Assessment of the diversity of *Bacteria* and methanogenic *Archaea* in Zebra faeces

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PREFACE

The experimental work described in this dissertation was carried out in the School of Life Sciences, University of KwaZulu-Natal, Pietermaritzburg, under the supervision of Professor Stefan Schmidt.

These studies represent original work by the author and have not otherwise been submitted in any form for any degree or diploma to any other University. Where use has been made of the work of others, it is duly acknowledged in the text.

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DETAILS OF CONTRIBUTION TO PUBLICATIONS that form part and/or include research presented in this dissertation (include publications in preparation, submitted, *in press* and published and details of the contributions of each author to the experimental work and the writing of each publication).

NOT APPLICABLE

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ABSTRACT

The need to develop a renewable, environmentally friendly source of energy has become a primary focus in modern science, with biogas showing considerable potential. Interest in the methanogenic *Archaea* has therefore grown in recent years and extensive studies have been carried out to investigate the population diversity in various habitats. Presently, there are only a few studies that have evaluated the microbial communities inhabiting the gastrointestinal tract of wildlife native to southern Africa. This study aimed to investigate the microbial diversity, in particular the bacterial and methanogen communities involved in fermentative digestion in the gastrointestinal tract of zebra.

Assessment of the microbial diversity in zebra faeces included both culture-based techniques and nucleic acid targeting analysis via 16S rRNA gene sequencing. Quantitative analysis using selected solid media revealed high counts for aerobic and anaerobic *Bacteria* ($7.51x10^8$ and $2.45x10^9$/gram of faecal sample respectively). The majority of aerobic colonies that were detected exhibited *Bacillus*-like morphology.

Nucleic acid based analysis of the diversity of both *Bacteria* and methanogenic *Archaea* in zebra faecal material was performed. Both manual and kit based extractions were used for DNA isolation in order to compare the efficiency of the two methods. Results show that a vigorous mechanical treatment was best for the release of DNA from the faecal matter. Amplification of target gene regions was carried out using established primer pairs (ARCH69F/ARCH915R and EUB338F/EUB907R) for methanogen and bacterial DNA respectively. Amplified 16S rRNA gene regions were cloned into a high copy number vector and random clones were selected for evaluation. Clones containing the target gene were further analysed by ARDRA and were assigned to a specific phylotype. Two bacterial (105 clones in total) and three methanogen (178 clones in total) clone libraries were constructed, of which 24 phylotypes were established for *Bacteria* and 25 for methanogenic *Archaea*. A representative of each phylotype was analysed by sequencing and further phylogenetic analysis was conducted.

Six bacterial phylotypes, which represented 56% of all bacterial clones, exhibited 99% sequence similarity to *Bacillus* species. Six methanogen phylotypes, which exhibited 99% sequence similarity to the hydrogenotrophic species *Methanobrevibacter gottschalkii* strain PG, were established to be predominant in zebra faeces. These phylotypes represented 71% of all archaeal clones selected for analysis in this study.

TABLE OF CONTENTS

PAGE

LIST OF TABLES

LIST OF FIGURES

PAGE

CHAPTER 1
Introduction

Substitution of fossil fuels, such as coal and oil, has gained much attention in recent years. This divergence from traditionally used fuels is owed not only to ever depleting resources, but also to the increased awareness of global warming from greenhouse gases resulting directly from the combustion of these fossil fuels. Alternative energy sources, with emphasis on renewable and environmentally friendly, have therefore become a focus of modern scientific research (Chynoweth *et al*., 2001; Demirel & Scherer, 2008; Weiland, 2010; Biswas *et al*., 2011; Liebetrau *et al*., 2011; Demirel, 2013).

The production of biogas, the main constituent of which is methane gas, from biomass substrates via anaerobic digestion has considerable potential. Substrates that can be utilised include a variety of biodegradable organic materials, such as paper waste (Yen & Brune, 2007), fruit and vegetable waste (Mandal & Mandal, 1997), kitchen waste (Lee *et al*., 2009), supermarket wastes (Alkanok *et al*., 2013), agricultural waste and plant biomass (Jagadish *et al*., 1998), dairy waste (Demirel *et al*., 2013), animal manure (Vijayaraghavan *et al*., 2006; Zhu *et al*., 2011; Nasir *et al*., 2012) and even the organic fraction of municipal solid waste (Owens & Chynoweth, 1993; Bareither *et al*., 2013). Biogas generation potential differs between the various organic substrates. Although mono-digestion of energy crops has gained more attention in recent years (Demirel, 2013), co-digestion of energy crops with supplementation of manure is also regarded as having a high biogas generation potential (Mandal & Mandal, 1997; Weiland, 2010; Westerholm *et al*., 2012).

The use of faecal matter as an inoculum or additional co-substrate for biogas production has its advantages. With this type of organic substrate, the microbial community required for anaerobic digestion already exists. Both methanogenic *Archaea* and hydrolytic fermentative *Bacteria* naturally inhabit the gastrointestinal tract of various animals, including cattle (Wright *et al*., 2007; Lwin *et al*., 2012), pigs (Zhu *et al*., 2009), sheep (Wright *et al*., 2008), buffaloes (Lwin *et al*., 2012) chickens (Saengkerdsub *et al*., 2007) and humans (Fricke *et al*., 2006).

Faecal matter also serves as a supply of essential trace elements such as iron, nickel, cobalt and selenium that are required by various members of the anaerobic food chain, including methanogens (Angelidaki & Ellegaard, 2003; Demirel & Scherer, 2011). Both anaerobic fermentation and microbial growth rate are dependent on the availability of adequate micronutrients (Weiland, 2010; Demirel & Scherer, 2011), which would otherwise have to be supplemented in cases such as mono-digestion of energy crops (Weiland, 2010) where the availabilty of nutrients is deficient (Demirel, 2013). The importance of nutrient availabilty was

demonstrated by Pobeheim *et al*. (2011) where the supplementation of nickel to the mono-digestion of energy crops resulted in a 20% increase in methane production.

Anaerobic digestion is considered as a favourable option in the treatment of biological wastes (Demirel *et al*., 2010) and shows several advantages over its aerobic counterpart. These advantages include a volume reduction in biomass, thereby minimising biomass disposal costs. Most organic wastes accumulate in landfills, which leads to the release of harmful gases into the atmosphere. A volume reduction of biomass through the use of anaerobic biotechnology would also help in the reduction of pollutants released into the atmosphere, which may ultimately mitigate the effect of hazardous gases on global warming (Chynoweth *et al*., 2001). Anaerobic digestion has the ability to biodegrade and stabilise organic matter (Chen *et al*., 2008), thereby assisting in reducing the toxicity levels of many organic wastes. Anaerobic digestion also results in the production of beneficial resources, an environmentally friendly fuel source in the form of biogas and, if the organic waste is treated under thermophilic conditions (Gong, 2007), a potentially pathogen-free digested residue that may be utilised as a compost or fertiliser on agricultural crops (Six & De Baere, 1992; Möller & Müller, 2012).

The harnessing of operational factors such as substrate feedstock, temperature, salinity and pH in anaerobic digesters would need to be incorporated to optimise or enhance the methane producing capacity of the microbial community (Sekiguchi, 2006). However, in order to optimise methane production, an initial understanding of the microbial community that is present in the inoculum or the organic substrate is firstly required, in particular the methanogenic *Archaea* that are involved in the final stage of the anaerobic food chain.

Extensive research studies have been carried out to investigate the diversity of both the general microbial community and the methanogenic population in manures of numerous domesticated animals including cows, sheep, goats (Shi *et al*., 2008) and horses (Miller & Lin, 2002). However, only a few studies have focused on the microbial communities inhabiting the gastrointestinal tract of wildlife native to southern Africa (Nelson *et al*., 2003; Ley *et al*., 2008).

Studies which investigated the equine gastrointestinal tract (Mackie & Wilkins, 1988; Daly *et al*., 2001; Yamona *et al*., 2008) established the presence of an extremely diverse microbial community, suggesting high fermentative potential and therefore substantial biogas generation potential (Jensen, 1996; Mandal & Mandal, 1997).

Owing to their similarities to horses, zebras are of particular interest as it may be presumed that the microbial communities that inhabit the gastrointestinal tract of zebras may have comparable fermentative potential. While Ellis & Schmidt (2011) recently reported the biogas production potential of faeces from South African indigenous ungulates such as zebra and wildebeest, other

authors showed that the total gas produced from maize stover via fermentation was comparable when using either horse or zebra faeces as inoculum (Fon & Nsahlai, 2012).

This study aimed to investigate the microbial diversity in zebra faecal matter. It focused particularly on the methanogenic community present in these materials that is potentially involved in methane production by employing a culture-independent approach. The specific objectives were to:

- Quantitatively assess the general non-archaeal microbial community via cultural techniques by use of selected solid media.
- Extraction of genomic DNA using both kit and manual based methods in order to compare these two methods.
- Amplification of both methanogen and bacterial partial 16S rRNA genes by use of specific primer pairs.
- Establishment of methanogen and bacterial clone libraries followed by restriction digestion and diversity analysis.

Background Information

1.1 Methane

Methane is the major constituent of biogas, which is a product of methanogenesis. Chynoweth (1992) reported that the primary source of atmospheric methane is of biological origin, namely methanogen bioactivity (Kubota *et al*., 2008). Other sources, as indicated in Figure 1.1, include methane gas emissions derived from coal mine resources and incomplete combustion of biomass. As a result, the concentration of atmospheric methane has steadily increased and it is now considered as a significant greenhouse gas (Chynoweth, 1992). In fact, the effect of methane as a greenhouse gas is greater than that of CO_2, with the global warming potential of methane measured at 72 times greater than CO_2 over a 20 year period (IPCC, 2007).

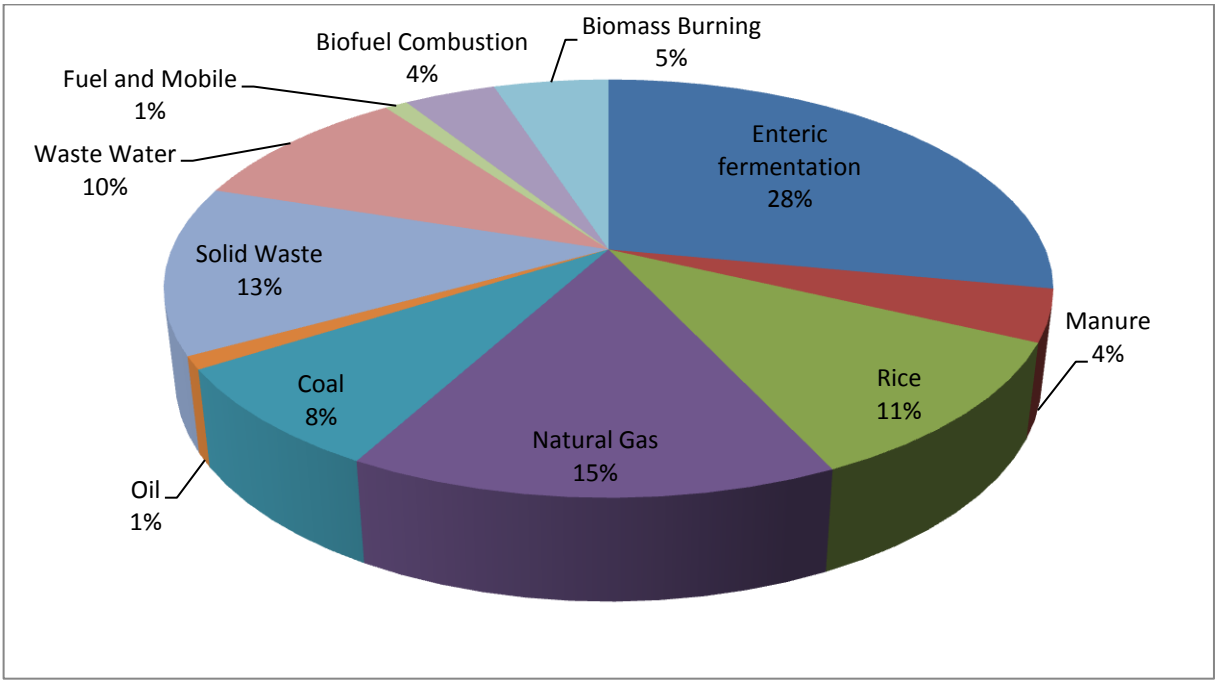

Figure 1.1: Sources of atmospheric methane gas (Values adapted from Chynoweth, 1992)

The odourless, colourless hydrocarbon is found in the gaseous state at ambient temperatures (Table 1.1). It is readily combustible in an environment where the oxygen to methane ratio exceeds that of 2 (Chynoweth, 1996), and is therefore regarded as a potentially high energy content fuel (Chynoweth, 1992) with a calorific value of 783 kJ/mol. It is important to note that because methane is the simplest hydrocarbon, it has little adverse effects when combusted, as it burns without the release of hazardous gases such as oxides of sulphur and nitrogen (Chynoweth, 1992).

Table 1.1 Basic properties of methane

Property	Value
Molecular Formula	CH_4
Molar Mass	16.04 g mol^{-1}
Appearance	Colourless Gas
Odour	Odourless
Density	0.6556 g L^{-1}
Melting Point	-183˚C
Boiling Point	- 164˚C to - 160˚C
Solubility in water	22.7 mg L^{-1}
Molecular Shape	Tetrahedron

(Matheson *et al*., 2013)

According to Chynoweth *et al*. (2001), a conventional reactor (mixed, fed once or more times a day, run under mesophilic conditions, with a hydraulic retention time of 20-30 days) can achieve a methane yield of 0.24 m^3 per kg volatile solids (organic matter as ash-free dry weight). Because of its high energy content, methane has been widely used as a fuel in many countries. Common applications include the use of methane for generation of electricity, heating and cooking. Methane has also been used in the operation of small engines (Chynoweth, 1992) and in the manufacture of many organic chemicals.

Biogas production via anaerobic digestion is advantageous compared to other bioenergies (Weiland, 2010) as it is considered as one of the more energy efficient and environmentally friendly bioenergies. The number of biogas systems in the United States is said to have increased by 30% between 2005 and 2007 (Abraham *et al*., 2007). Biogas usage in European Union countries have also increased in recent years with a projected energy production from biogas estimated at 209 TWh/year by 2020 (Abraham *et al*., 2007). More recently, the BP Statistical Review of World Energy (2011) reported the most rapid increase in global consumption of natural gas in almost three decades, with the United States being the world's largest producer. Although the use of fossil fuels accounted for 87% of global energy consumption in 2012, it was recorded that the global production and consumption of natural gas grew by 3.1% and 2.2% respectively (BP, 2012).

The use of domestic anaerobic digesters has increased considerably in developing countries with an estimated 5 million household digesters being operated in China in 2007 and an estimated 3.8 million household digesters that were being operated in India by the end of 2005 (Abraham *et al*., 2007). Biogas technology is also gaining favour in Africa, from plastic digester units on Tanzanian farms to larger scale fermenters that are operated in Rwanda (Brown, 2006). It is

estimated that biomass usage for biogas production could provide approximately a third of the global energy required in 2050 (Dornburg *et al*., 2010).

With the ever growing global need for renewable energy, controlled methanogenesis could prove to be the major approach in providing a sustainable and environmentally friendly energy source (Chynoweth *et al*., 2001; Weiland, 2010) and in the process reduce atmospheric methane emissions. The use of renewable biomass (such as energy crops) in an anaerobic digester to produce methane potentially represents a closed and balanced carbon cycle, meaning that this activity would not contribute to increasing levels of atmospheric carbon dioxide (CO_2). Although CO_2 is emitted during biogas production, an equivalent amount of CO_2 would have been initially required for the production of the plant biomass thereby creating a closed and CO_2 neutral system (Chynoweth, 1992). The methanogenic *Archaea*, the micro-organisms responsible for methane production during anaerobic digestion, have therefore received much attention in recent years.

1.2 The methanogens

Methanogenic *Archaea* are a large and diverse group of micro-organisms which have three common features:

- They produce methane as an end-product of anaerobic respiration / energy metabolism.
- They are strict anaerobes.
- They belong to the *Archaea* which constitute the third domain of life next to that of *Bacteria* and *Eukarya* (Woese *et al*., 1990).

1.2.1 Methanogen diversity

Methanogens are a phylogenetically diverse group of anaerobic *Archaea*. Historically, methanogen taxonomy was not reflective of this phylogenetic diversity owing to the fact that a revision of this taxonomy was created following conventions common to other prokaryote groups (Garrity & Holt, 2001).

The current taxonomy of methanogens is based primarily on 16S rRNA gene sequence similarity (Whitman *et al*., 2006; Liu & Whitman, 2008). Methanogens grouped into different orders have less than 82% 16S rRNA gene sequence similarity. Methanogens have also been categorised into orders according to phenotypic differences such as shape and cell wall structure, as represented in Table 1.2. Methanogen cell surface structures are diverse and are different to those of *Bacteria* (Kubota *et al*., 2008). Substrate utilisation and other biological properties such as metabolic pathways are also indicative of order groupings.

According to Bergey's Manual of Systematic Bacteriology (Brenner *et al*., 2001), orders may be further classified into families where there is less than 88-93% 16S rRNA gene sequence similarity. Families are further divided into genera where gene sequence similarity is less than 93-96%, and a gene sequence similarity of less than 98% is considered as a separate species (Whitman *et al*., 2001; Liu & Whitman, 2008). These numerical thresholds for phylogenetic classification of *Archaea* are derived from the comparison of DNA-DNA hybridisation studies and the rRNA sequence analysis (Schleifer, 2009). Generally organisms with less than 98.7% 16S rRNA gene sequence similarity can be considered as separate species (Schleifer, 2009). However, organisms that have more than 98.7% sequence similarity, can still be distinct species as Scheifer (2009) argues that with rRNA sequencing it is not always possible to define a separate species as the rRNA molecule is highly conserved and sequencing may not recognise closely related species as separate species as in the case of *Bacillus globisporus* W25^{T}, *Bacillus psychrophilus* W16A^{T} and *Bacillus psychrophilus* W5, where more than 99.5% sequence similarity is exhibited between different species (Fox *et al.*, 1992).

At present five well-established orders, *Methanobacteriales*, *Methanococcales*, *Methanomicrobiales*, *Methanosarcinales* and *Methanopyrales*, are recognised. These orders are further divided into 10 families and 28 genera (Table 1.2) with approximately a hundred species that have been isolated (Kubota *et al*., 2008). To date only a few cultured strains are known to have been completely sequenced (Janssen & Kirs, 2008). Sizes of genomes of the methanogens completely sequenced to date vary from 1.6 Mbp to about 5.8 Mbp, with species from the order *Methanosarcina* showing the greatest genome size (Liu & Whitman, 2008).

Table 1.2: Characteristics of recognised taxonomic groups of methanogenic *Archaea* (Whitman *et al.*, 2001; Liu & Whitman, 2008)

Order	Morphology	Cell Wall	Family	Genus	Major Methanogenesis Substrates	Temperature (˚C)
Methanobacteriales	range: short lancet-shaped cocci to long filamentous rods	Predominantly Pseudomurein	*Methanobacteriaceae*	*Methanobacterium*	Carbon dioxide/Hydrogen, (Formate)	37-45
				Methanobrevibacter	Carbon dioxide/Hydrogen, Formate	37-40
				Methanosphaera[*]	Carbon dioxide/Hydrogen & Methanol	37
				Methanothermobacter	Carbon dioxide/Hydrogen, (Formate)	55-65
			Methanothermaceae	*Methanothermus*	Carbon dioxide/Hydrogen	80-88
Methanococcales	irregular cocci	S-layer proteins, may contain glycoproteins in low abundance	*Methanococcaceae*	*Methanococcus*	Carbon dioxide/Hydrogen, Formate	35-40
				Methanothermococcus	Carbon dioxide/Hydrogen, Formate	60-65
			Methanocaldococcaceae	*Methanocaldococcus*	Carbon dioxide/Hydrogen	80-85
				Methanotorris	Carbon dioxide/Hydrogen	88
Methanomicrobiales	diverse: cocci, rods, sheathed rods	Protein, some cells surrounded by sheath containing glycoproteins	*Methanomicrobiaceae*	*Methanomicrobium*	Carbon dioxide/Hydrogen, Formate	40
				Methanoculleus[**]	Carbon dioxide/Hydrogen, Formate	20-55
				Methanofollis	Carbon dioxide/Hydrogen, Formate	37-40
				Methanogenium	Carbon dioxide/Hydrogen, Formate	15-57
				Methanolacinia[**]	Carbon dioxide/Hydrogen	40
				Methanoplanus[*]	Carbon dioxide/Hydrogen, Formate	32-40
			Methanospirillaceae	*Methanospirillum*	Carbon dioxide/Hydrogen, Formate	30-37
			Methanocorpusulaceae	*Methanocorpusculum*	Carbon dioxide/Hydrogen, Formate	30-40
				Methanocalculus	Carbon dioxide/Hydrogen, Formate	30-40
Methanosarcinales	diverse: cocci, pseudosarcinae, sheathed rods	Protein, surrounded by sheath / acidic heteropolysaccharides / glycoproteins	*Methanosarcinaceae*	*Methanosarcina*	Methylamine, Acetate	35-60
				Methanococcoides	Methylamine	23-35
				Methanohalobium[*]	Methylamine	40-55
				Methanohalophilus[*]	Methylamine	35-40
				Methanolobus	Methylamine	37
				Methanomethylovorans	Methylamine	20-50
				Methanimicrococcus	Carbon dioxide/Hydrogen & Methylamine	39
				Methanosalsum	Methylamine	35-45
			Methanosaetaceae	*Methanosaeta*	Acetate	35-60
Methanopyrales	Rod	Pseudomurein	*Methanopyraceae*	*Methanopyrus*	Carbon dioxide/Hydrogen	98

[*]Not formally placed within the family. [**]Not formally placed within family but includes species originally placed within a genus of a family.

1.2.2 Methane production

Anaerobic digestion is a naturally occurring process which results in the degradation of particulate organic matter, or biomass to the principle product, biogas. Biogas is typically comprised of mainly methane (50 – 75%) (Weiland, 2010) and CO_2.

A predominant feature of methanogens is their substrate range which is limited to a few simple, usually one or two-carbon compounds. Such substrates include C_1-substrates such as CO_2 and the C_2-substrate acetate. Methanogens do not possess the ability to catabolise longer-chain volatile organic acids which contain three or more carbon atoms such as propionate and butyrate (Whitman *et al*., 2006). As a result, most methanogens are very much dependent on other microbes in their environment to metabolise polymeric substrates to their simple precursors via anaerobic digestion.

Exceptions to this dependence on other microbial communities for reduced substrates are a few hydrogenotrophic methanogens such as *Methanothermobacter thermoautotrophicus* and *Methanococcus thermolithotrophicus* which inhabit terrestrial and marine hot springs respectively (Zinder, 1993). Both hydrogen (H_2) and CO_2 are amongst the gases which flush terrestrial hot springs, making it an ideal habitat for thermophilic, hydrogen-utilising methanogens. Marine hot springs also have the ability to support communities of hydrogen-utilising methanogens as the hydrothermal fluid contains compounds in the form of H_2 and CO_2 (Zinder, 1993).

Production of methane is a complex process and in most environments requires the interaction of various groups of anaerobes, namely syntrophic relationships. One such syntrophic relationship involves the interaction between carbohydrate utilising fermentative anaerobes and H_2 and formate utilising methanogens (Zinder, 1993). During sugar and amino acid metabolism (acidogenesis), the production of acetate by fermentative *Bacteria* is favoured. This is due to the presence of an extra ATP that is produced from acetyl-CoA (Whitman *et al*., 2006). When acetate is produced through this pathway, NADH + H^+ is generated and re-oxidation to NAD^+ + H_2 is thereafter required. NADH + H^+ oxidation to NAD^+ + H_2 is only favourable under conditions of very low H_2 concentrations (less than 1 µM) as an increase in partial pressure of H_2 may hinder the metabolism of acetate-utilising *Bacteria* (Weiland, 2010). Formate concentrations also need to be maintained at low (less than 100 µM) levels (Whitman *et al*., 2006). If methanogens that utilise H_2 and formate are present, these substances remain at a constant low concentration, thereby allowing these substances to be favourable products of NADH and H^+oxidation. Hydrogen and formate utilising methanogens are considered as the regulators of the fermentation process (Schink, 1997). An inhibition of hydrogenotrophic methanogens would result in a high H_2 partial pressure which in turn leads to an inhibition of volatile fatty acid and ethanol production via fermentation and thus ultimately the failure of complete fermentation (Schink, 1997). In environments where this type of syntrophic interaction exists, fermentative *Bacteria* are known to produce more acetate (Zinder, 1993; Whitman *et al*., 2006). This interaction is extremely important and advantageous to fermentative *Bacteria* as it results in greater

energy conservation (Zinder, 1993). However, since fermentative *Bacteria* are able to grow and survive without the presence of methanogens, this type of syntrophic interaction is referred to as non-obligate interspecies electron transfer.

Another type of syntrophic relationship, referred to as obligate interspecies electron transfer, involves the interaction between a group of *Bacteria* known as obligate syntrophs which are able to oxidise fatty acids such as propionate and longer-chain volatile organic acids, and H_2 and formate utilising methanogens. Accumulation of electrons results from the catabolism of organic substrates by these obligate syntrophs. Excess electrons must be disposed of and this occurs either by reduction of protons to H_2 or the reduction of CO_2 to formate (Whitman *et al*., 2006). Low concentrations of both H_2 and formate are therefore required. High concentrations of these substances would result in end-product inhibition and prevent substrate oxidation. The activities of H_2 and formate utilising methanogens maintain an environment with low concentrations of these substances, thereby allowing continued catabolism of organic substrates by obligate syntrophs (Whitman *et al*., 2006). Because these obligate syntrophs are limited to a few fermentative reactions and therefore do not possess the ability to oxidise other organic substrates, and methanogens are unable to grow on the longer-chain organic substrates, these two groups of microbes are dependent of the other's activities for survival (Zinder, 1993).

The effectiveness and efficiency of anaerobic respiration and hence biogas production is therefore highly dependent on the microbial community, namely *Bacteria* and *Archaea*, which is present in the environment (Sekiguchi, 2006; Demirel & Scherer, 2008). Despite a variation in the gastrointestinal microbial community between animals, certain microbes tend to be present. These include Enterococci, Bacteriodes, Clostridia and Bacilli (Daly *et al*., 2001; Józefiak *et al*., 2004; Klocke *et al*., 2007; Yokoyama *et al*., 2007; Ley *et al*., 2008;), while methanogenic *Archaea* tend to comprise the majority of the archael community in the rumen (Janssen & Kirs, 2008).

The overall conversion to methane, as depicted in Figure 1.2, can be divided into four key catabolic stages: Hydrolysis, Acidogenesis, Acetogenesis and Methanogenesis.

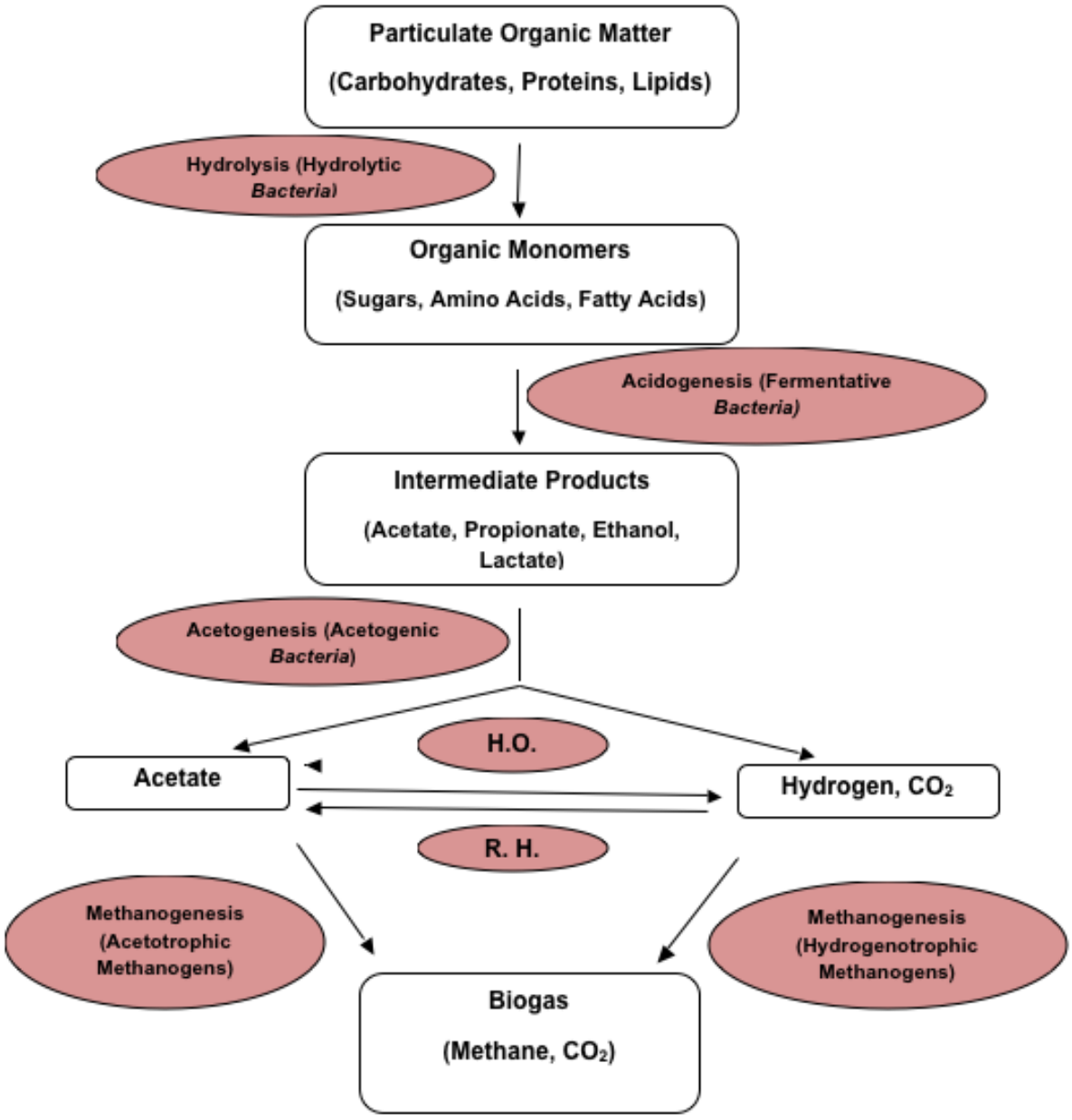

Figure 1.2: Anaerobic conversion of particulate organic matter to biogas (Demirel & Scherer, 2008)

H.O. = Homoacetogenic Oxidation / Syntrophic Acetate Oxidation;

R.H. = Reductive Homoacetogenesis

Reductive Homoacetogenesis (R.H.) (and hydrogenotrophic methanogenesis) is favoured at high H_2 partial pressures >500Pa. Acetate Oxidation by syntrophs to CO_2 and H_2 is favoured at low H_2 partial pressures <40Pa (Demirel & Scherer, 2008).

The digestion process begins with the microbial hydrolysis of polymeric substrates. In most cases the initial biomass is composed of complex polymeric organic compounds such as carbohydrates, proteins

and lipids. In order for micro-organisms to access the energy potential of the particular organic matter, these polymers must first be broken down into their smaller constituents or monomers. Hydrolytic-fermentative *Bacteria* are responsible for the breakdown of insoluble carbohydrates, proteins and lipids to their respective monomers – sugars, amino acids and fatty acids. This is regarded as the rate limiting step in anaerobic digestion because cellulose is not easily degradable (Noike *et al*., 1985). Key cellulose degrading *Bacteria* of the rumen were reported to belong to the *Firmicutes*. Wang *et al*. (2010) found that *Clostridium*-like species were predominant in the fermentation of grass silage.

Organic monomers resulting from hydrolysis are utilised as substrates by fermentative organisms and anaerobic oxidisers (Demirel & Scherer, 2008). The process known as acidogenesis results in the production of acetate or other intermediate products such as propionic acid and butyric acid. Hydrogen, CO_2 and hydrogen sulphide are also emitted. The consumption of O_2 by facultative anaerobes during acidogenesis is important since methanogens are strict anaerobes. Acidogenesis is typically carried out by members of the *Bacteria* such as *Clostridium* spp.

The acetate, H_2 and CO_2 that are produced in these initial stages can be directly utilised by methanogens. Other molecules however, such as volatile fatty acids with a greater chain length to that of acetate, require catabolism before being used by methane producers. These molecules are further digested by acetogenic *Bacteria* to produce largely acetate, as well as CO_2 and H_2, a process known as acetogenesis.

The final stage of anaerobic digestion is the production of methane. Here, either of two independent pathways may be employed, namely hydrogenotrophic (syntrophic) and aceticlastic methanogenesis (Schnürer & Nordberg, 2008). In aceticlastic methanogenesis, acetotrophic (acetate-utilising) methanogens can directly utilise acetate as a substrate for methane production. In the second pathway, known as hydrogenotrophic methanogenesis or the syntrophic pathway, acetate is first utilised by fermentative *Bacteria* resulting in the production of H_2 which is oxidised by using CO_2 or formate as a carbon source. These substrates are subsequently utilised by hydrogenotrophic (hydrogen-utilising) methanogens to produce methane. Many factors are believed to influence the degree to which either of these pathways proceeds. The syntrophic pathway has been demonstrated to be favoured under thermophilic conditions with high ammonium concentrations (Schnürer & Nordberg, 2008). Hydrogenotrophic methanogenesis is dominant in the rumen (Janssen & Kirs, 2008), with extremely limited metabolism of acetate to methane via the aceticlastic pathway. This is probably because the rate at which the rumen contents pass through the rumen is greater than the growth rate of acetotrophic methanogens (Wolin, 1979), and also because acetate is used as an energy source by the host (Janssen & Kirs, 2008).

1.2.2.1 Methanogenesis

Substrates that can be utilised by methanogens can in principle be divided into three types: CO_2, other one-carbon compounds (such as methylamine and formate) and acetate.

a) Carbon dioxide and hydrogen

Most methanogens, with the exception of a few obligate methylotrophic and acetotrophic species, are hydrogenotrophic. That is, CO_2 and molecular H_2 are the principal energy substrates utilised. The reduction of carbon to methane is important in anaerobic habitats such as the gastrointestinal tract as it assists in the maintenance of an environment low in H_2 and formate concentrations, thereby promoting hydrogen interspecies transfer (Whitman *et al*., 2006).

Carbon dioxide (electron acceptor) is reduced to methane gas with predominantly H_2 as the electron donor, with other substrates such as formate, having also been recognised as electron donors (Whitman *et al*., 2006; Liu & Whitman, 2008). Formate is considered as a major electron donor, although concentrations of formate are low in natural methanogenic environments (Demirel & Scherer, 2008). Here, four molecules of formate are oxidised by formate dehydrogenase to CO_2 which is subsequently reduced to methane.

To a lesser extent, alcohols such as 2-propanol, 2-butanol, cyclopentanol and even ethanol, by a select few methanogens, can be used as substrate (Widdel, 1986; Frimmer & Widdel, 1989). Alcohols as electron donors are scarce and growth on such a substrate is slow, however it is important to note as it is atypical to a group of micro-organisms that are otherwise not known for directly breaking down organic compounds (Liu & Whitman, 2008). Widdel (1986) showed that 2-propanol and ethanol could be used directly by mixed culture methanogens under anaerobic, sulphate-free conditions. Utilisation of secondary alcohols is made possible by oxidation of these alcohols via secondary alcohol dehydrogenases to respective ketones (Widdel, 1986; Widdel *et al*., 1988). It has been confirmed that *Methanogenium organophilium* can oxidise ethanol through the use of a nicotinamide adenine dinucleotide phosphate ($NADP^+$) – dependent dehydrogenase, to acetate (Frimmer & Widdel, 1989).

Carbon monoxide (CO) is also known to be used for CO_2 based methanogenesis. Two species, *Methanothermobacter thermoautotrophicus* and *Methanosarcina barkeri*, reportedly oxidise four molecules of carbon monoxide to one molecule of CO_2, before CO_2 in turn is reduced to methane (Liu & Whitman, 2008; Ferry, 2010). Daniels *et al*. (1977) stated that CO is oxidised by *M. barkeri*, although growth of the organism in a CO environment is slower, with a growth rate estimated at only 1% of the growth observed in a CO_2 and H_2 environment. When grown on CO, the doubling time was recorded at 200 hours for *M. thermoautotrophicus* and at least 65 hours for *M. barkeri* (Daniels *et al*., 1977; O'Brien *et al*., 1984).

Progression through steps known as the formyl, methylene and methyl levels result in the reduction of CO_2 to methane. The reaction makes use of a number of unique C_{-1} compound carrying cofactors,

namely methanofuran (MFR), tetrahydromethanopterin (H_4MPT) and coenzyme M (CoM). Overall, under standard conditions, this is thermodynamically a highly exergonic process according to the equation: $4H_2 + CO_2 \rightarrow CH_4 + 2H_2O$ ($\Delta G^{\circ'} = -135$ kJ/mol) (Liu & Whitman, 2008).

The two reactions essential in energy conservation are the transfer of methyl from H_4MPT to CoM and the final reduction of the heterodisulphide (Liu & Whitman, 2008). However, it is important to note that in natural methanogenic environments such as the rumen, the partial pressure of H_2 is kept at a low concentration, generally between 1 Pa and 10 Pa. The free energy change associated with fermentation of CO_2 and H_2 is therefore highly decreased to about 30 kJ/mol. Given that under physiological conditions, 50 kJ/mol is required for the synthesis of ATP from ADP and inorganic phosphate, less than 1 mol of ATP can be generated per 1 mol of methane that is formed (Müller *et al*., 1993; Thauer *et al*., 1993) under such conditions.

The initial reduction results from the binding of CO_2 by MFR to form formyl-MFR (Figure 1.3A), otherwise known as the formyl level. Here, ferredoxin (Fd), which is reduced with H_2, acts as the direct electron donor (Liu & Whitman, 2008). Under standard conditions this reaction is endergonic, with the required ATP being derived from the final step of methane production (Whitman *et al*., 2006).

The formyl group is transferred to H_4MPT, thereby forming formyl-H_4MPT. Subsequently dehydration and reduction results in the methylene and methyl levels via the production of methylene-H_4MPT and methyl-H_4MPT respectively. Electrons for both these reactions are sourced from reduced coenzyme F_{420} ($F_{420}H_2$). Coenzyme M binds to the methyl group to form methyl-CoM (Whitman *et al*., 2006; Liu & Whitman, 2008).

Finally, methyl-CoM is reduced via the methyl coenzyme M reductase system (Mcr) to methane, with coenzyme B (CoB) directly supplying the electrons. Coenzyme M is transferred to the oxidized CoB to form a heterosulphide, which in turn is reduced for the regeneration of thiols (Liu & Whitman, 2008). Energy in the form of ATP is released due to the reduction of the disulphide bond. Energy derived from this final stage completes the cycle by actively promoting the reduction of CO_2 to formylmethanofuran which is an endergonic reaction (Whitman *et al*., 2006).

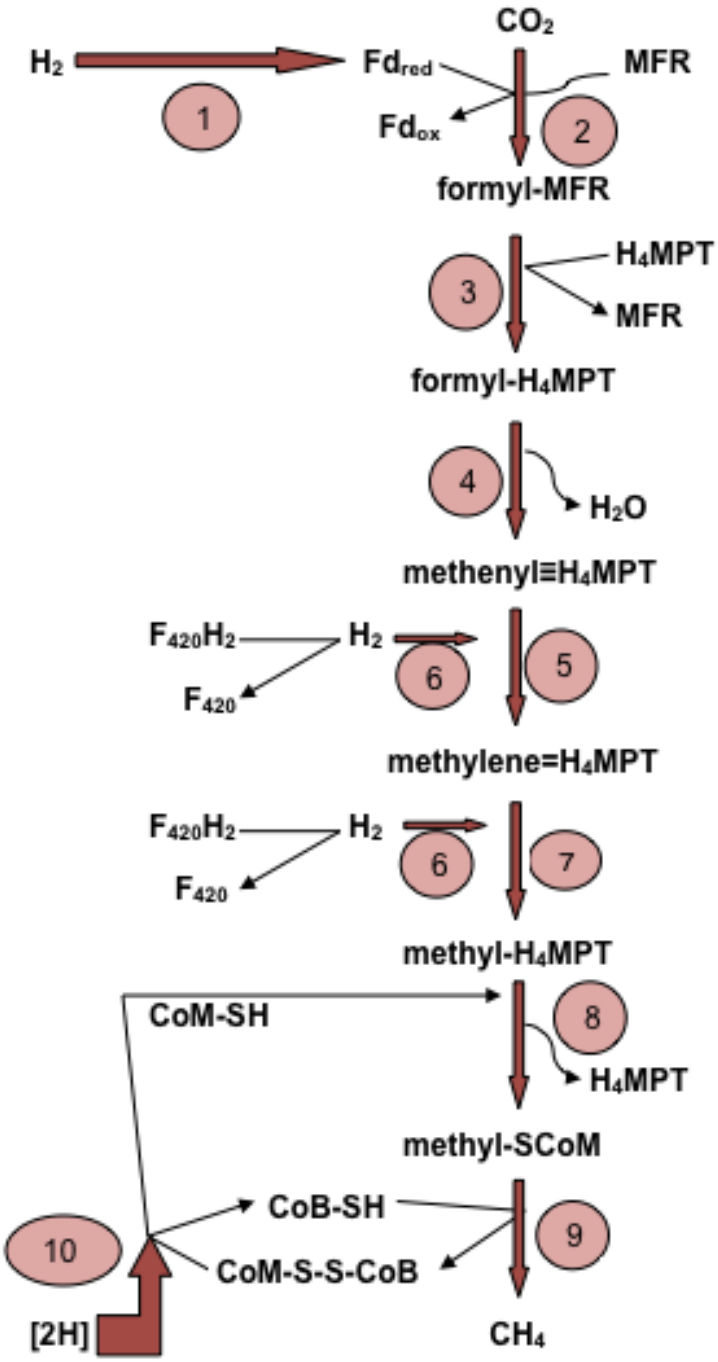

Figure 1.3A: Methanogenesis from hydrogen / carbon dioxide (Liu & Whitman, 2008) **Abbreviations:** Fd_{red}= reduced form of ferredoxin; Fd_{ox}= oxidised form of ferredoxin; $F_{420}H_2$= reduced form coenzyme F_{420}; MFR= methanofuran; H_4MPT= tetrahydromethanopterin; CoM-SH= coenzyme M; CoB-SH= coenzyme B; CoM-S-S-CoB= heterodisulphide of CoM and CoB; CoA-SH= coenzyme A. **Enzymes:** 1= energy-conserving hydrogenase (Ech); 2= formyl-MRF dehydrogenase; 3= formyl-MFR:H_4MPT formyltransferase; 4= methenyl-H_4MPT cyclohydrolase; 5= methylene-H_4MPT dehydrogenase; 6= F_{420}-reducing hydrogenases; 7= methylene-H_4MPT reductase; 8= methyl-H_4MPT:HS-CoM methyltransferase; 9= methyl-CoM reductase; 10= heterodisulfide reductase.

b) One-carbon methylated compounds

Methylotrophic methanogens are those methanogens that can use methyl-group containing compounds as substrates for methanogenesis. These compounds include methanol, methylated amines (such as monomethylamine, dimethylamine, trimethylamine and tetramethylammonium (Burke & Krzycki, 1997)) and methylated sulphides (which include methanethiol and dimethylsulphide) (Whitman *et al*., 2006; Liu & Whitman, 2008).

Methylotrophy seems to be restricted to the order *Methanosarcinales*, with about 20 species showing capability to use methylated compounds as substrate (Boone *et al*., 1993). A high number of these species have been isolated from marine or salt lake environments, which normally have increased sulphate concentrations (Keltjens & Vogels, 1993). Hydrogenotrophic methanogens do not thrive under these conditions as they are easily outcompeted by *Bacteria* capable of reducing sulphate. However, because of their ability to grow on methylated compounds, methylotrophic methanogens are able to compete with sulphate-reducing *Bacteria* in these environments (Keltjens & Vogels, 1993). Two exceptions of *Methanosphaera* species, *M. stadtmanae* (Miller & Wolin, 1983) and *M. cuniculi* (Biavati *et al*., 1988) also show growth on methanol with H_2 as the electron donor.

During this type of methanogenesis (Figure 1.3B), methyl groups from substrates are transferred to a corrinoid protein (Ferguson *et al*., 2000) and are then subsequently transferred to coenzyme M (Burke & Krzycki, 1997). Both reactions require methyltransferases, which are substrate specific (Burke & Krzycki, 1997; Ferguson *et al*., 2000). The methyl-coenzyme M structure then enters the methanogenesis pathway, resulting in the production of methane.

Electrons that are needed for the reduction of the methyl-group to methane can come from two sources. The two species of the genus *Methanosphaera* are dependent on H_2 to provide electrons (Whitman *et al*., 2006). However, in most cases, in the absence of H_2, electrons are obtained when methyl-groups are oxidised to CO_2. Here, additional methyl-groups are oxidised through a stepwise reverse reaction of hydrogenotrophic methanogenesis. This is known as disproportionation (Liu & Whitman, 2008), where a part of the substrate is oxidised to obtain electrons, which are then utilised in the reduction of remaining substrate. The oxidation of one methyl group is enough to reduce 3 methyl groups to methane according to the equation: $4CH_3OH \rightarrow 3CH_4 + CO_2 + 2H_2O$ ($\Delta G^{\circ'}$ = -105 kJ/mol) (Liu & Whitman, 2008).

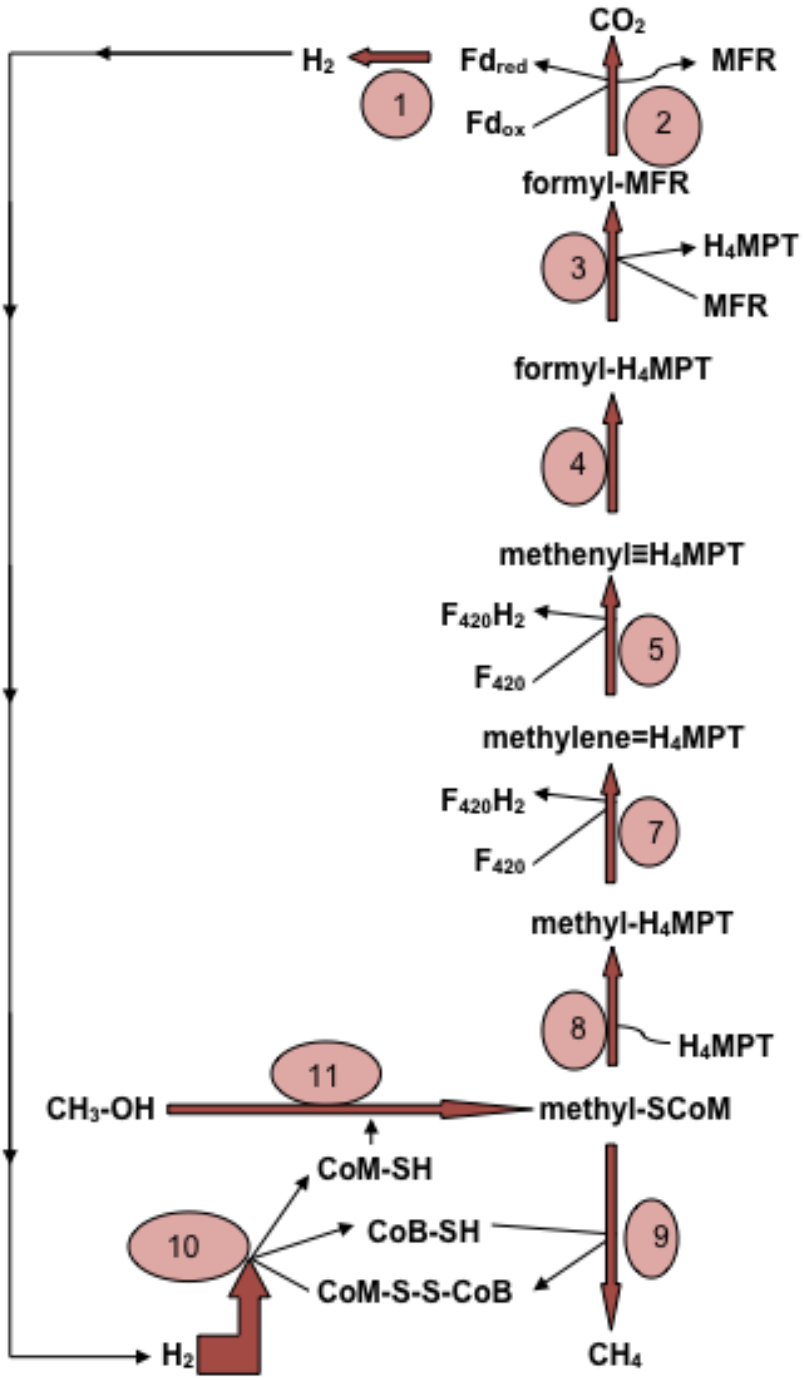

Figure 1.3B: Methanogenesis from one-carbon methylated compounds (Liu & Whitman, 2008). **Abbreviations:** Fd_{red}= reduced form of ferredoxin; Fd_{ox}= oxidised form of ferredoxin; $F_{420}H_2$= reduced form coenzyme F_{420}; MFR= methanofuran; H_4MPT= tetrahydromethanopterin; CoM-SH= coenzyme M; CoB-SH= coenzyme B; CoM-S-S-CoB= heterodisulphide of CoM and CoB; CoA-SH= coenzyme A. **Enzymes:** 1= energy-conserving hydrogenase (Ech); 2= formyl-MRF dehydrogenase; 3= formyl-MFR:H_4MPT formyltransferase; 4= methenyl-H_4MPT cyclohydrolase; 5= methylene-H_4MPT dehydrogenase; 6= F_{420}-reducing hydrogenases; 7= methylene-H_4MPT reductase; 8= methyl-H_4MPT:HS-CoM methyltransferase; 9= methyl-CoM reductase; 10= heterodisulfide reductase; 11= methyltransferase.

c) Acetate

Acetate, a methylated two-carbon compound, is the final type of substrate that can be used by certain methanogens. Acetate is considered the chief end-product of anaerobic fermentation in natural habitats, and 65-70% of methane that is produced is derived from acetate (Liu & Whitman, 2008). In a study investigating the methanogenic community within biogas plants containing manure or sludge, Karakashev *et al*. (2005) identified acetotrophs as the dominating methanogens regardless of the inital inoculum populations. However, Karakashev *et al*. (2005) did note that the apparent lack of hydrogenotrophic methanogens such as *Methanobacteriales* identified in the samples could be due to the difficulty of visualising these methanogens using fluorescence in situ hybridisation. In a more recent study conducted by Krakat *et al*. (2010[c]), biogas fermenters containing fodder and sugar beet silage, the hydrogenotrophic methanogens (*Methanobacteriales* and *Methanomicrobiales*) were found to be the dominanting methanogens, while acetotrophs made up less than 10% of the population.

Acetotrophic methanogens are limited to two genera, namely *Methanosarcina* and *Methanosaeta*. Of these, *Methanosarcina* seems to grow more rapidly when acetate concentrations are high (Ferry, 1993), although growth of *Methanosaeta*, which is considered the superior utiliser, is favoured at low acetate concentrations, with the minimum threshold concentrations for growth estimated at 5– 20 µM (Liu & Whitman, 2008).

The catabolism of acetate proceeds via the acetoclastic reaction (Figure 1.3C). Initially, acetate is activated to acetyl-CoA (Ferry, 1993). Thereafter, carbon-carbon bonds and carbon-sulphur bonds are cleaved by a CO dehydrogenase enzyme system. The carbonyl-group is oxidised by a nickel/sulphur component of the CO dehydrogenase system to form CO_2, whilst the methyl-group is transferred to coenzyme M via H_4MPT and a corrinoid/iron-sulphur component of the enzyme complex (Ferry, 1993; Whitman *et al*., 2006). The CO enzyme complex is further oxidised to generate H_2 and CO_2, with H_2 providing the electrons to reduce methyl-coenzyme M to methane. Methane is produced from acetate according to the equation: $CH_3COOH \rightarrow CH_4 + CO_2$ ($\Delta G^{\circ'} = -33$ kJ/mol) (Liu & Whitman, 2008).

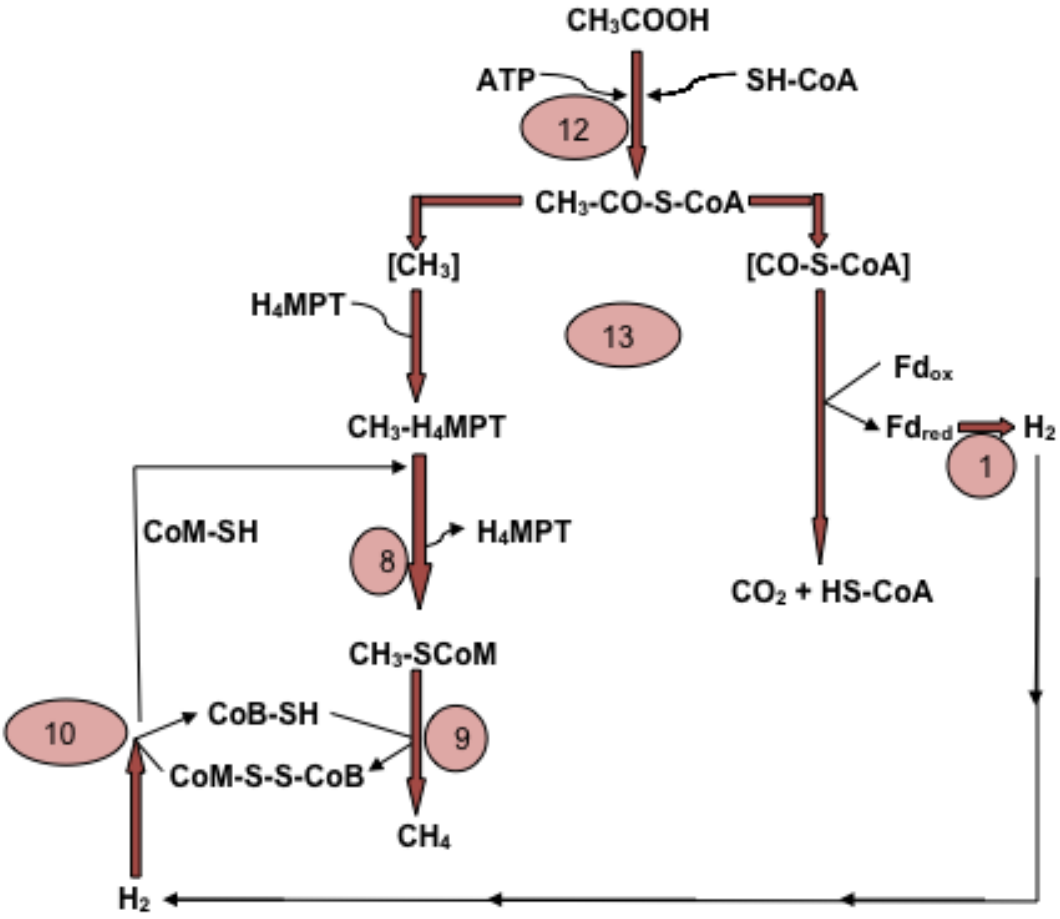

Figure 1.3C: Methanogenesis from Acetate (Liu & Whitman, 2008). **Abbreviations:** Fd_{red} =reduced form of ferredoxin; Fd_{ox}= oxidised form of ferredoxin; $F_{420}H_2$= reduced form coenzyme F_{420}; MFR= methanofuran; H_4MPT= tetrahydromethanopterin; CoM-SH= coenzyme M; CoB-SH= coenzyme B; CoM-S-S-CoB= heterodisulphide of CoM and CoB; CoA-SH= coenzyme A. **Enzymes:** 1= energy-conserving hydrogenase (Ech); 8= methyl-H_4MPT:HS-CoM methyltransferase; 9= methyl-CoM reductase; 10= heterodisulfide reductase; 12= acetate kinase (AK)-phosphotransacetylase (PTA) system / AMP-forming acetyl-CoA synthetase; 13= CO dehydrogenase/acetyl-CoA synthase.

1.2.3 Methanogen ecophysiology

Methanogens have been found to naturally occupy a wide variety of anaerobic environments. These habitats range from marine and freshwater sediments and geothermal areas to the gastrointestinal tract of animals and humans. They have also been shown to thrive in landfills and anaerobic digester systems.

1.2.3.1 Adaptations to environment

a) Salinity

Methanogens have been found to inhabit environments with a wide range of salinities, extending from freshwater to hypersaline habitats. Characteristically, methanogens inhabiting freshwater environments require at least a 1 mM sodium concentration. The reason for this is due to a sodium motive force that is employed by methanogens. This sodium motive force, which is inwardly directed, functions in the bioenergetics of the organism's methanogenesis cycle (Kaesler & Schönheit, 1989; Zinder, 1993).

Both freshwater and marine methanogen communities show extensive diversity, although only a few halophilic methanogens, all of which belong to the order *Methanosarcinales*, are commonly known (Zinder, 1993). In a recent study investigating the sediment and plankton from a freshwater pond, Briée *et al*. (2007) found a diverse achaeal community including *Methanosarcinales*, *Methanomicrobiales* and a few divergent linages.

It has been suggested that the increased quantity of methylated osmoprotectants, such as betaine and dimethylpropiothetin, present in methylotrophic methanogens, enables this select group to tolerate extreme salt concentrations (Mathrani *et al*., 1988). It has also been shown that methanogens have the ability to accumulate betaine if it exists in the surrounding environment (Robertson *et al*., 1990[a]).

Common to most organisms, adjustment to salinity occurs by accumulation of suitable compounds in the cytoplasm to create an equilibrium between internal and external osmolarity (Zinder, 1993). α-Glutamate, β-glutamate and N^{ε}-acetyl-β-lysine are such compatible solutes that have been detected in methanogens (Robertson *et al*., 1990[b]). α-Glutamate seems to be the primary solute in the cytoplasm under conditions of low osmolarity, while the occurrence of N^{ε}-acetyl-β-lysine in the cytoplasm is proportional to salinity (ie increasing N^{ε}-acetyl-β-lysine quantities in higher salinities) (Zinder, 1993).

b) Temperature

Methanogen populations are known to tolerate extreme temperatures, ranging from about 2˚C in marine sediments to geothermal areas which reach temperatures above 100˚C (Zinder, 1993). Both mesophilic and thermophilic methanogens show great diversity. However, it has been noticed that

thermophilic species grow at a quicker rate when compared with corresponding mesophiles. Jones *et al*. (1983) reported that the doubling time for *Methanococcus voltae* in a H_2-CO_2 enriched environment at 37˚C is nearly twice that of *Methanococcus thermolithotrophicus* (now *Methanothermococcus*) at 65˚C, while the shortest doubling time was recorded at 25 minutes for *Methanococcus jannaschii* (now *Methanocaldococcus*) at 85˚C.

The ability to optimally function under thermophilic conditions is common among methanogens (Zinder, 1993). Although methanogens appear to survive extremities in temperature, there seems to be an impediment on methanogenesis. Thummes *et al*. (2007) investigated the optimum temperature required for methanogensis in different compost materials. Results indicated that in six compost materials, optimal methanogenic activity was achieved at 50˚C (thermophilic), while only two compost materials achieved optimal methanogenic activity at 65˚C (hyperthermophilic). Lee *et al.* (2009) evaluated the performance of anaerobic digester systems with the same substrate feedstock at hyperthermophilic (70˚C), thermophilic (55˚C) and mesophilic (35˚C) conditions. Results showed that although optimal temperatures for treating substrates during the acidogenesis stages were in the hyperthermophilic range, thermophilic conditions were favoured for methanogenesis. It is important to note that together with temperature, the partial pressure of H_2 present in the environment plays a role in acidogenesis and methanogensis. As earlier indicated in Figure 1.2, a high H_2 partial pressure will favour hydrogenotrophic methanogenesis, whilst a low H_2 partial pressure will favour acidogenesis (Demirel & Scherer, 2008). Although biogas production was greatly diminished at hyperthermophilic and mesophilic conditions as compared to thermophilic conditions, the fact that the methanogen population was still able to survive and produce methane demonstrates the ability to adapt to adverse conditions (Lee *et al*., 2009). It has been suggested that the ability of prokaryotes, including methanogenic *Archaea*, to adapt to an unfamiliar or adverse environment is a result of a phenomenon known as horizontal gene transfer, where prokaryote cells are capable of transferring genetic information between each other. This is achieved by means of transformation, transduction and conjugation (Gribaldo & Brochier, 2009).

Several studies have also stressed the importance of temperature on methanogen diversity. In a study investigating mesophilic fermentation of beet silage (Krakat *et al*., 2010[b]) it was shown that hydrogenotrophic methanogens were dominant in a digester that was initially fed with a mixed acetotrophic and hydrogenotrophic methanogen inoculum. These results correspond to numerous studies where hydrogenotrophic methanogens were established to be the dominanting methanogens in the gastrointestinal tracts, which in fact represents a mesophilic fermenter (Krakat *et al*., 2010[b]), of various animals (Wright *et al*., 2004; Wright *et al*., 2007; Janssen & Kirs, 2008; Chaudhary & Sirohi, 2009; Evans *et al*., 2009). In the mono-digestion of swine faeces under mesophilic conditions, Zhu *et al*. (2011) reported that 57.7% and 34.2% of the clones assessed were affiliated to *Methanobacteriales*

and *Methanomicrobiales* respectively, meaning that over 90% of the clones assessed were affiliated to hydrogenotrophic methanogens.

Krakat *et al*. (2010[a]) also demonstrated that only hydrogenotrophic *Methanobacteriales* were present in a thermophilic biogas fermentor operated at 60˚C, with a slight change in populaion diversity at 55˚C, where *Methanobacteriales* still dominated but *Methanomicrobiales* and *Methanosarcinales* were also identified. Similarly, Bauer *et al*. (2008) reported a dominance of *Methanobacteriales* in thermophilic digesters, with a shift to a dominace of *Methanomicrobiales* in mesophilic digesters. This shift in methanogen diversity could be explained by the availability of hydrogen. In a study investigating hydrogen production in a mixed culture digestion of starch, Akutsu *et al*. (2009) reported that hydrogen production was not stable at mesophilic temperatures, but rather there was dominance of mesophilic non-hydrogen producing *Bacteria* such as hydrogenotrophic homoactogens. At thermophilic temperatures, hydrogen producing *Bacteria* were more easily cultivated, allowing for sustainable hydrogen production. Hydrogen would therefore be constantly available under thermophilic conditions which would result in the dominance of hydrogentrophic methanogens for the conversion of hydrogen and carbon dioxide to methane (Krakat *et al*., 2010[c]).

While Karakashev *et al*. (2005) showed greater methanogenic diversity in mesophilic operated reactors, Xing *et al*. (2007) descibed the lowering of temperatures, from 20˚C to 15˚C, resulted in a drastic change in methanogenic diversity in anaerobic sludge. There was a progressive decrease of most methanogen species, with the exception of possible psychrophilic methanogens such as *Methanocorpusculum parvum*. However, after continued operation, it was observed that species that initially decreased later recovered and increased in number, suggesting that temperature does govern methanogen diversity (Xing *et al*., 2007).

c) pH

A pH optimum near neutral is common to most methanogens (Jones *et al*., 1987), although there have been cases of methanogens inhabiting environments with extreme pH levels (Zinder, 1993). More recently, Bräuer *et al*. (2006) isolated an acidiphilic methanogen, belonging to the order *Methanomicrobiales*, from an acidic (pH 4.1) peat bog habitat.

An optimum of pH 6 has been typically reported for methanogenesis. Goodwin & Zeikus (1987) demonstrated that methanogenesis using both CO_2 and acetate as substrates was possible at pH 4, however methanogenesis reached an optimum when the pH value approached 6. Low pH, which can be established by an accumulation of fatty acids or an accumulation of acetate in the environment (Russell, 1991) inhibits methanogenesis probably due to acetate or acetic acid build-up inside the cells (Russell, 1991).

Maintenance of an environment with a pH within the optimum range for micro-organism growth and metabolism is essential since pH directly influences the toxicity of inhibitors such as ammonia or

hydrogen sulphide. Ammonia is present in two forms, the ammonium ion (NH_4^+) and free ammonia (NH_3), the latter of which is considered to be the primary cause of inhibition. Hydrophobic free ammonia can passively permeate the cell membrane, resulting in either a proton imbalance or potassium deficiency (Sprott & Patel, 1986; Chen *et al*., 2008). At an increased temperature and pH there is an adjustment to a higher free ammonium to ammonium ion concentration ratio, which subsequently leads to increased toxicity (Angelidaki & Ahring, 1993; Borja *et al*., 1996). Angelidaki & Ahring (1993) observed that under thermophilic conditions, digestion of cattle manure was inhibited at ammonia concentrations of 4 g N/l or more. It was also reported that acetotrophic methanogens were more susceptible to increasing ammonia concentrations, where the growth rate of aceticlastic and hydrogentrophic methanogens were halved at ammonia concentrations of 3.5 g N/l and 7 g N/l respectively (Angelidaki & Ahring, 1993).

Hydrogen sulphide (H_2S) has been reported as the toxic form of sulphide owing to its easy diffusion into the cell, where it may have the ability to denature proteins (Conn *et al*., 1987). More recently, it was observed that sulphide inhibition was related to non-ionised sulphide and total sulphide inhibition concentrations at certain pH ranges (O'Flaherty *et al*., 1998), where sulphide inhibition of aceticlastic methanogen activity was dependent on non-ionised sulphide concentration or total sulphide concentration within the pH range of 6.4-7.2 and 7.8-8.0 respectively.

d) Oxygen

Methanogens are strict anaerobes. Although they are unable to grow or produce methane in the presence of oxygen, they have been shown to be reasonably tolerant to oxygen exposure. Substantial variability of sensitivity to oxygen exists among methanogens. In a study undertaken by Kiener & Leisinger (1983) it was documented that the viability of methanogens such as *Methanococcus voltae* and *Methanococcus vannielii* dropped about 100-fold after 10 hours of oxygen exposure. Others, such as *Methanobrevibacter arboriphilus* and *Methanothermobacter thermoautotrophicus*, sustained viability for several hours, and after a period of 24 hours *Methanosarcina barkeri* still maintained viability. Leadbetter & Breznak (1996) observerd the oxygen tolerance of two hydrogentrophic *Methanobrevibacter* spp. isolated from gut homogenates of a subterranean termite. It was reported that both strains possessed catalase-like activity and were able to initate growth in H_2-CO_2 oxygen gradient tubes. In a study investigating the presence of thermophilic methanogens in compost materials (Thummes *et al*., 2007), oxic drying of the compost material was carried out. When compared to fresh compost, the methane production in the dried compost material was found to have slightly increased and demonstrated the abilty of the methanogens to survive stress stimuli (Thummes *et al*., 2007).

Adaptations to oxygen, including the presence of superoxide dismutase in low levels, have been reported in some methanogens (Kirby *et al*., 1981). Another adaptation found in

Methanothermobacter thermoautotrophicus is the formation of an ester between the AMP or GMP and deazaflavin ring in coenzyme F_{420} (Hausinger *et al*., 1985). This modification which only occurs when exposed to O_2 acts by "switching off" reductive metabolism in the organism.

e) Metabolic regulations

Interaction between an organism and the surrounding environment is important as activities of the organisms are regulated as a result of the ever changing environmental conditions. These responses include short-term regulation of enzyme activity (Zinder, 1993) and a few noted long-term regulations, which entail alteration of gene expression. Although many methanogens are known to utilise only one or two subtrates, enzyme activity regulation has been particularly noted in response to substrate availability.

Methanobacterium formicicum showed a slight increase in formate dehydrogenase (FDH) activity when grown on formate as compared with growth on H_2 and CO_2 (Schauer & Ferry, 1980), and *Methanococcus* (now *Methanocaldococcus*) *thermolithotrophicus* showed an almost 10-fold increase in FDH activity when grown on formate when compared to a mixed substrate environment (Sparling & Daniels, 1990). Limitation of H_2 in the environment can induce the synthesis of pseudomureinendopeptidase in the hydrogenotrophic *Methanothermobacter wolfei* (Kiener *et al*., 1987).

Regulation of methanogenesis from acetate has been well studied. Smith & Mah (1978) proposed that catabolism of acetate by *Methanosarcina* strain 227 was highly regulated by the presence of more easily metabolised substrates in the environment. In the presence of substrates such as methanol and H_2-CO_2 there was a suggested repression of acetate catabolism, with preference of methanol or H_2-CO_2 as a substrate for methanogenesis (Smith & Mah, 1978). As earlier mentioned, growth of acetotrophic methanogens is influenced by acetate concentrations. *Methanosarcina* spp. grow more rapidly when acetate concentrations are high, while low acetate concentrations are favoured for *Methanosaeta* spp. growth (Ferry, 1993).

f) Mobility and gas vesicles

Both *Methanococcales* and *Methanomicrobiales* display mobility via flagella that show distinct resemblance to their eubacterial counterpart (Jones *et al*., 1987). *Methanococcus voltae* and *Methanospirillum hungatei* show chemotaxis toward required substrates, namely acetate, leucine and isoleucine (Migas *et al*., 1988; Sment & Konisky, 1989). In prokaryotes, the response to non-uniform distribution of substrates occurs through use of components known as methyl-accepting chemotaxis proteins (MCPs). It has been suggested that the location of these MCPs within the cell may influence the regulation of chemotaxis (Bray *et al*., 1998). Gestwicki *et al*. (2000) showed that in a varied panel of both bacterial and archaeal elongated cells, the MCPs were located primarily at the poles and along the length of the cells, suggesting that the location of these MCPs, and therefore the regulation of chemotaxis, is some what conserved between *Bacteria* and *Archaea*.

Adjustments of position can also be achieved through the use of gas vesicles for floatation. To date only some *Methanosarcina* strains (Kamagata & Mikami, 1991) exhibit the presence of these vesicles. The formation of biofilms within anaerobic digesters allows for the accumulation of certain micro-organisms on solid materials. Zheng & Raskin (2000) demonstrated the dominance of filamentous *Methanosaeta* species in anaerobic bio-digesters with low acetate concentrations. This was due to the formation of a dense biofilm in a high solids and high retention time digester. Krakat *et al*. (2010[b]) investigated the effect of hydraulic retention time on the diversity of methane producing methanogens in a mesophilic digestor. It was demonstrated that a reduction in hydraulic retention time allowed for an increase in organic matter availability, which permitted the increased abundance of methanogens that were previously under represented in the digestor, and therefore resulted in increased methanogen diversity (Krakat *et al*., 2010[b]).

g) Reserve materials

A substitution for unavailable exogenous sources of energy and nutrients is required by all organisms. Endogenous or reserve materials are normally polymers which can be used as a source of energy. Glycogen (Murray and Zinder, 1987) and polyphosphate (Rudnick *et al*., 1990) have both been found in various methanogens including *Methanosarcina.* The classic limitation of nitrogen and carbon excess resulted in increased accumulation of glycogen in *Methanolobus tindarius* (Murray & Zinder, 1987), while the accumulation of stored polyphosphate in *Methanosarcina frisia* was found to be proportional to the phosphate concentration in the environment (Rudnick *et al*., 1991).

1.2.3.2 Competition for substrates

In natural habitats, methanogens have been found to compete for substrates, specifically H_2, with mainly three anaerobic groups of the *Bacteria*. These are the sulphate-reducing *Bacteria*, the

acetogens and the iron-reducing *Bacteria* (Zinder, 1993; Liu & Whitman, 2008). When electron acceptors like NO_3^-, Fe^{3+} and SO_4^{2-} are found in high concentration, hydrogenotrophic methanogens are usually outcompeted by these other hydrogen utilising *Bacteria*.

Hydrogen is an important substrate of sulphate-reducing *Bacteria*, acetogens and hydrogenotrophic methanogens. Growth kinetic parameters suggest that methanogens are potentially able to compete with other anaerobic groups when H_2 and CO_2 are the sole available substrates. However, it has been shown that in the presence of excess sulphate, sulphate-reducing *Bacteria* and acetogens completely outcompeted methanogens (Weijma *et al*., 2002). This is probably due to the fact that the reduction of these compounds is thermodynamically more favourable when compared to CO_2 reduction to methane (Liu & Whitman, 2008). Hydrogenotrophic methanogens are therefore usually found in environments where such electron acceptors are limited and therefore dominates in environments where CO_2 is the only abundant electron acceptor for anaerobic respiration.

1.2.3.3 Symbiosis

Symbiosis between *Bacteria* and *Archaea* is well documented. These syntrophic relationships, as described earlier, are considered to be either non-obligate interspecies electron transfer or obligate species electron transfer. The former involves fermentative *Bacteria* that utilise carbohydrates to produce substrates to be utilised by methanogens. In such a syntrophic interaction, where both *Bacteria* and *Archaea* benefit, the environment is kept at favourable low H_2 concentrations for carbohydrate fermentation by the hydrogen-utilising methanogens. With the latter interaction, both *Bacteria* and methanogens are dependent on each other for survival. Syntrophic *Bacteria* are responsible for oxidising longer-chain organic substrates while methanogens maintain a low H_2 and formate concentration environment required for continual organic substrate reduction (Zinder, 1993; Whitman *et al*., 2006).

Another type of symbiosis occurs between methanogens and protozoa. Vogels *et al*. (1980) first suggested an association between methanogens and rumen ciliate protozoa. Since then many associations with either methanogens on the surface (ectosymbionts) and inside (endosymbionts) protozoa have been observed in various anoxic environments including anaerobic sediments and gastrointestinal tracts (Zinder, 1993; Narayanan *et al*., 2009). Endosymbiotic associations have been observed in protozoa such as *Pelomyxa palustris* (Narayanan *et al*., 2009) and ciliates such as *Trymyema compressum* (Holler & Pfennig, 1991). The advantages of such symbiotic associations to the host are not clearly understood, although it has been suggested that in the presence of symbiotic methanogens the host grows more rapidly (Zinder, 1993; Narayanan *et al*., 2009) which might be due to an increased production of volatile fatty acids in the presence of H_2-consuming methanogens.

1.2.3.4 The gastrointestinal tract as a habitat

The gastrointestinal tract of many animals provides a potential environment for fermentation. Organic matter content in the tract is high, especially in herbivores where continual grazing is required. This, coupled with the fact that oxygen diffusion is low and both pH and temperature are stable (Zinder, 1993), make it the ideal environment for anaerobic microbial processes. Such an anaerobic microbial community may include *Bacteria*, fungi and methanogens (Wright *et al*., 2004; Yokoyama *et al*., 2007; Liu & Whitman, 2008) that work together in a symbiotic relationship to degrade complex organic matter that would otherwise be poorly digested.

Extensive studies have been carried out on the gastrointestinal microbial communities of ruminants. In ruminants, which include cows, sheep, goats and deer, fermentation of cellulosic plant material occurs in the foregut. In a study conducted by Wright *et al*. (2007) the diversity of methanogens found in the rumen of cattle was investigated. From the phylogenetic analysis of 16S rRNA gene libraries that were generated, it was found that almost 33% of clones were of the species *Methanobrevibacter ruminantium*, with about 56% of all clones exhibiting a ≥89.9% similarity to methanogens belonging to the *Methanobacteriales*, *Methanomicrobiales* and *Methanosarcinales* (Wright *et al*., 2007). Similarly, Wright *et al*. (2004) found that members of the order *Methanobacteriales*, i.e. hydrogenotrophic methanogens, were the predominant methanogens in sheep rumen, with over 90% of all clones identified as a *Methanobrevibacter* strain.

The microbial communities of monogastric animals, such as horses (Mackie & Wilkins, 1988), pigs (Zhu *et al*., 2009) and chickens (Saengkerdsub *et al*., 2007), have also been studied. Although methane production has been shown to be lower in monogastric animals when compared to ruminants, large herbivorous monogastric animals such as horses and mules have shown substantial methane production (Mandal & Mandal, 1995; Jensen, 1996). Species of *Methanobrevibacterium* tend to be the predominant methanogens present in monogastric animals (Jensen, 1996), with the exception of chickens and turkeys (Scupham *et al*., 2008), where *Methanogenium* is predominant.

The zebra, together with horses and donkeys, belong to the family *Equidae* (Mackie & Wilkins, 1988). Although domesticated animals have been well studied, little or no investigations have explored the microbial communities, in particular the methanogen populations, in wild herbivore gastrointestinal tracts (Nelson *et al*., 2003).

1.3 Culture based techniques

Culture methodology is an important technique in analysis of prokaryote diversity. Isolation and culturing allows for the phenotypic characterisation, such as morphology, physiological and biochemical identification, of micro-organisms (Pontes *et al*., 2007). Both liquid and solid media containing nutrients required for cell growth are widely used, although Schoenborn *et al*. (2004) demonstrated that culturing of soil *Bacteria* on solid media was superior to liquid serial dilution media.

However, many micro-organisms, especially those that are slow-growing or fastidious, cannot be isolated and cultured due to the limitations of currently available culturing methods, restricting recovery of diversity of micro-organisms found in an environmental sample (Pontes *et al*., 2007).

1.4 Culture independent techniques

To overcome the limitations and difficulties of culture-based techniques to assess the microbial diversity in environmental samples, a nucleic acid based technique was used for the analysis of both *Bacteria* and methanogenic *Archaea*.

1.4.1 DNA extraction from microbial cells

DNA extraction is both fast and convenient when compared to traditional isolation and culture methods. This, together with the fact that many microorganisms are currently not easily cultured from environmental samples, makes DNA extraction the preferred technique to collect DNA for molecular diversity analysis (Yeates *et al*., 1998). The method chosen for both the extraction and purification steps highly influences the quantity and quality of the nucleic acid extracted (Thakuria *et al*., 2009) which subsequently affects the performance of downstream molecular techniques such as Polymerase Chain Reaction (PCR). DNA extraction has therefore been described as one of the most important stages in molecular based investigations. A study carried out by Thakurai *et al*. (2009) showed that DNA of poor quality resulted in a modified or distorted interpretation of the sample's microbial diversity.

DNA extraction begins with the disruption of the cellular envelope which allows for the release of DNA. Lysis of the cellular envelope can be achieved through either mechanical or chemical treatment. Enzymatic lysis involves the addition of chemicals such as lysozyme. Lysozyme targets the 1,4-beta linkages between N-acetyl-D-glucosamine (NAG) and N-acetylmuramic acid (NAM) residues in bacterial peptidoglycan, making this type of treatment effective in the lysis of Gram-positive bacterial cells. However, in the case of Gram-negative *Bacteria*, lysis of the lipid bilayer is initially required to provide access for lysozyme to the murein layer. Here, ethylene diamine tetra acetic acid (EDTA) can be used as it removes $Mg^{++}$and Ca^{++}ions which are needed to preserve the

overall structure of the outer membrane. However, methanogenic *Archaea* do not have a cell wall that is composed of peptidoglycan and therefore are not susceptible to lysozyme treatment.

Physical treatments, such as French press, bead-beating and sonication, result in the release of cellular contents due to a mechanical force being applied to the cell. Past research shows that mechanical treatments are not only less expensive when compared to chemical treatments, but also more effective. A study carried out by Yeates *et al*. (1998) demonstrated that an extraction method that incorporated bead-beating consistently extracted DNA of higher quantity and quality when compared to other methods that included the use of lysozyme for enzymatic lysis and sonication. The use of beads in the lysis step in a study carried out by Thakuria *et al*. (2009) increased DNA yield by up to 50%.

Thereafter, the removal of membrane lipids is then required. This is achieved by addition of a detergent such as sodium dodecyl sulphate (SDS). This is followed by removal of contaminating protein with pronase or proteinase K treatment which breaks polypeptides into smaller units (i.e. amino acids) which are then easily removed. Cetyl trimethyl ammonium bromide (CTAB) is commonly used as it forms complexes with both proteins and polysaccharides. The lipid precipitates are removed upon centrifugation by phenol/chloroform extraction.

The final step involves the precipitation of the DNA by a short chain alcohol, usually ethanol or isopropanol. The DNA is insoluble in these alcohols and will therefore aggregate and form a pellet upon centrifugation.

However, quantitative extraction of nucleic acids from environmental samples including faeces has proven problematic (Yeates *et al*., 1998; Peršoh *et al*., 2008; Tang *et al*., 2008), owing mainly to the presence of co-extracted inhibitors. Faeces are complex samples that can inhibit subsequent molecular based manipulations such as amplification by PCR (Tang *et al*., 2008). Humic substances, a major contaminant, have similar chemical properties to that of nucleic acids and are therefore not removed during standard extraction procedures (Peršoh *et al*., 2008). Treatment with polyvinylpolypyrollidone (PVP) has been shown to effectively remove co-extracted inhibitors (Tang *et al*., 2008) and dilution of the extracted DNA has also been suggested to reduce inhibition by contaminants (Yeates *et al*., 1998).

In recent years commercial extraction kits have been favoured over traditional manual extractions because of high nucleic acid purity, convenience, speed and simplicity. Five commercially available kits were assessed by Dauphin *et al*. (2009) for DNA recovery from environmental samples. Results showed that a kit that combined bead-beating together with a silica spin column had the greatest efficiency regarding DNA recovery, although it has also been indicated that use of such kits is problematic as DNA yield efficiency could be impeded (Tang *et al*., 2008).

1.4.2 16S rRNA target gene amplification

The sequence analysis of 16S small sub-unit ribosomal nucleic acid molecules has allowed for examination of the phylogenetic and evolutionary relationships between prokaryotes (Woese, 1987). The 16S rRNA gene region is stable, ubiquitous, functionally constant, conserved and homologous, making it an ideal molecular marker (Schleifer, 2009). Because this method omits the need for culturing, it enables the isolation of novel and uncultured microorganisms, thereby providing knowledge of the diversity of microbial communities from various environments (Macrae, 2000). Although 16S rRNA gene amplification is considered as the "gold standard" (Schleifer, 2009) in inferring phylogenetic relationships between micro-organisms, multiple 16S rRNA genes do exist in a single organism, potentially creating a 16S rRNA sequence divergence of up to 1-2% (Schleifer, 2009) within the same organism.

Molecular based methods are widely preferred over culture based methods, however there are potential problems that could be encountered with the use of these techniques. One such problem is unequal amplification which distorts the distribution of PCR products. PCR bias, which results in unequal amplification, is thought to occur due to either using templates with differing amplification efficiency or self-annealing of templates in the final cycles of amplification which results in amplification inhibition (Acinas *et al*., 2005). Sequence artifacts can also occur due to PCR errors. The formation of these artifact sequences seems to occur from a *Taq* polymerase error, heteroduplex molecule formation or chimeric molecule formation (Acinas *et al*., 2005). Possible solutions to these problems include minimising the number of amplification cycles and introducing a reconditioning PCR step (Acinas *et al*., 2005).

1.4.3 Cloning

Cloning is the insertion of a foreign DNA fragment into a cloning vector or plasmid which often contains an antibiotic resistance gene marker. By applying an initial restriction digest, the foreign DNA may subsequently be ligated into the vector. Recombinant plasmids are then inserted into competent carrier cells such as *E. coli*. Thereafter, cells are exposed to an appropriate antibiotic such as ampicillin for selection of transformed cells. Only cells that carry the recombinant plasmid will be able to grow on media containing the antibiotic. Clones can then be selected to confirm the presence of the correct target insert by colony PCR.

1.4.4 Amplified ribosomal "DNA" restriction analysis

Amplified Ribosomal "DNA" Restriction Analysis (ARDRA) involves amplification of the conserved 16S rRNA gene with the use of specific primers that target this region. Amplicons of the targeted regions are then digested with restriction enzymes. Restriction enzymes have specific recognition sites (Roberts, 1976) and therefore the amplified gene regions are digested into varying fragment sizes. These fragments are detected as different size bands on an agarose gel, thereby creating a

pattern unique to the amplicon and therefore representing that specific 16S rRNA region. Each dissimilar pattern suggests a different operational taxonomic unit (OTU) (the term used to indicate an individual strain) or phylotype. Sequence analysis of representative OTUs is then performed to establish phylogenetic relationships. Although this technique is usually complemented with a probe hybridisation technique, Krakat *et al*. (2010[a]) verified the reliability of ARDRA by spiking experiments.

1.4.5 Other culture independent techniques that can be used to assess diversity

Other techniques that can be used to analyse diversity in environmental samples by targeting nucleic acids include an alternative target gene to the 16S rRNA gene. For methanogenic *Archaea* and alternative target gene is the *mcr*A gene which codes for the terminal enzyme complex in the methane generation pathway. Therefore this type of gene targeting is methanogen-specific (Luton *et al*., 2002). It was shown that the analysis of methanogen diversity via the 16S rRNA and *mcrA* gene regions were highly comparable (Luton *et al*., 2002; Kormas *et al*., 2008).

Other culture independent techniques that can be used to assess diversity include Denaturing gradient gel electrophoresis (DGGE), Temperature gradient gel electrophoresis (TGGE), Terminal-restriction fragment length polymorphism (T-RFLP) and Fluorescence *in situ* hybridisation (FISH).

DGGE is used to separate PCR amplified DNA via differential mobility through the denaturing gradient of the gel based on dissimilar G-C content of the sequences. With DGGE, the chemical denaturing gradient compounds are usually urea and formamide. The increasing denaturing gradient along the gel allows for the separation of double-stranded amplified DNA into single-stranded DNA, and the sequence becomes increasingly denatured as it passes throught the gel and comes to a stop when it is nearly fully denatured. Therefore different sequences will be visualised at different positions on the gel (Spiegelman *et al*., 2005; Li *et al*., 2009). TGGE works on the same principle as DGGE, except that the denaturing gradient is obtained through the use of heat (Li *et al*., 2009).

T-RFLP is an adapted version of ARDRA, where fluorescently labeled PCR primers are used to amplify rDNA genes and restriction digestion is applied, resulting in a fluorescently labelled fragment (Spiegelman *et al*., 2005). An automated scanner is then used to read the size and the intensity of the different restriction fragements. Fluorescence *in situ* hybridisation detects the presence or absence of specific RNA sequences by using fluorescent labelled specific probes which bind only to parts of the ribosome that have a high degree of sequence similarity. Thereafter, fluorescent microscopy can be used to locate the ribosome-bound fluorescent probe. This technique is often used to compare genomes of two species to deduce evolutionary relationships.

Since both the methanogenic and bacterial diversity were to be assessed in this study, appropriate primers specific to the 16S rRNA gene regions were chosen. This was followed by construction of clone libraries, restriction digestion of the amplified 16S rRNA gene regions from selected clones and

sequencing of a respresentative from each phylotype. Ramos *et al*. (2010) have shown that this combined methodology was advantageous since only a representative of each phylotype would require analysis and therefore decreased the sequencing effort required. It was also demonstrated that the sequencing of one representative per phylotype was reliable by randomly selecting three clones with the same restriction pattern profile, the resulting sequences of which were identical (Ramos *et al*., 2010).

CHAPTER 2

Materials and Methods

2.1 Zebra faecal sample collection

Samples of fresh zebra faeces (*Equus quagga* formerly *Equus burchelli*) were collected into sterile bags from the Queen Elizabeth Park, Pietermaritzburg, KwaZulu-Natal, South Africa in June and July. These are the winter months in South Africa. This is typically the dry season in the KwaZulu-Natal region. Samples were collected at least every two weeks, in the mornings before temperatures rose above 20˚C. The number of samples that were collected per day depended on availability and the freshness of the sample and ranged between 1 and 3 samples. The freshness of the wet weight samples was approximated by on-site temperature readings with a standard lab thermometer (cleaned prior to use with 70% ethanol) inserted into the faecal matter. The samples were transported to the laboratory in a polystyrene box where pH-Fix test strips (Macherey-Nagel) were used to determine the pH of the sample.

2.2 Quantification of selected *Bacteria* and fungi

10 g of wet weight faecal sample was diluted in 90 ml of sterile saline solution (Oxoid Saline Tablets, 1 tablet dissolved in 500 ml distilled water) in a Nerbe Plus (Germany) filter bag to achieve a particle-free effluent in a 1:10 dilution (10^{-1}) of substrate to total volume. Further dilution in saline solution was carried out to obtain a decimal dilution series in the range of 10^{-2}-10^{-8}. Thereafter, 100 µl of each decimal dilution was pipetted and spread plated under aseptic conditions on to solid media in triplicate unless otherwise stated. After the required incubation times and conditions for each medium (as described below), the colony forming unit (CFU) counts were established for plates (ie: 10 < CFUs < 300) for at least two neighbouring decimal dilutions. The weighted mean was calculated where possible (ie: 10 < CFUs < 300). Between 10 and 20 random colonies were microscopically examined by Gram stain.

2.2.1 Viable heterotrophic *Bacteria* (Plate Count Agar (PCA))

Preparation of PCA: 5.0 g tryptone, 2.5 g yeast extract, 1.0 g glucose in 800 ml distilled H_2O, adjusted to pH 7.0 (± 0.2). Addition of 15.0 g agar, adjusted to a final volume of 1 L with distilled H_2O. Medium autoclaved at 121˚C for 15 minutes, cooled to 50˚C prior to pouring plates. Prepared medium was beige in colour (Martley *et al*., 1970).

For the quantification of total aerobic heterotrophic *Bacteria*: Medium was poured into sterile petri dishes and allowed to set. Plates were inoculated by spread plating as described in 2.2. Inoculated plates were aerobically incubated at 35˚C for 48 hours.

For the quantification of spore forming aerobic heterotrophic *Bacteria*: Plates were inoculated by spread plating using a second dilution series where the original 10^{-1} dilution was first heat treated at 70˚C for 10 minutes and thereafter diluted in sterile saline solution to obtain a 10^{-2}-10^{-8} dilution series. Inoculated plates were aerobically incubated at 35˚C for 48 hours.

For the quantification of total and spore forming anaerobic *Bacteria*: Both serial dilutions from above were further utilised for the inoculation of PCA (pour plating). 100 µl of the decimal dilution samples were aseptically added to empty petri dishes, 15 ml PCA kept at 45˚C was then added and the petri dishes were swirled in order to mix the sample and medium and allowed to set. Plates were then anaerobically incubated in anaerobic jars at 35˚C for 48 hours. An anaerobic environment was created using an anaerobic jar (Oxoid, SA) and the Oxoid Gas Generating Kit Anaerobic System (Oxoid, SA).

All visible colonies that were present after the required incubation period were included in the final CFU value.

2.2.2 Gram-negative lactose and non-lactose fermenting *Bacteria* (MacConkey with crystal violet (CV) agar)

Preparation of MacConkey agar: 50 g of MacConkey agar with CV (Biolab Diagnostics, India) was added to 1 L distilled H_2O and boiled until completely dissolved. Medium autoclaved at 121˚C for 15 minutes, allowed cooled to 50˚C prior to pouring plates. Prepared medium was deep red in colour (MacConkey, 1905). Plates were inoculated by spread plating using the decimal dilution samples as described under 2.2 and were incubated aerobically at 37˚C for 24 hours.

2.2.3 Yeasts and moulds (Rose Bengal Chloramphenicol (RBC) agar)

Preparation of RBC agar: 5.0 g mycological peptone, 10.0 g glucose, 1.0 g potassium dihydrogen phosphate, 0.5 g magnesium sulphate, 0.05 g rose bengal, 0.1 g chloramphenicol in 800 ml distilled water and heated to boiling point until completely dissolved and allowed to cool, adjusted to pH 7.2 (± 0.2). Addition of 15.5 g agar, adjusted to a final volume of 1 L with distilled H_2O. Medium autoclaved at 121˚C for 15 minutes, cooled to 50˚C prior to pouring plates. Prepared medium was pinkish-red in colour (Jarvis, 1973). Plates were inoculated by spread plating using the decimal dilution samples as described under 2.2 and were incubated aerobically in the dark at room temperature (22˚C) for 4 days.

2.2.4 *Clostridium* spp.

Clostridium spp. have been previously described as an important group of the *Bacteria* present in the gastrointestinal tract (Daly *et al*., 2001) and are known to contribute to hydrolysis, acidogenesis and acetogenesis in the anaerobic food chain. Selective *Clostridium* solid media (Differential Clostridial

Agar (DCA) and Shahidi-Ferguson Perfringens (SFP) agar) were used to determine whether *Clostridium* would also be present as an important fermentative member of the *Bacteria* in the gastrointestinal tracts of zebra. The decimal dilution samples used for the *Clostridium* quantification were prepared where the original 10^{-1} dilution was initially heat treated at 70˚C for 10 minutes before subsequent dilution.

2.2.4.1 Differential Clostridial Agar (DCA)

Preparation of DCA agar: 5.0 g tryptone, 5.0 g peptone from meat, 8.0 g meat extract, 1.5 g yeast extract, 1.0 g starch, 1.0 g glucose, 0.5 g L-cystein, 5.0 g sodium acetate, 1.0 g sodium sulphite, 0.7 g ammonium iron (III) citrate, 0.002 g resazurin in 800 ml distilled H_2O, adjusted to pH 7.1 (± 0.2). Addition of 14 g agar, adjusted to a final volume of 1 L with distilled H_2O. Medium autoclaved at 121˚C for 15 minutes, cooled to 50˚C prior to pouring plates. Prepared medium was brown in colour (Weenk *et al*., 1991). Plates were inoculated by spread plating 100 µl of heat treated decimal dilution samples and were then anaerobically incubated in anaerobic jars at 35˚C for 72 hours. An anaerobic environment was created using an anaerobic jar (Oxoid, SA) and the Oxoid Gas Generating Kit Anaerobic System (Oxoid, SA). If no visible colony growth was detected after 72 hours incubation, the plates were incubated for a further 72 hours.

2.2.4.2 Shahidi-Ferguson Perfringens (SFP) agar

Preparation of SFP agar: 15.0 g tryptose, 5.0 g yeast extract, 1.0 g ferric ammonium citrate, 1.0 g sodium metabisulfite in 800 ml distilled H_2O, adjusted to pH 7.6. Addition of 20 g agar, adjusted to a final volume of 950 ml with distilled H_2O. Medium autoclaved at 121˚C for 15 minutes and cooled to 50˚C. 30,000 units Polymyxin B sulphate, 0.012 g kanamycin sulphate and 50 ml of 50% egg yolk emulsion was aseptically added to the cooled medium and mixed well before pouring plates. Prepared medium was creamy-beige in colour (Shahidi & Ferguson, 1971). 100 µl of heat treated decimal dilution samples were used to inoculate plates by spread plating. Inoculated plates were anaerobically incubated in anaerobic jars at 35˚C for 24 hours. An anaerobic environment was created using an anaerobic jar (Oxoid, SA) and the Oxoid Gas Generating Kit Anaerobic System (Oxoid, SA). If no visible colony growth was detected after 24 hours incubation, plates were incubated for a further 24 hours.

2.3 Phenotypic confirmation of random colonies

2.3.1 Gram stain technique

Between 10 and 20 randomly selected colonies from each medium type were microscopically examined after performing a Gram stain as follows: A sterile loop was used to transfer material from a colony to a drop of distilled H_2O on a glass slide, mixed and allowed to air dry before passing the

slide through the Bunsen flame 2-3 times without directly exposing the dried film to the flame. The slide was flooded with crystal violet for 1 minute and rinsed in a gentle stream of distilled H_2O. The slide was flooded with Lugol's iodine for 1 minute and was rinsed with distilled H_2O as before. The slide was then flooded with 95% ethanol for 10 seconds and rinsed with distilled H_2O. Finally, the slide was flooded with safranin for 30 seconds, rinsed with distilled H_2O, dried and examined by light microscopy. *E.coli* and *B. subtilis* were used as Gram-negative and Gram-positive controls respectively.

2.3.2 Malachite green stain technique

Between 10 and 20 randomly selected colonies were microscopically examined after performing the malachite green stain to check for the presence of endospores. A sterile loop was used to transfer material from a colony to a drop of distilled H_2O on a glass slide, mixed and allowed to air dry. The slide was placed over a steam bath and flooded with malachite green. The slide was kept over the steam bath for 5 minutes and re-covering with malachite green as it evaporated. The slide was then rinsed in a gentle stream of distilled H_2O to remove excess stain. Thereafter, the slide was flooded with safranin (counter stain) for 30 seconds, rinsed with distilled H_2O, dried and examined by light microscopy.

2.4 DNA extraction from zebra faeces

Three approaches were employed for the extraction of genomic DNA from zebra faecal samples. A manual approach, with two different sample pre-treatments, and a commercial kit were used in order to ascertain which type of approach would be best in the extraction of methanogenic DNA. Only the commercial kit was used for the extraction of bacterial DNA.

2.4.1 Commercial kit

The ZR Soil Microbe DNA Kit™ (Zymo Research, USA) was used for genomic DNA extraction according to the manufacturer's protocol. Extracted DNA was stored at -20˚C until further use.

2.4.2 Manual approach

Two procedures were employed for the initial treatment of faecal sample, followed by a single DNA extraction procedure according to Tang *et al*. (2008).

Pre-treatment A: 1.6 g (wet weight) faecal sample was aseptically added to 30 ml sterile ice-cold 50 mM phosphate-buffered saline (PBS) (Preparation of 50 mM PBS: 150 mM NaCl, 10 mM Na_2HPO_4, 20 mM NaH_2PO_4 in 800 ml distilled H_2O, adjusted to pH 7.4 and a final volume of 1 L with distilled H_2O, autoclaved at 121˚C for 15 minutes, stored at 4˚C) in a sterile 50 ml centrifuge tube and vortexed (Vortex-Genie, Scientific Industries) at maximum speed for 30 seconds. The sample was centrifuged in an Avanti® J-26 XPI Centrifuge (Beckman Coulter) at 500 x g for 4 minutes at room temperature. The supernatant was transferred to a new sterile centrifuge tube. The resulting pellet was re-suspended in 30 ml PBS buffer, briefly vortexed and centrifuged again. This procedure was repeated twice. Combined supernatants were centrifuged at 9000 x g for 5 minutes. The supernatant was discarded, while the pellet was re-suspended in 4 ml Tris-EDTA (TE) buffer and stored at -20˚C until further use. (Preparation of TE Buffer: 10 ml 1 M Tris (pH 8.0) and 200 µl of 0.5M Na_2EDTA (pH 8.0) in 800 ml distilled H_2O, mixed and adjusted to a final volume of 1 L with distilled H_2O, autoclaved at 121˚C for 15 minutes, stored at room temperature.)

Pre-treatment B: 1.6 g (wet weight) faecal sample was added to 10 ml sterile ice-cold 50 mM PBS buffer (as above) in a sterile 50 ml centrifuge tube and homogenised by addition of 5 sterile glass beads (5 mm in diameter) and vortexed at maximum speed for 3 minutes. Thereafter, the sample was centrifuged at 400 x g for 2 minutes to remove both glass beads and larger particles from the sample. The resulting suspension was transferred to a new sterile centrifuge tube. Addition of 3 volumes of 4% paraformaldehyde to the suspension was followed by incubation on ice for 1 hour. The cell suspension was centrifuged at 8000 x g for 3 minutes at room temperature. The supernatant was discarded, while the pellet was re-suspended in 4 ml PBS buffer and mixed with 4 ml absolute ethanol and incubated at -20˚C for 20 minutes. Thereafter, the sample was centrifuged at 8000 x g for 3 minutes at room temperature and the pellet was re-suspended in 4 ml TE buffer (as above) and stored at -20˚C until further use.

DNA extraction: Genomic DNA extraction was carried out on samples from both pre-treatment A and pre-treatment B in parallel according to the method described by Tang *et al.* (2008) with modifications as specified below. 323.5 µl of pre-treated sample was transferred to a new sterile 1.5 ml Eppendorf tube, centrifuged in a GenFuge 24D (Progen) bench-top centrifuge at 16,000 x g for 10 minutes. 646.5 µl of pre-heated (at 65˚C in a water bath) cetyl trimethyl ammonium bromide (CTAB) extraction buffer was added and the mixture vortexed at maximum speed for 30 seconds. (Preparation of CTAB extraction buffer: 100 mM Tris-HCl, 2.0 M NaCl, 20 mM EDTA, 2% CTAB in 800 ml distilled H_2O, adjusted to pH 8.0 and a final volume of 1 L with distilled H_2O, autoclaved at 121˚C for 15 minutes.)

10 µl (1%) polyvinylpyrrolidone (PVP) solution was added and vortexed at maximum speed for 30 seconds, followed by the addition 20 µl (2%) β-mercaptoethanol (98% solution) and vortexed at maximum speed for 30 seconds. The sample was incubated for 2 hours in a 65˚C water bath (the sample was mixed by inversion of the centrifuge tube every 15 minutes). After incubation, the sample was centrifuged at 16,000 x g for 10 minutes. The supernatant was transferred to a new sterile 1.5 ml Eppendorf tube.

An equal volume of chloroform:isoamyl alcohol (24:1, v/v) was added to the collected supernatant and centrifuged at 16,000 x g for 10 minutes. Thereafter, the supernatant was transferred to a new sterile Eppendorf tube, an equal volume of chloroform added, and centrifuged at 16,000 x g for 10 minutes at 4˚C. The resulting supernatant was transferred to a new sterile Eppendorf tube, 2 volumes of ice-cold absolute ethanol added, and incubated at -20˚C for 30 minutes. Following centrifugation at 16,000 x g for 10 minutes at 4˚C, the supernatant was removed by pipetting.

The remaining pellet was washed with ice-cold 70% ethanol. The pellet was completely air dried (~ 30 minutes on benchtop), re-suspended in 50 µl sterile milli-Q H_2O with added RNase (20 µg/ml, incubated at 37˚C for 30 minutes). The DNA was stored at -20˚C until further use.

2.4.3 Verification and analysis of genomic DNA extraction

Genomic DNA extraction was verified on a 0.8% agarose gel (0.24 g molecular grade agarose in 30 ml of 1 x Tris-Borate-EDTA (TBE) buffer). (Preparation of 10 x TBE buffer: 108 g Tris, 55 g boric acid dissolved in 900 ml distilled H_2O. 40 ml 0.5 M Na_2EDTA (pH 8.0) was added and final volume adjusted to 1 L with distilled H_2O, autoclaved at 121˚C for 15 minutes. 1 x TBE was prepared by adding 100 ml of 10 x TBE to 900 ml distilled H_2O.)

An O'GeneRuler 100 bp DNA Ladder (Fermentas, USA) was used as a molecular weight marker. For each sample that was loaded on the gel, 10 µl of sample was first mixed with 2 µl of 6x loading dye solution (Fermentas, USA) and then pipetted into the well. The gel was run at 80 V for 90 minutes. The gel was post-stained in ethidium bromide (EtBr) solution (100 µg EtBr in 200 ml 1 x TBE buffer) and visualised under UV light.

Spectrophotometer readings (BioRad SmartSpec^{TM}Plus Spectrophotometer) of manual based DNA extractions were taken at the following wavelengths: A_{230}, A_{260} and A_{280}.

2.5 Amplification of the 16S rRNA gene

The 16S rRNA genes of methanogens and the 16S rRNA genes of *Bacteria* present in the faecal sample were amplified from extracted DNA using the commercial kit and the maunal based extraction using pre-treatment B. (The manual based extraction using pre-treatment A was not used in further downstream applications.)

2.5.1 PCR parameters and primer pairs

Polymerase chain reactions (PCRs) were carried out with the use of KAPATaq™ HotStart (KAPABiosystems, South Africa) in a MultiGene™ II Personal Thermal Cycler (Labnet International, Inc, USA). Both component and cycling parameters for the amplification of methanogen and bacterial 16S rRNA genes (Tables 2.1 & 2.2) were used for all PCRs unless otherwise stated.

The following primer pairs, obtained from Inqaba Biotechnical Industries (Pty) Ltd, (South Africa), were used for the amplification of the methanogen partial 16S rRNA genes (Krakat *et al*., 2010[a]) and bacterial partial 16S rRNA genes (Amann *et al*., 1990; Muyzer *et al*., 1995) respectively.

i) Methanogen: Forward: ARCH 69F (5'-YGAYTAAGCCATGCRAGT-3')

Reverse: ARCH 915R (5' TCGTCCCCCGCCAATTCCT 3')

ii) *Bacteria*: Forward: EUB338F (5' GCTGCCTCCCGTAGGAGT 3')

Reverse: EUB907R (5' AAACTCAAAGGAATTGAC 3')

Table 2.1: PCR master mix per reaction (for both *Bacteria* and methanogens)

Component	**Final concentration**	**Volume per reaction**
5x KAPATaq HotStart Buffer	1x	5 µl
$MgCl_2$ (25mM)	5 mM	5 µl
dNTP mix (10mM each)	0.2 mM each dNTP	0.5 µl
Forward primer (10µM)	0.1 µM	0.25 µl
Reverse primer (10µM)	0.1 µM	0.25 µl
KAPA DNA Polymerase (5units/µl)	0.625 units	0.125 µl
Autoclaved MilliQ water	-	13.875 µl
Total		**25 µl**

A PCR master mix containing the above components was initially made and aliquoted (25 µl each) into PCR tubes. Thereafter, 1 µl DNA samples were added to each tube before amplification. Sterile water samples were employed as negative controls.

Table 2.2: Thermal cycling parameters used for PCR

Step	Temperature (˚C)	Time	No. of cycles
Initial denaturation	95	2 min	1
Denaturation	95	30 sec	30
Primer annealing*			
(methanogen)	53	60 sec	
(bacterial)	72	60 sec	
Extension	72	60 sec	
Final extension	72	5 min	1
Resting	4	∞	-

* Primer annealing temperatures differed according to the primer pair utilised. For the methanogenic primer pair an annealing temperature of 53˚C was used, whilst an annealing temperature of 72˚C was used for the bacterial primer pair.

2.5.2 Verification and analysis of amplified 16S rRNA genes

The presence and length of amplified 16S rRNA gene PCR products after PCR was verified on a 2% agarose gel (0.6 g molecular grade agarose in 30 ml 1 x TBE Buffer). An O'GeneRuler™ 100 bp DNA Ladder (Fermentas, USA) was used as a molecular weight marker. For each sample that was loaded on the gel, 3 µl of the PCR sample was mixed with 1 µl of 6x loading dye and then pipetted into the well. The gel was run at 100 V for 30 minutes. The gel was post-stained in EtBr solution for 15 minutes as specified under 2.4.3 and visualised under UV light.

2.6 Purification of amplified 16S rRNA gene PCR product

Purification of the amplified 16S rRNA gene PCR product was carried out by use of the Wizard® SV Gel and PCR Clean-Up System (Promega, USA) as per manufacturer's instructions. To verify the presence of the amplified product after purification, gel analysis was done as described under 2.5.2.

2.7 Cloning

A total of five clone libraries, 3 clone libraries based on amplified 16S rRNA genes of methanogens and 2 clone libraries based on the amplified 16S rRNA genes of *Bacteria*, were constructed as summarised in Table 2.3. For each of the 5 clone libraries only one of two DNA extractions were used (commercial kit or manual based extraction using pre-treatment B). After amplification of the 16S rRNA gene regions (as described under 2.5) and ligation of PCR gene products into a high copy plasmid vector, two types of competent cells were used as described under 2.7.2.

Table 2.3: Summary of clone libraries constructed

Clone Library	DNA extraction	Amplification primers	Ligation	Competent Cells
Methanogen1	Commercial kit	ARCH 69F/ARCH 915R	CloneJet	Lucigen E. cloni
Methanogen2	Commercial kit	ARCH 69F/ARCH 915R	CloneJet	$CaCl_2$-E.coli ATCC 8739
Methanogen3	Manual*	ARCH 69F/ARCH 915R	CloneJet	Lucigen E. cloni
Bacterial1	Commercial kit	EUB 338F/ EUB 907R	CloneJet	Lucigen E. cloni
Bacterial2	Commercial kit	EUB 338F/ EUB 907R	CloneJet	Lucigen E. cloni

* = The manual based extraction using pre-treatment B was used for DNA extraction.

2.7.1 Preparation of ligation mixture

The amplified 16S rRNA gene PCR products were ligated into the high copy plasmid vector pJET1.2/BLUNT with the use of the CloneJet™ PCR Cloning Kit (Fementas, USA) according to manufacturer's instructions with the following amendments:

- 2 µl of purified PCR product was used instead of 1 µl.
- The mixture was not vortexed in order to prevent shearing of the PCR product. Instead the Eppendorf tube was gently inverted to mix contents.
- Final incubation of the ligation mixture was extended to 30 minutes at room temperature (22-25˚C).

2.7.2 Transformation of competent cells and selection of clones

Two types of competent cells were employed, laboratory prepared competent cells using the calcium chloride method and the commercially available E. cloni® Chemically Competent Cells (Lucigen Corporation, USA). Commercially available E. cloni® Chemically Competent Cells were utilised with 4 of the prepared ligation mixtures, while laboratory prepared competent cells were utilised with a single preparation of the ligation mixture (Table 2.3).

2.7.2.1 Preparation of competent cells by the calcium chloride method

Day 1: 10 ml of Luria-Bertani (LB) medium was inoculated with *E. coli* ATCC 8739 and cultured in a shaking incubator (250 rpm) at 37˚C overnight. (Preparation of LB medium: 10 g tryptone, 5 g yeast extract, 10 g sodium chloride in 800 ml distilled H_2O, adjusted to pH 7.0 and a final volume of 1 L with distilled H_2O. Medium was autoclaved at 121˚C for 15 minutes.)

Day 2: 25 ml of LB medium was inoculated with 1 ml of an *E.coli* (ATCC 8739) overnight culture and was allowed to grow in a shaking incubator (250 rpm) at 37˚C to an $OD_{578} = 1$. Culture was transferred to a sterile 50 ml centrifuge tube and thereafter all procedures were performed on ice with

chilled apparatus. A pellet was obtained by centrifugation (5 minutes, 5000 rpm, 0˚C). The supernatant was discarded and cells were re-suspended in 10 ml chilled 50 mM $CaCl_2$-solution and incubated on ice for 30 minutes, centrifuged again, supernatant discarded and the pellet re-suspended in 0.5 ml chilled 50 mM $CaCl_2$-solution. Competent cells were stored in an ice-bath in the refrigerator for at least 12 hours prior to use.

Transformation of E. Cloni® Chemically Competent Cells was carried out according to the manufacturer's instructions with the following amendments:

- The ligation mixture was heat treated at 70˚C for 5 minutes according to the CloneJet™ PCR Cloning Kit Protocol.
- 2.5 µl of ligation reaction was added to 50 µl competent cells on ice.
- 900 µl of room temperature Recovery Medium was added to the cells and incubated at 37˚C.
- Transformed cells were plated on LB agar containing ampicillin. (LB agar was prepared as described for LB medium as above with the addition of 15 g agar before autoclaving. Medium cooled to 50˚C, 2 ml of sterile filtered ampicillin stock solution (50 mg/ml) was added and mixed prior to pouring plates.)

Transformation of competent cells prepared by the calcium chloride method was carried out as follows: 50 µl of competent cells were added to a chilled reaction tube. 2.5 µl of ligation mixture was added and mixed gently by pipette. Cells were transformed for 30 minutes in an ice-bath. Heat shock was applied for 45 seconds at 42.5˚C and thereafter the suspension was immediately returned to the ice-bath. 900 µl of LB-ampicillin medium was added to the transformed cells and incubated in a shaking incubator (250 rpm) for 90 minutes at 37˚C and 100 µl samples of the suspension were then plated onto LB-ampicillin agar and incubated at 37˚C overnight.
After overnight incubation at 37˚C, plates were checked for the presence of colonies. For each clone library (Table 2.3), one hundred colonies grown on LB-ampicillin agar were randomly selected and sub-cultured on LB-ampicillin agar at 37˚C overnight.

2.7.3 Verification of presence of 16S rRNA inserts in plasmid vector by colony PCR

After overnight incubation of sub-cultured colonies, a colony suspension for each colony was prepared by submerging material from the colony in 100 µl sterile distilled H_2O and vortexing at maximum speed for 10 minutes. The PCR master mix (Table 2.1) was prepared and aliquoted into PCR reaction tubes. Primer pairs, which allowed for amplification of 16S rRNA vector insert, were as follows:

Methanogen clone libraries:

- Forward: ARCH 69F (5' YGAYTAAGCCATGCRAGT 3')

- Reverse: ARCH 915R (5’ TCGTCCCCCGCCAATTCCT 3’)

Bacterial clone libraries:

- pJET1.2 Forward Primer, 23-mer (5’-CGACTCACTATAGGGAGAGCGGC-3’)
- pJET1.2 Reverse Primer, 24-mer (5’-AAGAACATCGATTTTCCATGGCAG-3’)

(The pJET1.2 primer pair was provided with CloneJet™ PCR Cloning Kit (Fermentas, USA))

1 µl of each colony suspension was added to aliquoted master mix and was amplified according to parameters described in Table 2.2 with the following amendment:

- The pJET1.2 primer pair was used for the bacterial clone libraries. This primer pair had an annealing temperature of 60˚C.

Thereafter, PCR products were analysed by running samples on a 2% agarose gel at 80 V for 2 hours. An O’GeneRuler™ 100 bp DNA Ladder (Fermentas, USA) was used as a molecular weight marker. For each sample that was loaded on the gel, 3 µl of sample was mixed with 1 µl of 6x loading dye and then pipetted into the well. The gel was post-stained in EtBr solution for 15 minutes (as described under 2.4.3) and visualised under UV light.

2.8 Amplified ribosomal “DNA” restriction analysis

Colonies containing inserts of the expected size (ie: 800-900 base pairs for methanogens and 600-700 base pairs for *Bacteria*) were considered positive clones and were further evaluated by restriction digestion of the amplified 16S rRNA gene.

2.8.1 Restriction digestion of cloned 16S rRNA genes

Two restriction enzymes, FastDigest® HinP1I (Hin6I) (Fermentas, USA) and FastDigest® HaeIII (BsuRI) (Fermentas, USA) were used for digestion according to manufacturer’s protocol with the following amendments:

- 0.5 µl of each enzyme solution was used instead of 1 µl.
- Incubation at 37˚C was carried out for 1 hour instead of 5 minutes.
- After incubation at 37˚C, the reaction was immediately transferred to ice to stop enzymatic activity.

2.8.2 Phylotype analysis

After enzymatic digestion, products were run on a 2.5% gel at 80 V for 2.5 - 3 hours. An O’GeneRuler™ 100 bp DNA Ladder (Fermentas, USA) was used as a molecular weight marker. For

each sample that was loaded on the gel, 15 µl of sample was mixed with 3 µl of 6x loading dye and then pipetted into the well. The gel was post-stained in EtBr solution for 15 minutes (as described under 2.4.3) and visualised under UV light.

For each sample, restriction patterns were analysed on agarose gel. From these patterns, samples with identical patterns were grouped as a single phylotype. A representative of each phylotype was sequenced (Inqaba Biotechnologies (Pty) Ltd, South Africa). The sequences were aligned using *CLUSTAL W* and compared to those of known methanogenic and bacterial species from GenBank. Phylogenetic evolutionary analysis was conducted for each clone library using *MEGA* version 5.2 (Tamura *et al*., 2011). The trees were constructed using the Neighbour-Joining (Saitou & Nei, 1987) and Maximum Likelihood based on the JTT matrix-based model (Jones *et al*., 1992) methods (1000 replicates) and evolutionary distances were computed using the p-distance method.

2.8.3 Rarefaction analysis and indices of species diversity

Rarefaction analysis of both the methanogen and bacterial clone libraries was performed to determine whether the number of clones picked for each microbial population were sufficient to give a reliable reflection of the microbial population diversity. Rarefaction curves were constructed using the *Mao Tau* values from output data obtained from *Estimate S* Version 8.2 (Colwell, 2009). Chao-1, Shannon and Simpson indexes of diversity were also calculated using *Estimate S*.

CHAPTER 3

Results

3.1 Collection of zebra faecal samples

On-site temperature readings of wet weight zebra faeces ranged from 26˚C to 35˚C, whilst pH readings ranged from 6-8. Samples collected on two occasions were used for further analysis as these samples were considered to be the freshest and the most suitable since the temperature (33˚C and 35˚C) of these samples best reflected the environment of the gastrointestinal tract.

3.2 Quantification of selected micro-organisms from zebra faecal samples

Media for target micro-organisms were inoculated with serially diluted sample solutions and incubated according to specific conditions for each medium. After incubation, colonies that were formed on the plates were macroscopically (size, colour, shape) evaluated. Microscopic analysis of at least 20 randomly selected colonies was conducted after performing staining techniques as descibed in section 2.3. Colony formation on representative agar plates are shown in Figures 3.1 – 3.4. The data for the quantification of target micro-organisms in zebra faeces are summarised in Table 3.1.

3.2.1 Viable heterotrophic *Bacteria*

Plate Count Agar (PCA) was used under four different conditions (as described in 2.2.1) to quantify viable heterotrophic *Bacteria* in the zebra faecal sample as A) aerobic, B) aerobic spore forming, C) anaerobic and D) anaerobic spore forming *Bacteria* as shown in Figure 3.1.

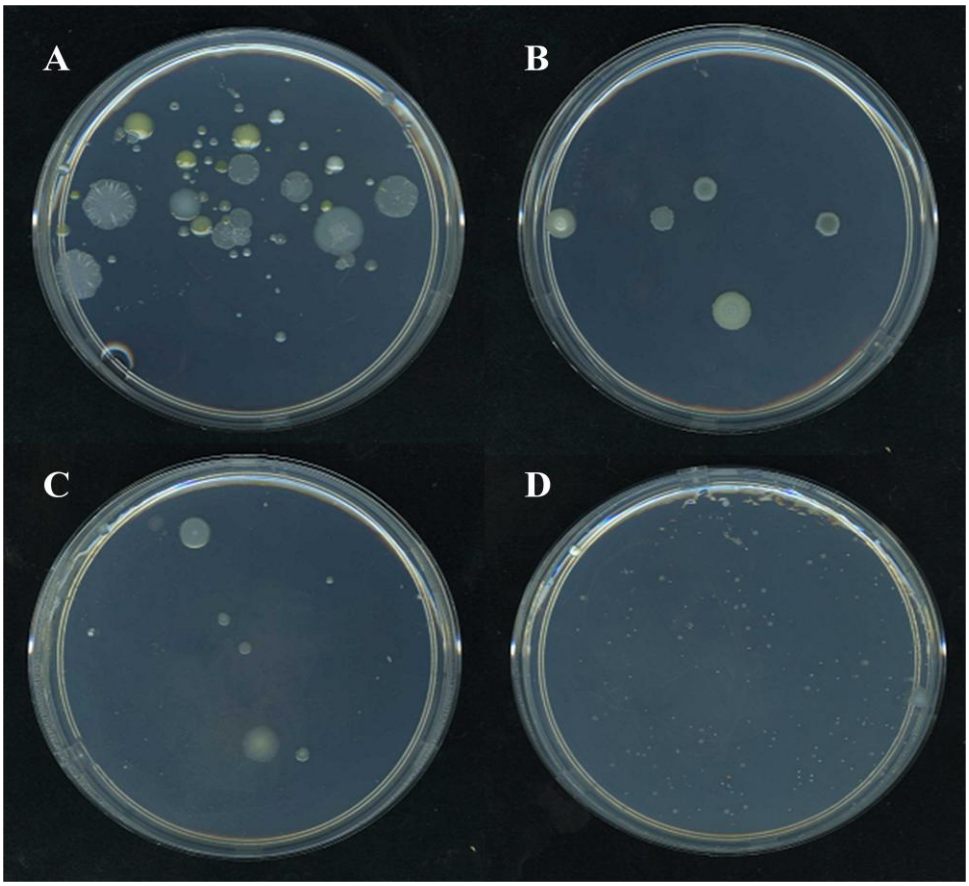

Figure 3.1: Representative PCA plates inoculated with the decimal dilutions of zebra faecal sample showing distinct colonies for A) aerobic, B) aerobic spore forming, C) anaerobic and D) anaerobic spore forming viable heterotrophic *Bacteria*.

Three types of colonies were detected for aerobic conditions, 1) creamish-white, circular colonies with irregular edges, colonies were 6-12 mm in diameter 2) yellow, circular colonies with a smooth, shiny surface which were 3-6 mm in diameter 3) white, circular colonies with a smooth, shiny surface which were 2-4 mm in diameter. Aerobic spore forming bacterial colonies were 5-10 mm in diameter, creamish-beige in colour and circular with irregular edges. An apparently high proportion of the colonies that were visualised exhibited *Bacillus*-like morphology (motile, Gram-positive rods, endospore positive).

Colonies formed on plates under anaerobic conditions were 3-8 mm in diameter, beige in colour and circular, with a smooth and shiny surface, while anaerobic spore forming bacterial colonies were 0.5-2 mm in diameter, circular and cream in colour. The edges and surface of all colonies were smooth.

Microscopic analysis of randomly selected colonies showed that these colonies were Gram-positive rods (Gram stain) and endospore positive (malachite green stain).

3.2.2 Gram-negative lactose and non-lactose fermenting *Bacteria*

MacConkey agar was used to evaluate the presence of Gram-negative *Bacteria* in the zebra faecal sample. Because this medium contains the pH indicator neutral red, further distinction could be made between Gram-negative *Bacteria* that can or cannot ferment lactose. Gram-negative lactose fermenting *Bacteria* will form pink colonies while Gram-negative non-lactose fermenting *Bacteria* will form opaque colonies.

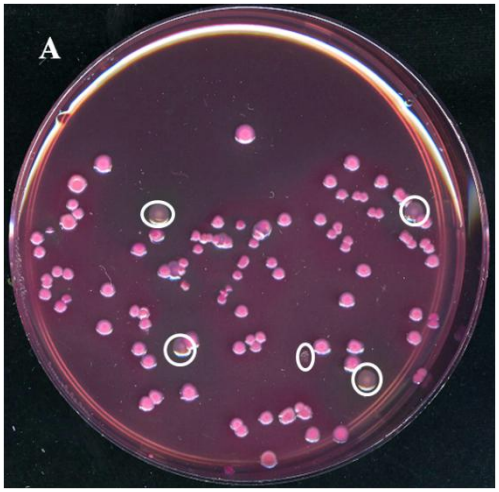

Figure 3.2: Representative MacConkey agar plate (A) inoculated with the decimal dilutions of zebra faecal sample showing distinct Gram-negative lactose fermenting and non-fermenting (circled in white) bacterial colonies.

As shown in Figure 3.2, two distinct colony types were present after 24 hours incubation at 37˚C. Pink colonies (Gram-negative lactose fermenting *Bacteria*) that were 2-6 mm in diameter formed on the plate. These colonies were circular with a raised margin. The surface of each colony was smooth and shiny. Gram staining confirmed that cells from pink colonies were Gram-negative rods.

Larger, opaque colonies (Gram-negative non-lactose fermenting *Bacteria*) that were 7-8 mm in diameter were also present on the plate. These colonies were also circular with a raised margin. The surface of each colony was smooth. Gram staining confirmed that cells from opaque colonies were Gram-negative rods.

3.2.3 Presumptive sulphite reducing clostridia

Differential Clostridial Agar (DCA) and Shahidi-Ferguson Prefringens (SFP) agar plates were used to evaluate the presence of sulphite reducing clostridia in the zebra faecal sample. Sulphite reducing clostridia produce sulphide from sulphite and will give a black precipitate since iron is present in the medium and therefore presumptive clostridia colonies formed on both media are expected to appear black in colour.

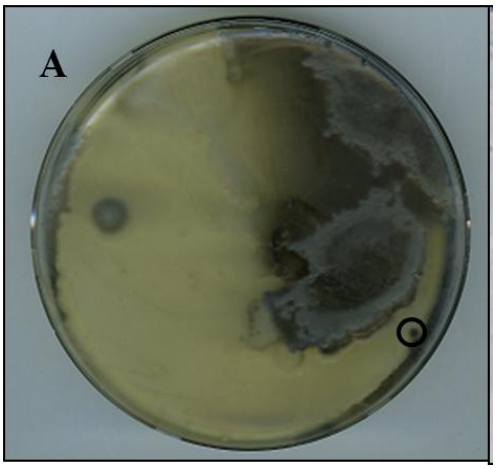

Figure 3.3: Representative DCA plate (A) inoculated with the decimal dilutions of zebra faecal sample showing sulphite reducing black colonies (circled in black).

Only a few distinct black colonies were visible on DCA plates after 6 days incubation at 35˚C under anaerobic conditions inoculated with the lowest dilution of faecal sample (10^{-1}) after numerous attempts. Colonies were circular, 2-3 mm in diameter and were black in colour. The surface of each colony was shiny and smooth. Microscopic evaluation of black colonies showed these colonies to be Gram-positive rods (Gram stain) and endospore positive (malachite green stain).

SFP agar plates inoculated with decimal dilutions were checked for colony formation after incubation for 48 hours at 35˚C under anaerobic conditions. No growth was observed on any of the plates after numerous attempts.

3.2.4 Yeast and moulds

Rose Bengal Chloramphenicol (RBC) agar was used for the enumeration of fungi (yeast and mould) in the zebra faecal sample. Because rose bengal is incorporated into the cell, yeast colonies will appear pink in colour and mould will grow as filamentous colonies with shades of pink on the reverse.

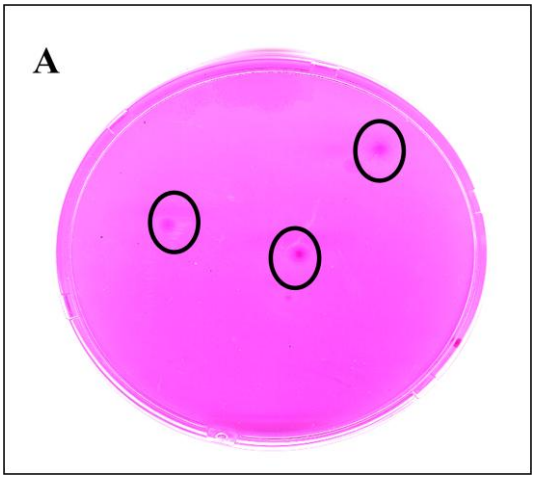

Figure 3.4: Representative RBC Agar plate (A) inoculated with the decimal dilutions of zebra faecal sample showing distinct yeast colonies (circled in black).

The uptake of rose bengal dye from the agar by the yeast colonies resulted in the colonies appearing pink in colour. Colonies were circular, 2-5 mm in diameter with a slight opaque zone surrounding each colony, while the margin appeared convex. The surface of each colony was shiny and smooth. Microscopic analysis of random pink colonies confirmed that cells of these colonies were spherical yeast cells (presence of a nucleus) with a cell diameter of 5-7 µm.

Table 3.1: Calculated colony forming units (CFUs) per gram of zebra faecal sample for selected micro-organisms

TARGET ORGANISM	MEDIUM	CFU/g#
Aerobic heterotrophic B*acteria*	PCA	**7.51×10^8**
Aerobic spore forming heterotrophic *Bacteria*	PCA	**1.74×10^6**
Anaerobic heterotrophic *Bacteria*	PCA	**2.45×10^9**
Anaerobic spore forming heterotrophic *Bacteria*	PCA	**1.57×10^8**
Gram-negative lactose fermenting *Bacteria*	MC(pink colonies)	**9.6×10^6**
Gram-negative non-lactose fermenting *Bacteria*	MC(opaque colonies	**<100**
Presumptive sulphite reducing clostridia	DCA	**<100**
Yeasts	RBC	**8.4×10^6**
Moulds	RBC	**Not detected**

#:g refers to gram (wet weight)

The colony forming units (CFUs) as mentioned in 2.2 were established via weighted mean where possible (Table 3.1). Plate count agar (PCA) was used to establish counts for both total aerobic and anaerobic heterotrophic *Bacteria*. As expected, the CFU/g of zebra faecal sample for total anaerobic *Bacteria* (2.45×10^9/g) was greater than the CFU/g of zebra faecal sample for total aerobic *Bacteria* (7.51×10^8/g). Following heat treatment of decimal dilutions of zebra faecal sample as described in 2.2.1, quantification of spore forming heterotrophic *Bacteria* formed on PCA was possible. Here, as with the total viable counts, the CFU/g of zebra faecal sample for anaerobic spore forming *Bacteria* (1.57×10^8/g) was greater than aerobic spore forming *Bacteria* (1.74×10^6/g). From this data it could be established that spore forming heterotrophic *Bacteria* found in the zebra faecal sample made up <1% and 6.4% of the total aerobic and anaerobic heterotrophic *Bacteria* respectively. MacConkey (MC) agar was used to establish the CFU/g for Gram-negative *Bacteria* in the zebra faecal sample. The CFU/g of zebra sample for Gram-negative lactose fermenting *Bacteria* was established as 9.6×10^6/g, while the CFU/g for Gram-negative non-lactose fermenting *Bacteria* was <100/g. Yeast counts established with Rose Bengal Chloramphenicol (RBC) agar was 8.4×10^6/g, while there was no detectable growth of moulds on the plates. The CFU/g for presumptive sulphite reducing *Clostridium* spp. (DCA) was <100/g.

3.3 DNA extraction from zebra faeces

DNA was isolated from zebra faeces using a commercial kit extraction procedure and a manual extraction procedure with two differing pre-treatments as described in 2.4. Successful DNA extraction from the zebra faecal sample using the commercial kit is shown in Figure 3.5. No spectophotometric analysis was carried out for the kit DNA extraction since column particles from the kit interferes with these measurements.

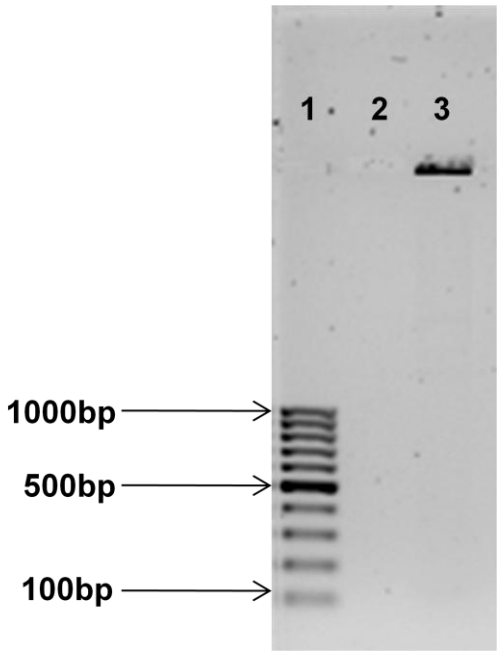

Figure 3.5: Gel electrophoresis showing extracted genomic DNA from zebra faecal sample using the commercial ZR Microbe Soil Kit. Lane 1 = O'GeneRuler™ 100 bp DNA Ladder (100 base pairs – 1000 base pairs.) Lane 2 = Negative control. Lane 3 = Extracted genomic DNA visible in well.

Gel electrophoresis of the extracted DNA from manual based extraction procedures showed no visible bands (figure not shown). This indicated that no or little DNA was extracted using the manual extraction procedures. Spectrophotometric analysis was carried out to check for the presence of extracted DNA, the values of which are summarised in Table 3.2.

Table 3.2: Spectrophotometric analysis of quality of DNA obtained from manual based extractions

Extraction	A230	A260	A280	A260/A230	A260/A280
MA1	1.00	0.45	0.31	0.45	1.48
MA2	1.04	0.63	0.43	0.61	1.47
MB1	1.76	1.41	1.11	0.80	1.27
MB2	1.46	0.96	0.75	0.66	1.29

MA1, MA2 = Manual extraction using pre-treatment A in duplicate. MB1, MB2 = Manual extraction using pre-treatment B in duplicate.

All manual extractions, regardless of the pre-treatment used, showed that DNA was extracted. However, the purity of the isolated DNA was not within the ideal value range according to the calculated A260/A230 and A260/A280 ratios where values of >2 and >1.7 respectively are indicative of high purity DNA (Yeates *et al*., 1998). This was expected since faecal sample material is a highly complex organic substrate containing many inhibitors (Tang *et al*., 2008) and relatively low DNA yields are usually obtained (Yu & Morrison, 2004). Only MA2 and MB1 were utilised for downstream applications and will subsequently be referred to as MA and MB respectively.

3.4 Amplification of the partial 16S rRNA gene

Amplified partial methanogenic (Figure 3.6) and bacterial (Figure 3.7) 16S rRNA gene products were analysed by gel electrophoresis and detected under UV light after staining with ethidium bromide.

A size of approximately 860 base pairs was expected for the amplified partial methanogenic 16S rRNA gene products (Westphal *et al*., 2007). A distinct band between 800 and 900 base pairs was visible for amplified 16S rRNA gene products from both the commercial kit extraction procedure (Figure 3.6 lane 2) and the manual extraction procedure that was preceded by pre-treatment B (Figure 3.6 lane 3). No distinct band was visible for the manual extraction procedure that was preceded by pre-treatment A (Figure 3.6 lane 4).

This result demonstrates that both the commercial kit procedure and manual extraction procedure that was preceded by pre-treatment B were successful in extracting methanogenic DNA from the zebra faecal sample, whereas the manual extraction procedure that was preceded by pre-treatment A was not successful. This result suggests the importance of a bead beating technique for the release of methanogenic DNA from the faecal samples, since beads were common to both the commercial kit and the manual extraction preceded by pre-treatment B, while the manual extraction preceded by pre-treatment A did not employ a bead based technique.

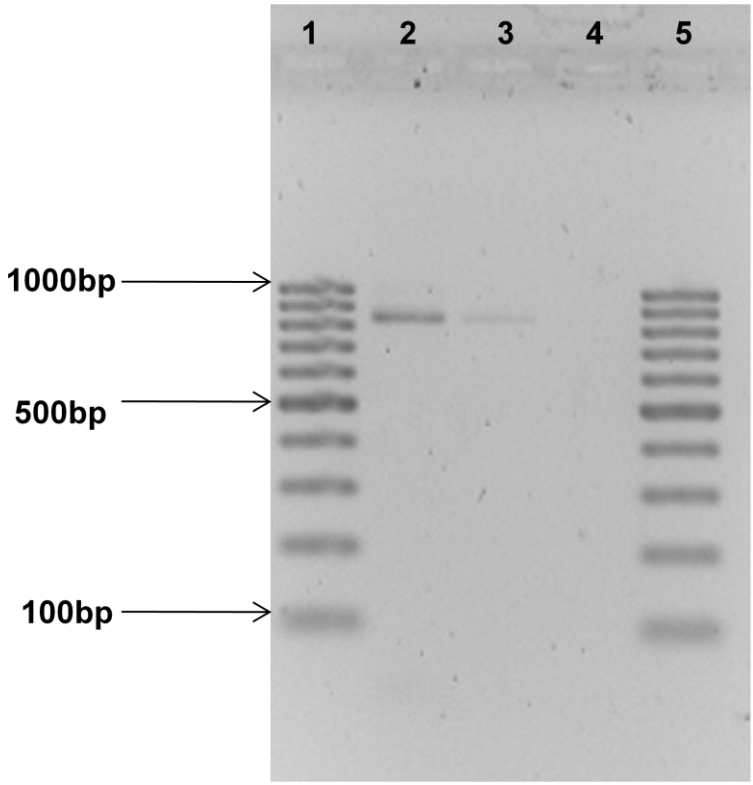

Figure 3.6: Gel electrophoresis showing amplified partial methanogenic 16S rRNA gene products by PCR from extracted DNA employing both the commercial and manual based extraction procedures. Lane 1, 5 = O'GeneRuler™ 100 bp DNA Ladder (100 base pairs – 1000 base pairs.) Lane 2 = Amplified partial methanogenic 16S rRNA gene product of DNA extracted with commercial ZR Soil Microbe DNA Kit. Lane 3 = MB (Amplified partial methanogenic 16S rRNA gene product of diluted (1:10) DNA extracted using the manual extraction procedure preceded by pre-treatment B. Lane 4 = MA (Amplified partial methanogenic 16S rRNA gene product of diluted (1:10) DNA extracted using the manual extraction procedure preceded by pre-treatment A).

As expected, bands exhibiting a size between 400 and 500 base pairs were visible for the amplified partial bacterial 16S rRNA gene products from DNA extracted with the commercial kit procedure (Figure 3.7 lane 2) and the positive control (Figure 3.7 lane 3). This result demonstrates that the commercial kit procedure was successful in extracting bacterial DNA from the zebra faecal sample.

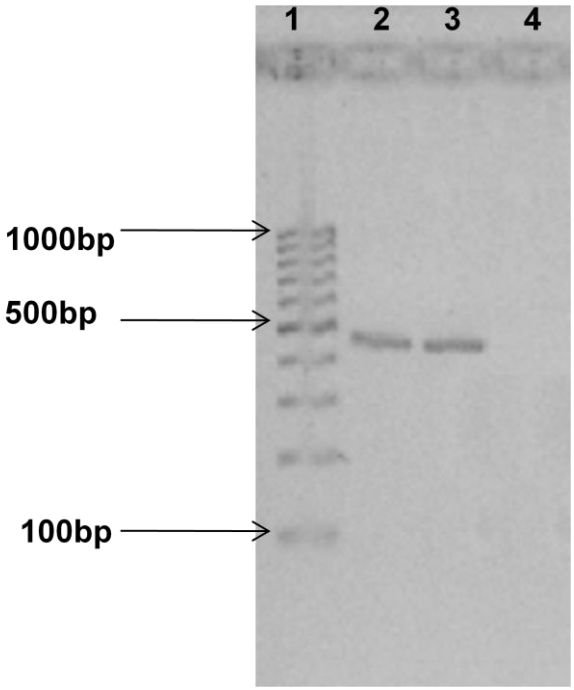

Figure 3.7: Gel electrophoresis showing amplified partial bacterial 16S rRNA gene products by PCR from extracted DNA employing the commercial ZR Soil Microbe DNA Kit. Lane 1 = O'GeneRuler™ 100 bp DNA Ladder (100 base pairs – 1000 base pairs.) Lane 2 = Amplified partial bacterial 16S rRNA gene products of DNA extracted with commercial ZR Soil Microbe DNA Kit. Lane 3 = Positive control of amplified partial 16S rRNA gene product of *B. subtilis* DNA extracted by freeze-thaw. Lane 4 = Negative control.

3.5 Selection of clones following cloning and colony PCR

As previously described in 2.7, five clone libraries were constructed. To verify the presence of the partial 16S rRNA inserts in the plasmid vector, 10 randomly selected colonies from both methanogen clone library 1 and bacterial clone library 1 were used for amplification of the partial 16S rRNA gene (colony PCR) and thereafter analysed by gel electrophoresis under UV light (Figure 3.8 and Figure 3.9).

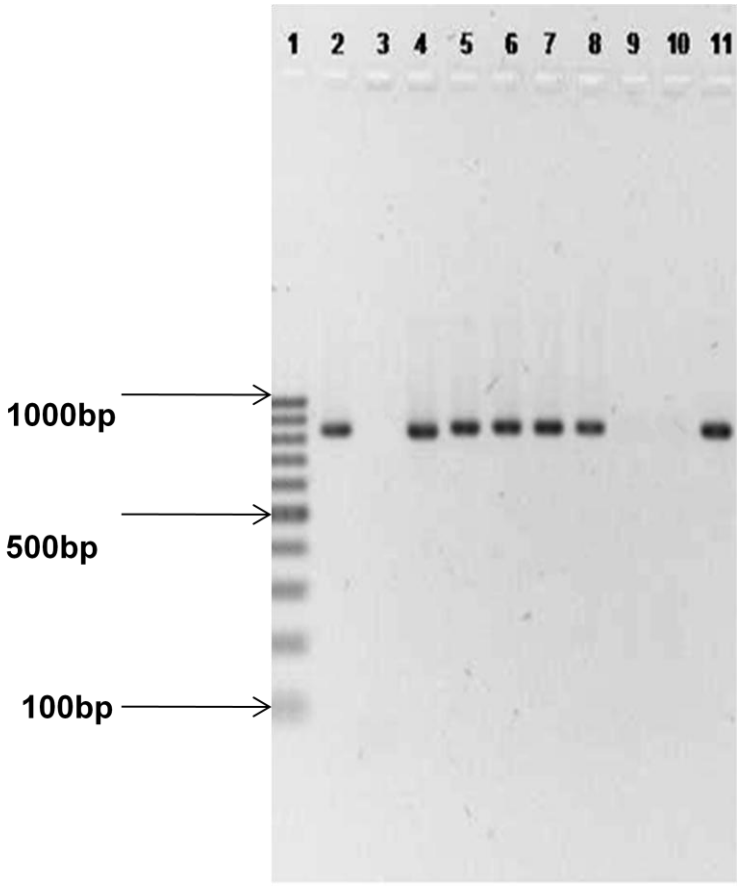

Figure 3.8: Representative gel electrophoresis showing PCR amplification of partial 16S rRNA gene vector inserts of 10 randomly selected colonies from methanogen clone library 1 using primer pair ARCH 69F/ARCH 915R. Lane 1 = O'GeneRuler™ 100 bp DNA Ladder (100 base pairs – 1000 base pairs.) Lanes 2-11 = Randomly selected clones 3, 9, 18, 20, 23, 29, 33, 39, 48 & 50 respectively.

For colonies selected from methanogen clone library 1, the detection of PCR amplicons between 800-900 base pairs was considered a positive sample (a clone carrying a methanogenic partial 16S rRNA gene insert). The absence of detectable PCR amplicons (ie: no visible band) was considered a negative sample (a clone carrying no methanogenic partial 16S rRNA gene insert). In Figure 3.8, samples 3, 18, 20, 23, 29, 33 & 50 (lanes 2, 4, 5, 6, 7, 8 and 11) had visible bands between 800-900 base pairs and were therefore considered positive samples, whereas samples 9, 39 & 48 (lanes 3, 9 & 10) formed no visible band between 800-900 base pairs and were therefore considered negative samples. Thereafter, a total of 100 colonies from each of the three methanogen clone libraries were selected for verification of a partial methanogenic 16S rRNA gene insert. Only positive clones were subsequently used for amplified ribosomal "DNA" restriction analysis (ARDRA).

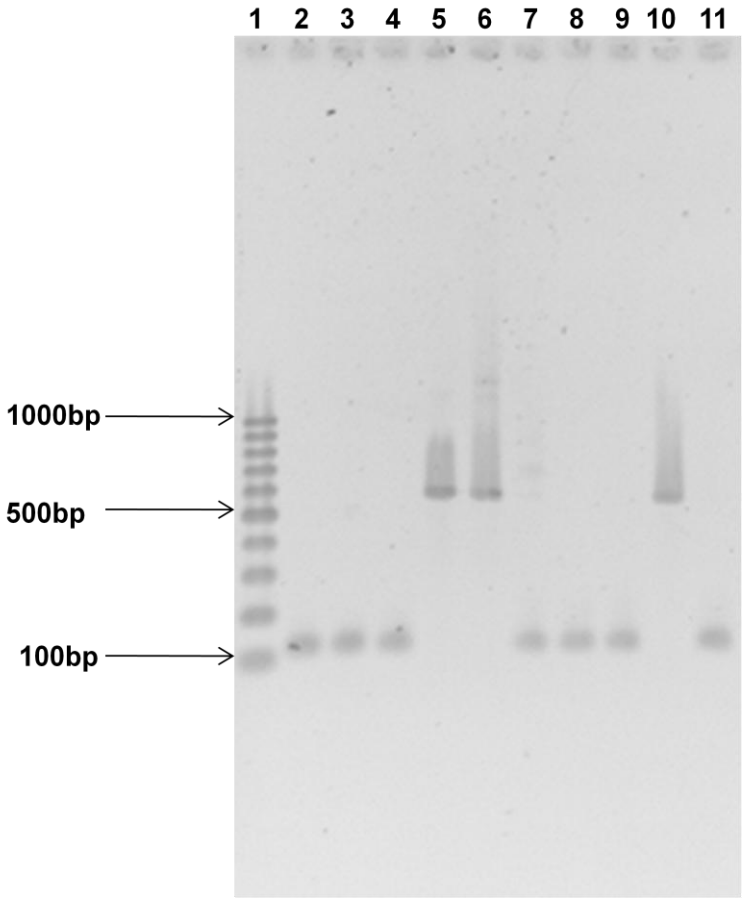

Figure 3.9: Representative gel electrophoresis showing PCR amplification of partial 16S rRNA gene vector inserts of 10 randomly selected colonies from bacterial clone library 1 using primer pair pJET1.2For-23-mer/pJET1.2Rev-24-mer. Lane 1 = O'GeneRuler™ 100 bp DNA Ladder (100 base pairs – 1000 base pairs). Lanes 2-11 = Randomly selected clones 4, 6, 8, 9, 12, 13, 14, 16, 20 & 23 respectively.

The expected size of the amplified products for bacterial clone library 1 was between 600-700 base pairs (size of amplified partial bacterial 16S rRNA gene plus the multiple cloning site). Amplified products with a band size between 600 and 700 base pairs were considered to be from a positive colony. However, amplified products with a band size of approximately 150 base pairs were considered a negative sample as the multiple cloning site of the plasmid would still be amplified in the absence of an insert to yield a product of 150 base pairs.

In Figure 3.9, samples 9, 12 & 20 (lanes 5, 6 & 10) had visible bands in the 600-700 base pair region and were therefore considered positive samples. Samples 4, 6, 8, 13, 14, 16 & 23 (lanes 2, 3, 4, 7, 8, 9 & 11) showed bands in the 150 base pair region and were therefore considered as negative samples. Thereafter, a total of 100 colonies from each of the two bacterial clone libraries were selected for verification of a partial bacterial 16S rRNA gene insert. Only positive clones were subsequently used for amplified ribosomal "DNA" restriction analysis (ARDRA).

3.6 Amplified ribosomal "DNA" restriction analysis

Amplified partial 16S rRNA gene inserts of positive clones for methanogenic and bacterial clone libraries were then digested with the two restriction enzymes Hin6I and BsuRI as suggested by Krakat *et al*. (2010[a]), resulting in unique band patterns for each positive clone. Each unique pattern indicates a different operational taxonomic unit (OTU) (the term used to indicate an individual strain) or phylotype. Colonies that were verified as positive in Figure 3.8 (methanogen clone library 1) were further analysed. Patterns of the digested product were detected by gel electrophoresis under UV light as shown in Figure 3.10.

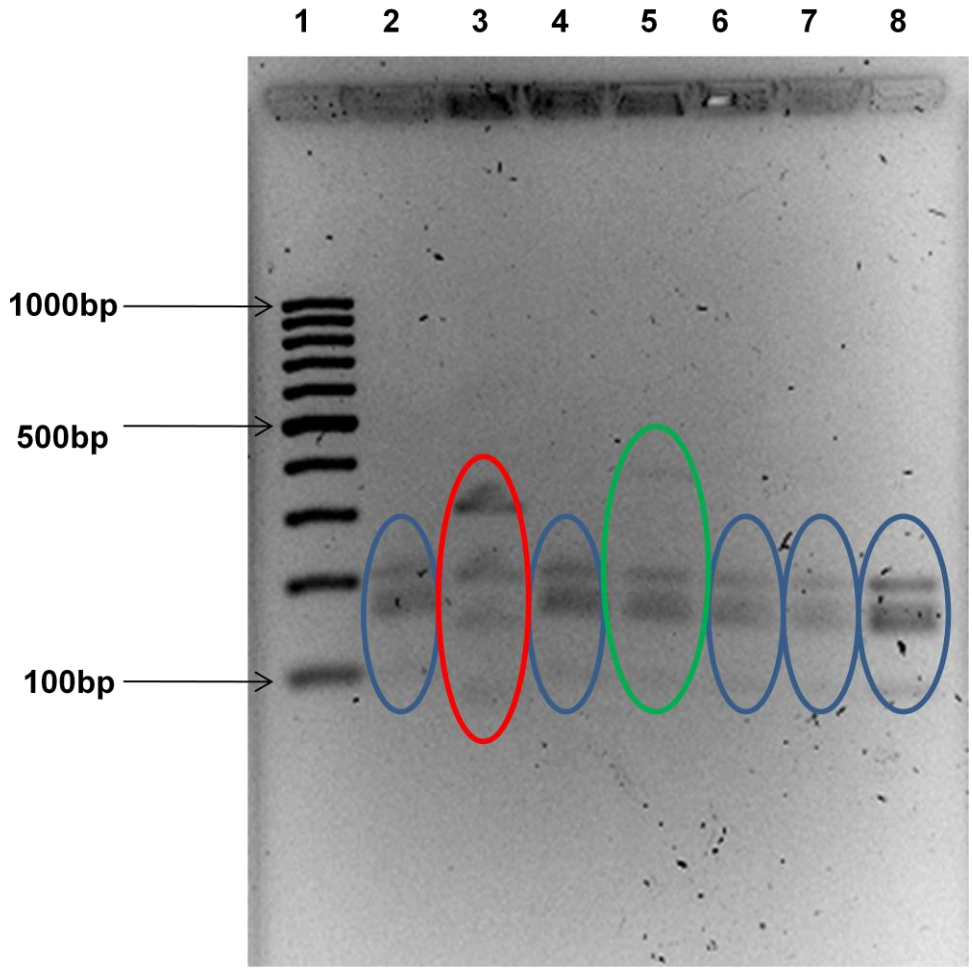

Figure 3.10: Representative gel electrophoresis showing restriction digest patterns of partial amplified 16S rRNA gene products from randomly selected positive clones from methanogen clone library 1 using restriction enzymes Hin6I and BsuRI. Lane 1 = O'GeneRuler™ 100bp DNA Ladder (100 base pairs – 1000 base pairs.) Lanes 3-8 = Samples 3, 18, 20, 23, 29, 33 & 50 respectively.

Samples 3, 20, 29, 33 & 50 (lanes 2, 4, 6, 7 & 8) show identical band patterns (ringed in blue, bands at 100, 170, 225 base pairs) and can therefore be grouped together as a single phylotype, while samples 18 & 23 (lanes 3 & 5, with bands at <100, 150, 200, 320 base pairs for sample 18 and bands at 100,

170, 225, 380 for sample 23) show different band patterns and can each be considered as a different phylotype (ringed in red and green respectively).

Table 3.3: Summary of number of positive clones assigned to a specific phylotype for each clone library

Clone Library	M1	M2	M3	B1	B2
Positive clones / phylotype	76 / 16	53 / 13	49 / 9	52 / 16	53 / 12

M1, M2, M3 = methanogen clone library 1, 2 and 3 respectively. B1, 2 = bacterial clone library 1 and 2 respectively.

Table 3.3 summarises the number of positive clones that were assigned to a specific phylotype for each of the clone libaries constructed. For the three methanogen clone libraries, 76 positive clones from methanogen clone library 1 were assigned to 16 phylotypes, 53 positive clones from methanogen clone library 2 were assigned to 13 phylotypes and 49 positive clones from methanogen clone library 3 were assigned to 9 phylotyes. For the two bacterial clone libraries, 52 positive clones from bacterial clone library 1 were assigned to 16 phylotypes and 53 positive clones from bacterial clone library 2 were assigned to 12 phylotypes. A total of 178 positive clones from methanogen clone libraries 1, 2 & 3 were assigned to 25 phylotypes and 105 positive clones from bacterial clone libraries 1 & 2 were assigned to 24 phylotypes (Table 3.4). A representative of each phylotype was sequenced.

Table 3.4: Band Patterns for each identified methanogen and bacterial phylotype

Phylotype	Band Sizes (base pairs)	Phylotype	Band Sizes (base pairs)
MA	100, 170, 225	BA	100, 200
MB	375, 500	BB	100, 225, 320
MC	100, 225, 475	BC	100, 225
MD	50, 100, 400	BD	200, 250, 400
ME	<100, 150, 200, 320	BE	120, 250
MF	100, 170, 225, 380	BF	100, 150, 375
MG	<100, 160, 250	BG	250, 375
MH	150, 225, 280	BH	300, 350
MI	100, 150, 200	BI	275, 350
MJ	100, 160, 600	BJ	160, 200, 275
MK	<100, 100, 200, 500	BK	150, 200
ML	160, 200, 250	BL	150, 180, 225
MM	100, 160	BM	180, 200
MN	<100, 700	BN	120, 160
MO	250, 330	BO	140, 160
MP	<100, 100, 150, 250, 400	BP	200, 300
MQ	140, 160, 225	BQ	100, 150
MR	<100, 200, 550	BR	250
MS	100, 180, 400	BS	<100, 120, 250
MT	100, 170, 500	BT	<100, 160
MU	160, 225, 300	BU	140, 250
MV	<100, 160, 300	BV	<100 X2, 100, 250
MW	<100, 150, 250, 350	BW	<100, 100, 250
MX	100, 180	BX	100, 250
MY	<100, 250, 350		

Methanogen phylotypes = MA-MY. Bacterial phylotypes = BA-BX

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The sequencing results for each phylotype from the methanogen clone libraries were compared to sequences in GenBank by using the NCBI BLAST search algorithm, the results of which are summarised below.

Table 3.5: Clones assigned to a specific phylotype for methanogen clone library 1

Phylotype	No. Of clones	% of Clone Library	Nearest known and uncultured Archaea	% Sequence Similarty
MA	46	60.5	*Methanobrevibacter gottschalkii* strain PG	99
MB	1	1.3	*Methanobrevibacter gottschalkii* strain PG	99
MC	1	1.3	Uncultured archaeon clone GHLW-A59	98
MD	1	1.3	Uncultured archaeon clone arc93	93
ME	10	13.1	Uncultured Methanomicrobiales archaeon clone 17-1F	96
MF	5	6.6	*Methanobrevibacter gottschalkii* strain PG	99
MG	1	1.3	Uncultured archaeon clone GHLW-A59	98
MH	2	2.6	Unidentified methanogen ARC25	99
MI	1	1.3	Uncultured archaeon clone ma77	90
MJ	2	2.6	Uncultured archaeon clone sy-904231058-87-i	83
MK	1	1.3	Uncultured archaeon clone WN-FWA-8	82
ML	1	1.3	Uncultured archaeon clone A0-260405-109F-32	85
MM	1	1.3	*Methanobrevibacter gottschalkii* strain PG	99
MN	1	1.3	Uncultured archaeon clone sy-904231058-87-i	84
MO	1	1.3	Uncultured archaeon clone ER2_11	82
MP	1	1.3	Uncultured archaeon clone A0-260405-109F-32	84

GenBank accessed: 14/11/2013

In methanogen clone library 1, 76 positive clones were assigned to 16 phylotypes. Table 3.5 outlines the nearest known or unknown *Archaea* to which each phylotype had the greatest sequence similarity from BLAST searches. Four phylotypes (MA, MB, MF and MM) had a 99% sequence similarity to *Methanobrevibacter gottschalkii* strain PG. These four phylotypes represented the majority (69.7%) of positive clones (53 out of 76) in methanogen clone library 1. The remaining 12 phylotypes (23 positive clones) showed between 82 – 99% sequence similarity to uncultured archaeon clones, of which phylotype ME represented 43.5% (10 out of 23).

Table 3.6: Clones assigned to a specific phylotype for methanogen clone library 2

Phylotype	No. Of clones	% of Clone Library	Nearest known and uncultured Archaea	% Sequence Similarty
MA	34	64.1	*Methanobrevibacter gottschalkii* strain PG	99
ME	6	11.3	Uncultured Methanomicrobiales archaeon clone 17-1F	96
MH	1	1.9	Unidentified methanogen ARC25	99
MK	1	1.9	Uncultured archaeon clone WN-FWA-8	82
MQ	2	3.8	Uncultured archaeon clone A0-080607-344Fa-b3	89
MR	1	1.9	Uncultured archaeon clone ER2_11	83
MS	1	1.9	*Methanobrevibacter gottschalkii* strain PG	99
MT	1	1.9	Uncultured archaeon clone 081030-OL-KR13:7I:1	91
MU	1	1.9	Uncultured archaeon clone MC118_31D15	83
MV	2	3.8	Uncultured archaeon clone K09_0_17	93
MW	1	1.9	Uncultured archaeon clone sy-904231058-87-i	83
MX	1	1.9	*Methanobrevibacter gottschalkii* strain PG	99
MY	1	1.9	Uncultured archaeon clone ER2_11	82

GenBank accessed: 14/11/2013

In methanogen clone library 2, 53 positive clones were assigned to 13 phylotypes. Table 3.6 outlines the nearest known or unknown *Archaea* to which each phylotype had the greatest sequence similarity

from BLAST searches. Three phylotypes (MA, MS and MX) had a 99% sequence similarity to *Methanobrevibacter gottschalkii* strain PG. These three phylotypes represented the majority (67.9%) of positive clones (36 out of 53) in methanogen clone library 2. The remaining 10 phylotypes (17 positive clones) showed between 82 – 99% sequence similarity to uncultured archaeon clones, of which phylotype ME represented 35.3% (6 out of 17).

Table 3.7: Clones assigned to a specific phylotype for methanogen clone library 3

Phylotype	No. Of clones	% of Clone Library	Nearest known and uncultured Archaea	% Sequence Similarty
MA	35	71.4	*Methanobrevibacter gottschalkii* strain PG	99
MB	1	2	*Methanobrevibacter gottschalkii* strain PG	99
ME	7	14.3	Uncultured Methanomicrobiales archaeon clone 17-1	96
MF	1	2	*Methanobrevibacter gottschalkii* strain PG	99
MG	1	2	Uncultured archaeon clone GHLW-A59	98
MN	1	2	Uncultured archaeon clone sy-904231058-87-i	84
MV	1	2	Uncultured archaeon clone K09_0_17	93
MX	1	2	*Methanobrevibacter gottschalkii* strain PG	99
MY	1	2	Uncultured archaeon clone ER2_11	82

GenBank accessed: 14/11/2013

In methanogen clone library 3, 49 positive clones were assigned to 9 phylotypes. Table 3.7 outlines the nearest known or unknown *Archaea* to which each phylotype had the greatest sequence similarity from BLAST searches. Four phylotypes (MA, MB, MF and MX) had a 99% sequence similarity to *Methanobrevibacter gottschalkii* strain PG. These four phylotypes represented the majority (77.5%) of positive clones (38 out of 49) in methanogen clone library 3. The remaining 5 phylotypes (11 positive clones) showed between 82 – 98% sequence similarity to uncultured archaeon clones, of which phylotype ME represented 63.6% (7 out of 11).

To assess the distribution of each phylotype across the three methanogen clone libraries, the data from Tables 3.5 – 3.7 are graphically presented below.

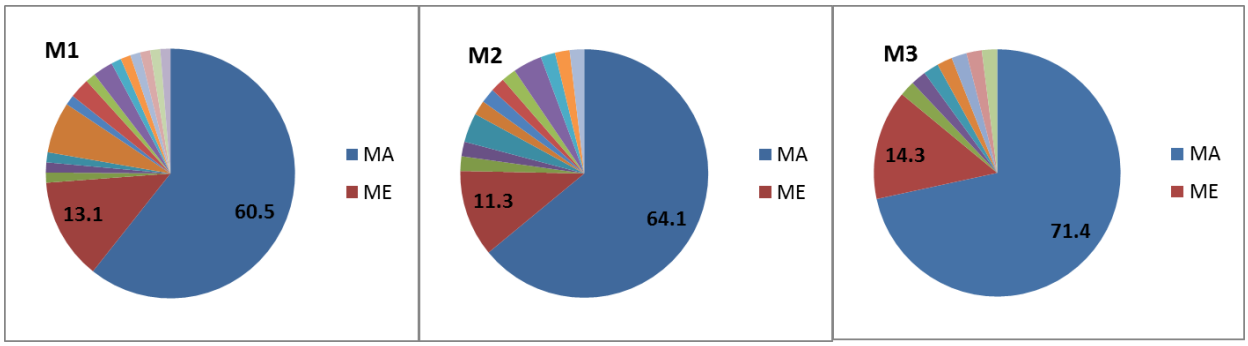

Figure 3.11: Distribution of phylotypes between methanogen clone libraries. M1-M3 = Methanogen clone libraries 1-3.

The clones assigned to phylotype MA formed the primary phylotype group across all three methanogen clone libraries (M1 = 60.5%, M2 = 64.1% and M3 = 71.4%). This phylotype exhibited

99% sequence similarity to *Methanobrevibacter gottschalkii* strain PG. Phylotype ME, which exhibited 96% sequence similarity to an uncultured *Methanomicrobiales* archaeon clone, also formed a distinct phylotype group across all methanogen clone libraries (M1 = 13.1%, M2 = 11.3%, M3 = 14.3%).

However, as mentioned above, certain phylotypes in each methanogen clone library exhibited an equal sequence similarity to the same known or unknown *Archaea*. (ie: in methanogen clone library 1, phylotypes MA, MB, MF and MM all had a 99% sequence similarity to *Methanobrevibacter gottschalkii* strain PG.) Because these phylotypes were identical, they were grouped together to give a corrected distribution of the different phylotypes for each clone library (Figure 3.12).

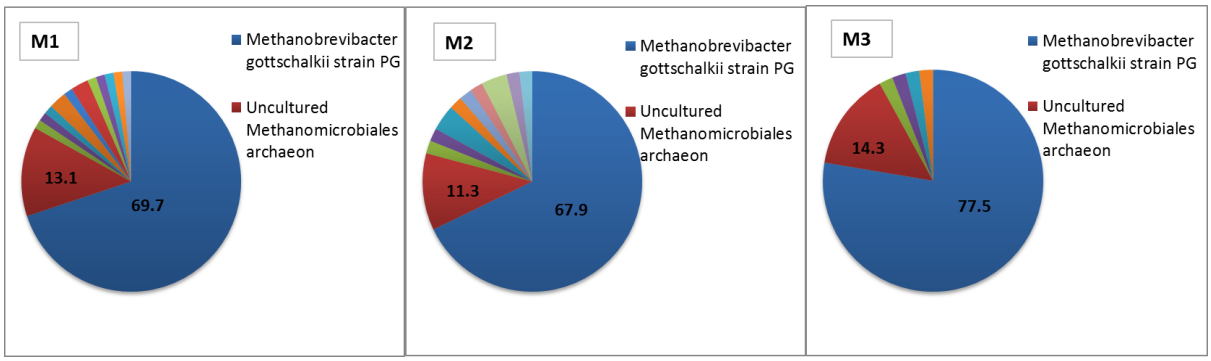

Figure 3.12: Corrected distribution of phylotypes between methanogen clone libraries. M1-M3 = Methanogen clone libraries 1-3.

The corrected distribution of phylotypes showed that the majority of the clones that were assessed in each clone library were affiliated with *Methanobrevibacter gottschalkii* (M1 = 69.7%, M2 = 67.9% and M3 = 77.5%). A large proportion of clones (M1 = 13.1%, M2 = 11.3%, M3 = 14.3%) were affiliated with an uncultured *Methanomicrobiales* archaeon clone. These results indicate that the three clone libraries are structurely comparable despite differing DNA extraction methods (methanogen clone library 1 & 3), differing clone numbers (M1 = 76, M2 = 53, M3 = 49) and the two different types of competent cells used (methanogen clone library 1 & 2).

The sequencing results for each phylotype from the bacterial clone libraries were compared to known sequences in GenBank by using the NCBI BLAST search algorithm, the results of which are summarised below.

Table 3.8: Clones assigned to a specific phylotype for bacterial clone library 1

Phylotype	No. Of clones	% of Clone Library	Nearest Valid Taxon	% Sequence Similarty
BA	3	5.8	*Bacillus* sp. FSL h8526	99
BB	1	1.9	*Bacillus odysseyi* strain 3P01SB	99
BC	2	3.8	*Treponema maltophilum* strain BR	94
BD	1	1.9	*Bacillus* sp. S7-3	99
BE	1	1.9	*Chryseobacterium daecheongense* strain CPW406	88
BF	1	1.9	*Blautia schinkii* strain B	93
BG	17	32.7	*Bacillus* sp. FSL h8526	99
BH	13	25	*Bacillus* sp. FSL h8526	99
BI	1	1.9	*Bacillus* sp. S7-3	99
BJ	4	7.7	*Holdemania filiformis* strain J1-31B-1	87
BK	2	3.8	*Desulfitibacter alkalitolerans* strain sk.kt5	91
BL	1	1.9	*Holdemania filiformis* strain J1-31B-1	87
BM	1	1.9	*Succiniclasticum ruminis* strain DSM 9236	92
BN	1	1.9	*Gluconacetobacter europaeus* strain DESII	84
BO	1	1.9	*Prevotella dentalis* strain ES2772	84
BP	2	3.8	*Shuttleworthia satelles* strain VPI D143k-13	93

GenBank accessed: 14/11/2013

In bacterial clone library 1, 52 positive clones were assigned to 16 phylotypes. Table 3.8 outlines the nearest known taxon to which each phylotype had the greatest sequence similarity from BLAST searches. Six phylotypes (BA, BB, BD, BG, BH and BI) had a 99% sequence similarity to *Bacillus* spp. These six phylotypes represented the majority (69.2%) of positive clones (36 out of 52) in bacterial clone library 1. The remaining 10 phylotypes (16 positive clones) showed between 84 – 94% sequence similarity to known taxa.

Table 3.9: Clones assigned to a specific phylotype for bacterial clone library 2

Phylotype	No. Of clones	% of Clone Library	Nearest Valid Taxon	% Sequence Similarty
BC	1	1.9	*Treponema maltophilum* strain BR	94
BG	11	20.7	*Bacillus* sp. FSL h8526	99
BH	12	22.6	*Bacillus* sp. FSL h8526	99
BJ	3	5.7	*Holdemania filiformis strain* J1-31B-1	87
BQ	1	1.9	*Gluconacetobacter europaeus* strain DESII	84
BR	1	1.9	*Clostridium orbiscindens* strain 265	96
BS	7	13.2	*Prevotella dentalis* strain ES2772	85
BT	1	1.9	*Gluconacetobacter europaeus* strain DESII	84
BU	4	7.5	*Holdemania filiformis* strain J1-31B-1	87
BV	2	3.8	*Prevotella dentalis strain* ES2772	85
BW	1	1.9	*Prevotella dentalis* strain ES2772	85
BX	9	17	*Prevotella dentalis* strain ES2772	85

GenBank accessed: 14/11/2013

In bacterial clone library 2, 53 positive clones were assigned to 12 phylotypes. Table 3.9 outlines the nearest known taxon to which each phylotype had the greatest sequence similarity from BLAST searches. Two phylotypes (BG and BH) had a 99% sequence similarity to *Bacillus* sp. FSL h8526.

These two phylotypes represented the majority (43.4%) of positive clones (23 out of 53) in bacterial clone library 2. The remaining 10 phylotypes (30 positive clones) showed between 84 – 96% sequence similarity to known taxa.

To assess the distribution of each phylotype across the two bacterial clone libraries, the data from Tables 3.8 and 3.9 are graphically presented below.

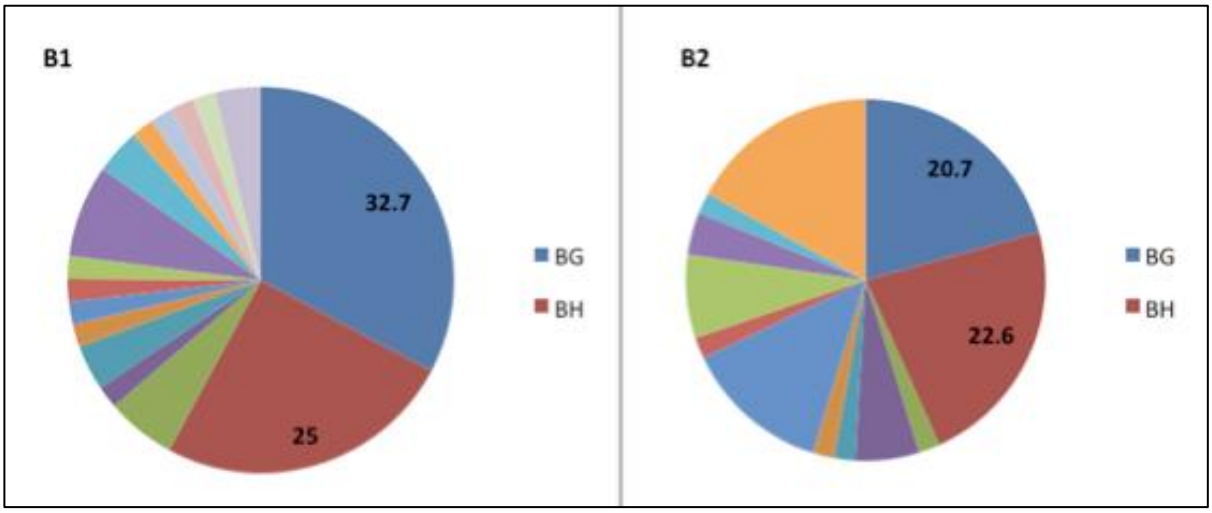

Figure 3.13: Distribution of phylotypes in bacterial clone libraries. B1-B2 = Bacterial clone libraries 1-2.

The clones assigned to phylotypes BG and BH formed the primary phylotype groups across both bacterial clone libraries (B1 = 32.7% and 25% respectively, B2 = 20.7% and 22.6% respectively). Both these phylotypes exhibited 99% sequence similarity to *Bacillus* sp. FSL h8526. These two phylotypes accounted for >40% of the total clones in both bacterial libraries.

As with the methanogen clone libraries, certain phylotypes showed identical sequence similarity to the same taxon and were therefore grouped together to give a corrected distribution of the different phylotypes for each clone library (Figure 3.14).

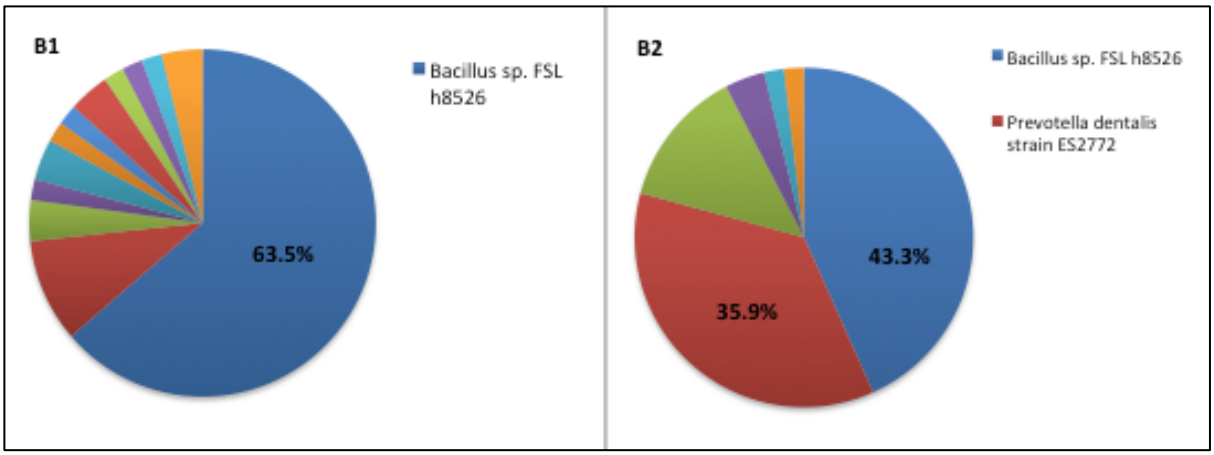

Figure 3.14: Corrected distribution of phylotypes in bacterial clone libraries. B1-B2 = Bacterial clone libraries 1-2.

The corrected distribution of phylotypes showed that the majority of the clones that were assessed in both bacterial clone library were affiliated with *Bacillus* sp. FSL h8526 (B1 = 63.5%, B2 = 43.3%). This result indicates that the two bacterial clone libraries are comparable. A great proportion of clones (35.9%) in bacterial clone library 2 were affiliated with *Prevotella dentalis* strain ES2772.

3.7 Phylogenetic analysis

To assess phylogenetic relationships for the clones representing methanogens and *Bacteria* in the differnent clone libraires, *MEGA* Version 5.2 (Tamura *et al*., 2011) was used to create phylogenetic trees for each clone library as discussed in 2.8.2.

For the three individual methanogen clone libraries (Figure 3.15 –Figure 3.17), phylotypes MA, MB, MF, MM, MS and MX (127 clones of 178 clones) grouped with *Methanobrevibacter gottschalkii*. These phylotypes represented 70.7% of all methanogen clones. Phylotype ME (23 clones of 178 clones) grouped with an uncultured *Methanomicrobiales* clone in each tree, while the remaining phylotypes (29 clones of 178 clones) grouped with the uncultured archaeon clones to which they had showed 82 – 99% sequence similarity in Table 3.5 – Table 3.7.

For the bacterial clone libraries (Figure 3.18 and Figure 3.19), phylotypes BA, BB, BD, BG, BH and BI (59 clones of 105 clones) grouped with *Bacillus* species. These phylotypes represented 56% of all bacterial clones. The other bacterial phylotypes grouped with the micro-organisms to which they showed 84 – 96% sequence similarity in Table 3.8 and table 3.9.

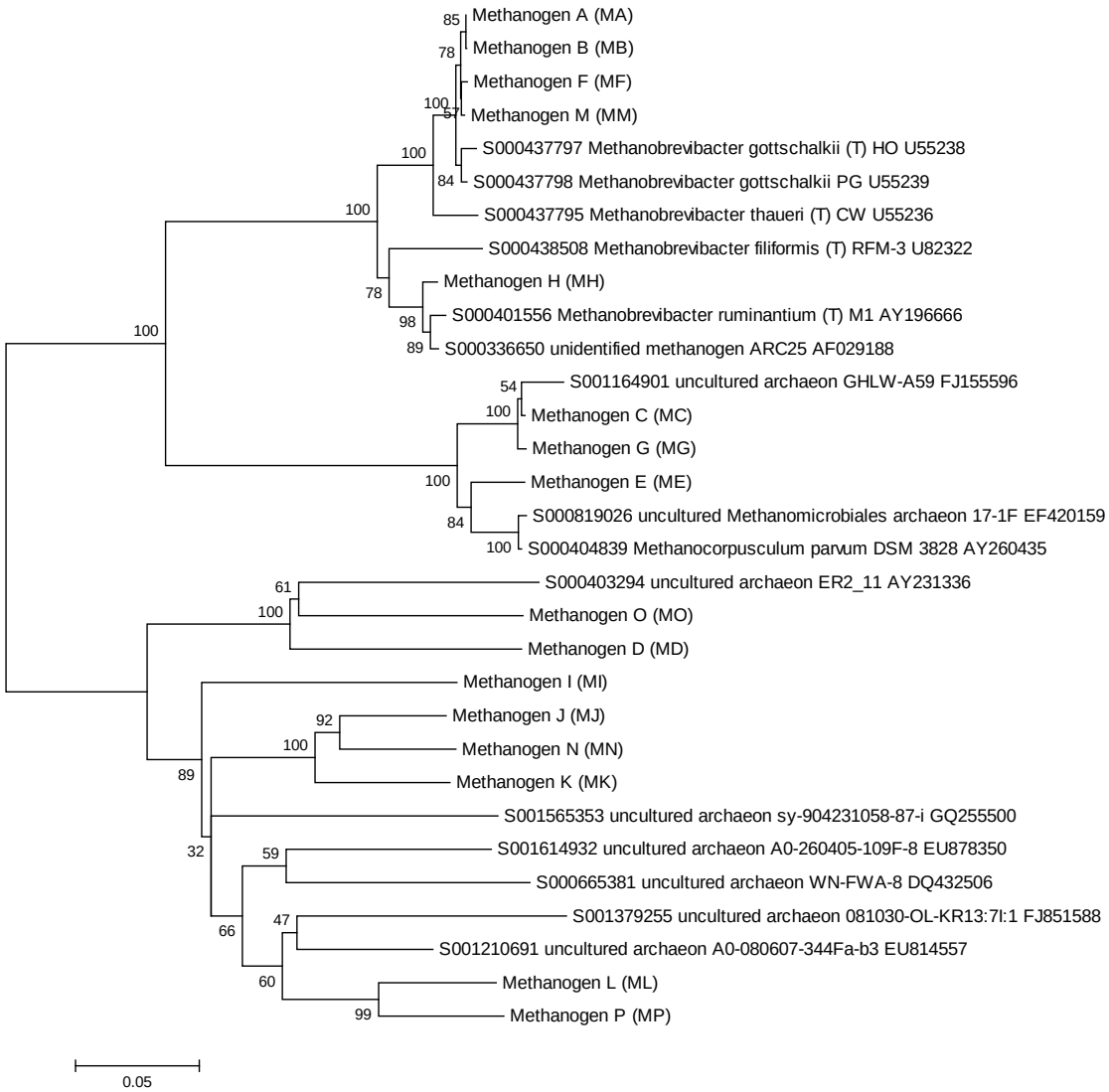

Figure 3.15: Phylogenetic relationships within the methanogenic *Archaea* for methanogen clone library 1. Methanogenic *Archaea* sequences for the unrooted neighbour-joining tree were obtained from GenBank. Methanogenic *Archaea* referenced Methanogen A – P were representative of all phylotypes that were identified in the zebra faecal sample for methanogen clone library 1. The tree was constructed according to specifications indicated in 2.8.2. The scale bar indicates 5 nucleotide changes per 100 nucleotides.

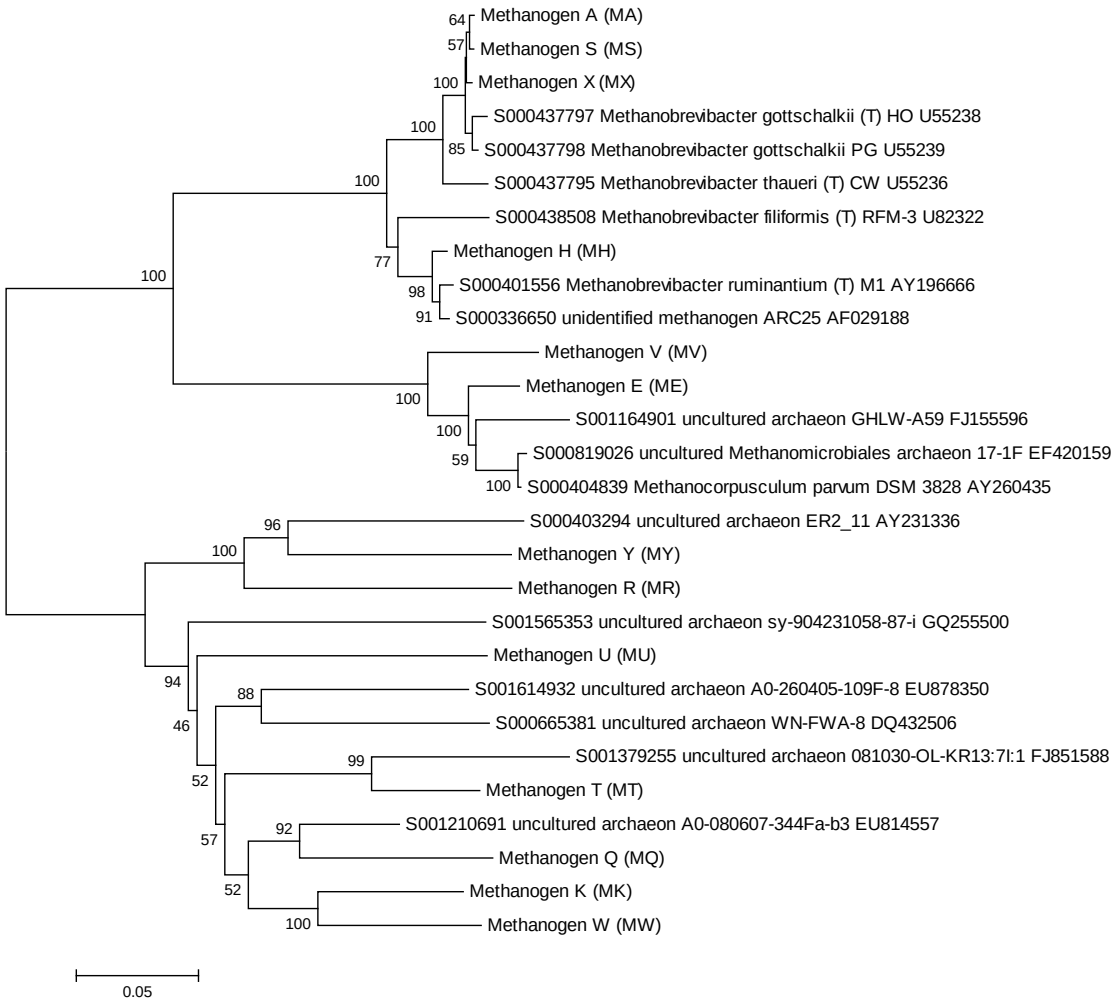

Figure 3.16: Phylogenetic relationships within the methanogenic *Archaea* for methanogen clone library 2. Methanogenic *Archaea* sequences for the unrooted neighbour-joining tree were obtained from GenBank. Methanogenic *Archaea* referenced Methanogen A – Y were representative of all phylotypes that were identified in the zebra faecal sample for methanogen clone library 2. The tree was constructed according to specifications indicated in 2.8.2. The scale bar indicates 5 nucleotide changes per 100 nucleotides.

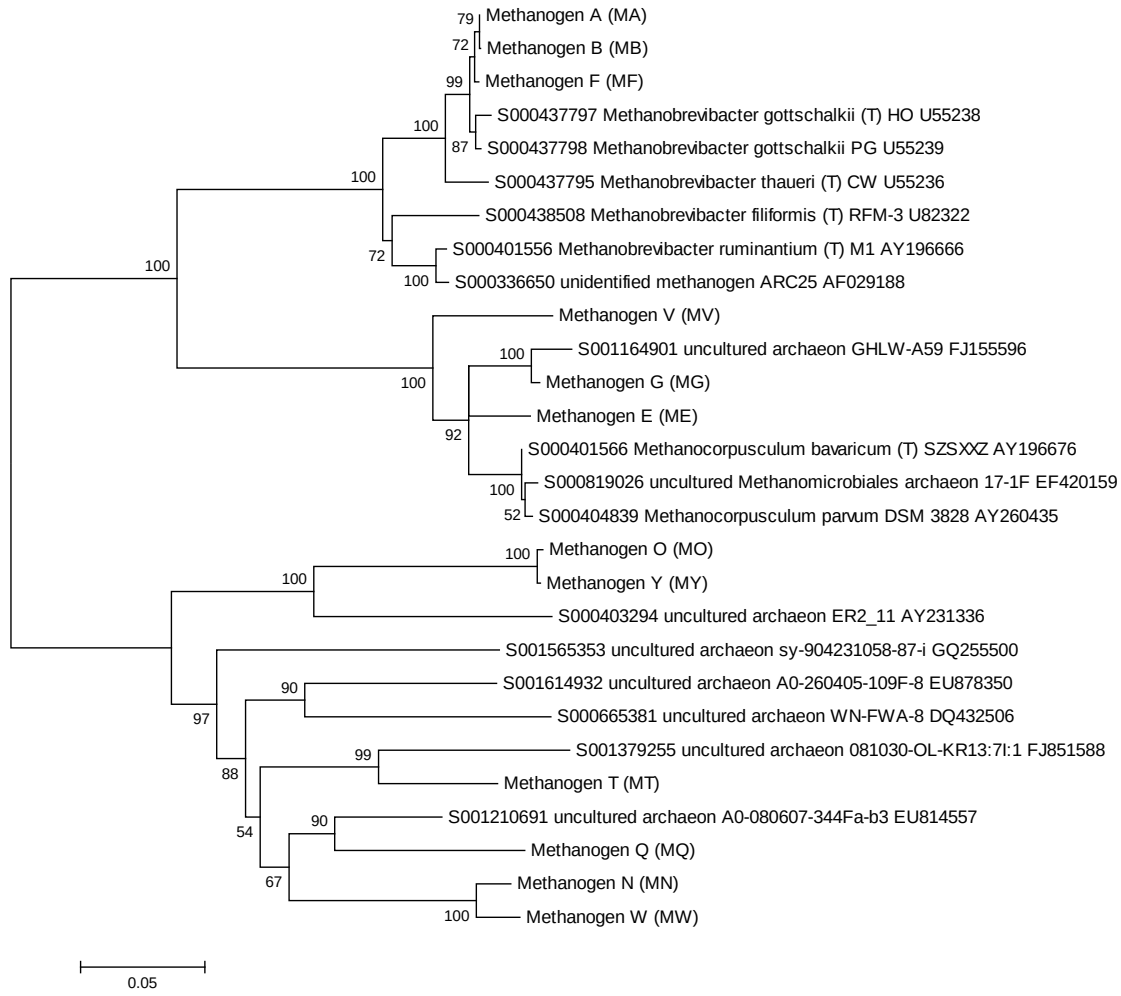

Figure 3.17: Phylogenetic relationships within the methanogenic *Archaea* for methanogen clone library 3. Methanogenic *Archaea* sequences for the unrooted neighbour-joining tree were obtained from GenBank. Methanogenic *Archaea* referenced Methanogen A – Y were representative of all phylotypes that were identified in the zebra faecal sample for methanogen clone library 3. The tree was constructed according to specifications indicated in 2.8.2. The scale bar indicates 5 nucleotide changes per 100 nucleotides.

The tree topologies for the three different methanogen clone libraries were similar using the neighbour-joining method. When the alternative maximum likelihood method was used to construct phylogenetic trees (Appendix), the tree topologies were also similar and each phylotype still grouped with the known or unknown *Archaea* to which they showed the highest sequence similarity. This indicates that the three clone libraries are a reliable reflection of the methanogen diversity in the sampled zebra faeces.

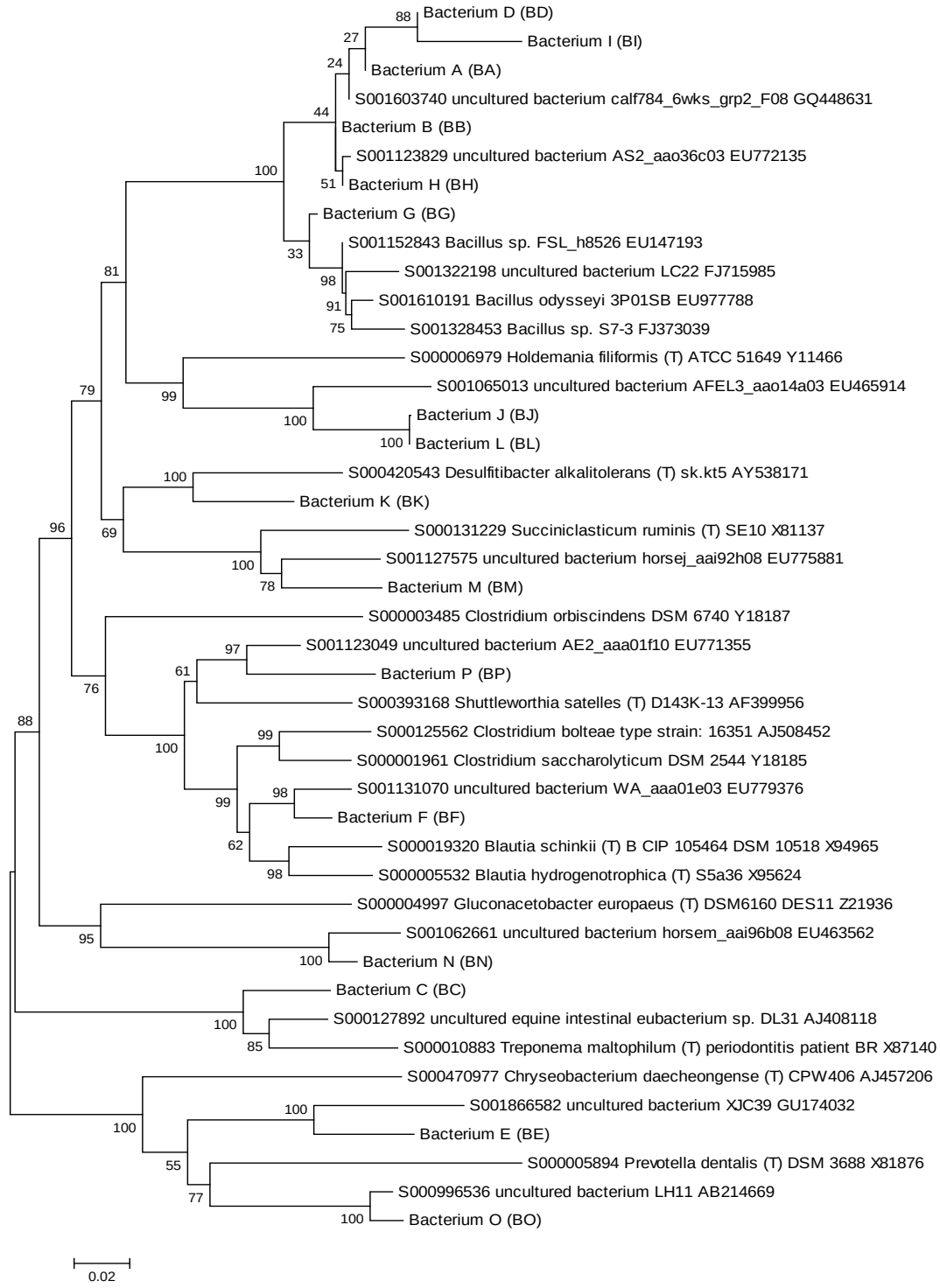

Figure 3.18: Phylogenetic relationships within *Bacteria* for bacterial clone library 1. *Bacteria* sequences for the unrooted neighbour-joining tree were obtained from GenBank. *Bacteria* referenced Bacterium A – P were representative of all phylotypes that were identified in the zebra faecal sample for bacterial clone library 1. The tree was constructed according to specifications indicated in 2.8.2. The scale bar indicates 2 nucleotide changes per 100 nucleotides.

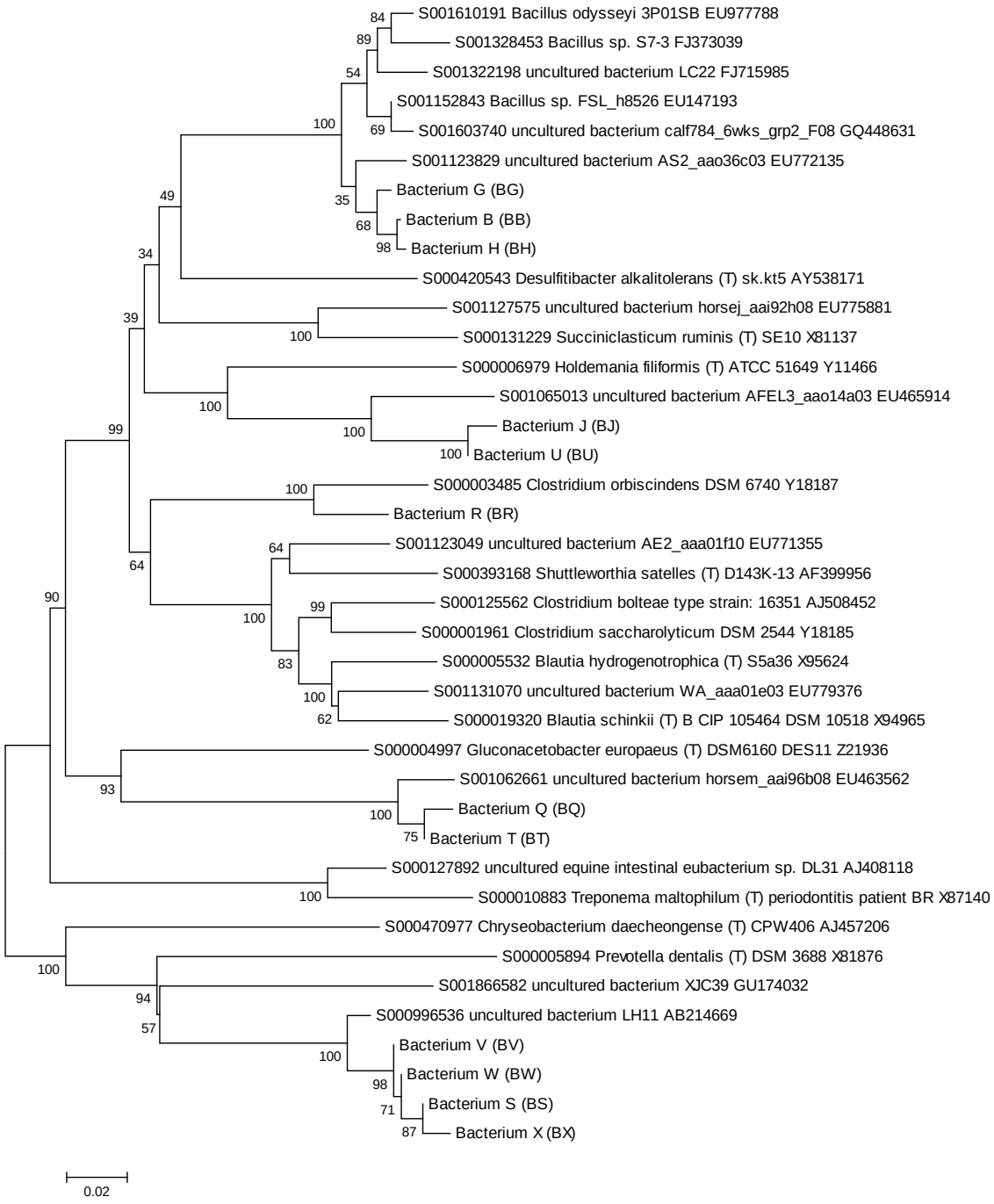

Figure 3.19: Phylogenetic relationships within *Bacteria* for bacterial clone library 2. *Bacteria* sequences for the unrooted neighbour-joining tree were obtained from GenBank. *Bacteria* referenced Bacterium C – X were representative of all phylotypes that were identified in the zebra faecal sample for bacterial clone library 2. The tree was constructed according to specifications indicated in 2.8.2. The scale bar indicates 2 nucleotide changes per 100 nucleotides.

The tree topologies for both of the bacterial clone libraries were similar using the neighbor-joining method. When the alternative maximum likelihood method was used to construct phylogenetic trees (Appendix), the tree topologies were also similar and each phylotype still grouped with the known

taxon to which they showed the highest sequence similarity. This indicates that the clone libraries are a reliable reflection of the bacterial diversity in the sampled zebra faeces.

3.8 Rarefaction analysis and indexes of species diversity

Rarefaction analysis for both the methanogen clone libraries and the bacterial clone libraries were performed to determine whether the number of clones selected for evaluation were a sufficient number to give a reliable representation of the diversity of microbial population of the faecal sample.

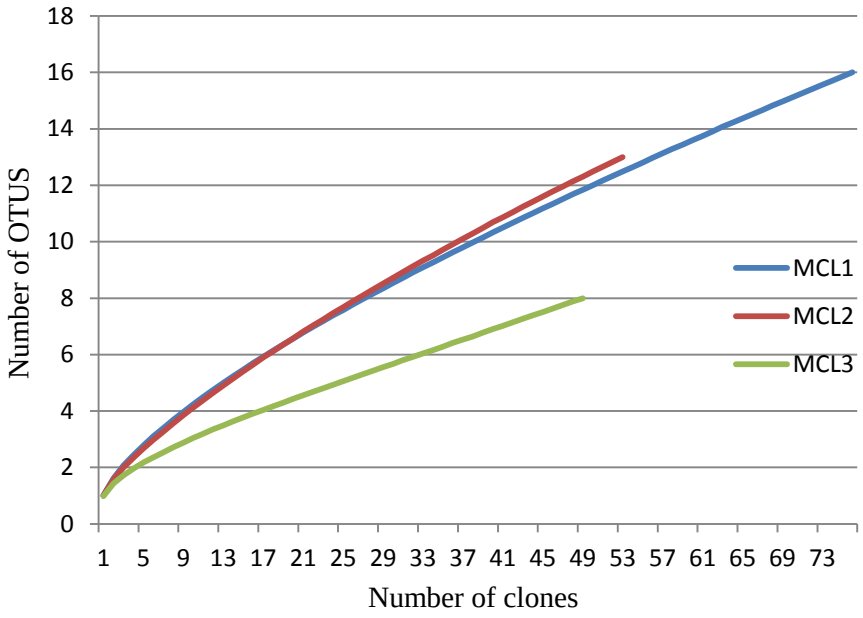

Figure 3.20: Rarefaction curves of the methanogen clone libraries (MCL1; MCL2; MCL3 = Methanogen clone libraries 1-3.)

The rarefaction analysis for the three methanogen clone libraries indicates that there was insufficient sampling. This was confirmed by comparison of Chao-1 calculations (Table 3.10) and from the number of phylotypes assigned to each clone library (MCL1 = 16 phylotypes; MCL2 = 13 phylotypes and MCL3 = 9 phylotypes). The diversity of methanogen clone library 1 was the highest of the three clone libraries as estimated by Shannon and Simpson's reciprocal indexes (Table 3.10).

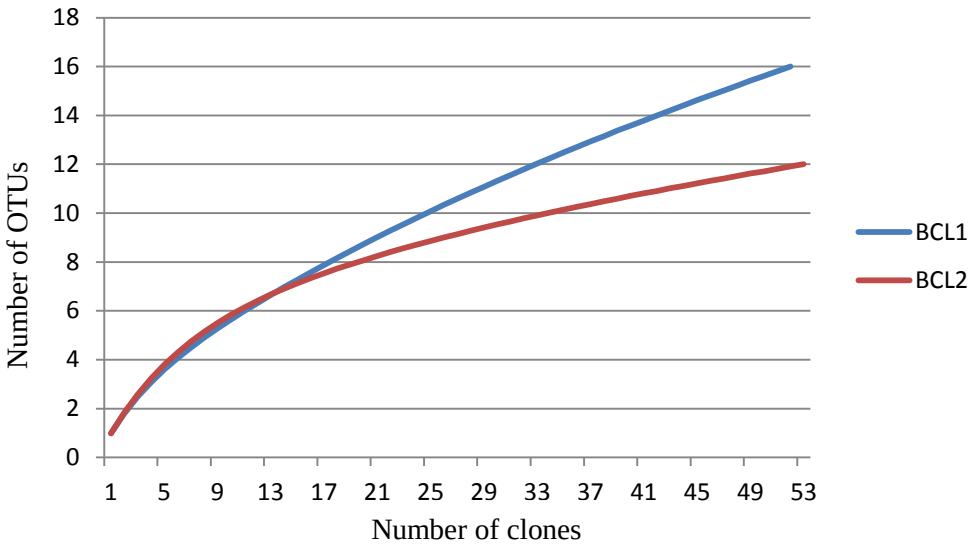

Figure 3.21: Rarefaction curves of bacterial clone libraries. (BCL1; BCL2 = Bacterial clone libraries 1-2.)

The rarefaction analysis for the two bacterial clone libraries indicates that an insufficient number of clones were sampled for bacterial clone library 1. This was confirmed by comparison of Chao-1 calculations (Table 3.10) and the number of phylotypes assigned to this clone library (BCL1 = 16 phylotypes). The rarefaction curve for bacterial clone library 2 indicates that an almost sufficient number of clones were sampled as a representation of the zebra faecal sample as the rarefaction curve approached saturation and this was confirmed by comparison of Chao-1 calculations (Table 3.10) and the number of phylotypes assigned to bacterial clone library (BCL2 = 12 phylotypes). The diversity of bacterial clone library 2 was the higher of the two clone libraries as estimated by Shannon and Simpson's reciprocal indexes (Table 3.10).

Table 3.10 Chao-1, Shannon and Simpson's Reciprocal calculations

Clone Library	MCL1	MCL2	MCL3	BCL1	BCL2
Chao-1	34	25	23	31	17
Shannon Index	1.56	1.45	0.99	2.10	2.09
Simpson's Reciprocal Index	2.60	2.38	1.83	5.66	7.36

CHAPTER 4
Discussion

Collection of zebra faecal samples

Many studies have investigated the microbial diversity of the gastrointestinal tract (GIT) of ruminant herbivores (Wright *et al*., 2004; Wright *et al*., 2007; Lwin *et al*., 2012; St-Pierre & Wright, 2012; de Oliveira *et al*., 2013). Investigation of the microbial diversity of the GIT of herbivorous monogastric animals have however in large been carried out in horses (Mackie & Wilkins, 1988; Daly *et al*., 2001; Yamano *et al*., 2008) with few studies investigating their wildlife counterparts (Nelson *et al*., 2003; Ley *et al*., 2008). Animal faecal matter, which contains the intestinal microbia, has been long used as a source to study the diverse microbial community of the gastrointestinal tract (Zhang *et al*., 2006; Tang *et al*., 2008). Although de Oliveira *et al*. (2013) recently showed that there was high variation in the microbial community between the faecal and rumen samples of a single Brazilian Nelore steer, faecal matter still remains a popular sample source used to study the microbial community of the gastrointestinal tract of animals (Ley *et al*., 2008; Tang *et al*., 2008; Yamano *et al*., 2008; Mao *et al*., 2011). The purpose of this study was to therefore investigate the microbial diversity of the GIT of zebras, a monogastric herbivore that is closely related to horses, using faecal matter as a sample source.

Zebra faecal samples collected from the Queen Elizabeth Park were analysed for freshness by on-site temperature readings. Temperature values ranged between 26˚C and 35˚C, with fresher samples exhibiting the higher temperature values (between 30˚C and 35˚C). This range of temperature values was expected as it is in close accordance with the normal body temperature associated with zebras which averages between 37˚C – 38˚C (Fuller *et al*., 2000). However, it is important to note that slight changes in faecal sample temperature could be attributed to environmental conditions. Elements such as air temperature, humidity and direct exposure to sunlight would contribute to both the temperature and moisture content of the sample.

Culture based quantitative analysis of selected micro-organisms

An initial particle-free dilution of the zebra faecal sample (substrate) was obtained through use of Nerbe Plus filter bags. The presence of a filter division within the filter bag permitted for the addition of solid substrate to saline solution on one side and the collection of effluent that was free of larger solid particles on the other side of the filter bag. This subsequently allowed for preparation of a dilution series as an inoculation of the various media.

Plate count agar (PCA) is a non-selective, general purpose agar that was used to evaluate the total bacterial count of the faecal sample. Enzymatic digests of casein (tryptone) provided amino acids and other complex nitrogenous compounds while yeast extract and glucose supplied B-complex vitamins and an energy source respectively, all of which are necessary to support bacterial growth. Both the

total aerobic and anaerobic bacterial populations were assessed. As mentioned previously, samples were also heat treated to assess the viable aerobic and anaerobic spore forming *Bacteria*. As expected, the analysis of the zebra faecal sample revealed that colony forming units for viable heterotrophic anaerobic *Bacteria* (2.45×10^9/g) were about 3-fold higher than those of viable heterotrophic aerobic *Bacteria* (7.51×10^8/g). The number of colony forming units for spore forming heterotrophic anaerobic *Bacteria* (1.57×10^8/g) was also greater than the number of colony forming units for spore forming heterotrophic aerobic *Bacteria* (1.74×10^6/g) in the zebra faecal sample. While spore forming aerobic heterotrophic *Bacteria* only contributed to <1% of the total viable aerobic *Bacteria* in the faecal sample, a greater proportion (6.4%) of the viable anaerobic heterotrophic *Bacteria* were spore forming.

In a study investigating the anaerobic bacterial community of eleven grass fed horses (Mackie & Wilkins, 1988) it was found that proteolytic *Bacteria* composed a high proportion of the total culturable *Bacteria* in the ruminal fluid taken from different locations in the gastrointestinal tract. The colony counts of total culturable anaerobic *Bacteria* in the cecum of horses (Mackie & Wilkins, 1988) was 2.5×10^9/gram of gut content which is comparable to 2.45×10^9/gram of faecal matter for anaerobic heterotrophic *Bacteria* that was calculated for zebra faeces in this study. In another study, Sorlini *et al*. (1988) showed that cattle had a considerably higher anaerobic bacterial count of 3×10^{11}/gram of dry weight faeces. In a more recent study, Al-Shadeedi *et al*. (2012) determined the total bacterial counts in the faeces of zoo animals. Carnivorous animals were found to have a higher bacterial count than ponies and ruminants.

MacConkey's agar was utilised for the quantification of Gram-negative *Bacteria* from the sampled zebra faeces, while the growth of Gram-positive *Bacteria* was expected to be inhibited due to the presence of both crystal violet and bile salts in the medium. By using this medium, which contains the pH indicator neutral red and the disaccharide lactose, it is also possible to differentiate between lactose fermenting and non-lactose fermenting Gram-negative *Bacteria* (Mossel *et al*., 1962). Gram-negative lactose fermenting *Bacteria* utilise the medium's lactose and in the process will produce an acidic end-product. This results in a colour change of the neutral red to pink and formed colonies will appear pink in colour. These colonies are typically coliform *Bacteria* which include the genera *Escherichia*, *Klebsiella*, *Enterobacter*, *Hafnia* and *Citrobacter* of the family *Enterobacteriaceae*. With Gram-negative non-lactose fermenting *Bacteria*, there is no fermentation of lactose and therefore the formed colonies will appear opaque in colour. These colonies are typically non-coliform *Bacteria* and may include species from the genera *Proteus*, *Salmonella* and *Shigella* of the family *Enterobacteriaceae* (Mossel *et al*., 1962; March & Ratnam, 1986).

Inoculation of MacConkey agar plates with diluted zebra faecal samples resulted in the cultivation of numerous pink coloured colonies and only a few opaque colonies. Microscopic analysis confirmed that both colony types were Gram-negative rods. Enumeration of Gram-negative lactose fermenting bacterial colonies (pink colonies) was possible with colony forming units established at 9.6 x

10^6/gram of faecal sample, while colony forming units of Gram-negative non-lactose fermenting *Bacteria* were <100 per gram in the zebra faecal sample. A study by Clauss *et al*. (2008) reported the colony forming units of *Enterobacteriaceae* formed on MacConkey agar inoculated with decimal dilutions of faecal sample from a captive black rhinoceros (*Diceros bicornis*) fed on a conventional diet of grass and lucerne hay. It was found that the number of colony forming units was 9.5 x 10^6/g of fresh rhinoceros faeces, which is comparable to the colony forming units per gram of zebra faecal sample found in this study. The principle type of colony formed on MacConkey agar in this study was pink in colour, which is indicative of coliform *Bacteria* including *E. coli*. Yukikiko *et al*. (1999) established that *E. coli* was the major coliform bacterium in foals with colonisation of the intestine occurring as early as three days.

Quantification of clostridia in the faecal sample was attempted by use of two selective media, namely Differential Clostridial agar (DCA) and Shahidi-Ferguson Perfringens (SFP) agar. Both media were inoculated with heat-treated dilutions of faecal sample. Clostridia produce spores that are able to withstand harsh environmental conditions including extremely high temperatures (Nakamura & Converse, 1967) of up to 120˚C. By heat-treating the samples at 70˚C for 10 minutes, most vegetative cells would have been destroyed. However, heat resistant spores would still be present and spore germination would proceed after tolerable conditions had been re-established. Because clostridia are obligate anaerobes, the inoculated agar plates were incubated under oxygen-free conditions in anaerobic jars.

Differential clostridial medium, developed by Gibbs and Freame in 1965, is supplemented with starch which promotes spore germination after heat treatment. It also contains peptones, beef extract and yeast extract that provide the nutrients and co-factors that are required for the growth of clostridia. Ferric ammonium citrate is included to detect sulphite reduction. Sulphite reducing *Clostridium* spp. produce sulphide from sulphite and will give a black precipitate when iron is present in the medium (Kawabata, 1980), therefore clostridia colonies will appear black in colour. Resazurin is used as a redox-indicator and the agar will turn from brownish-red to colourless when suitable anaerobic conditions are met. Differential clostridial medium promotes the growth of several *Clostridium* species including *C. perfringens*, *C. bifermentans* and *C. sporogenes*.

After 6 days of anaerobic incubation, growth was only observed on one of the plates that had been inoculated with samples from the lowest dilution factor (10^{-1} dilution). The few visible colonies (<10 distinct colonies) were black in colour, indicating that sulphite reduction did occur. Gram staining was performed on the colonies that were present. The cells of the Gram stained colonies were Gram-positive rods, typical of clostridia with this staining technique.

Shahidi-Ferguson Perfringens (SFP) agar was also used for the detection of clostridia present in the zebra faecal samples. This selective and differential medium was initially developed for both the quantification and identification of *Clostridium perfringens* in food samples (Shahidi & Ferguson, 1971). The agar contains sodium metabisulfite and ferric ammonium citrate which both display the

hydrogen peroxide producing ability of *Clostridium perfringens*, while the addition of egg yolk exhibits the generation of lecithinase (Shahidi & Ferguson, 1971). Typically, clostridia will produce black colonies on this agar.

After the recommended 24 hour anaerobic incubation period, no growth was viewed on the SFP agar plates. A further 24 hour anaerobic incubation period still did not yield growth in the form of black colonies. It was noted by Shahidi & Ferguson (1971) that the SFP agar promoted the black colony growth of seven *Clostridia* species namely *C. perfringens*, *C. bifermentans*, *C. botulinum*, *C. parabotulinum*, *C. sporogenes*, *C. novyi* and *C. haemolyticum*, all of which are capable of hydrogen peroxide and lecithinase production. As no growth was observed on SFP agar in this study, it may be assumed that the few colonies that formed on DCA did not belong to these above mentioned species, although it is important to note that the SFP agar plates were incubated for a much shorter time than the DCA plates.

Both the analysis based on selective media and the data from later molecular analysis, where only a single clone from the bacterial clone libraries showed a 96% sequence similarity to *Clostridium orbiscindens*, suggest that *Clostridium* species do not appear to be abundant in the microbial population in the zebra faecal sample and therefore do not play a significant role in digestion in the zebra gastrointestinal tract. This indicates possibly that anaerobic spore forming *Bacteria* (CFU = 1.57 x 10^8/g zebra faeces) other than *Clostridium* species are responsible for fermentation in the gastrointestinal tract of zebra. However, it is also possible that if *Clostridium* species were present in the zebra faeces that they were not sulphite reducing *Clostridium* species, and that the DNA extraction methods that were employed in this study were not effective in releasing DNA of *Clostridium* species from the faecal sample.

Daly *et al*. (2001) identified cluster XIVa of the *Clostridiaceae* (to which many cellulolytic clostridia species belong) as comprising 37% of sequenced clones generated from equine large intestine samples and therefore identifying this cluster as a central cellulolytic group in the gastrointestinal tract of horses. Although Yamano *et al*. (2008) recognised low percentage G+C Gram-positive (LGCGP) *Bacteria* as a main phylum in horse faeces, only two out of a total 104 sequenced clones could be assigned to a specific *Clostridium* species, with a majority of clones not corresponding to known sequences with similarity values of less than 90%. Other studies (Donaldson & Palmer, 1999; Bacciarini *et al*., 2003) have associated high *Clostridium* species counts, namely for *C. perfringens* and *C. difficile*, with intestinal disease in horses, where alteration in the intestinal flora structure in horses with colic might assist in *Clostridium* species proliferation (Donaldson & Palmer, 1999).

Rose Bengal Chloramphenicol (RBC) agar is a selective medium used for the quantification of fungi (yeasts and moulds) (Smith & Dawson, 1944). The inclusion of both rose bengal and chloramphenicol inhibits the growth of Gram-positive and Gram-negative *Bacteria*. Uptake of rose bengal dye by the growing yeast colonies means that these colonies will appear pink in colour, while

moulds will grow as filamentous colonies (Gamble & Orcutt, 1951). Distinct pink yeast colonies formed on RBC agar inoculated with the zebra faecal dilution series. Random colonies were also positively identified by microscopic evaluation where yeast cells appeared as large and spherical or oval in shape. No mould growth was visible on the plates.

The colony forming units for yeasts was calculated at 8.4×10^6/g of zebra faecal sample, which was considerably lower than that of total *Bacteria* (aerobic and anaerobic) of 3.2×10^9/g of zebra faecal sample. Lin *et al*. (1997) suggested that the eukaryotic population density in the gastrointestinal tract was typically lower than that of the prokaryote population. Al-Shadeedi *et al*. (2012) found that the prevalence of total *Bacteria* in zoo animals faeces (including bears, lions, ponies, camels and deer) were almost 3-fold higher than the total fungi count in all animals, with ponies and ruminant animals exhibiting higher fungi (yeasts and moulds) counts than the other animals. Lund (1974) showed that in bovine rumen, yeast counts increased to 1.3×10^5 per ml of rumen fluid depending on the type of feed the animal received. In a study investigating yeasts in caecal contents (van Uden *et al*., 1958) only about half of the 252 horses examined showed the presence of yeasts.

Anaerobic fungi, which include yeasts, are recognised as facilitators of plant degradation by either direct disruption of plant biomass or through interaction with cellulose degrading *Bacteria* present in the gastrointestinal tract (Bernalier *et al*., 1992; Lin *et al*., 1997). In a study by Procházka *et al*. (2012) there was a 4 – 22% biogas yield increase after the addition of rumen fungi isolated from cow and deer faeces and rumen fluid into an anaerobic reactor containing pig slurry and energy crops.

DNA extraction

The choice of DNA extraction and purification procedures are widely considered as one of the most important steps in determining the microbial diversity present in environmental samples when molecular techniques are applied. The method chosen for DNA extraction can influence both the quantity and quality (Thakuria *et al*., 2009) of the DNA extracted which may subsequently manipulate further downstream molecular techniques and consequently result in biased interpretations regarding microbial diversity of the sample (Thakuria *et al*., 2009).

A wide variety of methodologies have been described for the initial lysis of the cellular envelope to release nucleic acids from cells. This variety reflects the differences of cellular envelope composition among micro-organisms (van Huynh, 2008). An efficient extraction method, especially in the case of methanogenic DNA (Leitner *et al*., 2011) is required in order to extract adequate amounts of DNA. Two extraction methods, a commercially available kit and a method developed by Tang *et al*. (2008) for isolation of DNA from faeces, were assessed to determine whether the method utilised impacted on the quantity and quality of recovered DNA, and if so, establish which method was more suitable in yielding high quantity and quality DNA from the zebra faecal sample.

Firstly, the commercially available ZR Soil Microbe DNA Kit (Zymo Research) was utilised. The manufacturers state that the kit may be used to simply and effectively isolate tough-to-lyse bacterial,

fungal or protozoal high quality DNA that is free of humic acid contaminants by use of a bead beating spin column. Mechanical disruption of the cellular envelope by bead beating is considered as an efficient and consistently superior method for the release of nucleic acids from cells (Yeates *et al*., 1998; Thakuria *et al*., 2009).

DNA extraction with the kit was considered successful as a bright band was observed in the well loaded with isolated DNA on an agarose gel viewed under UV light. Because the isolated DNA remained in the well after a 90 minute run at 80 V, it may be assumed that the extracted DNA was of a high molecular weight (above 1 Kb in size). Spectrophotometric analysis of the DNA recovered from the kit extraction was not performed as the isolated DNA may have contained kit extraction particles from the procedure that would interfere with the readings.

The second methodology used for nucleic acid recovery was developed by Tang *et al*. (2008) for the isolation of PCR-quality DNA from pig faeces. The procedure employs two pre-treatments, one of which includes the use of glass beads and polyformaldehyde, followed by a common extraction protocol. The procedure makes use of CTAB for denaturation of proteins (Tang *et al*., 2008), while the inclusion of PVP for the removal of possible co-precipitating inhibitors eliminates the utilisation of hazardous reagents such as phenol.

Both manual extraction pre-treatments were carried out in duplicate. No band was visible on an agarose gel loaded with isolated DNA, although spectrophotometric analysis confirmed the presence of DNA at a wavelength of 260nm. Both the A260/A230 and A260/A280 ratios were below 2 and 1.7 respectively, which are the ideal values expected for high purity DNA (Yeates *et al*., 1998). The calculated A260/A230 and A260/A230 ratios indicate that the quality of the extracted DNA was compromised and that both humic acid and protein contaminants were present in the isolated DNA. However, this was expected given that the faecal sample is an organic substrate and contains many inhibitors (Tang *et al*., 2008) and relatively low DNA yields are usually obtained (Yu & Morrison, 2004) from such samples. Leitner *et al*. (2011) found that although CTAB-based manual extraction gave a higher DNA yield when compared to a commercial kit for the extraction of DNA from biogas reactor and sewage treatment samples, OD values for the manual extraction were not in the range of high purity DNA which would impede downstream applications. For each pre-treatment in this study, the DNA with the apparent higher purity was subsequently used for 16S rRNA gene region amplification.

16S rRNA gene amplification

Methanogenic 16S rRNA amplification products were attained by use of primers specific to the 16S rRNA gene regions of methanogens by polymerase chain reaction (PCR). The primers have been used previously (Westphal *et al*., 2007; Krakat *et al*., 2011) for the amplification of 16S rRNA gene regions of methanogens. The 16S rRNA gene amplification products were of the expected size, approximately 860 base pairs. Because PCR product of high concentration was obtained without the

necessity of any additional purification steps prior to amplification, it would imply that the DNA recovered from the commercial kit extraction was of high quality with minimal inhibiting contaminants.

However, with regard to the manual extraction developed by Tang *et al*. (2008), initial attempts to amplify DNA with primers specific to the partial 16S rRNA gene of methanogens were unsuccessful. It was only after DNA was diluted (1:10), which resulted in the dilution of inhibiting contaminants (Yeates *et al*., 1998), that amplification was achieved for the DNA that was isolated with the bead-beating pre-treatment. Amplification of partial 16S rRNA gene from DNA extracted which employed the pre-treatment without the use of glass beads was unsuccessful, even after dilution of eluted DNA.

Amplification of partial methanogenic 16S rRNA genes was only successful from DNA that was extracted with the use of bead-beating (commercial kit extraction and manual extraction preceded by pre-treatment B) which allowed for the release of sufficient quantities of nucleic acids from methanogen cells. This implies that a vigorous treatment is fundamental for the release of cells from the faecal matrix, disruption of cellular envelopes and the release of nucleic acids.

Amplification of partial bacterial 16S rRNA genes was successfully obtained by PCR (using well established primers) of DNA extracted with use of the commercial kit. The amplification products were between 400 – 500 base pairs when analysed by gel electrophoresis under UV light.

Ligation, transformation and ARDRA

The pJET1.2/BLUNT plasmid vector that was used is a positive selection vector that carries a lethal restriction enzyme gene, which is disrupted by the ligation of the DNA insert into the cloning site. As a result, only those vectors containing a DNA insert (recombinant plasmids) and then subsequently introduced into an *E. coli* host cell should be able to form colonies on ampicillin agar.

It was, however, observed that many of the randomly picked transformed clones carried a vector that contained no insert, regardless of the competent cell used. *E. coli* strains may express increased levels of *lac* repressor, a DNA-binding protein which suppresses the expression of the lethal restriction enzyme gene. The host cell can therefore continue to grow without ligation of DNA insert into the cloning site, allowing for the growth of smaller satellite colonies. However, the growth of considerably smaller colonies was not noticeable.

Another possibility is nuclease contamination of the ligation mixture. Using compromised components, such as low quality water, results in contamination which impairs the lethal gene, again allowing for the selection of false positive clones (Rand, 1996). However, this is unlikely as a new ligation mixture was prepared for each clone library and in this case the proportion of false positive colonies would have been higher.

Other possible reasons, according to the manufacturer, could be due to vector end damage by thermophilic polymerases or nucleases. Since PCR products were purified, it is unlikely that there was inhibition of ligation due to the presence of thermophilic polymerases. It is however possible that

there was damage to the vector ends by nucleases. The manufacturer recommends that the ligation mixture be incubated at room temperature (22˚C) for 5 minutes and this incubation can be extended to 30 minutes for larger PCR product inserts. In this study this incubation was extended to 30 minutes. However, this incubation at room temperature may have been too long and thereby increased the risk of nucleases damaging the vector ends and therefore preventing the ligation of inserts into the vector.

Both the E. cloni® Chemically Competent Cells (Lucigen Corporation) and the competent cells of *E. coli* 8739 prepared using the calcium chloride method were successfully transformed. Interestingly, the two clone libraries constructed with these two differing types of competent cells (methanogen clone library 1 and methanogen clone library 2) lead to very similar results (see Table 3.8 and 3.9) thereby indicating that the two methods are comparable.

Restriction analysis of the amplified ribosomal "DNA" was performed to assign clones to phylotypes and thus simplifing sequencing as only a representative of each phylotype required sequencing. The sequencing of one sample per phylotype (Ramos *et al*., 2010) and the reliability of ARDRA through spiking experiments (Krakat *et al*., 2010[a]) has been recently verified. Different phylotypes (ie: having differing restriction digest patterns) in this study were later found to display identical sequence similarity to the same reference micro-organism, thus showing the sensitivity of the ARDRA technique.

Methanogen diversity in zebra faeces

Three separate methanogen clone libraries were generated in this study. A total of 178 randomly selected methanogen clones were examined. Of these, 25 phylotypes were identified based on restriction analysis and a representative clone of each was submitted for sequencing. *MEGA* (Molecular Evolutionary Genetics Analysis) Version 5.2 (Tamura *et al*., 2011) was utilised for the construction of evolutionary trees to compare methanogenic 16S rRNA gene sequences from the zebra faecal sample and known methanogen species from GenBank.

Six of the phylotypes (MA; MB; MF; MM; MS and MX) which represent the majority of clones present in the three methanogen clone libraries (126 clones out of a total 178 methanogen clones) exhibited a high degree of sequence similarity (99%) to *Methanobrevibacter gottschalkii* strain PG. This dominance of clones with high sequence similarity to *Methanobrevibacter gottschalkii* strain PG was evident across all methanogen clone libraries and ranged from 67.92-75.51% (methanogen clone library 1 = 69.74%, methanogen clone library 2 = 67.92%, methanogen clone library 3 = 75.51%).

Various studies that have examined the methanogen diversity in the gastrointestinal tract environment, consistently identified *Methanobrevibacter* spp. as the dominant methanogen in various animals including chicken (Saengkerdsub *et al*., 2007), cattle (Whitford *et al*., 2001), sheep in Western Australia (Wright *et al*., 2004) and in Venezuela (Wright *et al*., 2008), cattle in Canada (Wright *et al*., 2007), horses (Yamano *et al*., 2008) and alpaca (St-Pierre & Wright, 2012). In the studies investigating sheep, *Methanobrevibacter ruminantium* (85 out of 241 clones) (Wright *et al*., 2007)

and *Methanobrevibacter* strains SM9, M6 and NT7 (>90% of clones) (Wright *et al*., 2004) were the dominant methanogens. In alpaca (St-Pierre & Wright, 2012), clones showed a high sequence similarity to *Methanobrevibacter millerae*, while in chicken 10 out of the 11 phylotypes identified showed 99% sequence similarity to *Methanobrevibacter woesei* (Saengkerdsub *et al*., 2007). *Methanobrevibacter gottschalkii* strain HO and strain PG have been isolated from horse and pig faeces respectively (Lin & Miller, 1998).

This study is consistent with other studies in identifing *Methanobrevibacter*, a hydrogenotrophic methanogen, as a key methanogen in the gastrointestinal tract. Hydrogen-utilising methanogens are known to thrive in the gastrointestinal environment mainly due to their co-exsistance with hydrogen-producing micro-organisms (Janssen & Kirs, 2008).

To a lesser extent, colonies that accounted for a single phylotype (ME) and exhibited a 95% sequence similarity to the uncultured *Methanomicrobiales* archaeon clone 17-1F (Penner & Foght, 2010) can also be considered as an important component of the methanogen population found in the zebra gastrointestinal tract. Over the three clone libraries, 23 clones represented this phylotype at very similar percentages (methanogen clone library 1 = 13.16%, methanogen clone library 2 = 11.32%, methanogen clone library 3 = 14.29%). Clones that resembled (albeit at less than 97% sequence similarity) known methanogens of the order *Methanomicrobiales* have also been noted in a previous study where Wright *et al*. (2007) investigated the methanogen population composition in cattle, while Lin *et al*. (2007) described *Methanomicrobiales* to be predominant in the ovine rumen.

Studies have reported the methanogen community present in plant based biogas fermenter systems (Klocke *et al*., 2007; Krakat *et al*., 2010[c]). In the long term mesophilic fermentation of beet silage, Krakat *et al*. (2010[b]) demonstrated that 50 out of 60 phylotypes were assigned to the hydrogenotrophic *Methanobacteriales* and *Methanomicrobiales*, while Krakat *et al*. (2010[c]) reported that in the digestion of fodder and sugar beet silage, hydrolytic *Bacteria* were responsible for the conversion of biomass to H_2 and CO_2 and this allowed for the dominace of hydrogen-utilising methanogens (for the conversion of H_2 and CO_2 to methane) in the digester. This study confirms that in the zebra (herbivore) gastrointestinal tract, which is similar to plant based biogas fermenter systems, the hydrogenotrophic methanogens are the most abundant *Euryarchaeota*.

The remaining 18 identified phylotypes represent 29 clones from the three methanogen clone libraries, with between 1 and 4 clones being assigned to each phylotype. Over the three clone libraries these phylotypes represented an average of 16% of the entire methanogen population in the zebra faecal sample (methanogen clone library 1 = 17.11%, methanogen clone library 2 = 20.75%, methanogen clone library 3 = 10.20%) and displayed between 81% - 99% sequence similarity to uncultured archaeon clones that have been recovered from various environmental samples. In a study investigating the methanogen community in pig faeces, Mao *et al*. (2011) reported that 55.4% of the clones assessed were not closely related to known *Euryarchaeota* sequences (77% - 96% sequence similarity).

Bacterial diversity in zebra faeces

To assess the bacterial diversity in the zebra faecal sample, two clone libraries were set up during this study. A total of 105 randomly selected clones were examined and were grouped according to restriction digest patterns into 24 phylotypes. A representative clone of each phylotype was analysed by sequencing and phylogenetic analysis was performed using *MEGA* (Molecular Evolutionary Genetics Analysis) Version 5.2 (Tamura *et al*., 2011) was utilised for the construction of evolutionary trees to compare bacterial 16S rRNA gene sequences from the zebra faecal sample and known bacterial species from GenBank.

Two dominant phylotypes (BG and BH) represented 28 clones and 25 clones respectively of the total 105 microbial clones analysed. Both of these phylotypes (BG and BH), when compared to known microbes, showed a 99% sequence similarity to *Bacillus* species FSL h8526. Phylotype BG (bacterial clone library 1 = 62.69%, bacterial clone library 2 = 20.75%) also demonstrated a 99% sequence similarity to the uncultured bacterium clone LC22 which Knapp *et al*. (2009) previously found to be present in the gut microbiota population of a species of earthworm, *Lumbricus rubellus*, while phylotype BH (bacterial clone library 1 = 25%, bacterial clone library 2 = 23.08%) also demonstrated a 99% sequence similarity to an uncultured bacterium clone isolated from Argali sheep (Ley *et al*., 2008).

Two phylotypes, BA and BB, displayed a 99% sequence similarity to uncultured bacterium clones isolated from neonatal calves (Paustain & Palmer, 2008) and an Argali sheep (Ley *et al*., 2008) respectively, which when compared to known microbes, exhibited a 99% sequence similarity to *Bacillus* species FSL h8526 and *Bacillus odysseyi* strain 3PO1SB. Two other phylotypes, BD and BI, demonstrated a 99% sequence similarity to *Bacillus* species S7-3.

In all, six phylotypes (BA; BB; BD; BG; BH and BI) which represented a majority (59 clones out of a total 105 microbial clones) displayed a high degree of sequence similarity to *Bacillus* species (bacterial clone library 1 = >69%, bacterial clone library 2 = >43%). Of these phylotypes, BG and BH accounted for 89.83% of the 59 clones.

Bacillus species have been identified as important inhabitants of the gastrointestinal tract that are capable of anaerobic metabolism and have been previously isolated from faecal material of broiler chickens (Barbosa *et al*., 2005). Although *Bacillus* spp. are widely considered as aerobic, Fakhry *et al*. (2008) observed that *Bacillus* spp. isolated from the gastrointestinal tract were not only able to survive but also proliferate in this presumably anaerobic environment, proposing that a select few should be regarded as facultative anaerobes rather than strict aerobes (Fakhry *et al*., 2008), that are able to use either oxygen or a different electron acceptor depending on environmental conditions.

The presence of these spore-forming *Bacteria* in the gastrointestinal tract was initially thought to be due to ingestion of soil, water and food sources, and remaining in the spore form until excretion. However, it has been suggested that *Bacillus* spp. might establish an endosymbiotic relationship with the host, being able to proliferate within the gut (Fakhry *et al*., 2008). The cellulolytic activity of

Bacillus spp. under anaerobic conditions has been reported in studies carried out by Toerien (1967) and Beukes & Pletschke (2006) in anaerobic digesters and soil respectively. In a study carried out by Klocke *et al*. (2007) it was shown that Bacilli of the phylum *Firmicutes* represented 22% clones analysed from a mesophilic, beet silage fed reactor. Daly *et al*. (2001) showed that low %G+C Gram-positive *Bacteria* (*Firmicutes*) accounted for 72% (from 272 clones analysed) of the bacterial diversity in the equine large intestine.

In a study by Ley *et al*. (2008) investigating faecal microbial communities, numerous uncultured bacterial clones were isolated from both domesticated and wild mammals. Five phylotypes determined in this study (BM; BN; BQ; BR and BT) corresponded to three uncultured bacterial clones which Ley *et al*. (2008) isolated from the gastrointestinal tract of horses. Phylotype BM displayed only 95% sequence similarity to these bacterial clones, whilst BN, BQ, BR and BT demonstrated a high sequence similarity of between 98% and 99%. However, when compared, none of these sequences from the zebra faecal sample demonstrated a high degree of sequence similarity to known microbial sequences from GenBank, with the closest resemblance displayed at 84% sequence similarity to *Gluconacetobacter europaeus* for phylotypes BN, BQ and BT, while phylotypes BM and BR (which each represented a single clone) exhibited a 92% sequence similarity to *Succiniclasticum ruminis* and a 96% sequence similarity to *Clostridium orbiscindens* respectively. This was in contrast to the findings of Daly *et al*. (2001) where 37% of all clones analysed from the equine large intestine were affiliated with clostridial group cluster XIVa.

Succiniclasticum ruminis, an anaerobic, non-spore forming rumen bacterium, is known for its ability to convert succinate to propionate (van Gylswyk, 1995), while *Clostridium orbiscindens*, an anaerobic, quercetin-degrading bacterium has been isolated from human faeces (Winter *et al*., 1991).

Another phylotype, BF, demonstrated a 98% sequence similarity to an uncultured bacterium clone isolated by Ley *et al*. (2008) from a Somali wild ass, while phylotypes BJ, BL, BP and BU showed between 91% and 94% sequence similarity to isolates from the gastrointestinal tracts of either African or Asian elephants (Ley *et al*., 2008). Three of these phylotypes, BJ, BL and BU, were affiliated with *Holdemania filiformis* with 87% sequence similarity when compared to known sequences from GenBank. *Holdemania filiformis* has been previously isolated from human faeces (Willems *et al*., 1997).

Yamano *et al*. (2008) investigated the phylogenetic composition of hindgut *Bacteria* in horses. It was found that most of the sequences found in either Hokkaido native horses or light horses did not correspond, with <90% sequence similarity, to any known sequences found in GenBank (Yamano *et al*., 2008). Five phylotypes (BO; BS; BV; BW and BX) indicated a 99% sequence similarity to an uncultured bacterium clone established by Yamano *et al*. (2008) from light horses. These phylotypes resembled between 84% and 85% sequence similarity to sequences of *Prevotella dentalis*, which is known to colonise the rumen and is responsible for degradation of carbohydrate to mainly acetate and succinate (Willems & Collins, 1995).

A single phylotype, BC, displayed a 98% sequence similarity to an uncultured eubacterium that was isolated from the equine large intestine (Daly *et al*., 2001). When compared to known sequences, BC could be affiliated to *Treponema maltophilum* with a 94% sequence similarity. Phylotype BE, which is represented by a single clone, showed a 94% sequence resemblance to a bacterial clone that was found in Bactrian camels (Huo *et al*., 2009).

The final phylotype, BK, which represented two clones, was assigned with 91% sequence similarity to the sulphite-reducing bacterium *Desulfitibacter alkalitolerans* which was isolated from a heating plant (Nielsen *et al*., 2006).

Rarefaction analysis of methanogen and bacterial clone libraries

Rarefaction analysis for the three methanogen clone libraries and the two bacterial clone libraries were performed to determine whether the number of clones selected for evaluation were sufficient to give a reliable representation of the microbial population diversity of the zebra faecal sample.

The rarefaction analysis for the three methanogen clone libraries indicates that the number of clones that were sampled for each of the clone libraries were insufficient. This was also clear from Chao-1 calculations and the high number of phylotypes assigned from the number of clones sampled for each clone library (MCL1 = 16 phylotypes out of 76 clones; MCL2 = 13 phylotypes out of 53 clones and MCL3 = 9 phylotypes out of 49 clones). This indicates that the zebra faecal sample had a greater methanogen diversity than what was established in this study. Although the number of clones sampled in this study was adequate to determine the dominant methanogen as *Methanobrevibacter gottschalkii*, a much larger number of clones will need to be sampled to better reflect the methanogen diversity in the sample. However, using the ARDRA technique, it may not be possible to identify less abundant methanogens since the detection limit of this technique was reported to be about 10^5 cells per ml (Krakat *et al*., 2010[a]).

The rarefaction analysis for the two bacterial clone libraries indicates that an insufficient number of clones were sampled for bacterial clone library 1 and was confirmed by Chao-1 calculations. The rarefaction curve for bacterial clone library 2 indicates that an almost sufficient number of clones were sampled as a representation of the zebra faecal sample. This indicates that a high percentage of the bacterial diversity in the zebra faecal sample was identified which was confirmed by the Shannon index value of 2.09. Although *Bacillus* spp. were identified as the dominant *Bacteria* in the zebra sample, further clone libraries would need to be constructed in order to better evaluate the bacterial diversity.

Limitations

This study was important in establishing the methanogenic and bacterial population present in zebra faecal matter as a means to assess the microbial community inhabiting the gastrointestinal tract of zebra. Although this study has provided an initial insight into the diversity of these populations, in

particular an insight into the principal methanogens and *Bacteria* present, additional studies are required to further analyse the microbial diversity. Future studies should increase the number of clones sampled for analyses in order to determine lesser occurring microbes.

Since all the potential media to quantitatively assess the microbial population could not possibly be employed in this study, only selected media were chosen. Most methanogens are difficult to culture or cannot be cultured. Therefore, only a nucleic acid targeting approach was used to evaluate the methanogenic diversity in this study. Other techniques that are used to assess the presence of methanogens in sample material such as FISH were not available for this study but could be employed for future investigations.

Conclusions

In this study, both the methanogenic and bacterial diversity in zebra faeces were assessed. The presence of methanogens in the zebra faecal sample was determined by sequencing of amplified partial 16S rRNA genes. Phylotypes exhibiting 99% sequence similarity to *Methanobrevibacter gottschalkii* strain PG, of the hydrogenotrophic order *Methanobacteriales*, were established to be predominant in the zebra faecal sample, representing >70% of archaeal clones selected for analysis in this study. A single phylotype demonstrating a 95% sequence similarity to an uncultured *Methanomicrobiales* archaeon clone, represented 12.9% of the total archaeal clones sampled. Therefore, it was established in this study that hydrogen-utilising methanogens dominate in the zebra faeces. Twelve phylotypes, representing 9% of archaeal clones displayed only 80-89% sequence similarity to previously uncultured archaeon clones which suggests the presence of unique *Archaea* that have not been recovered from previous studies.

Selected non-archaeal microorganisms were quantitatively assessed. As expected, the analysis of zebra faecal sample revealed that counts for anaerobic colony forming units were higher than that of aerobic colony forming units for both total and spore forming *Bacteria*.

Sequencing of amplified partial 16S rRNA genes, indicated that six phylotypes exhibited 99% sequence similarity to *Bacillus* species. These phylotypes accounted for 56% of all bacterial clones. Of the remaining clones, 12 phylotypes, which represents 34% of the selected bacterial clones, displayed only 95-99% sequence similarity to uncultured clones from various studies investigating gastrointestinal tract microflora. However, when compared, these phylotypes exhibited only 80-89% sequence similarity to known micro-organisms. Whether these phylotypes represent micro-organisms that are yet to be characterised and represented in culture collections requires further investigation.

Although the predominant methanogens and *Bacteria* present in this sample matter have been identified, further investigation is required to ascertain the potential of not only zebra faeces, but also other wildlife faecal matter, as potential inocula and co-digestion substrates for methane generation.

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APPENDIX

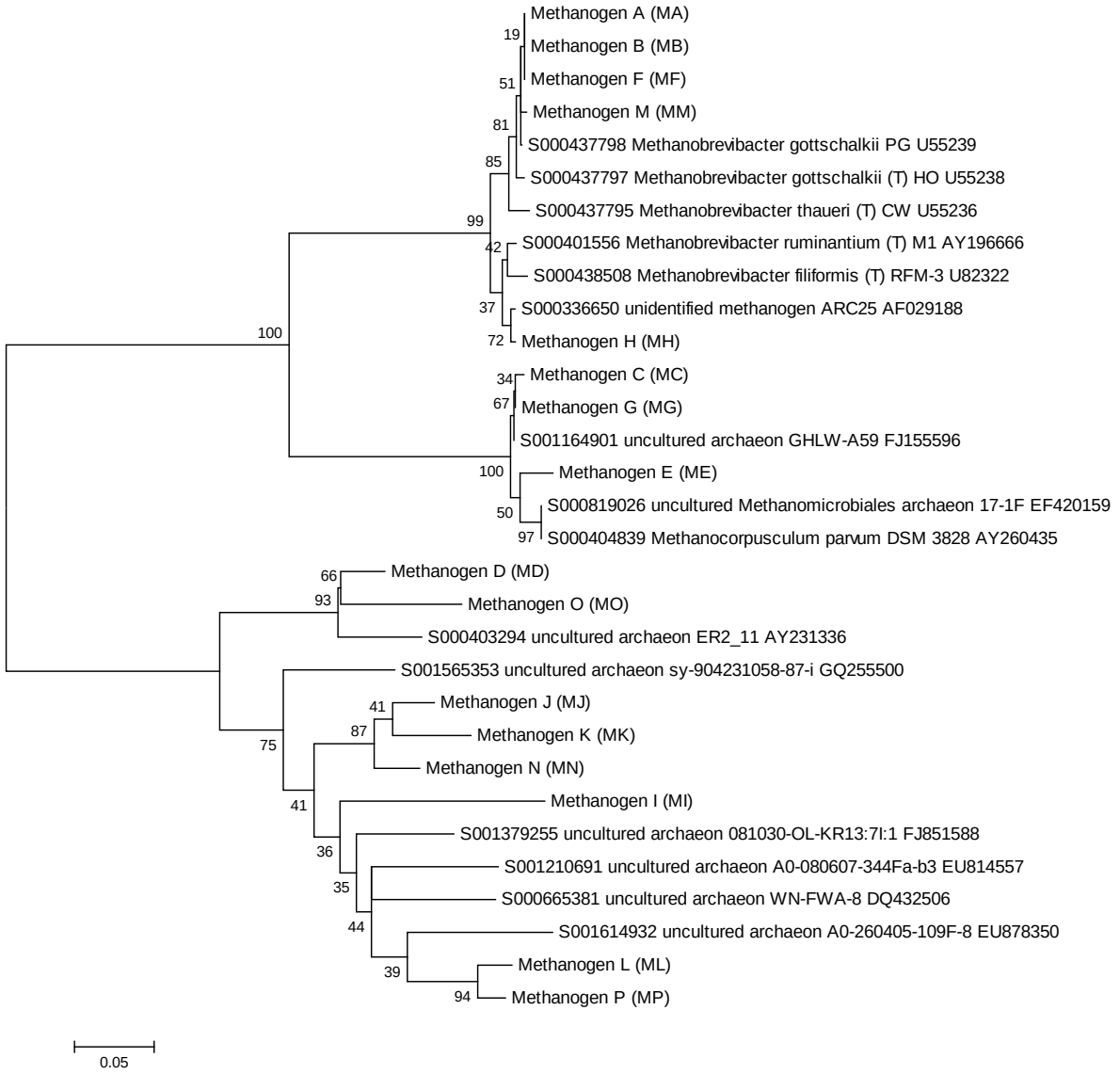

Figure A1: Phylogenetic relationships within the methanogenic *Archaea* for methanogen clone library 1. Methanogenic *Archaea* sequences for the unrooted maximum likelihood tree were obtained from GenBank. Methanogenic *Archaea* referenced Methanogen A – P were representative of all phylotypes that were identified in the zebra faecal sample for methanogen clone library 1. The tree was constructed according to specifications indicated in 2.8.2. The scale bar indicates 5 nucleotide changes per 100 nucleotides.

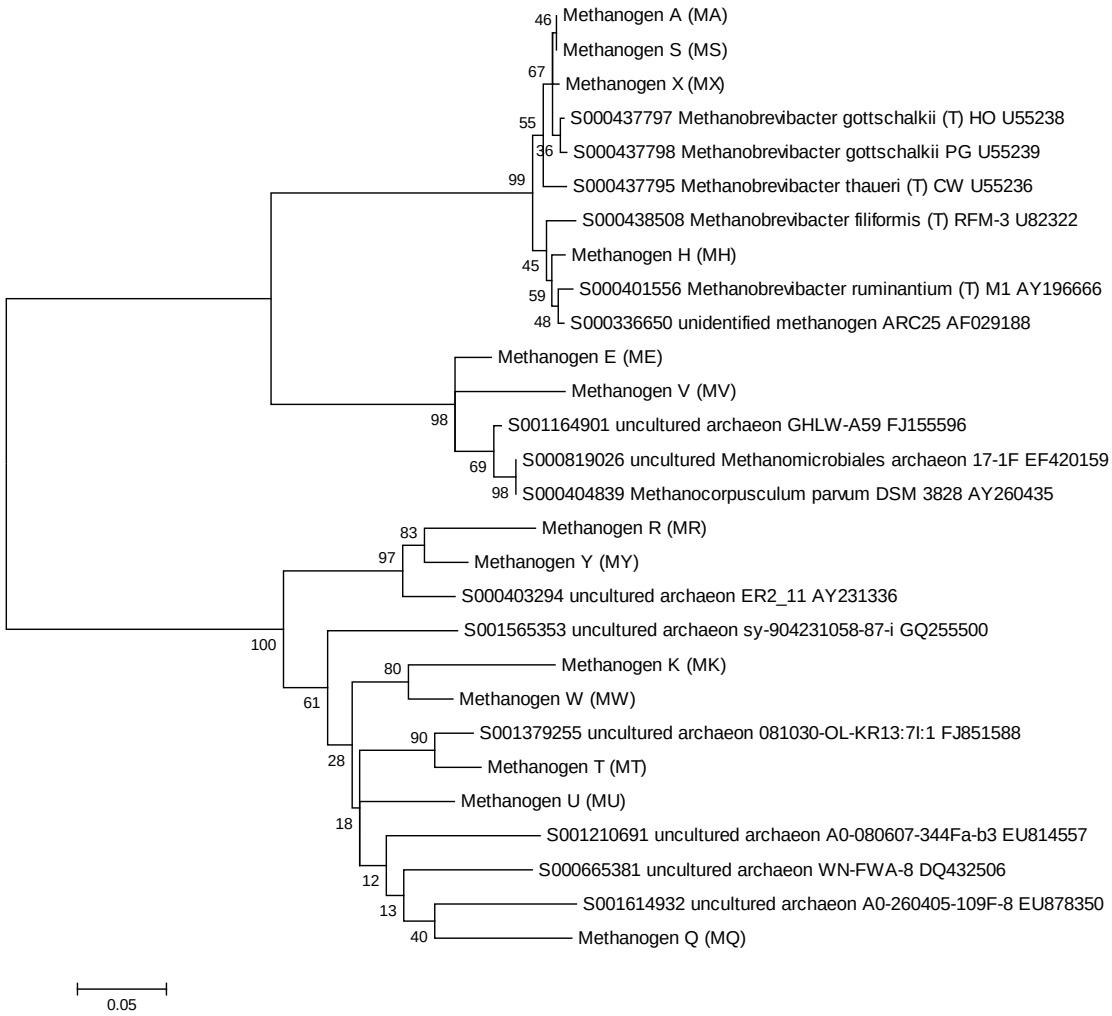

Figure A2: Phylogenetic relationships within the methanogenic *Archaea* for methanogen clone library 2. Methanogenic *Archaea* sequences for the unrooted maximum likelihood tree were obtained from GenBank. Methanogenic *Archaea* referenced Methanogen A – Y were representative of all phylotypes that were identified in the zebra faecal sample for methanogen clone library 2. The tree was constructed according to specifications indicated in 2.8.2. The scale bar indicates 5 nucleotide changes per 100 nucleotides.

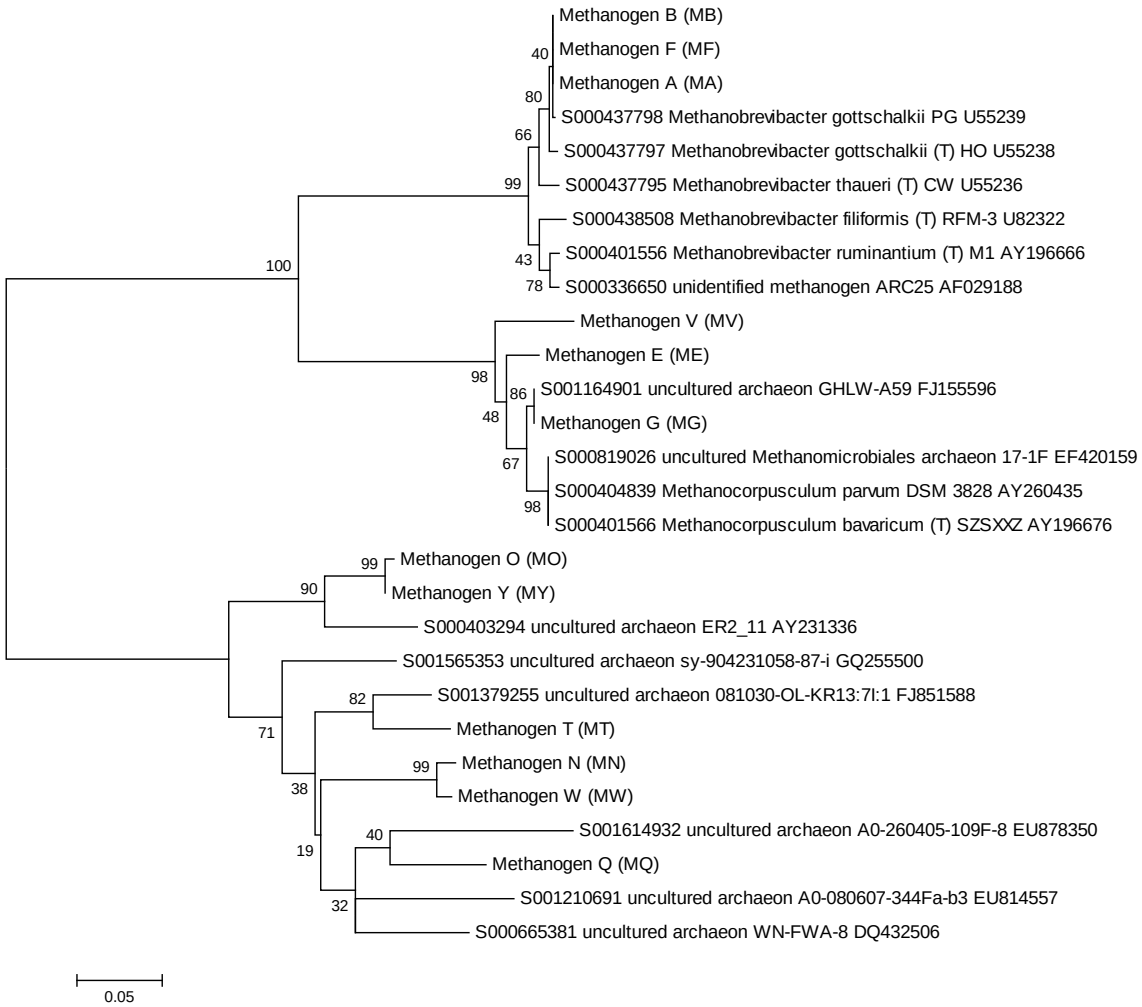

Figure A3: Phylogenetic relationships within the methanogenic *Archaea* for methanogen clone library 3. Methanogenic *Archaea* sequences for the unrooted maximum likelihood tree were obtained from GenBank. Methanogenic *Archaea* referenced Methanogen A – Y were representative of all phylotypes that were identified in the zebra faecal sample for methanogen clone library 3. The tree was constructed according to specifications indicated in 2.8.2. The scale bar indicates 5 nucleotide changes per 100 nucleotides

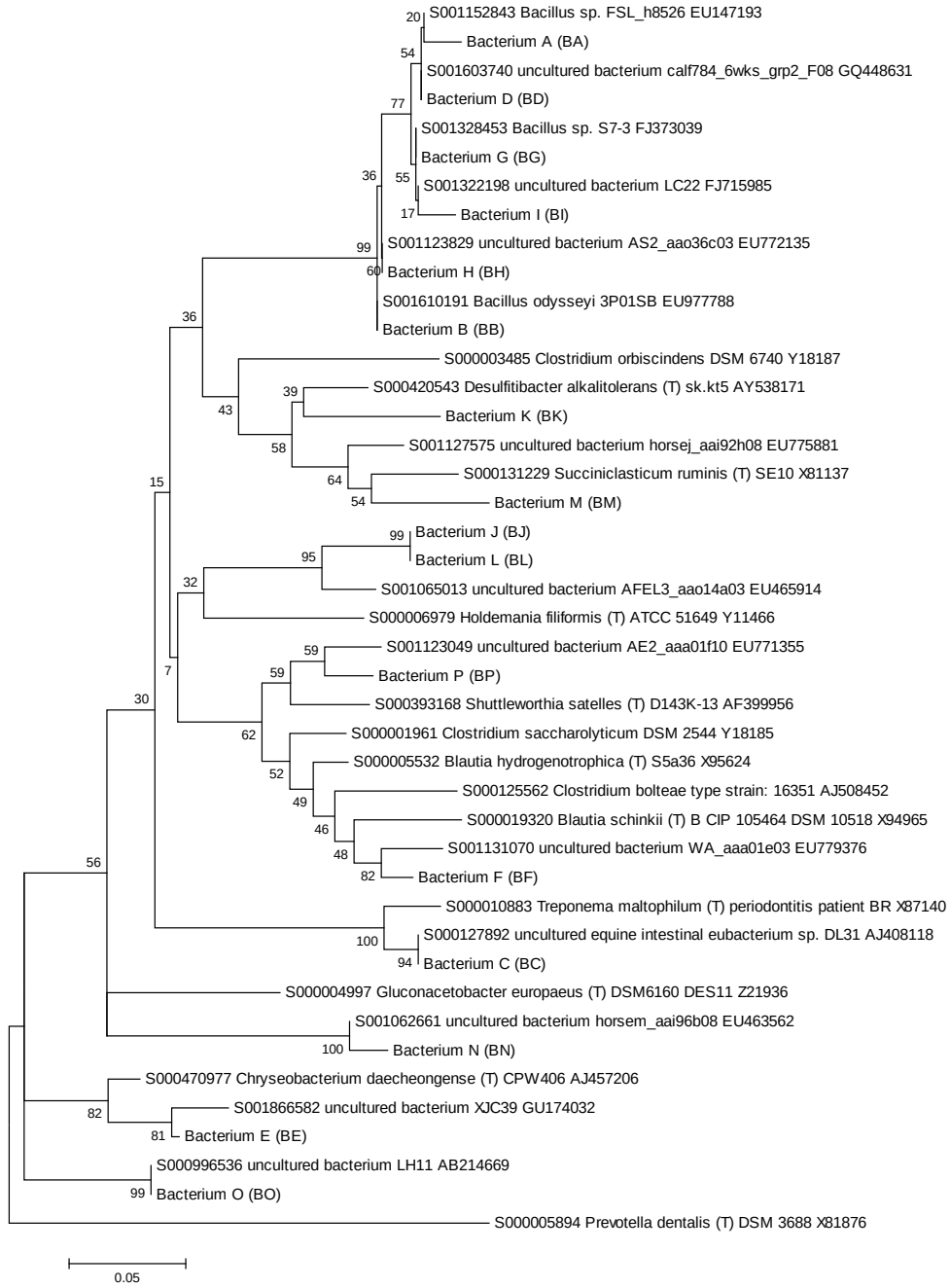

Figure A4: Phylogenetic relationships within *Bacteria* for bacterial clone library 1. *Bacteria* sequences for the unrooted maximum likelihood tree were obtained from GenBank. *Bacteria* referenced Bacterium A – P were representative of all phylotypes that were identified in the zebra faecal sample for bacterial clone library 1. The tree was constructed according to specifications indicated in 2.8.2. The scale bar indicates 5 nucleotide changes per 100 nucleotides.

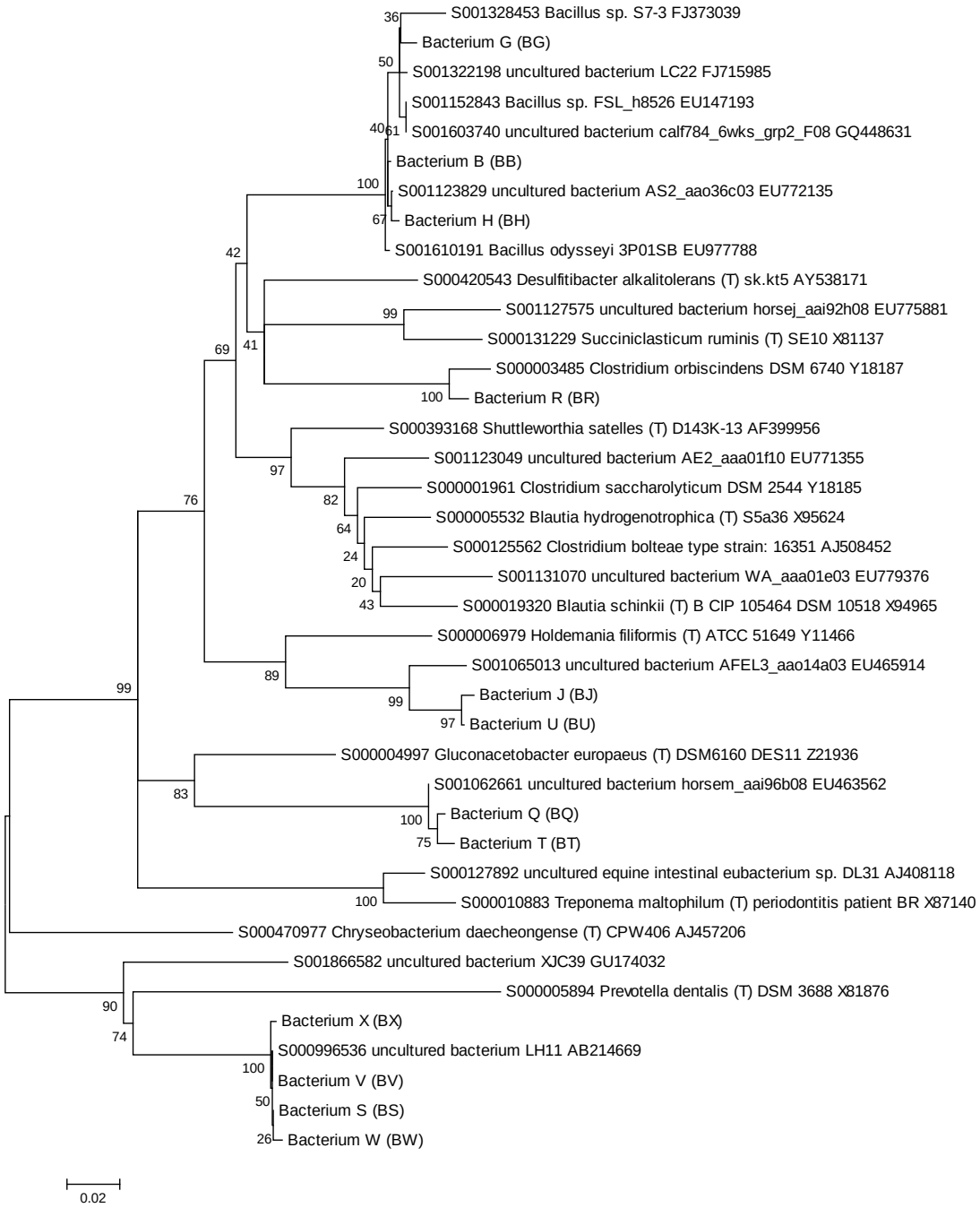

Figure A5: Phylogenetic relationships within *Bacteria* for bacterial clone library 2. *Bacteria* sequences for the unrooted maximum likelihood tree were obtained from GenBank. *Bacteria* referenced Bacterium C – X were representative of all phylotypes that were identified in the zebra faecal sample for bacterial clone library 2. The tree was constructed according to specifications indicated in 2.8.2. The scale bar indicates 2 nucleotide changes per 100 nucleotides.