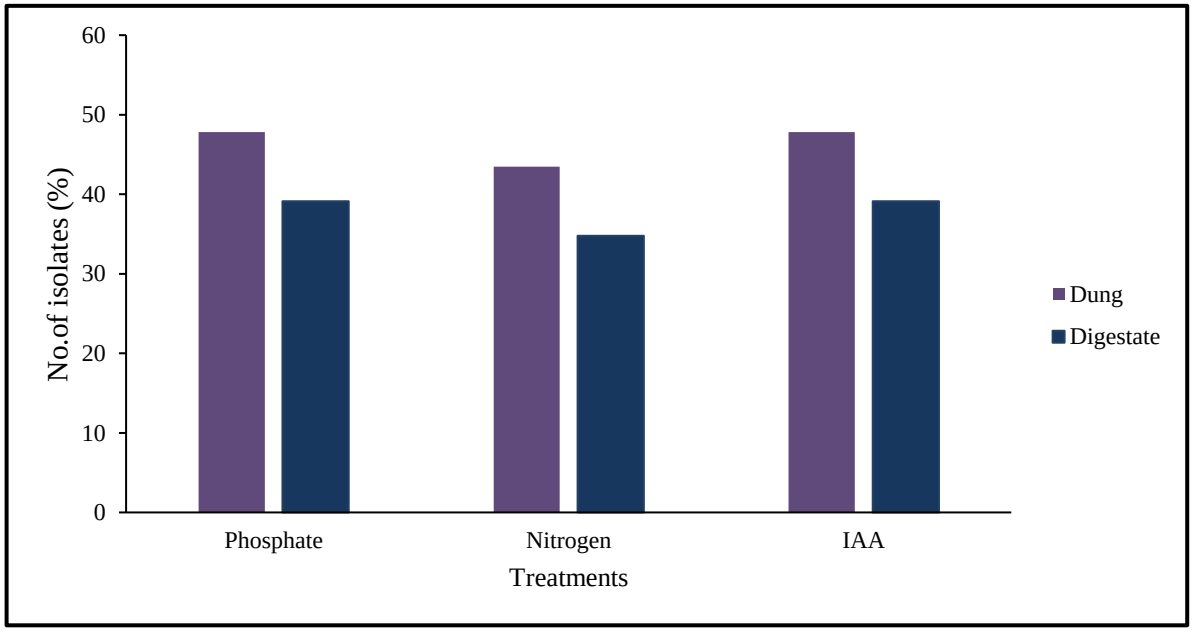


Figure 4.14: Phosphate solubilisation, nitrogen fixation and indole acetic acid production of bacterial isolates.

4.5.4 Community analysis for plant growth promoting abilities

Sequences of the isolates were clustered into seven OTUs (Table 4.7). The phylogenetic tree of the representative OTUs was constructed using the neighbour joining method (Figure 4.15). Four genera were obtained from the dung and three genera from the digestate. Shared genera from both samples included *Acinetobacter* and *Bacillus.*

Table 4.7: OTUs in relation to the phyla and sequence they contain

OTUs	**No. of sequences**	**Accession numbers of sequences**	**Representative isolates**	**Genera**	**Phyla**
OTU1	17	MF044461,MF044462,MF044463 MF044464,MF044465,MF044466, MF044467,MF044469,MF044471, MF044472,MF044473,MF044474, MF044474,MF044475,MF044477 MF044479,MF044480.	S7b	*Acinetobacter*	*Proteobacteria*
OTU2	2	MF044482,MF044476	D16	*Escherichia*	*Proteobacteria*
OTU3	1	MF044468	D19a	*Staphylococcus*	*Firmicutes*
OTU4	1	MF044478	D8	*Micrococcus*	*Actinobacteria*
OTU5	1	MF044484	D17c	*Bacillus*	*Firmicutes*
OTU6	1	MF044485	S20a	*Bacillus*	*Firmicutes*
OTU7	1	MF044470	S22	*Pseudomonas*	*Proteobacteria*

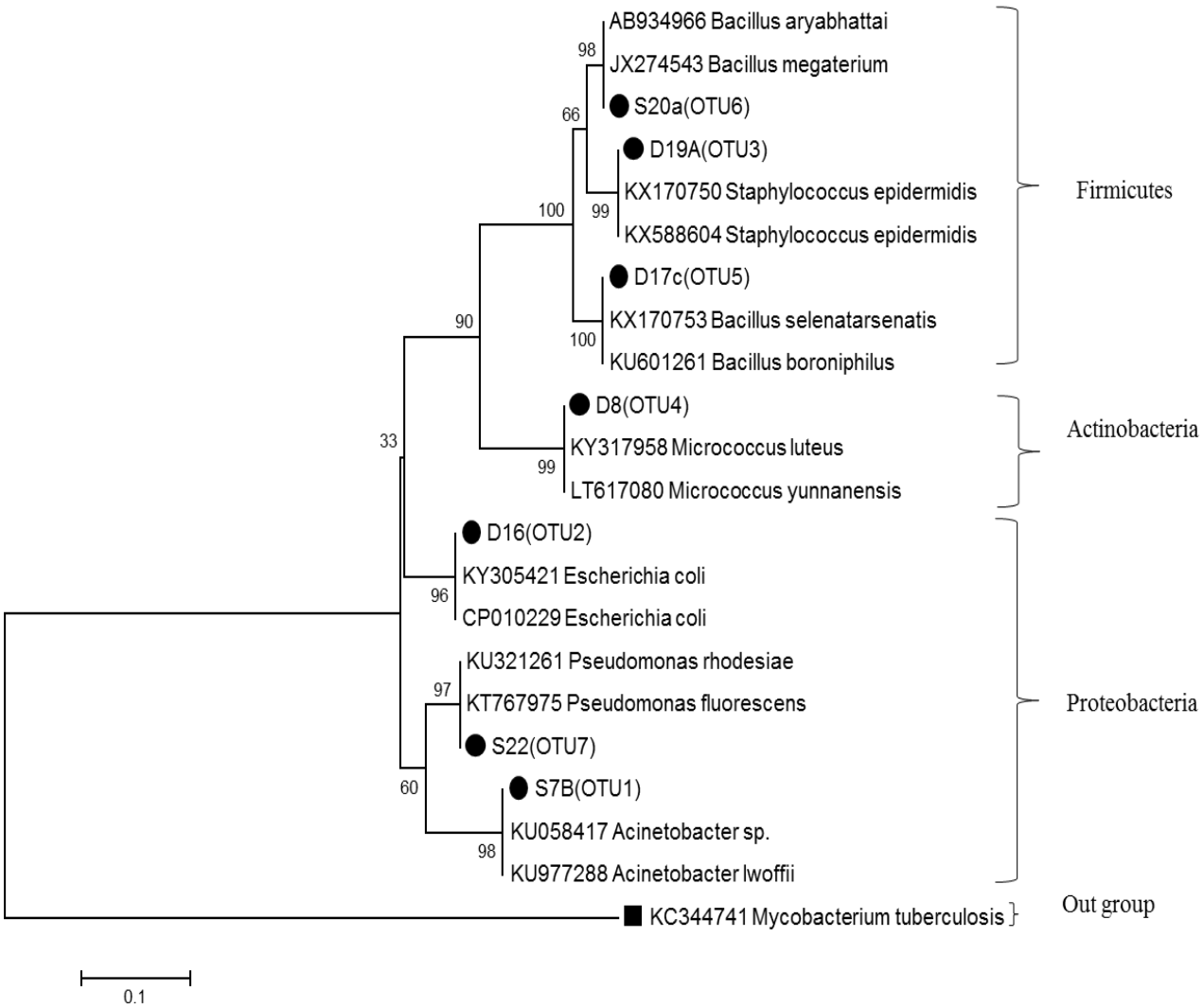


Figure 4.15: The evolutionary history was inferred using the Neighbor-Joining method. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches. The evolutionary distances were computed using the Jukes-Cantor method and *Mycobacterium tuberculosis* was used as anoutgroup. The analysis involved 24 nucleotide sequences. Evolutionary analyses were conducted in MEGA7. Seven OTUs were generated using MOTHUR software (97% similarities). The relatives of the OTUs were obtained from NCBI.

CHAPTER 5: DISCUSSION

5.1 Anaerobic digestion in the batch experiments

5.1.1 Compositional analyses

Characterisation of organic waste is crucial when designing and operating the digester (Garcia-Peña *et al*., 2011). In a study by Ward *et al*. (2008), it was found that FVWs mainly consist of low TS and high VS, findings that were also observed in this study. The values of the VS and moisture content of FW are comparable with those of Bouallagui *et al*. (2005). The VS and moisture content results revealed that all substrates used contained a high energy content, which is desirable for biogas production. A high moisture content facilitates the AD process (Boullagui *et al*., 2003), which indicates that CD and FW are good substrates for AD. Food waste contains more than 30% cellulose, 10% hemicellulose and lignin (Mata-Alvarez *et al*., 1992). Low lignin content was recorded in all substrates used. This is advantageous because substrates with high lignin contents tend to slow down the AD process due to a decreased decomposition rates (Nurliyana *et al*., 2015) because microorganisms cannot easily breakdown the lignin structure.

5.1.2 pH

pH has been reported as one of the most important parameters in AD (Mata-Alvarez *et al*., 1992). Whenever the pH is below 6.5 or above 8, the CH_4 forming microorganisms are inhibited. The low pH results observed are in agreement with previous study conducted by Bouallagui *et al*. (2005) who reported low pH in FW ranging between 3.5 -4.2. After the AD process, the pH increased slightly towards alkalinity in most treatments containing the inoculum. The alkalinity of CD probably neutralized the pH, hence increasing the buffering capacity. This was an indication of process stability during AD. However, the pH values of the digested fruit wastes were still below the range required for AD. A logical explanation is that the mixture of fruit waste used was mainly lemon, which is known to be acidic in nature. Perhaps the acid accumulated and inhibited the growth of methanogens which led to a reduction in the pH level.

5.1.3 Batch methane production

The CH_4 yield relies mostly on waste composition (protein, carbohydrates and lipid) and reactor design (Bouallagui *et al*., 2009). The range of CH_4 yields obtained from tomatoes and MFW is comparable with those obtained in studies conducted by Gunaseelan. (2004) and Bouallagui *et al*. (2005). A well-balanced mixture of fruit and vegetable wastes is ideal, since it reduces the chances of sugar accumulation (Scano *et al*., 2014). The lowest CH_4 production was obtained from fruit and potato peel wastes indicating the occurrence of inhibition. Fruits contain high sugar content that results in rapid hydrolyses and acidification, which in turn increase the CO_2 and reduce the CH_4 content during AD (Scano *et al*., 2014). Only CO_2 was produced in the controls which indicates that FW do not contain the microorganisms necessary to initiate methanogenesis. The CH_4 yield could also be affected by the C/N ratio. For instance, low cellulose content and C/N ratio associated with FW can accelerate the release of ammonia in large quantities. The vegetables, tomatoes, mixed food waste, CD and fruits had low C/N ratio ranging between 12-19, and potato peels had C/N ratio of 27 (Table 4.2). This is in line with the findings reported by Kumar *et al*.(2010) who obtained a C/N ratio ranging between 13.9-19.6 from composting of food wastes and green wastes.

5.2 Biogas production in semi-continuous mode

Elevated CH_4 yields are most important for economic efficiency of the process (Lauterböck *et al*., 2014). Based on the results of the batch experiments, no significant differences were observed between the highest CH_4 producers in CD, MFW, vegetables and tomatoes. Mixed food waste was selected because of the variety or mixture of wastes they contained and was selected CD for its potential to maintain the buffering capacity that favours the growth of methanogens. The semi-continuous digestion occurred in a 20L digester.

5.2.1 Mono and co-digestion of food waste and cow dung

The pH profile results showed a slight decrease at the initial stage. This was expected to happen at the beginning of the process (hydrolysis) when substrates are broken down to produce fatty acids and the microorganisms are still in the lag phase adapting to the environment. The pH began to increase within the optimum range required for AD and CH_4 yield started to increase due to the exponential growth of methanogens in CO and CD experiments. Macias-Corral *et al*. (2008) reported similar results. In their experiment, the pH initially dropped rapidly during

hydrolysis, but increased again when the fatty acids were utilised by the methanogens during CH_4 production. The increase in pH could be attributed to the degradation of proteins contained in CD (Nurliyana *et al*., 2015). Cow dung contains nitrogen, trace elements and has also been reported to maintain the stability of the process (Mata-Alvarez *et al*., 2000; Manyi-Loh *et al*., 2015). Accumulation of VFAs in large amounts might have caused the decrease in pH recorded in MFW. Mixed food waste also contain carbohydrates that undergo rapid decomposition which create greater chances of inhibition (Table 4.1). The study conducted by Zhang *et al*. (2014) stated that inhibition always occurs when food waste is digested alone. These findings are in agreement with those reported by Zhang *et al*. (2012) at two selected loading rates. Anaerobic microorganisms especially methanogens are known not to survive in a highly acidic environment (Ali Shah *et al*., 2014).

The digestion of single substrates is usually challenging owing to imbalance of nutrients, poor buffering capacity and a low pH substrate (Demirel and Scherer, 2008). Compared with the work of Macias-Corral *et al*. (2008), the CD results of the present study showed low CH_4 production. Whereas, a study by Abukar and Nasir (2012) reported 47% CH_4 yield which is lower than the results found in this study. This may be attributed to factors such as the environment, type of cattle feed and composition of the substrates (Leung and Wang, 2016).

To deal with the challenges of mono-digestion, several authors reported optimal biogas production with CO (Iyagba *et al*., 2009; Khalid *et al*., 2011; Gashaw and Teshita, 2014; Wang *et al*., 2014; Sebola *et al*., 2015). Furthermore, Molinuevo-Salces *et al*. (2013) reported the addition of vegetable processing codigested with animal waste has improved CH_4 yield with a balanced C/N ratio. This may be linked to balanced C/N ratio in a mixture of more than one substrate thus reducing the concentration of inhibitory products (Mshandete *et al*., 2004; Molinuevo-Salces *et al*., 2013). The CO of MFW with CD is therefore an effective approach to improve CH_4 production in the AD process.

5.3 Methane yield, pH and microbiology of the AD process

5.3.1 Denaturing gradient gel electrophoresis (DGGE)

Molecular techniques to monitor the microbial community have been developed and they provide an opportunity to link the microbial community structure and parameters in the AD process (Akarsubasi *et al*., 2005). Microbial communities involved in the AD process of organic waste are affected by changes in the environmental conditions. These community shifts also influence biogas yield and composition. Therefore, it is important to relate the microbial community to the AD process as this will help to optimise the digester operation (Nettmann *et al*., 2008). The PCR-DGGE results revealed higher diversity in CO which was confirmed with *H'* and 1/D indices. This observation agrees with previous studies that have shown great abundance and diversity of microbes when AD residues combined with chicken and pig manure are co-digested compared to mono-digestion (Song *et al*., 2014). Therefore, the higher the number of substrates, the higher the microbial diversity (Song *et al*., 2014).

The increase in bacterial abundance and diversity were observed over time in CO (lanes 9-12). It is likely that the conditions were sufficient to promote microbial growth (Su *et al*., 2015). Similar interpretations of the PCR-DGGE results were provided by Ye *et al*. (2007) and Shin *et al* (2010). The community shift may also be associated with the CH_4 yield and intermediate products produced during acidogenesis (Lin *et al*., 2012). The CH_4 yield in all digesters treating different substrates varied, which can be linked to the microbial communities that were present at each stage (Tale *et al*., 2011).

The study conducted by Ye *et al*. (2007) compared the DGGE banding pattern at different pH levels. They found that the banding patterns were much more similar although well-separated at pH 4-6 than in pH 7. In this study, similar patterns of bacterial DGGE bands were severally observed with mono-digestion of CD which was within the range of pH 6.5-7. This resulted in smearing of bands. Such smearing of bands in CD was not observed in MFW with low pH. The smear observed might be due to different bacterial species present in large quantities in the sample (elevated bacterial diversity). Cow dung also has a rich microbial diversity and contains many bacterial species (Randhawa and Kullar, 2011). The changes in microbial diversity were brought about primarily by changes in pH. A study conducted by Horiuchi *et al.* (2002) stated

that pH affects the microbial growth which may cause a community shift. Bouallagui *et al*. (2004) studied the microbial diversity in a two-stage AD of FVWs and found that the community changed due to acid inhibition.

Furthermore, the archaeal banding patterns in all treatments were less diverse compared to those of bacteria. Smaller archaeal diversity was also reported by Shin *et al.* (2010) and there is a relatively low diversity of archaea in most microbial complexes (Curtis and Sloan, 2004). It was evidenced that the banding and intensity of many bands in archaea did not change. This means that the archaeal community shift was minor (Ye *et al*., 2007). The inability to link CH_4 production and archaeal diversity in this study may be attributed to the low number of archaea with CH_4 producing potential in MFW.

5.3.2 Microbial community analysis during semi-continuous digestion

The alpha diversity analyses in terms of the Chao1 and Shannon indices showed greater diversity when the CH_4 production was in an intermediate range (26-45%) in CD, MFW and CO in that order. A higher microbial diversity enhances the stability of the process, especially during changes of environmental conditions (Wrighton *et al*., 2008). However, microbial diversity is not key in developing a functionally successful AD process but the structure or functionality of the community is more important (Li *et al*., 2015).

Results from the high-throughput sequence analyses further revealed that the *Bacteroidetes*, *Firmucutes*, *Actinobacteria* and *Proteobacteia* phyla were ubiquitous in all reactors. This observation agrees with previous studies that reported the abundance of the phyla *Bacteroidetes*, *Firmucutes* and *Proteobacteia* (Wan *et al*., 2013; Wang *et al*., 2014; Guo *et al*., 2015; Wang *et al*, 2017). The abundance of *Firmucutes* in CD and MFW may be due to the cellulose content of these substrates (Table 4.1). *Firmucutes* play a very important role in the degradation of cellulosic material (Garcia-Peña *et al*., 2011; Hanreich *et al*., 2013) and produce H_2, CO_2 and VFAs. The majority of bacteria belonging to *Bacteroidetes* are known to produce lytic enzymes and acetic acid (Riviere *et al*., 2009). Ariesyady *et al*. (2007) reported that bacteria belonging to the phylum *Proteobacteria* use glucose, acetate and propionate. *Proteobacteria* phylum was evident in all categories of MFW (L-3.32%, I-3.55%, H-8.15%, H-10.82%) in elevated numbers because bacteria belonging to this phylum can survive in acidic

environments. The abundance of *Bacteroidia* and *Clostridia* in the AD process was previously reported by Wang *et al*. (2014) and Lee *et al.* (2017) agrees with the results achieved in this study. The contribution of *Bacteroidetes* might be associated with acetic acid production for syntrophic bacteria and utilisation of organic matter (Ros *et al*., 2017). *Clostridia* is a gram positive, strict anaerobe known to play a very important role in the initial stage of the AD process (Ziganshin *et al*., 2013). Bacteria from the order *Clostridiales* also help in the digestion of recalcitrant cellulose and most importantly support acetogens and methanogens with nutrients needed for their growth (Ziganshin *et al*., 2013). The predominance of *clostridia* in the AD process was previously associated with VFA fermentation (Guo *et al*., 2015). Most of the *Syntrophomonas* members were present in large quantities in CO in comparison with other treatments. Members of *Syntrophomonas* genus are strict anaerobes that breakdown long chain of organic acids to form acetate and propionate (Zhao *et al*., 1993) and also have the ability to enhance VFA consumption (Zhang *et al*., 2017).

A study conducted by Nelson *et al*. (2011) found that the majority of archaea belonging to *Euryarchaeota*, *Methanosaeta* and *Methanosarcina* were predominant CH_4 producers (Patil *et al*., 2010). These species of these taxa were found in abundance in CO which can be linked to the highest CH_4 production observed and neutral pH. The major microbial communities in AD are prone to change in diverse conditions because microorganisms have different adaptabilities (Li *et al*., 2013). Similarly, the increased CH_4 production could be attributed to the partial shift from *Methanosaeta* to *Methanosarcina* and *Methanobacterium* and from *Firmicutes* to *Bacteroidetes* in bacterial communities observed in CO AD trials (Mu *et al*., 2017). A High concentration of acetic acid may have influenced the archaeal abundance shift from *Methanosarcina* to *Methanosaeta* species.

Species from *Methanosarcina* prefer methylated compounds for CH_4 production (Shin *et al*., 2010) and these species can also tolerate high VFA concentrations compared to *Methanosaeta* (Demirel and Scherer, 2008). *Methanosarcina* generate CH_4 via three different metabolic pathways using CO_2 or hydrogen and acetate (Galagan *et al*., 2002). In their study, Mu *et al*. (2017) analysed the microbial characteristics and AD performance using potato and cabbage wastes as substrates. They found that *Methanobacterium* and *Methanosarcina* were responsible for high CH_4 production in CO of the two substrates. Furthermore, similar to the outcome of

this study, the methanogenic archaea were previously reported to be abundant in stable AD processes (Cardinali-Rezende *et al*., 2009). *Methanobrevibacter* was present in abundance among other hydrogenotrophic methanogens in CD and MFW. The *Methanobrevibacter* population reached 81% in mixed food waste (MFW-H) when 42% CH_4 production was recorded. *Methanobrevibacter* species are naturally found in the rumen of ruminants and participate in CH_4 production (Resende *et al*., 2016). The high percentage of these species in MFW might be due to the addition of the CD inoculum at the beginning of the process. It further confirms that the adaptation of these microorganisms was successful when the MFW was mixed with inoculum.

However, it seems that when the CH_4 production was low (MFW-L), the *Firmucutes* outcompeted the *Bacteroidetes.* The bacteria belonging to the phylum *Firmucutes* are known to degrade various substrates and produce VFAs (Garcia-Peña *et al*., 2011). High concentration of VFAs can inhibit the growth or may even cause the death of certain methanogens. These findings are consistent with the results of Ros *et al*. (2017), who reported on the microbial community structure during CH_4 production using pig slurry and FVW. In stressed conditions such as high VFAs concentrations, most methanogens undergo hydrogenotrophic methanogenesis (Schnürer *et al*., 1999). In addition, the decrease of CH_4 production was closely related to the disappearance of acetoclastic methanogens which are more sensitive than hydrogenotrophic methanogens (Palatsi *et al*., 2010). Furthermore, *Methanosaeta* methanogens were not detected when the CH_4 production was low, intermediate and high (MFW-I, MFW-H and CD-L, CD-H). The absence of *Methanosaeta* has been reported when high ammonia concentrations occur (Karakashev *et al*., 2005). In contrast, the growth rate of acetoclastic methanogens is affected even when the concentration of the ammonia is low (Angelidaki and Ahring, 1993). This is why when there is inhibition, the hydrogenotrophic methanogens are predominant (Lauterböck *et al*., 2014).

Species of genera such as *Methanospirillum* and *Methanoculleus* are hydrogenotrophic CH_4 producers. This group of organisms produces CH_4 gas via the reduction of hydrogen and CO_2 (Jain *et al*., 2015). It has been observed that a lower CH_4 content is generated from hydrogenotrophic methanogenesis than from acetotrophic methanogenesis which can generate up to 70% of CH_4 (Čater *et al*., 2013; Jain *et al*., 2015). The high-throughput sequence results

show that the methanogenesis stage was carried out mainly by acetoclastic and hydrogenotrophic methanogens throughout the process. As previously mentioned, methanogens are inhibited in an acidic environment as compared to when the pH is neutral (6.5-7.5). Therefore, there is an interrelationship between the process parameters (pH), CH_4 and the microbial communities.

5.4 Potential plant growth promoting attributes of bacterial isolates

Utilisation of the digestate material as a source of nutrients for plants is important as it contributes to the sustainability of the AD process. However, the digestate may contain pathogens and heavy metals that might have negative effects on humans and crop yield during application. This aspect of the study was aimed at evaluating the suitability of the digestate as an organic soil amendment in comparison to the untreated substrate currently being used as feedstock for AD i.e. CD.

Decreases in the values of TS, VS, potassium and phosphorus content were evidenced in the digestate in comparison with the dung samples. Elevated levels of dry matter and volatile solids in the dung compared to the digestate were expected because the anaerobic bacteria utilise organic matter during the AD process for biogas production (Möller and Müller, 2012). However, in the present study, the NH_4^+ content increased after AD. This increase may be attributed to the conversion of organic nitrogen to ammonium in the course of AD, which is advantageous if the digestate is intended for use as a fertilizer, because nitrogen mineralisation ensures that nitrogen is available in a plant-accessible form. Similar results were obtained by Gómez-Brandón *et al*. (2016), who reports a higher ammonium content in digestate than in manure. Elevated ammonium levels in the digestate could also be attributed to mineralisation of nitrogen compounds in the substrate (Gómez-Brandón *et al*., 2016).

The AD process is carried out by microbial communities that are sensitive to pH fluctuations. According to Chen *et al.* (2010), anaerobic bacteria prefer a pH range of between 6.5 and 7.5 for optimal biogas production. The pH before and after the AD process did not vary ($P>0.05$), as shown in Table 1. Previous studies (Lukehurst *et al*., 2010; Alburquerque *et al*., 2012b; Astals et *al*. 2012) have established that the type of substrate used in biogas production influences the nutrient composition and quality of the digestate. The substrate used in this study

contained the macronutrients required by anaerobic bacteria. In their study, Singh *et al*. (2010) report a lower C/N ratio in the digestate than in untreated dung, which is similar to the results of the present study. A decrease in the C/N ratio after AD was expected owing to the breakdown of organic matter and the accumulation of inorganic nitrogen sources. However, the C/N ratio of the feedstock (cow dung) in the present study was higher than that reported by Kataki *et al*. (2017). An elevated feedstock C/N ratio may be detrimental because of the risk of nitrogen limitation for microbial growth, but a low C/N ratio may impede biogas production as a result of ammonia inhibition (Igoni *et al*., 2008; Rajagopal *et al.*, 2013). The elevated C/N ratio of the substrate in the present study may be attributed to the environmental conditions of the place where the cattle were raised, the type of cattle (dairy or beef) and the cattle feed.

The AD process also resulted in a reduction in the heavy metal content, which is beneficial to the environment if the digestate is intended for use as a fertiliser. Numerous heavy metals such as manganese, copper, nickel, zinc, chromium and cadmium are toxic to the environment when present in high concentrations (Meena *et al*., 2008). Heavy metal pollution poses significant environmental threats to the entire ecosystem. The bio-magnification of heavy metals along the food chain may exacerbate the associated risks (Yi *et al*., 2011). Furthermore, heavy metal accumulation has the ability to damage plants as it might affect the growth and metabolism of plant cells (Madu *et al*., 2011).

More isolates were obtained from the feedstock (dung) than from the digestate. This may be attributed to the environmental pressures imposed by the AD process. Some microorganisms in the feedstock (dung) were unable to survive the unfavourable AD conditions. The identification of isolates showed that the dung had a greater culturable microbial diversity than the digestate. However, genera such as *Escherichia* and *Staphylococcus* were obtained from the dung. Some of the species of these genera are known to be pathogenic. As reviewed by Carbone *et al*. (2002), the faeces of animals contain *E. coli*, a pathogen that naturally occurs in the intestinal tract. Therefore, the use of untreated manure as a fertiliser poses higher risks of environmental pollution than the digestate (Insam *et al*., 2015; Adesemoye and Kloepper 2009).

The isolates were further evaluated for their ability to enhance soil fertility. All isolates from the dung and the digestate had one or more soil-enhancing attributes. However, IAA production and the ability to solubilise phosphate were elevated in most of the isolates in comparison with the ability to fix atmospheric nitrogen. As mentioned before, the quality of the digestate depends on the type of substrate used (Mata-Alvarez *et al*., 2000). In this study, shared genera were obtained from both the dung and the digestate. However, *Acinetobacter* was found to be the most prominent genus in both the dung and the digestate. A study carried out by Gulati *et al.* (2010) shows that *Acinetobacter* is an efficient phosphate solubiliser. The availability of PGPB in the digestate is advantageous because *Acinetobacter* organisms enhance plant growth in a more environmentally friendly way than chemical fertilisers do. A longer-term study comparing the yield of barley fertilised with digestate and barley fertilised with compost, as reported by Odlare *et al*. (2011), found digestate gave a higher yield. Therefore, apart from making certain nutrients available to the plant, the presence of PGPB in digestate will also assist in plant nutrient uptake.

CHAPTER 6: CONCLUSIONS AND RECOMMENDATIONS

6.1 Conclusions

This study has provided more insights into microbial diversity and CH_4 production during the AD process using CD and MFW. The CH_4 production depends on the composition of the substrate used. Overall findings from semi-continuous AD showed that CD is a good feedstock for biogas production due to the presence of rumen microorganisms that play an important role in AD and the buffering capacity of CD. However, the use of MFW as a single substrate for biogas production is challenging due to accumulation of VFAs. Hence, CO of CD and MFW is ideal for reducing the waste in large quantity, production of high CH_4 content due to a balanced C/N ratio and less chance of facing inhibition.

The PCR-DGGE results showed higher diversity in CO as compared to mono-digestion of CD and MFW.The high-throughput sequence analyses revealed that the OTUs belonging to the phyla *Bacteroidetes* followed by *Firmicutes, Actinobacteria* and *Proteobacteria* were dominant in all treatments. The *Bacteroidia* followed by *Clostridia* were the major classes identified under phyla *Bacteroidetes* and *Firmicutes*. Highest CH_4 production in CO was because of the presence of methanogens (*Methanosaeta* and *Methanosarcina*).

In the plant growth promotion experiment, both CD and the digestate contained cultivable microbial communities with one or more soil fertility enhancing attributes. However, the dung might contain pathogenic microorganisms that can damage plants and cause diseases in humans. The AD process resulted in the reduction of heavy metal contents and some of the potentially pathogenic bacteria in CD. Furthermore, the AD process resulted in the mineralisation of organic N enabling the presence of plant-available N in the digestate. Hence, the quality of reported digestate in this study indicates that it could be used as a potential fertiliser.

6.2 Recommendations and future work

The CH_4 production in MFW fluctuated throughout the process and low CH_4 was recorded towards the end of the process. Less feeding to avoid acid accumulation is therefore recommended. It is also advisable to use a well-balanced mixture of fruit and vegetable waste with food sources that are rich in proteins and fats. Future studies could investigate the use of two-stage digesters that separate the hydrolysis and acidogenesis steps, which might reduce the chances of microbe inhibition occurring.

It is also recommended that studies be conducted on appropriate growth media that could be used to culture and isolate uncultured microorganisms. Such studies could provide better insight into the AD process, because many uncultured microorganisms were obtained from the high-throughput sequencing data. Further investigation is also required into the operating parameters that affect the microbial community during AD, for instance the mechanisms of ammonia inhibition and VFAs concentration. The bio-augmentation method (addition of specific microbes known to improve the AD performance) could be tested to increase CH_4 production and stabilise the digestion process.

The digestate contained plant-growth-promoting bacteria, lower heavy-metal content and nutrients needed by plants. Smallholder farmers should use the digestate as a fertiliser in order to increase crop yields and reduce the cost of buying chemical fertilisers. It is recommended that future studies examine the effects of long-term fertilisation with digestate on soil quality and soil microbial biomass. There is a need for further investigation into molecular techniques to identify genes responsible for phosphate solubilisation and nitrogen fixation, and into indole acetic acid assays from the digestate.

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APPENDIX

Nutrient agar

- 28g of dehydrated nutrient agar
- 1L distilled water
- Sterilisation of the medium by autoclaving at 121℃ for 15 min
- Dispense the medium into the petri dishes

Gel electrophoresis

- 0.5g agarose
- 50 ml 50x TAE buffer
- Heat until the agarose completely dissolve
- Add 2 µL ethidium bromide

DGGE

100% denaturant solution

- 40ml formamide
- 42g Urea
- Make to 100 ml with distilled water

Denaturing gradient on 8% polyacrylamide gel (60%-40%) for bacteria and (60%-25%) for archaea

Composition	High (60%)	Low (40%)	Stacking gel
40%acrylamide solution	2.4	2.4	0.6
50x TAE	0.24	0.24	0.08
Glycerol	0.48	0.48	0
100%denaturing solution	7.2	4.8	0
dH_20	1.68	3.84	3.32
Total	12ml	12ml	4ml

Composition	High (60%)	Low (25%)	Stacking gel
40%acrylamide solution	2.4	2.4	0.6
50x TAE	0.24	0.24	0.08
Glycerol	0.48	0.48	0
100%denaturing solution	7.2	3	0
dH_20	1.68	5.88	3.32
Total	12ml	12ml	4ml

Ammonium Persulfate 10% (m/v)

- 0.1g Ammonium persulfate
- Dissolve in 1ml dH_20

DNA extraction using Powersoil DNA extraction kit (MO BIO Laboratories)

Briefly, the kit provides PowerBead Tubes to which 0.25 mg of dung sample were added and the tubes were mixed by gentle vortexing. Then 60 µL of Solution C1 were added and the tubes were vortexed for several minutes. The tubes were centrifuged at 10,000 x g for 30 seconds at room temperature, then the supernatant was transferred to a clean 2 ml Collection Tube. Then 250 µL of Solution C2 were added and the tubes were vortexed for 5 seconds. Following that, the tubes were incubated at 4°C for 5 min and the centrifuged at room temperature for 1 min at 10,000 x g. Then up 600 µL of supernatant were transferred to a clean 2 mL Collection Tube avoiding the pellet. 200 µL of Solution C3 were added and the tubes were briefly vortexed and incubated at 4°C for 5 min. The mixtures were centrifuged at room temperature for 1 min at

10,000 x g. Then, up to 750 µL of supernatant were transferred to a clean 2 mL Collection Tube. Then 1200 µL of Solution C4 were added to the supernatant and the mixtures vortexed for 5 seconds. Approximately 675 µL of the mixture were loaded onto a Spin Filter and centrifuged at 10,000 x g for 1 min at room temperature. The flow through was discarded, an additional 675 µL of supernatant was added to the Spin Filter and centrifuged at 10,000 x g for 1 min at room temperature. The same was done with the remaining volume of supernatant. Then 500 µL of Solution C5 were added and centrifuged at room temperature for 30 seconds at 10,000 x g. After discarding the flow through, the samples were centrifuged again at room temperature for 1 min at 10,000 x g and the Spin Filters were placed in a clean 2 mL Collection Tube with care not to splash them with the flow through. Then 100 µL of Solution C6 were added to the centre of the white filter membrane. Centrifugation was performed at room temperature for 30 seconds at 10,000 x g and the Spin Filter was discarded.

Principles for the determination of fibre fractions (Irene Analytical Services)

- Neutral detergent fibre (NDF) method

The feed was extracted with a hot neutral solution of sodium lauryl sulphate.
(Robertson, J.B. and Van Soest, P.J., 1981)

- Acid detergent fibre (ADF) method

Method using heat treatment of the sample with sulphuric acid containing cetyltrimethyl ammonium bromide (Goering and Van Soest, 1970).

*The difference between NDF and ADF value gives an estimate of the content of non-cellulosic polysaccharides. The ADF residue contains mainly cellulose and lignin. The lignin content is determined by permanganate oxidation and the resulting residue gives the cellulose content.

NDF = lignin + cellulose + hemicellulose
ADF = lignin + cellulose
ADL = acid detergent lignin
NDF – ADF = hemicellulose
ADF – ADL = cellulose

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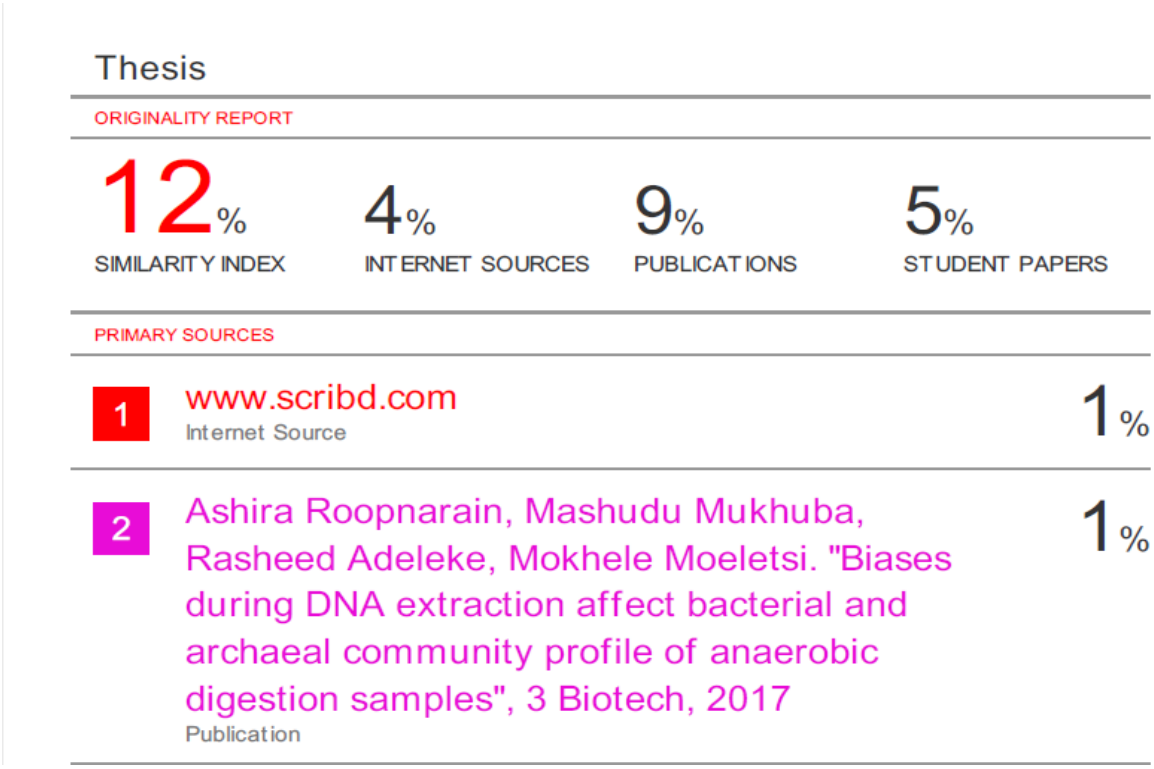
27 October 2017

To whom it may concern

This is to certify that I, Alexa Kirsten Barnby, ID no. 5106090097080, a language practitioner accredited by the South African Translators' Institute, have edited the research report, titled "Ecological guild of microbes that drive production of biogas from multiple feedstock", by Mukhuba Mashudu.

The onus is, however, on the author to make the changes and address the comments made.

Plagiarism



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UNISA

CAES RESEARCH ETHICS REVIEW COMMITTEE

Date: 13/10/2015

Ref #: **2015/CAES/092**
Name of applicant: **Ms M Mukhuba**
Student #: **55737560**

Dear Ms Mukhuba,

Decision: Ethics Approval

Proposal: The effects of microbial composition in the production of biogas from multiple feedstock

Supervisor: Dr R Adeleke

Qualification: Postgraduate degree

Thank you for the application for research ethics clearance by the CAES Research Ethics Review Committee for the above mentioned research. Final approval is granted for the duration of the project.

Please note point 4 below for further action.

The application was reviewed in compliance with the Unisa Policy on Research Ethics by the CAES Research Ethics Review Committee on 09 October 2015.

The proposed research may now commence with the proviso that:

1) *The researcher/s will ensure that the research project adheres to the values and principles expressed in the UNISA Policy on Research Ethics.*
2) *Any adverse circumstance arising in the undertaking of the research project that is relevant to the ethicality of the study, as well as changes in the methodology, should be communicated in writing to the CAES Research Ethics Review Committee. An amended application could be requested if there are substantial changes from the existing proposal, especially if those changes affect any of the study-related risks for the research participants.*
3) *The researcher will ensure that the research project adheres to any applicable*

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