A seasonal comparison of the gut microbiome of the Southern Lesser Galago, *Galago moholi* (A. Smith, 1836)

by

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Declaration of Originality

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Declaration

I, Channen Long declare that the thesis/dissertation, which I hereby submit for the degree of *Magister Scientiae* at the University of Pretoria, is my own work and has not been previously submitted by me for a degree at this or any other tertiary institution.

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Channen Long

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Date

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Summary

Increased anthropogenic activities such as urbanisation and the bushmeat trade increase the degree of contact humans have with non-human primates. Zoonotic pathogens have increased the risk of disease emergence significantly. Non-human primates are major reservoirs of zoonotic diseases. The close relatedness of primates, including humans, increases chances of sharing harmful pathogens.

The gut microbiome has been shown to contribute substantially to the health of its host. The composition of gut microbes is strongly affected by the diet of the host. The diet of the Southern Lesser Galago (*Galago moholi*) shows vast changes throughout the seasons. With the seasonal changes affecting diet and the continuous growth of human activities within their natural habitats, there is a great risk for transmission of zoonotic pathogens to occur between *G. moholi* and humans. This study investigated the effects that changes in season had on the gut microbiome of the Southern Lesser Galago (*G. moholi*) in isolated populations.

In order to assess the gut microbiome, a next generation sequencing approach was taken. Ion Torrent technology was used to sequence the 16S rRNA gene regions in order to quantify the diversity and abundance of the bacterial taxa. The hypervariable regions of the 16S rRNA gene were assessed in terms of the abundance and diversity of bacteria present.

The four major phyla present were *Proteobacteria*, *Firmicutes*, *Bacteroidetes* and *Actinobacteria*. The results showed only slight significances in variation in the taxonomic bacteria between seasons. However, the dominance of bacterial species from phylum *Proteobacteria* in summer and phylum *Firmicutes* in winter were indicative of shifting diets. Of the bacteria present, several potentially pathogenic genera were identified. The hypervariable region V3 proved to be the most consistent after an assessment of the 16S sequencing data from each individual. Conversely, hypervariable regions V2 and V9 proved to be the least informative.

The results indicate the diversity and composition of the microbial community during seasonal changes. These findings form a basis for future studies to assess gut health in primate species

Chapter 1

Literature Review

1.1 Introduction

Primates comprise one of the most species-rich orders of mammals (Wilson & Reeder, 2005; IUCN, 2016). They are found in mainland Africa, Madagascar, Asia and the Neotropics (Estrada *et al*., 2017). Current estimates are that 60% of primates are threatened or critically endangered owing to human activities (Groves, 2001; Groves, 2005; Estrada, 2013; IUCN, 2016; Estrada *et al*., 2017). Primates are threatened by a range of anthropogenic stressors including loss of habitat caused by, for instance, agricultural advances, selective logging, and urbanisation, the bushmeat trade, traditional medicine, and anthropogenic climate change (Butynski, 2001; Rijksen, 2001; Wilkie, 2001; Gillespie *et al*., 2005; Schloss *et al*., 2012). In addition to these effects, such ecological alterations could also cause an increase in susceptibility to infection as well as increase the risk of disease transmission between human and primate (Woodford *et al*., 2002; Gillespie *et al*., 2005; Calvignac-Spencer *et al*., 2012; Estrada *et al*., 2017).

The primary routes of pathogen transmission between primates and humans are either the faecal-oral route, or by airborne transferal (Kalema & Cooper, 1996; Butynski & Kalina, 1998; Homsy, 1999; Wallis & Lee, 1999; Calvignac-Spencer *et al*., 2012). Close contact between humans and animals causes contamination of clothes allowing for transmission of pathogens (Woodford *et al*., 2002). For example, coprophagy, the eating of faeces, has been documented in a number of non-human primate species, such as the ring-tailed Lemurs (*Lemur catta*; Fish *et al*., 2007) of Madagascar and the brown capuchin monkeys (*Cebus pella*; Prates & Bicca-Marques, 2005), demonstrating a method of transfer (faecal-oral) between individuals.

"Pathogen pollution", termed by Daszak *et al*. (2000), describes the spread of pathogens owing to the introduction of exotic flora and fauna to new regions and is seen as an anthropogenic form of invasion ("biodiversity invasion"; Vitousek *et al*., 1997; Daszak *et al*., 2000). An invasion of exotic pathogens would have the potential to cause biodiversity loss: new and vulnerable host populations will rapidly decline, the pathogens could then potentially become enzootic, causing significant mortality to less resistant host populations

(Hudson & Greenman, 1998); this has been exhibited by the introduction of the grey squirrel (*Sciurus carolinensis*) infected with helminth parasites to an area inhabited by the native red squirrel (*Sciurus vulgaris*; Romeo *et al*., 2015). Small populations in fragmented environments will be more susceptible to contracting and spreading diseases. Pathogens that can be transferred to more than one species are at risk of mortally reducing endangered species populations, as has been documented with rabies being passed from domestic dogs to African wild dog populations (*Lycaon pictus*; Alexander *et al*., 1993; Gascoyne *et al*., 1993).

A study conducted by Cooper *et al*. (2012) has suggested that humans share more host parasites with Old World monkeys and Strepsirrhines than with the great apes (chimpanzees and gorillas). This is interesting as humans are taxonomically and genetically more similar to the great apes (as seen in Fig. 1), where one would expect similarities between more similar clades (Davies & Pederson, 2008). However, as suggested by the authors, the overlap of the geographical range may lead to a pathway for distributing parasites between any primate species. Studies have shown a relationship between urban extensions, habitat fragmentation, decreases in biodiversity, and the transmission of zoonotic diseases (LoGiudice, 2003; Daszak *et al*., 2007) possibly supporting the close relationship between these primates and humans.

It is apparent that the emergence of diseases within the human population is increasing. One of the reasons is owing to the zoonotic abilities of these pathogens between animals and humans. It is therefore imperative to understand the dynamics of these zoonotic pathogens in order to prevent the spread of disease, and the potential for fatalities within animal and human populations.

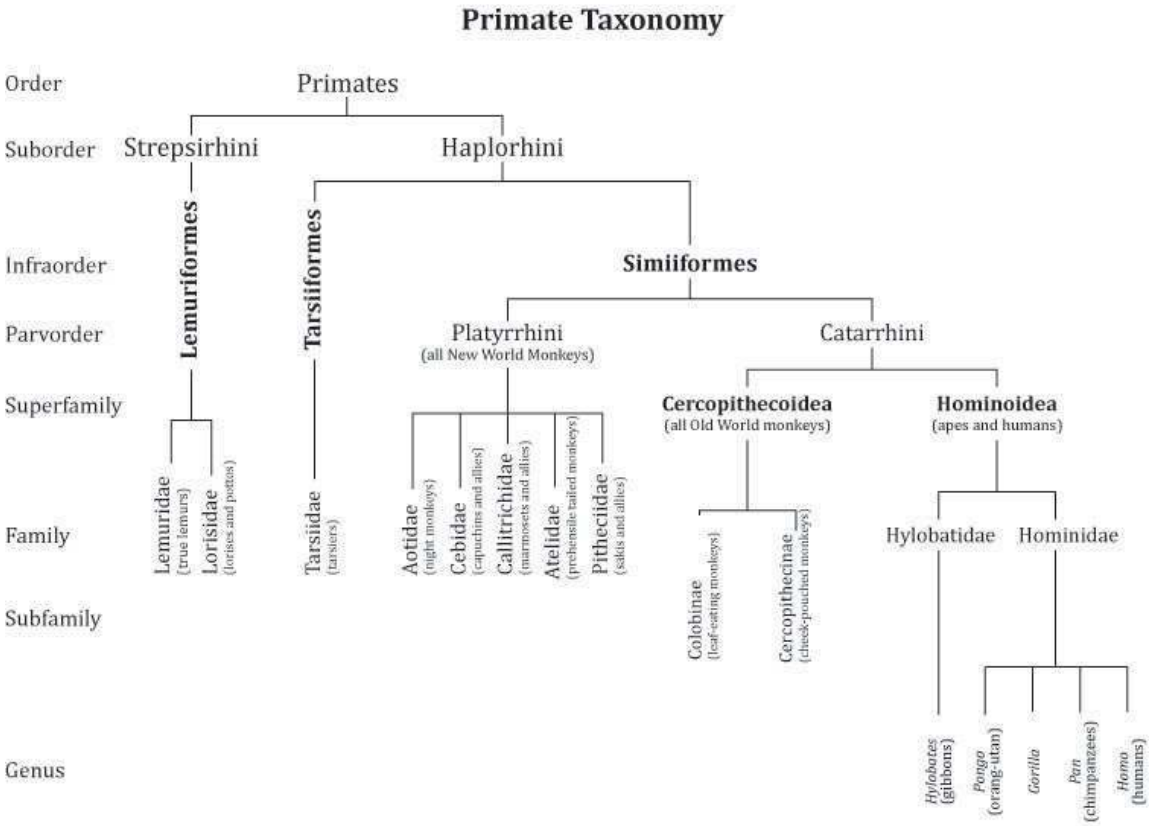

Figure 1. Taxonomic representation of the Order Primates. (Dean, 2017).

1.2 Host-Parasite Relationships

Parasitism refers to an ecological relationship between populations of different species (Crofton, 1971). The author has suggested several features defining this relationship: parasites are reliant on the host; parasite species have a higher reproductive potential; and thus, hosts are severely compromised when infected with the colonisation of harmful parasites, with a high possibility of death. Essentially these features are all necessary for the regulation of both parasite and host populations. There are several different relationships between the host and parasite and they share a variety of degrees of symbiotic relationships (Anderson & May, 1978). There can be a mutualistic bond, where both host and parasite benefit by the interaction. This can be described by the stabilising abilities of the vaginal microflora (Boskey *et al*., 1999). Commensalism is an interaction between host and parasite where one benefits but the other does not benefit nor is harmed. This is demonstrated by the relationship between multiple barnacle species (superorder Thoracica) and several cetacean species, mainly baleen whale and dolphin species, whereby the barnacles adhere to the bodies of whales assuming the skin as a place of residence while gaining access to food as the

cetacean travels. The cetacean experiences no harm from the presence of the barnacles (Fertl & Newman, 2009).

Bacterial pathogenesis is the ability of the bacteria to cause diseases in the host (Wilson *et al*., 2002). Several species of *Salmonella* are capable of infecting human and animal hosts causing enteric fever (Miller & Pegues, 2000; Ohl & Miller, 2001), *Streptococcus iniae* is a gram-positive bacterium and has been reported to cause disease in both human and multiple fish species (Baya *et al*., 1990; Eldar, & Bercovier, 1994; Eldar *et al*., 1995; Weinstein *et al*., 1997; Neely *et al*., 2002)

Microorganisms are capable of causing disease in a host that is already compromised and is known as opportunistic pathogenesis (Todar, 2000). An example of an opportunistic pathogen is *Pseudomonas aeruginosa*, an environmental bacterium (Bodey *et al*., 1983; Hardalo & Edberg, 1997). Members of the mycobacteria species, including atypical (for instance, *Mycobacterium marinum, M. genovense,* and *M. avium*) and tuberculosis (such as *M. bovis, M. tuberculosis, M. africanum,* and *M. canetti*) bacteria are known to be opportunistic (Grange, 1996; Stead, 1997). The *M. tuberculosis* is common in HIV-infected patients, *M. bovis* is found commonly in cattle (Brosch *et al*., 2002), and *M. avium* is seen in numerous bird species (Dhama *et al*., 2011). Many of these are transferrable between species and have been shown to have a high fatality in multiple animal species –for instance, the transmission of mycobacteria from humans to elephants (Michalak *et al*., 1998). They are distributed primarily in soil, water, faeces, and animal feed (Bercovier & Vincent, 2001; Biet *et al*., 2005; Rushton *et al.*, 2007). Additionally, a parasitic member of the normal flora can also become an opportunistic pathogen. For example, certain *Escherichia coli* strains have been found thriving in the mucosal layer of Crohn's disease patients (Darfeuille-Michaud *et al*., 2004; Sasaki *et al*., 2007).

1.3 Zoonoses and Pathogens

One of the major causes of emerging pathogenic infections is through the transmission from zoonotic sources (Wolfe *et al*., 1998). A zoonotic disease is defined by the WHO (2014) as any disease capable of natural transmission from animal to human. The Centre for Disease Control and Prevention (CDC) reported that approximately 75% of newly emerging pathogens are zoonotic in nature (CDC, 2014). With the outbreak of diseases such as Ebola, swine flu, and salmonella, it has become increasingly important to investigate the potential

reservoirs of zoonotic infection and their pathways into human populations as they could have severe environmental and socio-economic consequences (Cleaveland *et al*., 2001; Slingenbergh *et al*., 2004; Strinden, 2014). Non-human primates (NHP) are major reservoirs of zoonoses (Strinden, 2014) and, because they are taxonomically and genetically similar to humans, transmission is more direct (Guerrera, 2003). This, in addition to increased urbanisation, antimicrobial drug use, transportation, tourism, and relocation of animals to and from institutions, increases the amount of contact with humans (De Thoisy, 2001; Guerrera, 2003; Chapman *et al*., 2005; Engel & Jones-Engel, 2012; Karesh *et al*., 2012). Zoonotic pathogens are usually characterised by their transmission pathway, pathogen type, or degree of transmissibility (Lloyd-Smith *et al*., 2009).

Over 60% of emerging diseases are shared with animals (Jones *et al*., 2008), hence emerging infectious diseases are a major threat to the health of humans and animals (both domestic and wild) and are a major cause of population declines. Zoonotic pathogens can spread rapidly in the human population once infected from an animal source, thus causing many pandemics such as SARS, or localised outbreaks (Ebola virus; Wolfe *et al*., 2005; Wolfe *et al*., 2007; Jones *et al*., 2008; Newcomb *et al*., 2011). The transfer of pathogens between human and animal populations is a consequence of microbes entering into new niches and adapting to new host environments (Karesh *et al*., 2012). This was shown by Kilpatrick *et al.* (2006), where the risk of spread of the West Nile Virus was greater in urban areas owing to the wide variety of vectors able to transmit the virus to each other and to humans.

Bacteria in the gut have been shown to serve as reservoirs for antimicrobial resistance. Resistant genes have the capacity to be transferred to another virulent bacteria (Salyers *et al*., 2004; Sachs, 2005; Sommer *et al*., 2010; Sommer *et al.*, 2015). These genes can even be shared with species that only partially move through the gut, including respiratory bacteria (for example, *Streptococcus pneumonia*; Shoemaker, *et al*., 2001; Sachs, 2005). Sommer *et al.* (2015) found bacteria, previously deemed as harmless, having multiple resistant genes, making hosts more susceptible to this disease. This result supports the importance of understanding these bacteria and the relationship they share with their host. The transfer of these antimicrobial-resistant microbes occurs from direct contact with humans, for example in a slaughterhouse, which then allows for pathways to people in close contact with the infected body tissue (Levy *et al.* 1976; Marshall & Levy, 2011). Antibiotic resistance by *E. coli* increases in the intestinal tract of animals and humans when exposed to antimicrobial resistance (Gellin *et al*., 1989). Conversely, cold and heat stress has been shown to affect

antibiotic resistance in livestock that had not been exposed to antibiotics over a long period of time (Moro *et al*., 1998; Moro *et al*., 2000). If an animal is exposed to food (including feed additives) or water, contaminated with any form of antimicrobial resistance, the normal flora in the gut would dissipate and allow for harmful pathogens to cause infections (Sørum & Sunde, 2001).

The effects of zoonotic outbreaks can be seen in a wide range of species, such as bats (Luis *et al*., 2013; Brook & Dobson, 2014; O'Shea *et al*., 2014) which have been shown to be reservoirs for a large variety of zoonotic pathogens including leptospirosis, rabies, Hendra virus and the Ebola virus (Hartskeerl & Terpstra, 1996; Calisher *et al*., 2006). These authors have suggested that bats are good disease reservoirs because, as hosts, they are influenced by their ability to travel long distances, their extended life span, social structure, and dependency on torpor or hibernation.

Primates have also been linked to diseases such as Severe Acute Respiratory Syndrome (SARS), Ebola, Salmonellosis (*Salmonella* spp.), Shigellosis (*Shigella* spp.) and Campylobacteriosis (*Campylobacter* spp.; Walsh *et al*., 2003; Leroy *et al.*, 2004). Chapman *et al*. (2005) believe the transmission between NHPs and humans is caused by anthropogenic activities, for example, tourism, hunting and agricultural initiatives. A study conducted by Muriuki *et al*. (1998) established the presence of both helminths (such as *Trichuris* spp.) and protozoans (for instance, *Entamoeba coli*) in the gut of black and white colobus (*Colobus abyssinicus*), vervet monkeys (*Cercopithecus aethiops*), blue monkeys (*Cercopithecus mitis*), olive baboons (*Papio cyanocephalus anubis*), Debrazza's monkeys (*Cercopithecus neglectus*), grey mangabeys (*Cercocebus torquatus*) and black mangabeys (*Cercocebus albigena*) in Kenya. The authors suggested the most likely cause of transmission of the parasites is through an environmental medium or direct contact.

Parasites have shown a conversion from mostly zoophyllic to predominantly anthrophyllic orientation owing to the movements of humans and their activities into the habitats of animals. These changes will lead to the inevitable proliferation of emerging zoonotic pathogens (Patz *et al*., 2000).

1.3.1 Urban and Rural Habitats

Changes in the environment, whether inflicted by natural disturbances or human activities, have and will continue to cause the emergence of zoonotic diseases (Ambroise-Thomas, 2000; Patz *et al*, 2000). The improvement of the human quality of life has not always shown advantages for the natural environment (Mumford, 1968). A good example of this would be urbanisation. Urbanisation has led to an imbalance in the ecology of the ecosystem (Botkin & Beveridge, 1997; Tarsitano, 2006) owing to constant transformations. The abundance, diversity, behaviour and competence of parasites can be affected by environmental changes. These changes in habitats, owing to the increase in a number of buildings, industrialisation, and population growth, have all affected the natural dynamics of the ecological systems. These factors have given rise to the upsurge in pathogens and the ease of which they are spreading through the urban environment (Hancock, 2002).

Pets and synanthropic (living near and benefitting from humans) species have promoted the increase of and emergence of pathogens in these urban settings (Petney, 2001; Sutherst, 2001; Childs, 2004; Deplazes *et al*., 2004; Sutherst, 2004). Synanthropic animals have the capability of colonising the urban environment as various microenvironments close to each other are easily accessible for exchanges and transmission of pathogens from animal to animal (Parlange, 1998; Tarsitano, 2006). These changes have led to the inevitable proliferation of emerging zoonotic pathogens (Patz *et al*., 2000). Decreases in biodiversity may influence the transmission of vector-borne pathogens. The Dilution Effect is a process depicting that an increased diversity of vector species dilutes the effects of the reservoir host (LoGiudice *et al*., 2003). Conversely, if the opposite is implemented in an urban environment, the reduction of host species diversity could lead to more direct pathways of transmission from the vector host species (Schmidt & Ostfeld, 2001; LoGuidice, 2003; Bradley & Altizer, 2006; Keesing *et al.*, 2006).

1.4 The Gut Microbiome

Bacteria are the most diverse domain of life on Earth (Pace, 1997) with the ability to survive extreme conditions, for instance, extreme temperatures (Isaksen *et al.*, 1994; Carpenter *et al.*, 2000) and the internal body systems of animals (Shirkey *et al*., 2006; Tetlock *et al*., 2012; Kohl *et al*., 2013). In humans, bacteria have been discovered in several organs of the body: on the skin, vaginal and oral cavities, bladder, and –most predominantly, the gut (Dewhirst *et*

al., 2010; Grice & Segre, 2011; Wolfe *et al*., 2012; Li *et al*., 2013). Bacterial organisation varies lengthways down the digestive tract: in humans there are 10^3 to 10^4 microbes per gram in the stomach., the jejunum houses 10^5 10^6 g^{-1}, the ileum contains between 10^8 and 10^9 microbes per gram, and approximately 10^{11} microorganisms per gram inhabit the large intestine, (Whitman *et al.*, 1998; Backhed *et al*., 2005; Eckburg *et al.*, 2005; Van den Abbeele *et al*., 2011). The low number of bacteria in the stomach, compared to the colon, is a result of a low pH and fast movement of the contents and contributes to the digestion of food compounds to meet the nutritional needs of the host (Cummings & MacFarlane, 1991).

The gastrointestinal (GI) tract is responsible for various functions, including digestion and absorption of nutritional components (Cummings & MacFarlane, 1991) in the body, and in recent studies, it has also been shown to function as an immune defence mechanism (Sharma & Schumacher, 1995; Deplanke & Gaskins, 2001; Swidinski *et al*., 2005; Gaskins *et al*., 2008; Stecher & Hardt, 2008; Leser, & Mølbak, 2009; Doré *et al*., 2010). It houses large communities generally called microbiomes consisting mostly of anaerobic bacteria, eukarya, and archaea, (Rajilić-Stojanović, 2007; Baquero, 2012; Fogel, 2015); however, viruses, protozoa, and fungi are also present (Tlaskalovà-Hogenovà *et al*., 2011). The gut is mainly dominated by *Firmicutes*, *Bacteroidetes, Actinobacteria* and *Proteobacteria* species (Backhed *et al*., 2005; Eckburg *et al.*, 2005; Turnbaugh *et al*., 2009) which make up the normal or commensal microbiota (Davis, 1996).

The gut microbiome in a host stabilises at a young age and the dominant bacterial species generally remain in the body for the majority of the host life span (Doré *et al*., 2010). However, changes in age will alter the composition of the gut microbiota, as seen in the gut of tadpoles in comparison to adults (Kohl *et al*., 2013). The main functions of the gut microbiome are still being explored. In general, the microbiota function in nutrition and metabolism - the microbial community is used to metabolise resistant fibres and starches such as polysaccharides and oligosaccharides (Bugaut & Bentejac, 1993; Zoentendal *et al*., 2001) and to metabolise xenobiotics, modulate nutrient absorption, and produce short chain fatty acids which are a necessary energy source (Macfarlane & Macfarlane, 2003; Stumpf *et al*., 2016). These mechanisms are advantageous to an animal inflicted by stressful parameters. The wild western lowland gorillas (*Gorilla gorilla gorilla*) have been documented ingesting resistant starches during periods of low-quality food in order to maintain the energy supply (Gomez *et al*., 2015; 2016). The gut microbiome has also been shown to influence the immune responses, cell signalling and proliferation, and neural function (Newman &

Banfield, 2002; Backhed *et al*., 2004; Dethlefson *et al.*, 2006; Crawford *et al*., 2009; McFall-Ngai *et al*., 2013; Velagapudi *et al*., 2010).

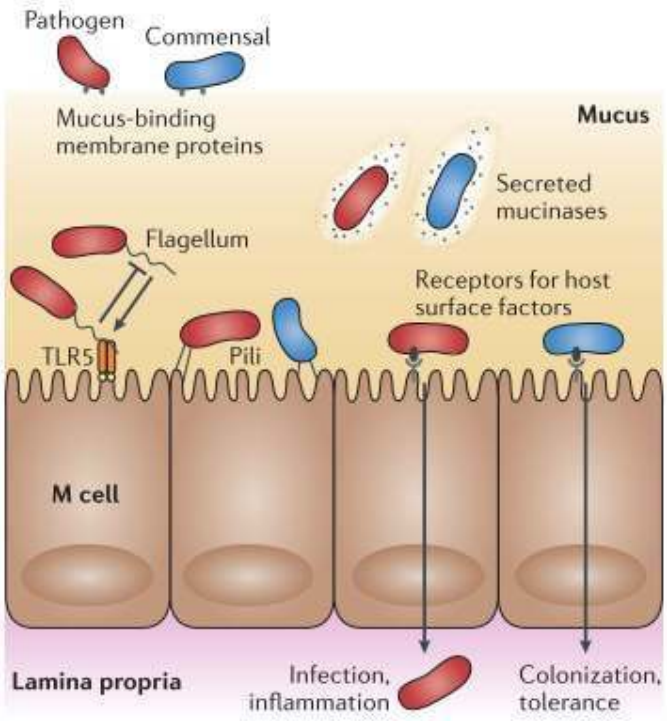

Figure 2. Bacterial access through the epithelial layer. Note both commensal and pathogenic bacteria are able to adhere to the epithelial wall, a method which has been suggested to increase risk of infection. Taken from Donaldson *et al.* (2015).

The diversity and abundance of bacteria have been studied longitudinally along the digestive tract, as well as laterally across the gut wall (Donaldson *et al*., 2015). Microbes reside in the luminal environment in the upper GI tract (where optimal digestion and absorption occurs). In the subsequent regions of the digestive tract, the microbiome also inhabits the mucosa as a distinct mucosal-associated microbial community (Zoetendal *et al*., 2002; Noverr & Huffnagel, 2004; Eckburg *et al.*, 2005; Van den Abbeele *et al*., 2011) whereby mostly functioning as a defence mechanism for the body. Additionally, gut-associated lymphoid tissues (GALT), which are critical for host defence against gastrointestinal pathogens, comprise more than half the immune cells in the host body (Gaskins, 1997). The lymphoid tissues would not develop and function normally without the presence of the microbiota (Vaishnava *et al*., 2008; Hooper *et al.*, 2012; Stumpf *et al*., 2016). The mucus layer acts as a filter limiting the passage of bacteria through the layer (Fig. 2). Various peptides in the mucus layer separate the commensal bacteria from the intestine (Hooper & Macpherson, 2010; McGuckin *et al*., 2011; Vaishnava *et al.*, 2011); however, commensal microbes have

adapted to this by deploying proteins, lectins and attachment pili to adhere to the epithelial cells (Fig. 2). Invasive pathogens also depend on these adherence methods to gain access to the lamina propria (Taylor *et al*., 1987; Sansonetti, 2004; Belkaid & Hand, 2014; Donaldson *et al.*, 2015). While the microbial community is healthy and homeostatic, the regulation of their populations outside the mucus layer is easily maintained. However, when there is dysbiosis and loss of diversity within the gut microbial composition, pathogenic species are more capable of gaining access through the epithelium (Belkaid & Hand, 2014). It can be concluded that the gut microbiome has a significant role in preventing and causing bacterial pathogenesis within the host (Stumpf *et al*., 2016).

The gut microbiome is influenced predominantly by the rhythmic changes (daily and seasonal) which then influence the physiology and behaviour of the host (Carey & Duddleston, 2014). A significant factor influencing pathogenic occurrence in a host is the microbiome; most commonly, from the intestinal tract and, oral and vaginal cavities (Yatsunenko *et al*., 2012; David *et al.*, 2014). Studies in these sites have been conducted in order to understand the bacterial abundance and diversity, as well as how these affect the health of the host individual (Li, 2013; Strinden, 2014). For example, Table 1 depicts the floral diversity of several species of domestic animals and humans. It is apparent that the diversity and abundance of microflora differs between species. *Bacteroides* are anaerobic, gram-negative rods, that can be passed from mother to offspring during birth and thus a relationship between host and bacteria commences in the early stages of life in symbiosis (Reed *et al.*, 2011). *Bacteroides*, in general, appears to be the most abundant species present in animals in which they are present. *E. coli* is the most widespread between the different animals. However, the relationship between all the bacterial species found in the gut is not well understood, although it has been shown that microbes will prevent pathogens colonising by competitive exclusion (Sekirov *et al*., 2010).

The microbiome is the major contributor to digestive efficiency although the composition diversity and abundance is greatly affected by the diet of the host (Wu *et al*., 2011; Deloris Alexander *et al*., 2006; Ley *et al*., 2006; Friswell *et al*., 2010; Campbell *et al*., 2012; Amato *et al*., 2013).

Table 1. The normal faecal flora of various animal species (Log viable cells per g faeces)[a].

Animal	*Escherichia coli*	*Clostridium perfringens*	Enterococci	*Bacteroides* spp.	Lactobacilli
Cattle	4.3	2.3	5.3	0	2.4
Sheep	6.5	4.3	6.1	0	3.9
Horses	4.1	0	6.8	0	7.0
Pigs	6.5	3.6	6.4	5.7	8.4
Chickens	6.6	2.4	7.5	0	8.5
Rabbits	2.7	0	4.3	8.6	0
Dogs	7.5	8.4	7.6	8.7	4.6
Cats	7.6	7.4	8.3	8.9	8.8
Mice	6.8	0	7.9	8.9	9.1
Humans	6.7	3.2	5.2	9.7	8.8

[a] Median values from ten individuals. Taken from Rosebury (1962) and Todar (2000).

1.4.1 Dietary Influences

As previously mentioned, the composition of the microbiota is influenced by birth. The initial colonisation period begins when infants are fed, and even in this preliminary stage, diet has a great effect on the microfloral species present, for instance, breastfed infants contain higher volumes of *Bifidobacteria* spp., while formula-fed infants have higher numbers of *Bacteroides* spp., *Clostridium coccoides*, and *Lactobacillus* spp. (Fallani *et al*., 2010). As the infant develops, the microbiome is suggested to stabilise (Doré *et al*., 2010; Brown *et al*., 2012). Zhang *et al*. (2010) found that changes in diet had a 57% effect on the variation of the composition of the gut microbiota; whereas, genetics only explained a 12% variation. These perturbations can have effects on the host and could even cause healthy gut microbiota to transition into disease-stimulating organisms (Brown *et al*., 2012). Walker *et al*. (2011) investigated the gut microbial composition in mice when changing from a more natural diet (low-fat, plant polysaccharide-rich), to a "Western" diet (high in sugar and fat). Several studies have declared that a diet rich in complex carbohydrates contain less pathogenic species than diets in higher fat and protein (Cummings *et al*., 1979; Birkitt *et al*., 1996; Smith & Macfarlane, 1997; Santacruz *et al*., 2009; Walker *et al*., 2011). It is possible that some diets stimulate the proliferation of pathogenic microbes while other dietary changes may promote more beneficial responses by other microbes (Ley *et al*., 2005; Nauman & Nanau, 2012; Parnell & Reimer, 2012; Martens *et al*., 2011; Tremellen & Pearce, 2012).

Previous studies have highlighted the influence of the gut microbiome on health and metabolic effects of humans (Turnbaugh *et al*., 2009; Sommer *et al.*, 2010; de Vos & de Vos., 2012; Penders *et al*., 2013; Manor *et al.*, 2014; Sommer *et al.*, 2015); although fewer studies

have investigated the gut microbiome of other mammals (Eckburg *et al*., 2005; Frey *et al*., 2006; Uenishi *et al*., 2007; Ley *et al*., 2008; Ochman *et al*., 2010; Xu *et al*., 2010; Yildirim *et al*., 2010; Degnan *et al*., 2012; Moeller *et al*., 2012; Amato *et al*., 2013; McCord *et al*., 2013; Fogel, 2015).

1.5 In Mammals

The majority of mammal populations are facing severe ecological pressures at present (Estrada, 2013) and many species are currently characterised as critically threatened or endangered (Walsh *et al*., 2003). Several tools (for instance, GIS) and conservation programs (for example, national parks and hunting regulations) have been established to help understand these animals in order to overcome conservation challenges. Analyses of the gut microbiome has become a potential tool to support conservation efforts (Stumpf *et al*., 2016).

The gut microbiome of mammals has a major effect on the metabolic activities of the host organism. In previous studies the gut microbiome systematics have been investigated predominantly in captive animals (Fogel, 2015), or shown as a comparison between the captive and wild animals (for instance, Ley *et al*., 2008; Strinden, 2014; Fogel, 2015), thus restricting our knowledge of the natural dynamics of the host gut microbiome. It is widely accepted that the diet of mammalian species has a great effect on the microbial assemblages. These effects result in changes in the gut contents and phylogeny (Ley *et al*., 2008). The authors also noted the microbial contents were similar between conspecific hosts (even while geographically distant).

Nocturnal behaviour is practiced by 44% of the mammal population worldwide (Jones *et al.*, 2009). It is a primitive activity and the adaptations incurred from this lifestyle are thought to be a driving force of mammal evolution (Crompton *et al.*, 1978). Many nocturnal species must adjust their feeding activities to correspond to the changes in the lunar phases (Bowden, 1973). These behavioural activities will be influenced either by predation risk, or food availability (Brown & Taylor, 1971). Williams & Singh (1951) first described marked differences between the prevalence of insect activity during the new moon week compared to the full moon week. Prugh & Golden (2014) has shown species-specific preferences for various cycles of the moon – the abilities of nocturnal primates during a high volume of illumination are enhanced, whereas in contrast, rodents and carnivores are at a disadvantage. This is also demonstrated for kangaroo rats (*Dipodomys spectabilis*; Daly *et al*., 1992), where

their activities were suppressed during the full moon week and more active during partial moon phases. Nocturnal bats use the moonlight to search for prey; however they have been recorded as active during nights with reduced moonlight and to have reduced activity close to the full moon in order to avoid predation (Eckert, 1978). Morrison (1978) termed this behaviour as 'lunar phobia'. Moonlight has also been shown to influence Galapagos fur seals (*Arctocephalus galapagoensis*) to feed at different depths as squid and fish migrate accordingly (Horning & Trillmich, 1999).

1.5.1 Seasonal Adaptations

Nocturnal animals highly dependent on vision, sound, and smell to detect prey are particularly affected by seasonal changes. Behavioural changes are an expected response to an extensive drop in temperature, such as the austral winter (Nowack *et al*., 2013). As a short-term reaction, smaller animals will increase metabolic rate (MR) in order to maintain a stable body temperature (Lyman, 1982; Carey *et al*., 2003). Heterothermy, the ability to alter between poikilothermic and endothermic strategies (Lyman, 1982), has been suggested as a sufficient response for various mammalian orders; all three extant classes of mammals have been revealed to undergo heterothermy (Lyman, 1982; Geiser, 1994; Nicol & Anderson, 2008). Smaller mammals will undergo shivering thermogenesis in order to increase body temperature –however– this is only a short-term solution owing to energy costs (Nowack *et al*., 2013). Another solution achieved by smaller mammals is to go into a state of hibernation or torpor (short periods of sleep) during winter in order to conserve energy and to cope with the harsh environment and reduction of food source (Mzilikazi *et al*., 2006; Nowack *et al*., 2013). These periods of inactivity are deemed as adaptations in colder environments. However, it can also be used to compensate for food scarcity during periods of high energy demands (Lovegrove, 2000).

Most primate species experience food shortages for certain periods of the year, for instance during periods of cooler temperatures. The food relied upon during these periods are known as fallback foods (Marshall & Wrangham; 2007). Fallback foods tend to be high in abundance, yet poor in nutritional quality. They are usually of low preference consumed only when preferred foods are scarce (Hanya, 2004; Lambert *et al*., 2004; Ungar, 2004; Yamakoshi, 1998; Knott, 2005; Laden & Wrangham, 2005).

1.6 Microbial Phylogenetics Analysis

Identifying and classifying bacterial species has been a challenge in biology (Kluyver & van Niel, 1936; Stanier & van Niel, 1941; Stanier & van Niel, 1962; Woese, 1994). The primary domains of life –Eucarya, Bacteria, and Archaea– were suggested by Woese *et al*. (1990). In the past, morphological characteristics such as flagellates, pathogenesis, cell size, and shape were used to identify and classify bacterial species (Woese, 1987). Molecular phylogenetics based on nucleic acids and selected proteins, specifically small subunit (SSU) rRNA molecules of a microorganism have established a classification system (Woese *et al*., 1990). Advances in DNA sequencing analyses have led to groups or divisions within Bacteria being suggested (Ludwig & Klenk, 2001). There is a relationship between the rate of evolution, its speed, and the type of changes that occur (Woese, 1987). However, microorganisms tend to have a high rate of evolution as they have a rapid reproductive rate and can exchange genes through lateral gene transfer (LGT; Woese, 1987).

LGT is the transfer of genetic material from one organism to another irrespective of their evolutionary distance to one another (Lawrence, 2005). This is comparable to vertical gene transfer, the flow of genes along the branches of the family tree, that is, parent to offspring (Lawrence, 2005). LGT has been shown to play a significant role in the evolution of bacterial genomes (Lawrence & Ochman, 1998) and has illustrated that microbial evolution is more non-linear and web-like than previously proposed (Doolittle, 1999). However, between unrelated species, LGT has led to uncertainty in the classification of bacterial species (Ochman *et al*., 2000).

The conserved signature indels (CSIs) inserts and deletions in protein and gene sequences provide a significant category of molecular markers that can be implemented in identifying microbes and understanding microbial phylogeny and systematics and different phylogenetic depths (Gupta, 1998; Baldauf, 1993). The CSIs can be defined as the indels surrounded on both sides by conserved regions as this ensures specific molecular markers are present (Gupta & Griffiths, 2002). Genetic changes resulting in CSIs may occur at different stages of evolution, and hence making it possible to identify CSIs at the various branch points (Griffiths & Gupta, 2004; Gupta, 2001; 2010; 2014; Bhandari & Gupta, 2014). Therefore, it is possible to identify specific clades from phyla to species. Additionally, CSIs provide the opportunity to determine branching order and the relationships between bacterial phyla (Griffiths & Gupta, 2004; Bhandari & Gupta, 2014; Gupta, 2014). It has been debated by

many scientists whether CSIs shared by unrelated taxa are the outcome of genetic changes, or from LGT. There are several different views proposed as to the degree of impact of LGT on CSIs. In general, it seems a large proportion of scientists with extensive knowledge of CSIs believe LGT have minimal impact on the phylogeny of prokaryotes (Beiko & Ragan, 2008; Gao & Gupta, 2012; Puigbo *et al*., 2010; Bhandari *et al*., 2012).

With the development of phylogenomics, whole-genome sequencing has provided an alternative method of identifying and categorising bacterial species. Despite improvements in technology, chimeric sequences, read inaccuracies, and low phylogenetic resolution have restricted tree construction to the use of full-length and near full-length 16S rRNA sequences (Kim *et al*., 2011).

Lane *et al*. (1965) first described the use of the 16S ribosomal (rRNA) gene. It has become the universal genetic marker for investigating bacterial community structure and composition (Huber *et al*., 2007). Technologies such as PCR amplification, cloning, and sequencing have been used to identify these 16S rRNA gene sequences (Cole *et al*., 2009). In the past twenty years several million bacterial 16S rRNA gene sequences have been identified from both cultured and, mostly, uncultured prokaryotes (Cole *et al*., 2009). Certain traits of this gene make it favourable for amplification. 16S rRNA is part of the translation process, therefore present in all bacteria. Also, it is a multi-copy gene which increases the detection sensitivity; and it consists of conserved and highly variable regions. The conserved regions allow primers to target all bacteria where they amplify the 16S gene through a hypervariable region. Differences in the sequence of bases allow for determination of various species while a constant rate of evolution allows the hypervariable region to infer phylogenetic relationships (Kim *et al*., 2011). 16S rRNA gene profiling is an efficient method for characterizing bacterial populations at low cost and with minimal bioinformatics effort (Chakravorty *et al*., 2008). The capacity of high-throughput sequencing to sequence multiple 16S rRNA gene hypervariable regions simplifies the analysis of microbial community dynamics in terms of spatial and temporal contexts (Huber *et al*., 2007; Scholz *et al.*, 2012; Hiergeist *et al*., 2015).

The 16S rRNA gene is comprised of 9 hypervariable regions (V1–V9) of varying lengths making up a total of 1542 base pairs (Lane *et al*., 1985; Woese, 1987; Clarridge, 2004). It is recognised that some hypervariable regions are more variable and reliable than others (Yu & Morrison, 2004; Liu *et al*., 2007; Wang *et al*., 2007; Youssef *et al.,* 2009). Several previous studies investigating the oral microbiome have suggested the use of regions V1–V3 and V7–

V9 (Kumar *et al*., 2011), however, currently the is a general understanding the V4 hypervariable region reveals consistent results while the V9 region poorly represents the bacterial diversity (Chakravorty *et al*., 2008; Kumar et al., 2011; Caporaso *et al*., 2012; McHardy *et al*., 2013; Davenport *et al*., 2014; Moreau *et al*., 2014; Birtel *et al*., 2015; Barb *et al*., 2016; Lund, 2016).

Generated 16S rRNA gene sequences are clustered into operational taxonomic units (OTUs) from varying distance levels depending on the classification level. Phylum, family, genus and species are defined with distance values 0.20, 0.10, 0.05 and 0.03, respectively (Schloss & Handelsman, 2004). Sequencing of hypervariable regions serves to partition large data sets into these taxonomic assignments.

Research in microbiomes is a relatively new context in science. Until recently investigations into the host's microbiota has been limited by the techniques available (Amann *et al*., 1995). Methodical advances have progressed beyond the necessity to cultivate using standard techniques as this has proved limited in its capability to identify bacterial species (Amann *et al*., 1995; Connon & Giovannoni 2002). Molecular methods have allowed for the revolutionary advances we see today in areas of microbiological research. Sanger sequencing, originally developed in 1977 (Sanger *et al.*, 1977), is one of the older of the sequencing approaches (DiGuistini, *et al*., 2009). The development of culture-independent high-throughput next generation sequencing (NGS) techniques (for example Roche 454, Illumina, and Ion Torrent) and metagenomic approaches (for instance, whole-genome shotgun sequencing) have allowed for more insightful analyses into the community structure, diversity and abundance, ecology, and functionality in the host (Lund, 2016; Allali *et al*., 2017).

There are several next generation sequencing platforms readily available. These all have various strengths and weaknesses pertaining to read-length, accuracy and throughput (Amato, 2016). Additionally, hypervariable region and primer biases, sequencing depth and error rates should be considered when selecting a platform (Kuczynski *et al*., 2011; Amato, 2016). Currently the Illumina sequencing platforms (HiSeq and MiSeq) is most commonly used as they show consistent results (Gloor *et al*., 2010; Caporaso *et al.,* 2012; Clooney *et al*., 2016), although they can only provide short reads (up to 350 bp) and the run-time is between five and 55 hours (Ansorge *et al*., 2017. Ion Torrent Technology is an alternative next generation sequencing platform owing to its simplicity, short run-time, and affordable pricing. Particles

are deposited into separate nano-wells and placed on a semiconductor sequencing chip (ThermoFisher Scientific; Shendure & Ji, 2008; Rothberg *et al*., 2011; Quail *et al*., 2012; Salipante *et al*., 2014). Nucleotides are individually incorporated with DNA polymerase. Hydrogen ions are released as a by-product. The hydrogen ions change the pH of the solution which is then detected by the instrument sensors (ThermoFisher Scientific; Quail *et al*., 2012; Salipante *et al*., 2014). The benchtop sequencers Ion S5 and Ion S5 XL were introduced in 2015 (ThermoFisher Scientific; Ansorge *et al*., 2017). These instruments can generate read lengths of approximately 200 bp (ThermoFisher Scientific; Ansorge *et al*., 2017). Ion Torrent has also simplified the next generation data analysis by the establishment of the Ion Suite and Reporter software.

Currently, the functions of these microorganisms are poorly understood, although these gut populations are thought to contribute substantially to the health of the host (Lund, 2016). New developments in microbial phylogenetics analysis will help resolve uncertainties regarding the effect microbes have within the host and, more specifically, the consequences of bacterial colonisation on the health of the host (Gilbert *et al.*, 2012; Eren *et al*., 2013; McFall-Nagai *et al*., 2013; Carey & Duddleston, 2014; Gilbert, 2014; Park *et al*., 2015; Green *et al*., 2016).

1.7 The Southern Lesser Galago

Primates are divided into two suborders (Fig. 1): haplorhines (dry-nosed) and strepsirrhines (wet-nosed). Strepsirrhines (prosimians) have adapted to an arboreal lifestyle by climbing and grasping. They have preserved much of the sensory features derived from primitive species including a developed sense of smell and a lack of colour vision, and a nocturnal lifestyle (Doyle *et al.*, 1969).

The Southern lesser galago (*Galago moholi*), also known as the Mohol Galago or the African Lesser Galago (Bearder *et al*., 2008), is part of the Galagidae family, from the Lorisiformes infraorder (Fig. 1). It is a nocturnal, arboreal mammal found throughout the northern provinces of the Republic of South Africa as well as Eastern Africa in countries such as, Mozambique, Botswana, Malawi, Tanzania, Kenya (Fig. 3; Bearder & Martin, 1980; Bearder, 1987; Nekaris & Bearder, 2007). They are generally found in savanna woodlands, *Acacia* woodlands and shrublands (Bearder & Martin, 1980). Commonly deemed solitary, galagos will feed individually at night, but sleep within a group during the day (Bearder, 1999). They

are the smallest primate species found in Southern Africa, with an average weight of 200g (Bearder, 1987; Nekaris & Bearder, 2011).

They have two pregnancy periods a year, approximately March to April and again September to October with the gestation period ranging between 120 and 125 days – births occurring, initially, in November and, secondly, in late January, early February (Lowther, 1940; Sauer & Sauer, 1963; Doyle *et al.*, 1969; Doyle *et al.*, 1971). Lowther (1940) documented twin births in *G. moholi*. Once given birth, females will potentially come into oestrous and conception is possible for the second mating season (Doyle, Pellettier & Bekker, 1969; Doyle, Andersson & Bearder, 1971; Pullen, 2000).

Figure 3. Distribution of *Galago moholi* throughout Africa indicated by the yellow shading (Bearder, Butynski, & Hoffmann, 2008).

1.7.1 Diet

The diet of *Galago moholi* shows vast changes throughout the seasons. They feed mainly on arthropods; however, in winter when the invertebrate population numbers decrease, galagos survive mainly on the remaining active insects and exudates from *Acacia* trees (Bearder & Martin, 1980; Harcourt, 1986; Nash, 1986; Nekaris & Bearder, 2011; Nowack *et al*., 2013; Scheun *et al*., 2014). In addition, in areas of low gum-producing vegetation, fruit-feeding and

even avian chick predation has been documented (Scheun *et al*., 2014; Engelbrecht, 2016). Gum is an important food source as it is found annually. Noticeably in winter; exudates of *Acacia* trees become a critical food resource for *G. moholi*. In light of the necessity for the galagos to feed, gum is a poor source of proteins, vitamins, and minerals (Bearder & Martin, 1980; Nash, 1986) having a high calcium-phosphorus ratio. Furthermore, even though gum provides calcium and carbohydrates, galagos lose weight and mortality rate increases (Bearder & Martin, 1980; Nash, 1986) possibly owing to limited protein-supplementation (Bearder & Martin, 1980) during the colder months. The GI tract of the southern lesser galago has been adapted for some digestion of gum exudates – an enlarged caecum allows for the fermentation of gum (Argenzio & Stevens, 1984; Nash, 1986; Caton *et al*., 2000), allowing for the digestion of β-polysaccharides (Nash, 1986; Caton *et al*., 2000; Porter *et al*., 2009). These carbohydrates are then converted into short chain fatty acids, which are directly absorbed and used as a major source of energy (Flint *et al*., 2008; Flint *et al*., 2012; Amato *et al*., 2016).

1.7.1.1 Insects

Invertebrates are the main food source in the Order Primates (Rothman *et al*., 2014). Galagos primarily capture insects using auditory senses (Charles-Dominique, 1977). Per unit of mass, insects provide a greater volume of energy than fruits and leaves as they contain more calories (approximately 350–500 kcal/100g; DeFoliart, 1995; Johnson *et al*., 2012; Isbell *et al*., 2013). Kay (1975) suggested insectivorous feeding as a major food source for small primate species weighing less than 500g, termed "Kay's Threshold". Only these small primates are able to survive as insectivores owing to the volume of energy expended capturing their prey (Kay, 1975; Kay & Sheine, 1979). Insects predominantly consist of proteins (McGrew, 2001; Verkerk *et al*., 2007), chitin and fats. It has even been suggested that reproductive seasonality in primates reliant on insects will be affected by the abundance of prey in order to coincide with the lactating period (Goldizen *et al*., 1998). This has been shown in night monkeys, *Aotus azarai*, in Argentina (Fernandez-Duque *et al.*, 2002). Chitin is a long-chain polymer of N-acetylglucosamine, derived from glucose (Finke, 2007). In most cases, chitin is seen as a deterrent as it not digestible in most primates. Nonetheless, Kay & Sheine (1979) found *Galago senegalensis* from Kenya as having the ability to partially digest chitin.

1.7.1.2 Gum Exudates - Arabinogalactin

The main component of gum exudates is a hemicellulose carbohydrate called arabinogalactin (D'Adamo, 1996). This is a non-starch polysaccharide that has recently been suggested to enhance the immune system and defend against pathogens in humans (D'Adamo, 1996; Kelly, 1999; Dion *et al.*, 2016) via the gut-associated lymphoid tissue (GALT). Arabinogalactin has been shown to increase the growth of *Bifidobacterium* and *Lactobacillus acidophilus* (Robinson *et al.*, 2001; Grieshop *et al.*, 2002; Kim *et al.*, 2002; Marzorati *et al*., 2010). Previous studies have shown an improvement in immunoglobulin response to infections (such as tetanus and influenza; as seen by Udani, 2013). Grieshop *et al.* (2002) have shown that the oral administration of arabinogalactin increases the white blood cell count in dogs. Thus, it can be suggested that even if gum has a nutritional value for the host, it may provide some immunity for the host during the times of low food availability (Dion *et al.*, 2016).

1.8 Research Aims and Objectives

1.8.1 Aims

We hypothesise that changes in dietary and lifestyle habits will alter the composition of the gut microbiome of *G. moholi*. The purpose of this investigation is to use modern molecular phylogenetics to investigate the effects of diet on the gut microbial community in Galagos, and to determine if this species is a potential reservoir for harmful pathogenic bacteria. This comparison can be achieved by sampling wild galago individuals in a less disturbed habitat, as well as in an anthropogenically disturbed area of Gauteng during seasons of high and low food availability.

1.8.2 Objectives

1. To assess the bacterial diversity present in the gut of the Southern lesser Galago.
2. To investigate whether changes in diet (seasonal changes) have a significant influence on the gut microbial community in the gut of *G. moholi*.
3. To determine whether habitat (urban and rural) has a significant influence on the gut microbiota of galagos.

Chapter 2

Materials & Methods

2.1 Study Sites

Study areas were selected based on a physical environment. Galago populations present in anthropogenically disturbed (urban) regions and a site which has been less accommodated by humans (rural; Table 2).

Table 2. Description of trapping sites

Site	GPS Coordinates	Categorisation
National Zoological Gardens	25°44'18" S, 28°11'21" E	Urban (disturbed)
Roodeplaat Dam Nature Reserve	25°37'14.99" S, 28°22'16.97" E	Rural (undisturbed)

The sites, the National Zoological Gardens of South Africa (NZG, 25° 44' 18" S, 28° 11' 21" E) and Roodeplaat Dam Nature Reserve (RDNR, 25°37'14.99" S, 28°22'16.97" E), are both situated in the northern region of the Gauteng Province in close proximity to one another. These sites have been selected based on the presence of substantial numbers of the study species, *Galago moholi*. The sites are approximately 20 km apart, separated by the Magaliesberg mountain range (Fig. 4) which acts as a geographical barrier preventing the overlap of population home ranges.

Figure 4. A map exhibiting the two sampling sites. Note the Magaliesberg mountain. Also note the difference in habitat, the NZG in an urban environment, while the Roodeplaat Dam Nature Reserve is a more rural setting. Taken from Google Earth©.

2.1.1 National Zoological Gardens of South Africa

The National Zoological Gardens (NZG) is situated in Pretoria CBD (Fig. 5). It is situated on 85-hectare plot comprising of more than 9000 animals from approximately 700 species as well as the third largest collection of exotic trees (NZG; 2016). The zoo itself hosts more than 600'000 visitors annually which disturbs the landscape and habitats unprotected by enclosures. Anthropogenic activities have had significant effects on this area leaving the natural habitat structure highly fragmented. Galagos have been sighted in the trees and scavenging from the dustbins located around the premises. In the NZG, two sites outside the animal enclosures were selected as galagos were commonly sighted: Site A with an elevation of 1336 m, and Site B at 1322 m (Fig. 5).

Figure 5. Trapping sites (A and B) at the National Zoological Gardens of South Africa, Pretoria CBD. Taken from Google Earth©.

2.1.2 Roodeplaat Dam Nature Reserve

The Roodeplaat Dam Nature Reserve (RDNR) is situated in the Dinokeng Region, north-east of Pretoria. Constructed in 1959 on the Pienaars River, before being established as a nature reserve in 1977, the dam was used as a water source for surrounding landowners. The dam is well-known for birding, angling, game viewing and water sports; however most human activity occurs on the northern side of the dam. Sampling took place on the southern side of the dam (Fig. 6). The vegetation type surrounding the dam is classified as Marikana

Thornveld (DWAF, 2008); the habitat is considered open savannah veld and dense savannah woodland. This vegetation type is characterized by open *Acacia karroo* woodland with dense shrub areas. Dominant tree species include *Acacia caffra*, *Acacia gerrardii*, *Acacia karroo*, *Rhus lancea* and *Ziziphus mucronata* and also contains several Red Listed plant species such as *Trachyandra eruthrorrhiza*, *Delosperma gautengense*, and *Schizoglossum umbelluliferum* (Hilton-Taylor, 1996; Germishuizen & Meyer, 2003; IUCN, 2003;). The southern side of the dam has over 250 species of birds recorded and numerous species of amphibians, reptiles, and large and small mammals (Barnes, 2000). The water quality of the dam is considered poor with the presence of *Cyanobacteria* presenting a health risk for both animals and humans if ingested, and water hyacinth (*Eichhornia crassipes*) suppressing the aquatic diversity and creating breeding sites for malaria vectors and bilharzia snail (DWAF, 2008).

Figure 6. Map of Roodeplaat Dam Nature Reserve during the winter season. Please note the markers for the sampling areas, Site A and Site B. Taken from Google Earth©.

2.2 Methodology

2.2.1 Seasonal Trapping

Sherman (7.62x8.89x22.86 cm and 10.16x11.43x38.1 cm; H.B. Sherman Traps, Tallahassee, FL, USA) and self-made, live traps were used to capture the galagos during February–March (summer) and May–July (winter) periods. For the summer sampling period, galagos were captured at the NZG only, whereas both the NZG and RDNR sites were used for galago

capture in the winter period. Traps were baited with peanut butter, bananas and syrup (Cuozzo & Sauther, pers. comm.; Nowack *et al*., 2010). Late in the afternoon, traps were secured to tree branches using zip ties, baited, and set. Initially, traps were checked every two hours and thereafter every four hours from 20:00 till 06:00. This was altered to increase the chances for capture per night as checking the traps too often disturbs the site hence deterring the galagos. It was also apparent that the animals were most active from 18:00 to 21:00, and then again from 04:00 till sunrise, thus influencing the checking times. Traps were moved to different locations if there were no captures or repeated captures of previously sampled individuals (or the capture of other species). To prevent recapture and resampling of an individual, each galago was marked by removing a large portion of hair on their inner leg. This site on the body was deemed a safe, non-invasive and short-term method of identifying sampled individuals.

For statistical accuracy purposes, a capture rate of 10 unique individuals per site and per season was determined. A total of 23 samples were collected: ten different individuals from the NZG site for both summer and winter seasons but only three different individuals were captured at the RDNR site.

2.2.2 Measurements and Sample Collection

Once removed from the trap, individuals were sampled at the Animal Hospital at the National Zoological Gardens of South Africa. Sampling was undertaken by a SAVC certified veterinarian to ensure correct and safe animal handling and sampling methods. The animals were restrained using a capturing bag (regularly used when sampling birds) while the body mass was determined using the Radwag® WLT 12/30/X/2 balance. Faecal samples were then acquired by swabbing intra-rectally using cotton-tipped FLOQSwabs™ (Copan Flock Technologies).

Age class (juvenile, sub-adult or adult) was estimated by size, behaviour, and canine length. The gender was also determined by checking for the presence of sexual organs. Morphometric measurements were recorded using a flexible measuring tape and calipers (Digital Caliper®) as per the methods of Nowack *et al*. (2010). This included total body length (from tip of the nose to tip of the tail), tail length, canine length, eye diameter, pupil- to-pupil length, and hind foot length). Dental images were taken using a Nikon® DSLR 3500. The hair was stored in envelopes to be later processed with all the other samples by the technician at the NZG Biobank.

The animals were handled appropriately to avoid any risk of injury or induce any additional stress. Individuals were handled for less than 15 minutes, without the use of anaesthesia and once the veterinarian was satisfied with the condition of the animal, before being released at the exact source of capture. All persons involved in the sampling procedure were required to wear protective masks and gloves during all activities.

2.2.3 DNA Isolation

DNA was extracted from faecal swabs using the QIAamp® DNA Investigator Kit (Catalogue number: 56504; Qiagen®). The swab head was removed using scissors and placed in a 2 ml microcentrifuge tube with 20 µl Proteinase K and 400µl Buffer ATL. The contents were mixed by vortexing. The tube was then placed in a heating block for one hour at 56°C with vortexing for ten seconds every ten minutes. Four hundred microlitres of Buffer AL was added to the tube and vortexed. The tube was placed in the heating block again, this time at 70°C for ten minutes, with the contents vortexed for ten seconds every three minutes. Binding of the DNA 200 µl was completed by adding 99% ethanol to the tube and then mixed by vortexing. The lysate was then transferred into a QIAamp MinElute column (provided by the manufacturer, in a 2ml collection tube) and centrifuged at 10,000 rpm for one minute. Once all the lysate had passed through the membrane of the column, the QIAamp MinElute column was then placed into a clean collection tube, while the used tube was discarded. Five hundred microlitres Buffer AW1 was added to the QIAamp MinElute column and centrifuged at 10,000 rpm for one minute. The column was then placed onto a clean collection tube. Seven hundred mircolitres of 99% ethanol was added to the column and centrifuged at 10,000 rpm for one minute. The column was then placed in a clean collection tube and centrifuged again at 14,000 rpm for three minutes to dry the membrane. The column was placed in a 1.5 ml microcentrifuge tube and incubated in a heating block for three minutes at 56°C. Seventy-five microlitres of Buffer ATE was added to the column for the final elution stage. It was essential to add the buffer onto the centre of the membrane to ensure complete elution. The column was incubated at room temperature for one minute before being centrifuged at 14,000 rpm for one minute.

The quality of the DNA was evaluated on 2% agarose gel (results shown in Appendix 1), and the concentrations of DNA were measured with a Nanodrop spectrophotometer ND-1000 (NanoDrop®). DNA was stored in -20°C freezer until next-generation sequencing.

2.2.4 16S rRNA gene PCR Amplification

Of the 20 samples collected from the NZG, eight (four from each season) were selected for sequencing. Genomic DNA was converted to a sequencing library by amplification of selected regions using the Ion 16S™ Metagenomics Kit (Catalogue no.: A26216) protocol as recommended by the manufacturers (Thermo Fisher Scientific, Inc). To prepare the amplicons, 2 µl of each sample DNA was added to a solution of 15 µl 2X Environmental Master Mix and 3µl 16S primer set (10X) for both forward and reverse in separate reactions. A positive (*E. coli* DNA) and negative reaction were also created. The plate was run in a thermal cycler (Bio-Rad T100™) under a specific protocol (Table 3).

Table 3. A table demonstrating the program used to amplify the 16S hypervariable regions. The samples were run in a thermal cycler.

Stage	**Step**	**Temperature**	**Time**
Hold	Denature	95°C	10 min
18 Cycles	Denature	95°C	30 sec
	Anneal	58°C	30 sec
	Extend	72°C	20 sec
Hold	–	72°C	7 min
Hold	–	4°C	∞

The selected primer set included V2, V3, V4, V6-7, V8, and V9 hypervariable regions of the 16S rRNA gene. The quality of the PCR product was evaluated on 2% agarose gel.

2.2.5 Purification of Amplification Products

Purification of the amplified products followed. Agencourt® AMPure® XP Reagent beads were mixed with the amplified product in Eppendorf® tubes. The mixture was incubated at room temperature for five minutes, and then placed on a magnetic rack (Bio-Rad SureBeads™ Magnetic Rack) for three minutes. The supernatant was carefully removed without disturbing the beads. Subsequently, 300 µl of 70% ethanol was added to each tube (still on the magnet), incubated for 30 seconds, and the supernatant removed without disturbing the beads. A second wash was completed to ensure the removal of supernatant. The beads were then air-dried on the magnet for 4 minutes. After removing the tubes from the magnet, 15 µl of Nuclease-free water was added to the beads and mixed with a pipette.

The tubes were placed on the magnet again for 1 minute and the supernatant was transferred to a 1.5 ml clean Eppendorf® tube.

2.2.6 Library Preparation

To prepare the library the Ion Plus Fragment Kit (Catalogue no.: 4471252) was employed following the manufacturer's protocol. First, the pooled amplicons were purified. 79 µl of the amplicons were mixed with 20µl 5X End Repair Buffer and 1µl End Repair Enzyme in an Eppendorf® tube. One hundred and eighty microlitres Agencourt® AMPure® XP Reagent beads were added, mixed and left to incubate for 5 minutes. The tube was placed on a magnetic rack (Bio-Rad SureBeads™ Magnetic Rack) for three minutes. The supernatant was then removed and discarded without disturbing the beads. While still on the magnetic rack, 500 µl of 70% ethanol was added. The mixture was incubated for 30 seconds, while mixing occurred. The supernatant was removed and discarded. A second wash was repeated. The tube was then pulse-spun, placed back on the magnet and any supernatant removed. This would ensure the complete removal of any remaining ethanol. The beads were air-dried for 4 minutes. Twenty-five microlitres of Low TE was added to the beads and the mixture vortexed. The tube was then placed back on the magnetic rack for one minute, and the supernatant (with eluted DNA) transferred to a clean 1.5 ml Eppendorf® tube.

For ligation and nick-repair, the Ion Xpress™ Barcode Adapters 1–16 Kit (Catalogue no.: 4471250) was used following the manufacturer's protocol. In a 0.2 ml PCR tube, 25 µl sample was mixed with a calculated proportion of 10X Ligase Buffer, Ion P1 Adapter, specified Ion Xpress™ Barcode, dNTP Mix, Nuclease-free water, DNA Ligase, and Nick-Repair Polymerase. The tube was placed in the thermal cycler (Bio-Rad T100™) to be run on a specific protocol (Table 4). Once completed, the product was placed in a clean 1.5 ml Eppendorf® tube.

In order to purify the DNA 140 µl of Agencourt® AMPure® XT Reagent beads were combined with the sample. Once mixed, the solution was incubated at room temperature for 5 minutes. The sample was spun down and placed on a magnetic rack (Bio-Rad SureBeads® Magnetic Rack) for three minutes.

Table 4. Thermal cycling necessary for the ligation and nick-repairing phase prior to DNA purfication.

Stage	**Temperature (°C)**	**Time (minutes)**
Hold	25	15
Hold	72	5
Hold	4	∞

The supernatant was carefully removed. Five hundred microlitres of 70% ethanol was added to the tube while on the magnetic rack. The wash was repeated, and any remaining ethanol was removed. The beads were then air-dried on the rack for 4 minutes. After removing the tube from the rack, 20 µl Low TE was added and the tube was vortexed for 10 seconds. After placing back on the rack for one minute the supernatant was removed and transferred to a clean 1.5 ml Eppendorf® tube.

2.2.7 Library Concentration Determination

This stage was achieved by adding 5 µl of Low TE to the purified adapter-ligated library (a total of 25 µl). Then, in a 2 µl microcentrifuge tube, 100 µl Platinum™ PCR SuperMix High Fidelity, 5 µl Library Amplification Primer Mix, and 25 µl of the library solution were combined. The reaction was divided between two 0.2 µl PCR tubes and placed in the Agilent® 2100 Bioanalyzer® instrument. The run protocol can be found in Table 5.

Table 5. A table representing the run protocol executed in order to determine the library concentration.

Stage	**Step**	**Temperature**	**Time**
Hold	Denature	95°C	5 minutes
7 Cycles	Denature	95°C	15 seconds
	Anneal	58°C	15 seconds
	Extend	70°C	1 minute
Hold	–	4°C	<60 minutes

Purification of the library followed by adding and mixing 195 µl of Agencourt® AMPure® XP Reagent beads with each sample. The mixture was then left to incubate for 5 minutes then placed on a magnetic rack for 3 minutes. The supernatant was removed and discarded. 500 µl of 70% ethanol was added to the tube while still on the magnetic rack. The mixture was

incubated for 30 seconds, with turning of the tube to mix the contents. Then the supernatant was removed. The wash was repeated. To remove all remaining ethanol from the tube, while still placed on the rack, the supernatant removed using a 20 µl pipette. The beads were air-dried for 4 minutes. Twenty microlitres of Low TE was added to the tube after being removed from the magnetic rack and vortexed to mix with the beads. The tube was placed back on the magnetic rack for 1 minute and then the supernatant was transferred to a 1.5 ml Eppendorf® tube.

And lastly, the quality of the library was assessed using the Agilent 4200 BioAnalyser® instrument. First, analysis of 1 µl aliquot of the amplified library was done using the Agilent 4200 BioAnalyser® instrument, diluted 1:10, with the Agilent® High Sensitivity DNA Kit.

2.2.8 Template Preparation and Sequencing

Initially, the dilution of sample libraries was done using recommendations based on qPCR quantification. Once library was diluted, 25 µl was added to the Ion Chef™ Library Sample tube and then loaded onto the Ion Chef™ System. The Ion 530 chip cartridge was loaded into the appropriate station. Once the Ion Chef™ run was completed, the chips were sequenced immediately using the Ion S5 sequencing platform: a reagent cartridge, buffer, cleaning solution, and waste container was loaded, the Ion 530 chip followed, and the run was started.

2.3 Sequence Analysis

Torrent Suite software was initially used to obtain preliminary data analysis. Once sequencing was completed, the sequenced raw data was then uploaded to the Ion Suite Software for annotation, filtering using Quantitative Insights into Microbial Ecology (QIIME; Caparoso *et al*., 2010). Reads from all samples were quality filtered using the Q20 quality value (Caparaso *et al*., 2010; Zhang *et al*., 2015); sequences with a mean quality score < 20 were excluded from the analysis, and chimeras were also excluded. Uparse v7.0.1001 was used for species analysis where sequences with ≥97% similarity were classified under the same OTU (Edgar 2013). A sequence of each OTU was selected and the taxonomic information was assigned to each respective read using Greengenes database (DeSantis *et al*., 2006) and Bayesian RDP Classifier (Wang *et al*., 2007). If a read does not receive a result through the BLAST process it is deemed “Unmapped”. Once all mapping was completed, the data was stored in TXT files and uploaded to the Ion Reporter™ Software system where it is accessible by the investigators.

The initial analyses included rarefaction curves, alpha diversity, specifically constructing plot graphs for chao1, Shannon Index, Simpson Index, and Observed Species. Beta diversity was also conducted in Ion Reporter: Bray Curtis, Manhattan, Euclidean, and Chi-Square PCoA graphs were determined and depicted using the Emperor tool (Vasquez-Baeza *et al*., 2013). Krona was used to visually display the consensus of the community structure, as well as the bacterial abundance and diversity found in each hypervariable region (Ondov, Bergman & Phillippy, 2011).

2.4 Statistical Methods

Preliminary sequence analyses were implemented by use of OTU tables, fasta files and abundance graphs determined and downloaded from the Ion Reporter™ Software.

Operational Taxonomic Units were used to cluster the bacterial species identified. From this, the characterisation of the microbiomes (Whittaker, 1972) was accomplished by various tools.

2.4.1 Diversity Analysis

In alpha diversity analysis, the OTU table was rarefied and calculated four metrics: chao1, estimating the total richness of species per sample; Shannon Index, estimating the diversity of species; Simpson Index, measuring the diversity of species while taking into account each species and their relative abundance; and Observed Species which calculates the total number of OTUs present per sample. Rarefaction graphs were generated based on these metrics.

In beta diversity, PCoA graphs were generated based on Bray-Curtis distance calculations, Manhattan distances, Euclidean distances, and Chi-square distance metrics. Three-dimensional graphs were analysed to assess the dissimilarities between species in summer and winter.

Heatmaps were created to show the occurrence of genera and phyla within each sample. This was conducted in RStudio© (Version 1.1.383), using the package “ggplot2”.

2.4.2 Seasonal Analysis

Analyses were conducted in RStudio© (Version 1.1.383), except for the ANOVA and T-tests which were calculated in Microsoft Excel®, in order to statistically and graphically display the data.

Venn diagrams were created using Venny 2.1.0 (Olivieros, 2015) in order to depict the shared and unique bacterial genera present between the samples and seasons.

Analysis of similarities was also determined using the "anosim" command from the "Vegan" package.

Non-metric Multidimensional Scaling (NMDS) was implemented to evaluate the community structure during each season. Additional packages required to statistically analyse and visualise these results include "ggplot2", "permute", "phangorn", "lubridate", "SPECIES", and "phyloseq". Permutational multivariate analysis of variance (PERMANOVA) was employed to test for significant variation in seasonal effects on the microbial composition. The "Vegan" package was used. The significance was set at $p \leq 0.05$. The number of permutations was kept at 999. The Mann-Whitney U-test was implemented to analyse the entire bacteria load (Amato *et al*., 2013) between seasons. In RStudio© the "Wilcoxon" command of the "Vegan" package was employed. The package "plotly" was used to construct the boxplot graphs. Additionally, the Spearman test was performed to assess the correlation between seasons.

2.4.3 Hypervariable Region Comparison

The Kruskal-Wallis test was used to determine any significant differences between the bacterial compositions found within each selected hypervariable region (V2, V3, V4, V6-7, V8, and V9). The Dunn test, a *post hoc* test was conducted on these results to determine the levels of significance between each hypervariable region. Packages installed for this statistical analysis include "FSA", "dplyr" "DescTools", "rcompanion", and "multcompview". The package "plotly" was used to construct the boxplot graphs.

2.5 Ethical Clearance

Ethics was approved by the University of Pretoria Animal Ethics Committee (reference ec012-16) and the NZG Research Ethics and Scientific Committee (reference number P16/17). Approval was given to collect samples at Roodeplaat Dam Nature Reserve by the Gauteng Province Department of Agriculture and Rural Development (GDARD; reference number RDNR 012017). Section 20 of the Animal Diseases Act of 1984 (Act no. 35 of 1984) was approved by the Department Agriculture, Forestry and Fisheries (Reference number 12/11/1/1/18).

Chapter 3

Results

3.1 Bacterial Community Composition

The individuals trapped from the NZG site during summer had an average body mass of 199.6g, while the winter individuals at the same site had a mean body mass of 194.0 ± 33.36g. The two individuals sampled at RDNR (RDNR 1 and RDNR 2; Appendix 2) had an average body weight of 142.1 ± 42.87g (Appendix 2). It was observed and documented by the certified veterinarian present during all sampling sessions, that NZG individuals suffered from oral ailments (such as broken canines and periodontitis) more so than the rural individuals (Appendix 2).

A total of 10 DNA samples were sequenced. A total of 13,860,966 amplicon sequences were generated. The total number of valid reads (reads present after and base pair cut-off and primer trimming; ThermoFisher Scientific) for all ten samples was 9,014,929, with 6,384,934 reads mapped (reads with a successful identification after BLAST mapping; ThermoFisher Scientific) and 515,768 reads unknown (7% of the total samples). Sequence length averaged 223 bp for all samples after filtering and trimming of erroneous reads.

At the phylum level, *Proteobacteria* was the dominant phylum comprising of 28% of all samples. *Firmicutes* were present in 24% of all the samples and, *Bacteriodetes* and *Actinobacteria* constituted 19% each of the combined sample population. *Chloroflexi* accounted for 0.9% of all samples, phyla *Fusobacteria* and *Tenericutes* contributing to 0.3%, *Spirochaetes* 0.2% of all samples, and finally, *Cyanobacteria* contributing 0.05% (Figure 7).

A total of 81, 115, 77 and 127 OTUs were obtained for the summer (S) samples S1, S2, S3, and S4, respectively, collected from the NZG site. A total of 152, 137, 123, and 196 OTUs for the respective winter (W) samples, W1, W2, W3, and W4, collected at the same site. A total of 161 and 81 OTUs were found in samples RDNR1 and RDNR2, respectively.

A Venn diagram (Fig. 8) was used to depict the shared and unique genera found between the summer samples. S1 contained only one unique OTU, *Aerococcus*. S2 contained 9 unique OTUs, namely, *Cellulomonas, Butyricimonas, Odoribacter, Eubacterium, Anaerostipes, Robinsoniella, Megasphaera, Victivallis,* and *Gemmiger*. S3 harboured only one unique

OTU, *Sebaldella*. S4 comprised of unique genera, including *Flavobacterium, Trichococcus, Bavarricoccus, Peptoniphilus, Ruminococcus, Peptococcus, Rhizobium, Pseudorhodoferax,* and *Cronobacter*. 11 genera were shared between all four samples, including *Bifidobacterium, Collinsella, Bacteroides, Sarcina, Butyrivibrio, Megamonas, Sutterella, Campylobacter, and Helicobacter*. 5 OTUs were shared between S2 and S4. Three bacterial genera were identified in both S2 and S3 samples: *Fusobacterium, Bilophila*, and *Acinetobacter*. S1, S2, and S3 shared no OTUs, while S1, S2 and S4 shared 5 OTUs: *Varibaculum, Corynebacterium, Clostridium, Blautia*, and *Flavonifracter*. Only one OTU was found in S2, S3, and S4: *Brachyspira*.

Fig. 9 displays the shared and unique bacterial genera found within and between the winter samples (W1–W4). Table 6 illustrates the OTUs shared between all winter samples. These including *Bifidobacterium, Bacteroides, Streptococcus, Clostridium, Sarcina, Blautia, Butyrivibrio, Megamonas, Sutterella, Campylobacter,* and *Helicobacter*. W1 contains 6 unique OTUs: *Aerococcus, Weissella, Turicibacter, Photorhabdus, Rickettsiella*, and *Nicoletella*. *Granulicatella, Filifactor, Serratia*, and *Acinetobacter* are only found in W2. W3 harbours *Singulisphaera, Plesiomonas*, and *Haemophilus*. In W4 22 genera are found. These include *Barnesiella, Odoribacter, Enterococcus, Vagococcus, Lactobacillus, Peptococcus, Peptoclostridium, Peptostreptococcus, Faecalibacterium, Phascolarctobacterium, Veillonella, Rhizobium, Desulfovibrio, Escherichia, Shigella, Klebsiella, Raoultella, Yokenella, Ignatzschineria*, and *Brachyspira*. W1 and W2 share only one bacterium: *Stomatobaculum*. W1 and W3 share *Avibacterium*. W3 and W4 share *Trichococcus* and *Ruminococcus*. *Neisseria* is shared only between W2 and W3. W2 and W4 have no common genera. W1, W2, and W3 share 6 OTUs: *Collinsella, Olsenella, Megasphaera, Actinobacillus, Mannheimia*, and *Pasteurella*. W2, W3, and W4 share only one genus: *Fusobacterium*. W1, W2, W4 share four genera including *Gemella, Abiotrophia, Lactococcus*, and *Bilophila*; and *Corynebacterium, Parabacteroides*, and are shared between W1, W3 and W4.

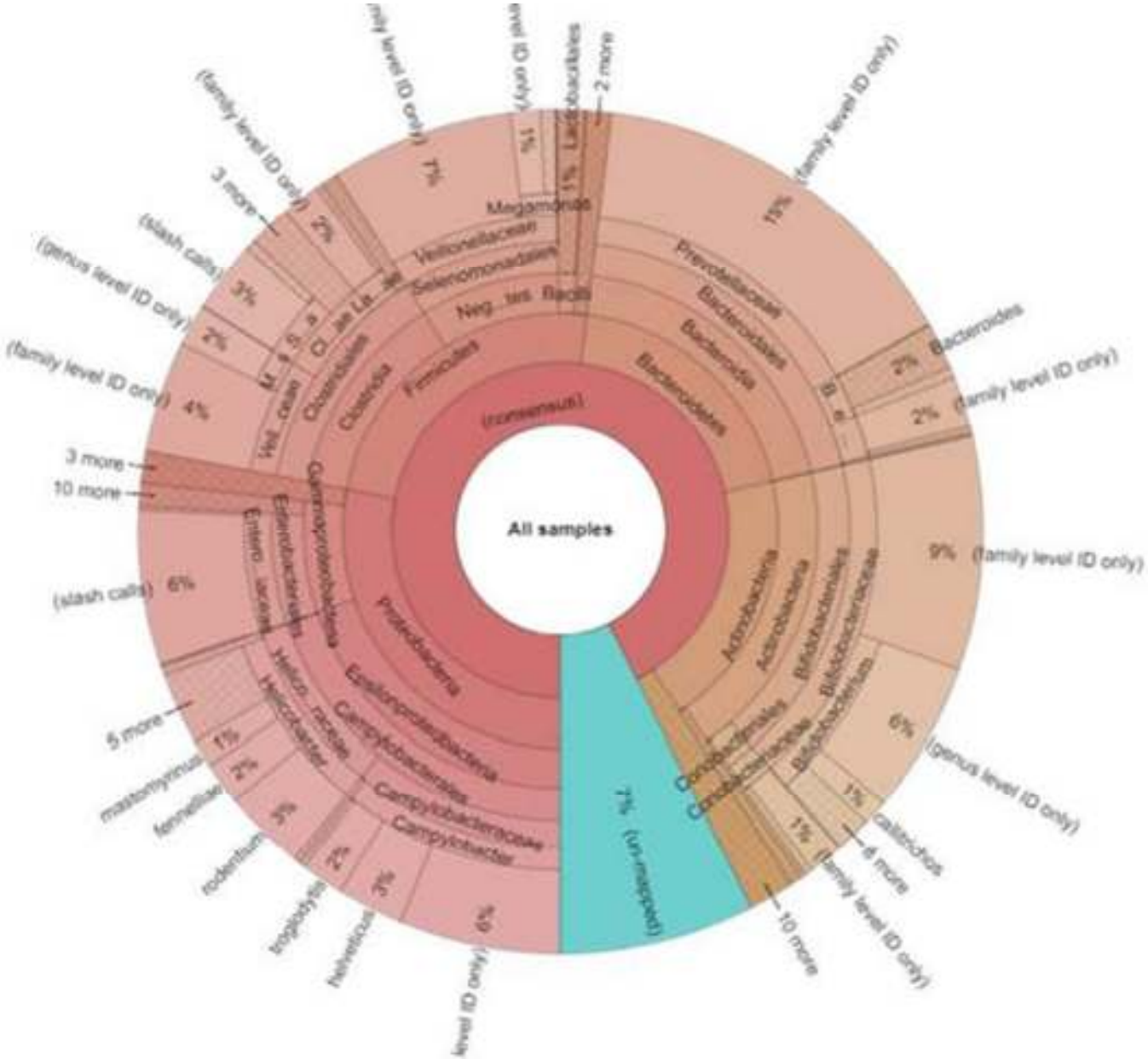

Figure 7. A pie chart demonstrating the array and abundance of bacterial species present within all samples sequenced. This chart displays taxonomic nomenclature from Phylum name to Family, and if possible, up to species name. The blue shading indicates the sequences that were unable to be identified.

From the Roodeplaat site (Appendix 3), samples shared 21 OTUs including *Bifidobacterium, Collinsella, Bacteroides, Parabacteroides, Lactobacillus, Leuconostoc, Streptococcus, Clostridium, Sarcina, Blautia, Butyrivibrio, Stomatobaculum, Ruminococcus, Megamonas, Flavonifractor, Bartonella, Sutterella, Campylobacter*, and *Helicobacter*. However, RDNR2 only contained two unique genera: *Abiotrophia* and *Pediococcus*; while RDNR1 harboured 20 unique OTUs: *Varibaculum, Barnesiella, Odoribacter, Alistipes, Enterococcus, Weissella,*

Lachnoclostridium, Roseburia, Ruminococcus, Peptococcus, Fusobacterium, Parasutterella, Bilophila, Desulfovibrio, Cronobacter, Enterobacter, Eschericia/Shigella, Pantoea, Salmonella, and *Trabulsiella*.

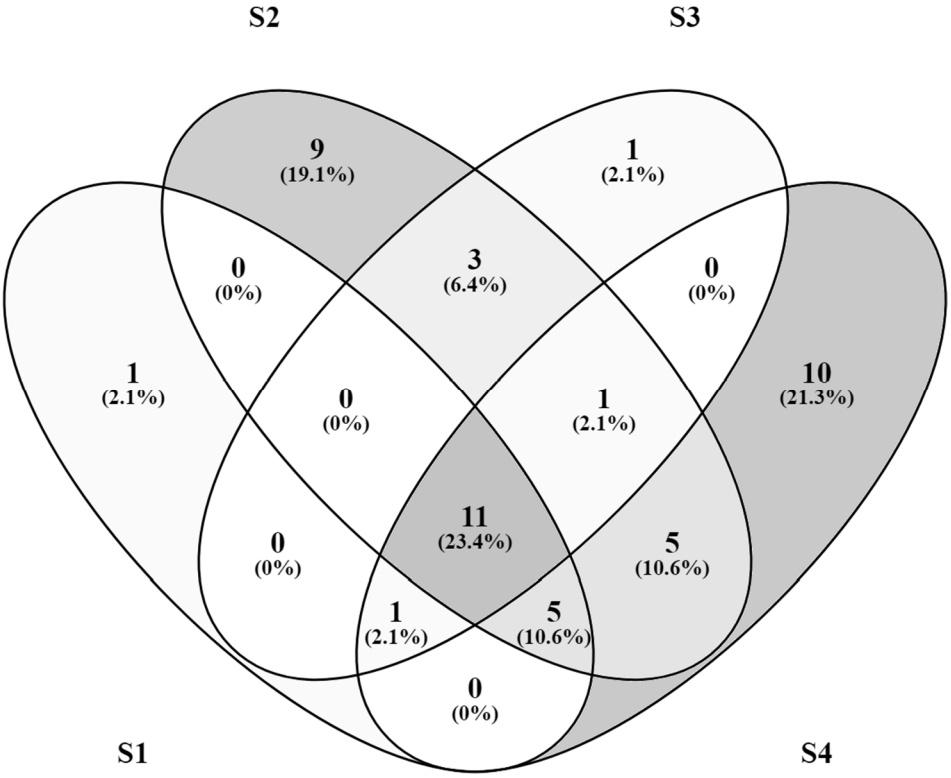

Figure 8. A Venn Diagram displaying the OTUs shared between the summer samples (S1, S2, S3, and S4) and the OTUs found uniquely to the specific Sample. The darker shading indicating a greater number of OTUs. This was conducted at Genus level. The numbers inside the diagram indicate the numbers of OTUs.

3.1.1 Species Present

A total of 151 species were identified from all samples (Appendix 10 & Appendix 14). One-hundred and thirty-six species were present in the individuals from the NZG site, while 64 species were identified from RDNR. *Campylobacter helveticus* was the most predominant species identified, it was present in all individuals with a total of 327,429 reads (24,7% of the total reads). *Helicobacter rodentium* (16.4%), *Campylobacter troglodytis* (8.5%), *H. fennelliae* (8.4%), *H. mastomyrinus* (5.4%), *Bifidobacterium callitrichos* (4.9%), *B. merycicum* (3.9%), *H. rappini* (3.8%), *Megamonas hypermegale* (3%), *Sutterella* sp. (1,9%), *B. angulatum* (1%), *B. cuniculi* (0.6%), and *B. reuteri* (0.4%) were present in all samples (Appendix 14).

Interestingly, *Sarcina ventriculi* (2%), *Streptococcus sanguinis* (0.4%), *S. cristatus* (0.07%), *S. alactolyticus* (0.01%), *S. infantarius* (0.005%), and *Stomatobaculum longum* (0.28%) were only present in the winter period. Inversely, *Campylobacter cuniculorum* was only found in the summer individuals at the urban site (NZG; Appendix 14). Curiously, members of *Lactobacillus* were predominantly seen in Individual W4 of the NZG site and overall present only in the winter period. *Bartonella rattaustraliani* was present in the RDNR individuals only. *Ruminococcus* members *R. gnavus* and *R. torques* constituted a total of 0.2% only.

3.2 Seasonal comparison at the NZG

In summer, the samples comprised a total of 5,148,378 reads. 3,838,983 of these were valid reads, 2,746,359 mapped, and 193,992 unmapped sequences. In winter, the samples had a total of 5,040,877 reads. 3,701,036 of these were valid, 2,633,465 mapped, and 41,104 unmapped. Fig. 10 visually depicts the genera present within each sample during summer (S1-S4) and winter (W1-W4), with the genera stacked and their level of abundance depicted by the amount of shading.

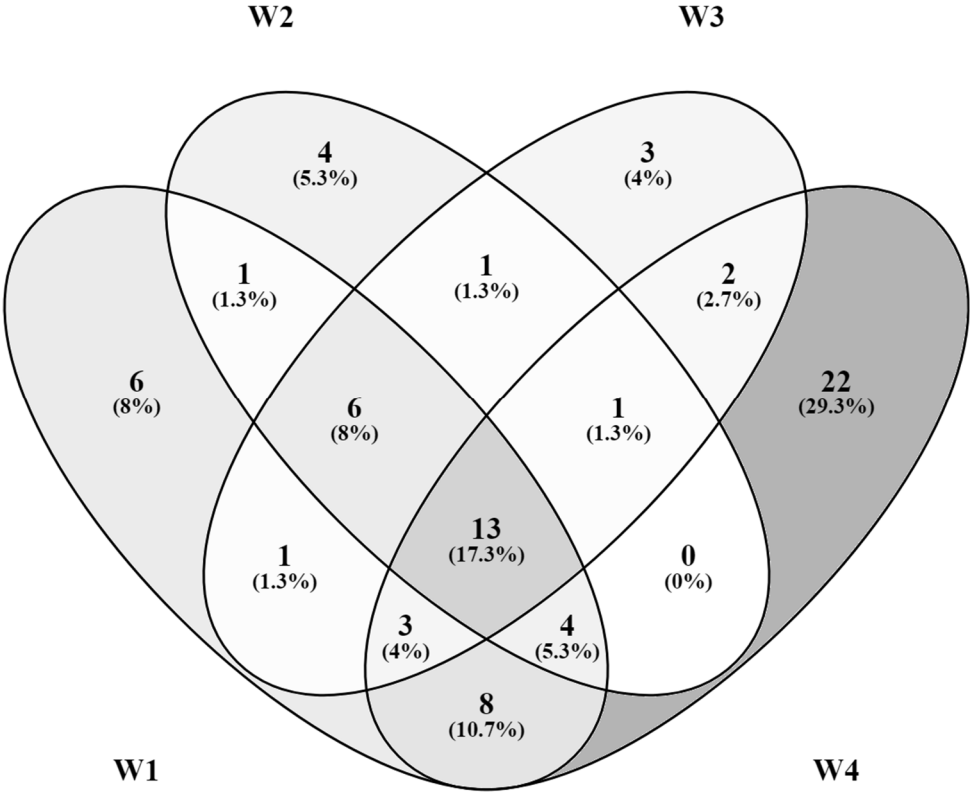

Figure 9. Venn Diagram describing the proportions of OTUs found between the winter samples (W1, W2, W3, and W4). The darker shading indicates the ascending number of samples. This figure describes Genus OTU level. The numbers inside the diagram indicate the numbers of OTUs.

Fig. 11 demonstrates the unique and shared bacterial genera present between both seasons. *Campylobacter* is the most prevalent genus (42.3%) present in summer, followed by *Helicobacter* (32.3%), *Bifidobacterium* (11.8%), *Bacteroides* (5%), *Megamonas* (4.9%), and *Collinsella* (1%; Fig. 10). In contrast in winter, *Bifidobacterium* is the most abundant at 32.2% of the total composition; *Sarcina* is also a dominant genus present with 20.5% of the total. *Megamonas* (13.3%), *Campylobacter* (11%) and *Helicobacter* (5.4%) are also abundant. Other more common genera in winter include *Sutterella* (3.2%), *Bacteroides* (3.1%), *Lactobacilli* (3%), *Clostridium* (2%), *Fusobacterium* (1.5%), and *Streptococcus* (1.5%; Fig. 10). All other genera account for less than 1% of the total in winter.

This Venn diagram highlights the differences in diversity between seasons (Figure 11); the winter samples have retained 39 unique bacterial genera, while in summer only fourteen unique OTUs are present. The shared genera include *Bifidobacterium, Bacteroides, Enterococcus, Lactobacillus, Clostridium, Sarcina, Fusobacterium, Campylobacter,* and *Helicobacter*. In winter, there were 39 unique genera (Figure 11). These include *Streptococcus, Faecalibacterium, Escherichia, Shigella, Plesiomonas, Salmonella,* and *Pasteurella*. In summer the unique genera include *Flavobacterium, Robinsoniella, Sebaldella*, and *Pseudomonas* (Table 6).

Between both seasons, *Proteobacteria, Firmicutes, Bacteroidetes*, and *Actinobacteria* were the most predominant phyla, whereas the other phyla accounted for less than 5% of the total composition. In summer, it can be seen that *Proteobacteria* was the dominant phylum as it constitutes 36% of the total reads, *Firmicutes* only accounting for 19%, and *Bacteroidetes* 17% (Fig. 12). Conversely in winter, *Firmicutes* was the most prevalent phylum being 33% of the total, while *Proteobacteria* is reduced to 24%, and *Bacteroidetes* lowest at 19% (Fig. 12). There was no change in the composition of *Actinobacteria* which accounted for 20% in both seasons, with the other phyla (*Chloroflexi*, *Chlamydiae, Lentisphaerae, Fusobacteria, Deinococcus-Thermus, Cyanobacteria, Planctomycetes, Spirochaetes, Synergistetes,* and *Teniricutes)* accounting for the residual volume.

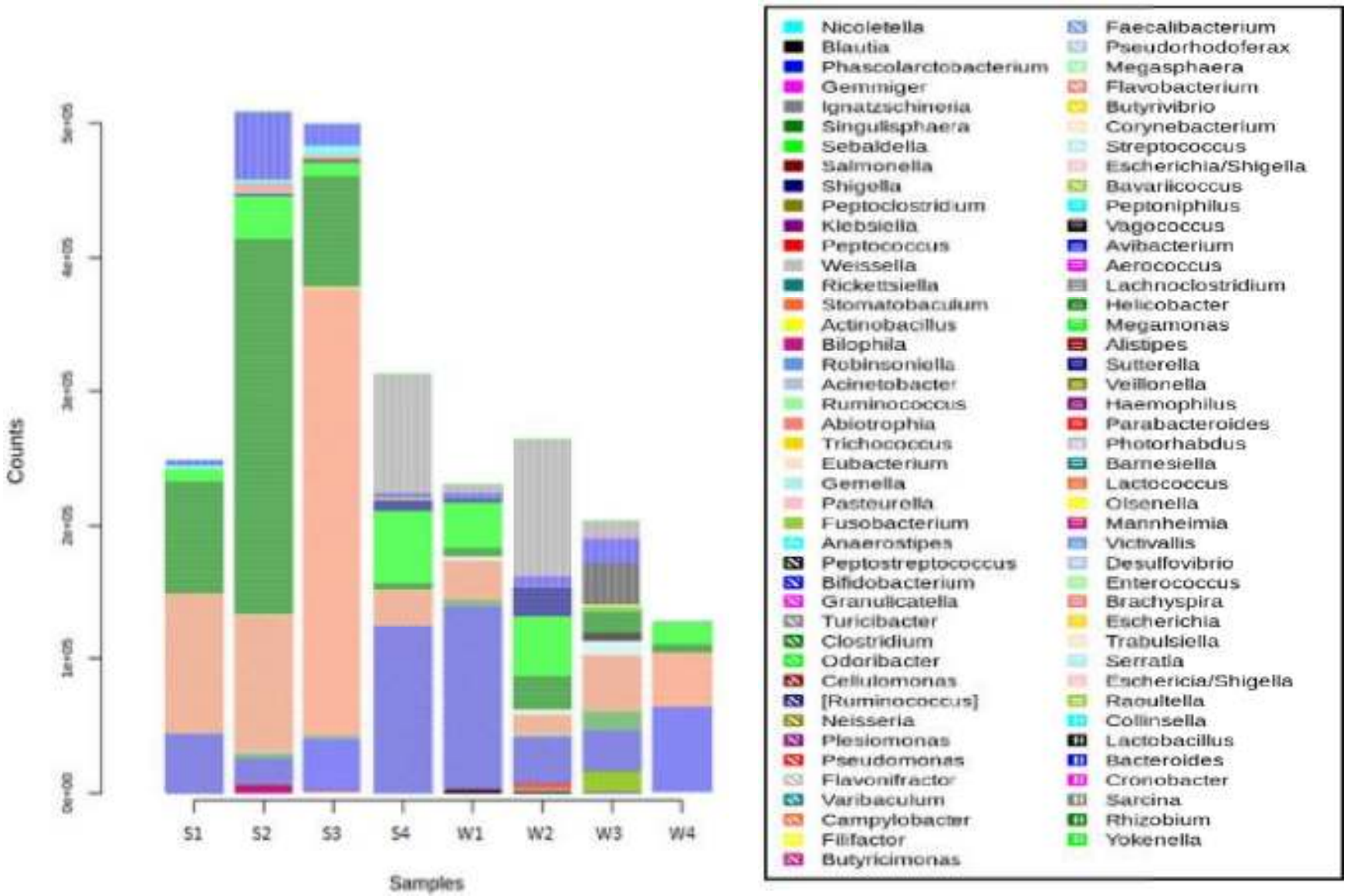

Figure 10. OTUs bar chart indicating the abundance and diversity of OTUs present in each sample of summer (S1-S4) and winter (W1-W4). This graph is based on the genus level.

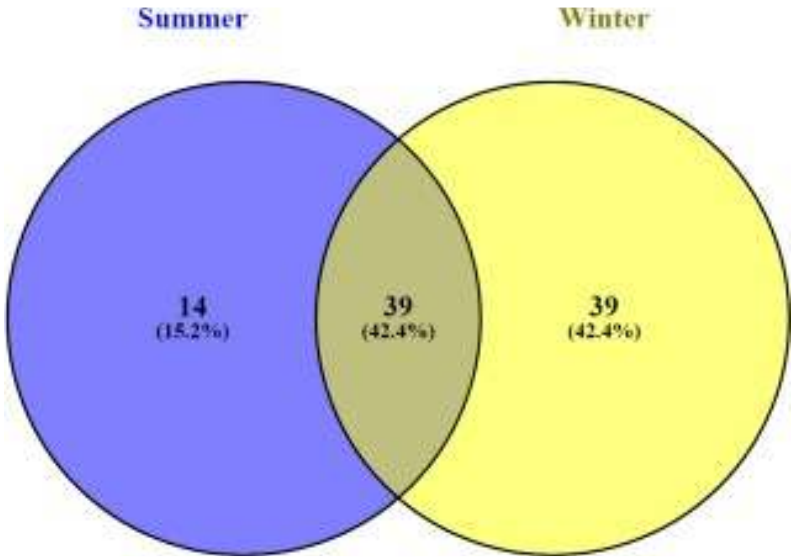

Figure 11. A Venn Diagram describing the differences in bacterial composition in both seasons at the NZG site. The numbers inside the diagram indicate the numbers of OTUs present.

Table 6. A table illustrating the total genera found within each season (winter and summer) at the NZG site (W1-W4; and S1-S4) during the winter period. Shared genera between both sites are also shown.

Winter	Summer	Shared
Barnesiella, Gemella, Abiotrophia, Granulicatella, Vagococcus, Weissella, Lactococcus, Streptococcus, Parvimonas, Filifactor, Peptoclostridium, Peptostreptococcus, Faecalibacterium, Turicibacter, Phascolarctobacterium, Veillonella, Singulisphaera, Methylobacterium, Neisseria, Escherichia, Shigella, Klebsiella, Photorhabdus. Plesiomonas, Raoultella, Salmonella, Serratia, Trabulsiella, Yokenella, Rickettsiella, Actinobacillus, Avibacterium, Haemophilus, Mannheimia, Nicoletella, Pasteurella, Ignatzschineria.	*Cellulomonas, Propionibacterium, Butyricimonas, Flavobacterium, Peptoniphilus, Anaerostipes, Lachnoclostridium, Robinsoniella, Allisonella, Sebaldella, Victivallis, Gemmiger, Pseudorhodoferax, Pseudomonas*	*Varibaculum, Corynebacterium, Bifidobacterium, Collinsella, Olsenella, Slackia, Bacteroides, Odoribacter, Parabacteroides, Alistipes, Aerococcus, Trichococcus, Bavariicoccus, Enterococcus, Lactobacillus, Clostridium, Sarcina, Eubacterium, Blautia, Butyrivibrio, Stomatobaculum, Ruminococcus, Peptococcus, Megamonas, Flavonifractor, Megasphaera, Fusobacterium, Rhizobium, Sutterella, Bilophila, Desulfovibrio, Campylobacter, Helicobacter, Cronobacter, Acinetobacter, Brachyspira.*

Chao1, Simpson and Shannon Index, and Observed Species plot graphs were based on 97% similarity. It can be seen the species richness in winter to be greater when calculating the mean estimates for each season (summer = 51.37 ±13.92; winter = 61.9 ±33.9; Table 6) and is supported by their respective graphs (Appendix 7). Table 7 demonstrates the individual values for each sample number (S1–S4 and W1–W4).

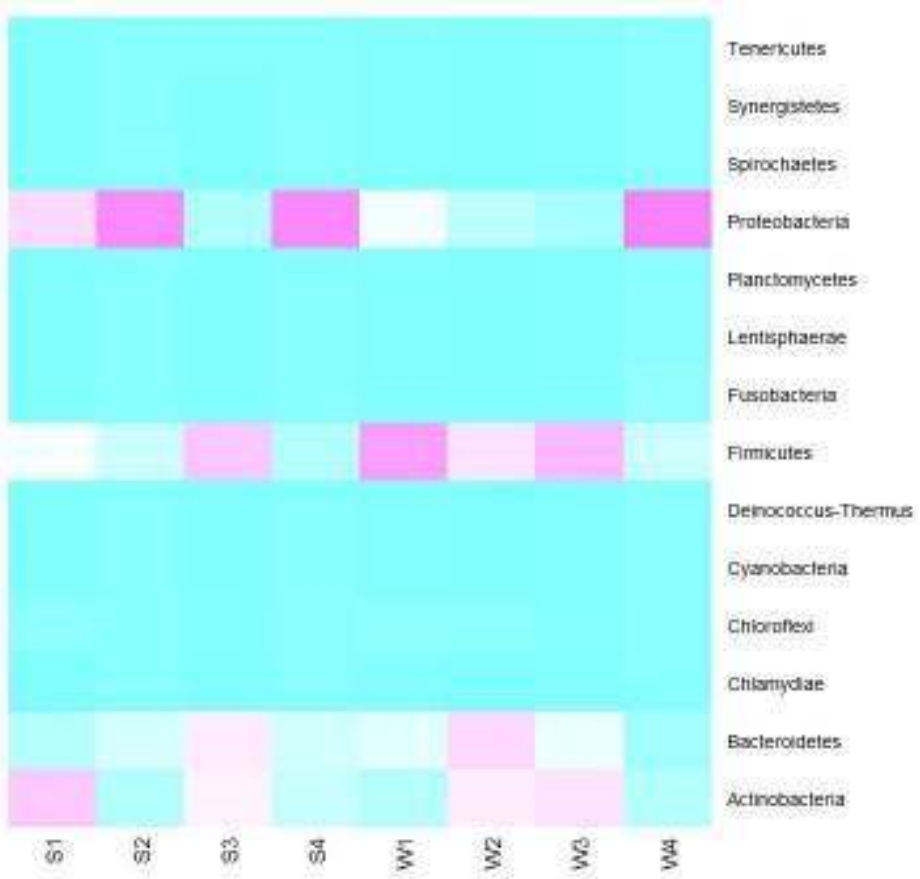

Figure 12. A heatmap representing the abundance of phyla present within each sample. This heatmap has generated values from the Phylum level. The blue shading indicates less abundance, and the red shading displays higher abundance.

Three-dimensional Principal Coordinates Analysis (PCoA) of Bray Curtis similarity matrix of samples collected during summer and winter showed clustering of the samples of the two seasons – summer samples S1, S2 and S4 grouped by PC3, W1, W2, and W3 grouped together by PC1. Interestingly, S3 and W4 grouped together separate of the two groups (Fig. 13).

NMDS ordination plot graph based on Bray Curtis dissimilarity matrix (Fig. 14) demonstrates the variation and clustering of the samples within the seasons in terms of their similarity to the bacterial genera present. A stress value of 0.13 indicates a good line of fit as

demonstrated in the Shepard graph (which demonstrates the stress values for each sample; Appendix 8). Two-way Bray Curtis ANOSIM between the seasons showed somewhat significant dissimilarity between the groups (R statistic = 0.5208; Significance = 0.061). Conversely, Adonis command described that a *p*-value of 0.051 indicates that at an alpha of 0.05, the grouping of individuals is somewhat significant; additionally, R^2 indicates that only approximately 33% of the variation in distances is explained by the grouping. Thus supporting the results achieved by ANOSIM.

The Mann-Whitney U test, after 999 permutations, described only a slight significance (*W*=2535.5; *Pr*>*F*=0.0006; as shown in Fig. 15) between the community structures of each season, and is supported by the results found in the ANOSIM analysis. Owing to the limited outcomes of the PERMANOVA, the Spearman test was performed which indicated a weak significance in the correlation between the samples from each season (*S*=73775; *p*=0.0096; ρ=0.279). T-tests were performed on the estimates of each season, there was no significant difference (T-test=0.12; *p*<0.05).

Table 7. A table showing the mean estimates of richness and diversity of all respective samples for both seasons based on Chao1 abundance, observed species, Shannon diversity, and Simpson diversity. There were no significant differences between the estimates of the two seasons (t-test= 0.12; *p*>0.05). Rarefaction plot graphs were constructed in correspondence (Appendix 12).

Sample	Chao1	Observed Species	Shannon	Simpson
S1	14.76 ± 3.92	14.74 ± 3.98	1.87 ± 0.13	0.67 ± 0.03
S2	29.12 ± 8.43	28.88 ± 8.48	2.03 ± 0.19	0.63 ± 0.02
S3	12.64 ± 5.17	12.60 ± 5.26	1.66 ± 0.12	0.63 ± 0.02
S4	28.56 ± 8.01	28.35 ± 8.31	1.64 ± 0.13	0.51 ± 0.01
W1	36.77 ± 10.21	36.55 ± 10.68	2.69 ± 0.29	0.78 ± 0.04
W2	25.34 ± 7.78	25.12 ± 8.15	1.95 ± 0.26	0.59 ± 0.04
W3	25.80 ± 6.73	25.56 ± 7.11	2.14 ± 0.13	0.72 ± 0.03
W4	45.36 ± 15.43	45.06 ± 16.00	3.33 ± 0.44	0.87 ± 0.05

3.3 Habitat Comparison

From a total of two samples (RDNR 1 and RDNR 2), there was a total of 2,013,160 reads (1,474,910 reads valid) with 996,630 mapped, and 60,655 unknown reads. RDNR 1 a total of 896,164 reads. RDNR 2 consisted of 1,116,996 total reads. *Bacteroidetes* dominating with 34% of the total bacteria present. *Firmicutes* at 23%, *Proteobacteria* 20%, *Actinobacteria* 16%, while the remaining phyla constituting 1%. 6% of the bacterial species were unknown.

NZG had a total of 5,040,877, 3,701,036 valid. 2,633,465 mapped, 41,104 unmapped. *Firmicutes* 33%, *Proteobacteria* 24%, *Actinobacteria* 20%, *Bacteroidetes* 19%, other 2%. Unmapped 2%.

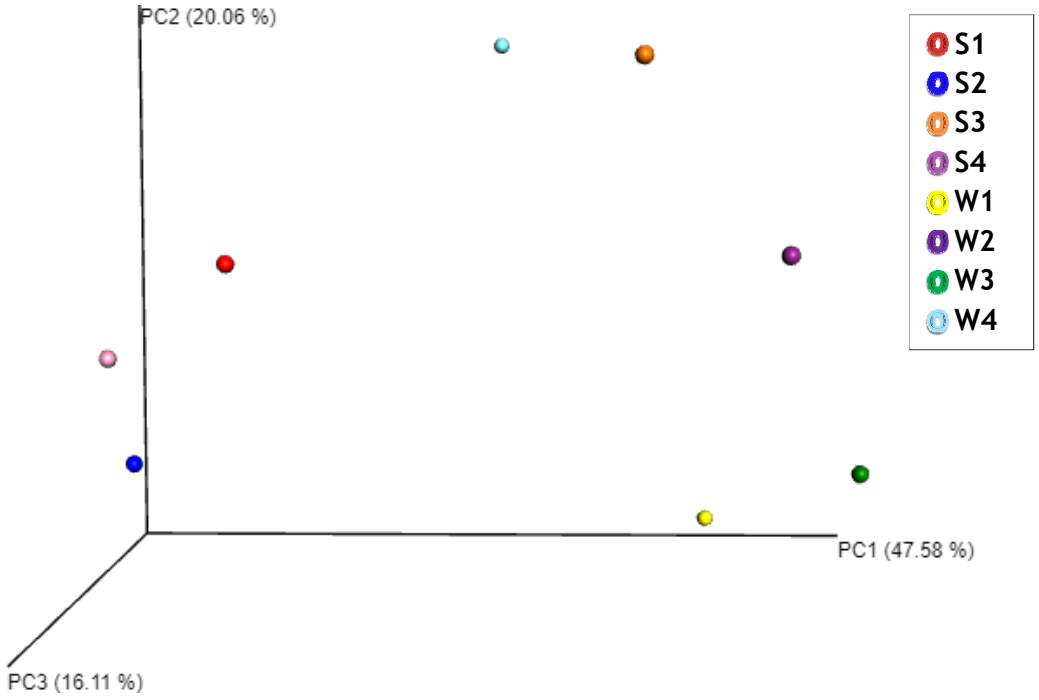

Figure 13. Bray Curtis PCoA graph describing the dissimilarities between the summer (S1-S4) and winter (W1-W4) samples. Note the grouping of S1, S2 and S4 close to PC3, and W1, W2, and W3 clustered together at PC1, and the outliers S3 and W4 grouped together.

The differences in composition between the NZG and RDNR samples were also compared in a Venn Diagram (Appendix 4). Between the two sites, 37 samples are shared (Table 8). These include *Bifidobacterium, Bacteroides, Lactobacillus, Fusobacterium, Bilophila, Campylobacter, Helicobacter, Escherichia,* and *Salmonella*. In the RDNR samples, eight genera are unique including *Bartonella* and *Enterobacter*. In the NZG samples, 41 are unique (Table 8) including *Faecalibacterium, Plesiomonas, Yokenella, Haemophilus, Pasteurella,* and *Acinetobacter*.

Owing to a small sample size for the RDNR site, statistical analyses were deemed redundant and no further in-depth analyses could be performed.

Table 8. A table illustrating the total genera found within each site (NZG and RDNR) during the winter period. Shared genera between both sites are also shown.

NZG	RDNR	Shared
Corynebacterium, Olsenella, Slackia, Gemella, Aerococcus, Granulicatella, Trichococcus, Bavariicoccus, Vagococcus, Lactococcus, Parvimonas, Eubacterium, Filifactor, Peptoclostridium, Peptostreptococcus, Faecalibacterium, Turicibacter, Phascolarctobacterium, Megasphaera, Veillonella, Singulisphaera, Methylobacterium, Rhizobium, Neisseria, Klebsiella, Photorhabdus, Plesiomonas, Raoultella, Serratia, Yokenella, Rickettsiella, Actinobacillus, Avibacterium, Haemophilus, Nicoletella, Pasteurella, Acinetobacter, and *Ignatzschineria*	*Pediococcus, Leuconostoc, Lachnoclostridium, Roseburia, Bartonella, Parasutterella, Enterobacter,* and *Pantoea*	*Varibaculum, Bifidobacterium, Collinsella, Bacteroides, Barnesiella, Odoribacter, Parabacteroides, Alistipes, Abiotrophia, Enterococcus, Lactobacillus, Weissella, Streptococcus, Clostridium, Sarcina, Blautia, Butyrivibrio, Ruminococcus, Stomatobaculum, Peptococcus, Megamonas, Flavonifractor, Fusobacterium, Sutterella, Bilophila, Desulfovibrio, Campylobacter, Helicobacter, Cronobacter, Eschericia, Shigella, Salmonella, Trabulsiella, Mannheimia*, and *Brachyspira*

3.4 Hypervariable Region Analysis

V3 was responsible for the most with 41%, V8 comprised of 16% of the total consensus, and V4 and V6/7 accounting for 15% respectively (Appendix 11). V2 and V9 hypervariable regions contributed the least number of taxa, both accounted for 3% each of the total consensus (Appendix 11). Kruskal-Wallis and *post hoc* Dunn test showed significant differences between various hypervariable regions (chi sq.= 68.9, p=1.74e^{-13}; Fig. 16).

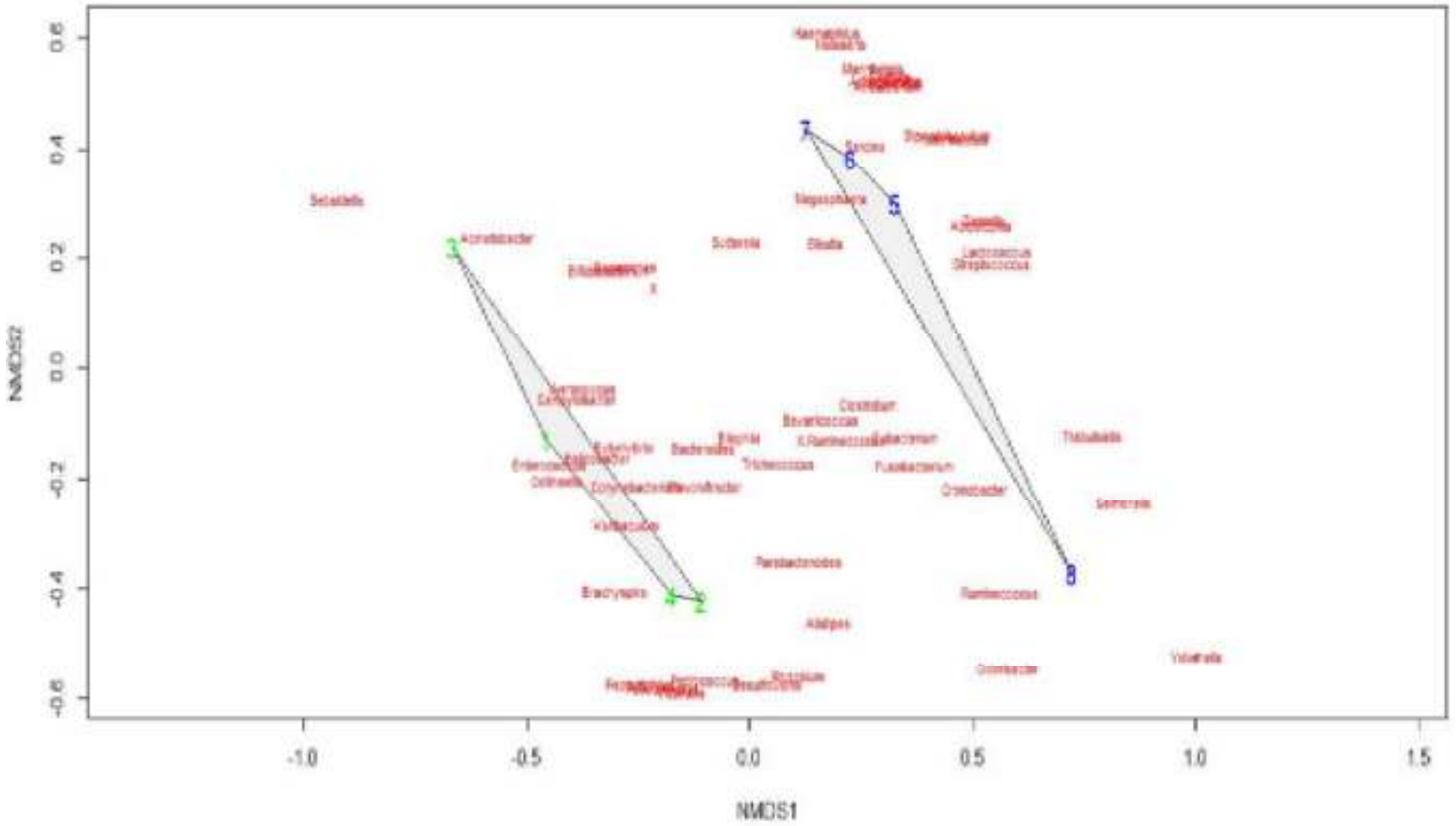

Figure 14. NMDS ordination plot graph indicating the samples (1 – 8) and their distances from each other in relation to the common bacterial genera. The shaded regions visualising the similarity in distances of samples of seasons. Green numbers identify the summer samples, and blue indicate winter samples.

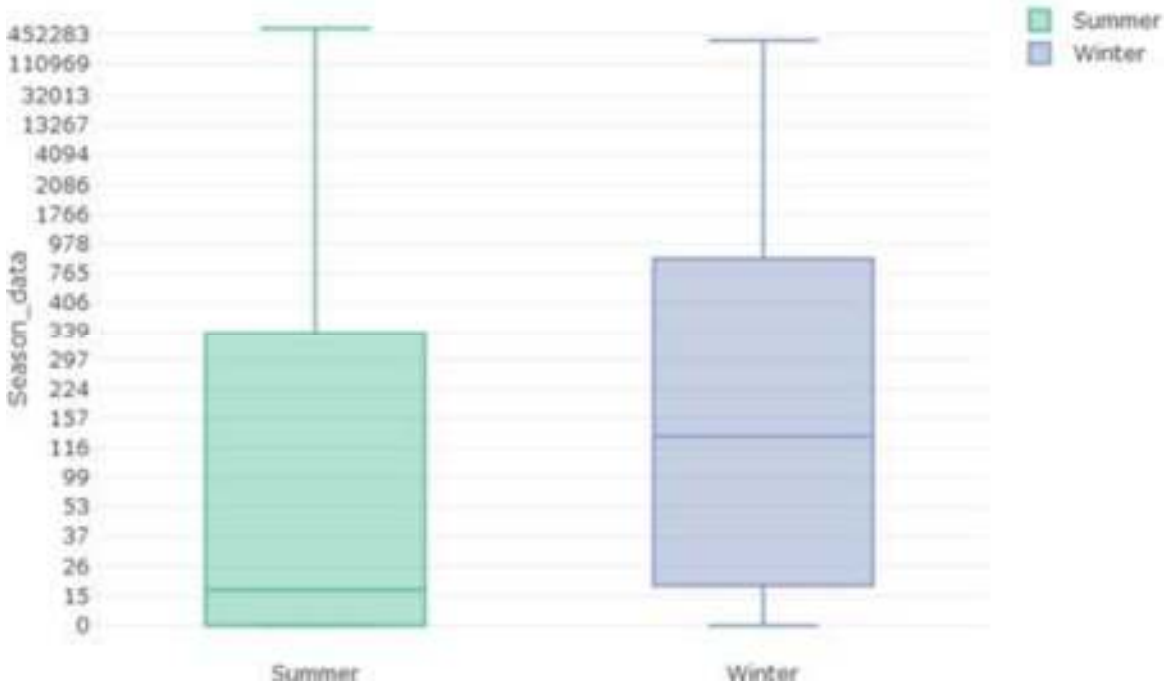

Figure 15. A box plot demonstrating the variations between seasons based off Mann-Whitney U test indicating a 0.1 level of significance between the seasons (W=2535.5; p=0.057)

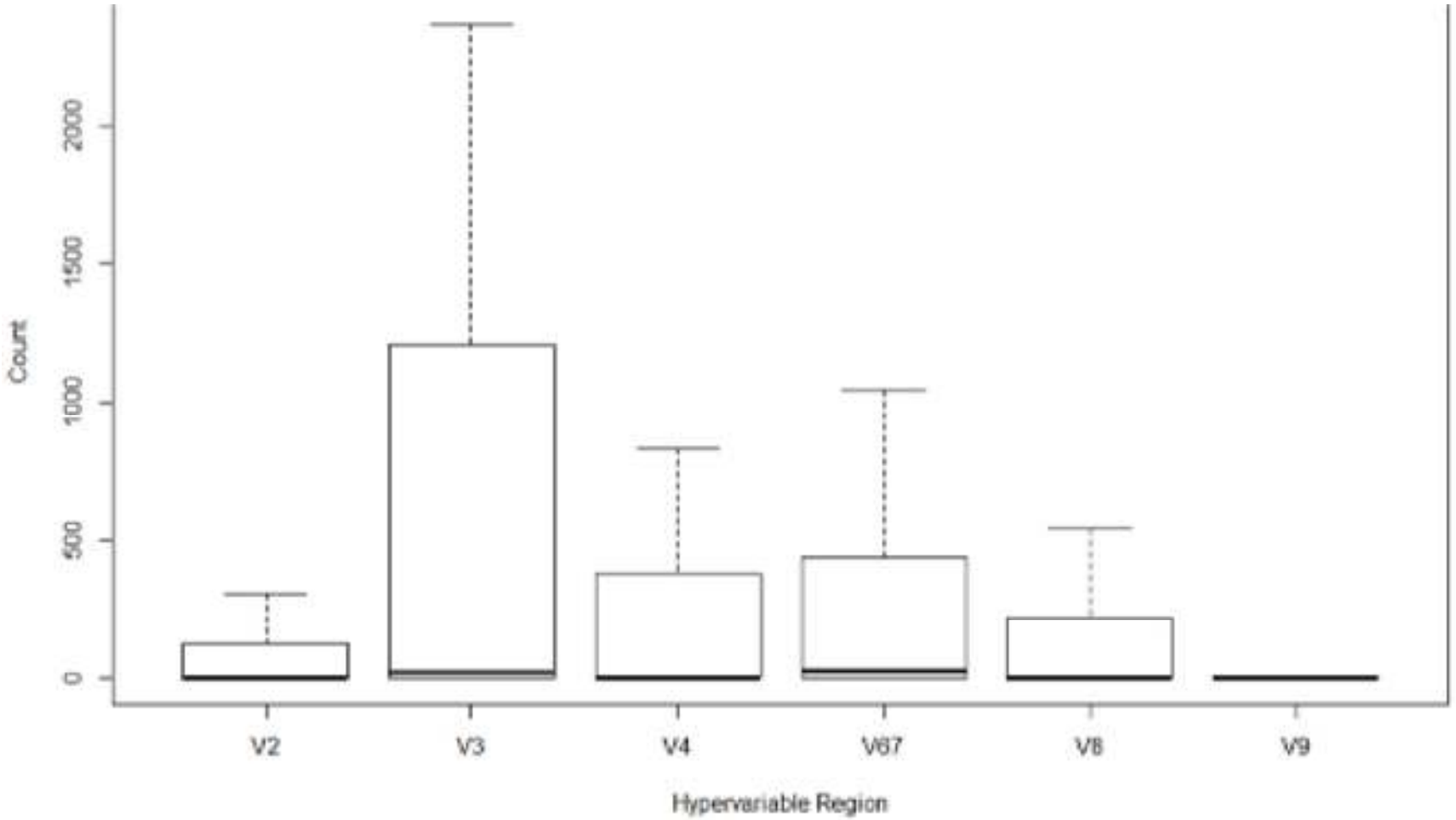

Figure 16. A boxplot representing the variations between the total read counts from each sample present within the various hypervariable regions (V2, V3, V4,V6/7,V8,V9) based on Kruskal-Wallis test and *post hoc* Dunn Test.

Chapter 4

Discussion

With an increasing awareness of anthropogenic impacts on primate population declines, researchers are now focusing on the human-associated stressors attributing to these species losses, such as climate change, urbanisation, and habitat fragmentation (Wallis & Lee, 1999; Gillespie *et al.*, 2005; Lee & Priston, 2005; Pederson *et al*., 2005; Davies & Pederson, 2008; Barreiro *et al*., 2010; Young *et al*., 2013; Barelli *et al.,* 2015; Stumpf *et al*., 2016; Estrada *et al*., 2017). With the increasing proximity of humans to non-human primates, the risk of disease transmission rises dramatically (Muriuki *et al*., 1998; Wolfe *et al*., 1998; Legesse & Erko, 2004; Goldberg *et al*., 2007; Davies & Pederson, 2008; Pederson & Davies, 2009; Pourrut *et al*., 2011; Mossoun *et al*., 2015). This study set out to determine whether gut microbiome composition is altered in response to seasonal changes (diet) and differences in lifestyle (urban and rural). The results indicate that diet has a major effect on the gut microbial flora and corresponds with previous studies (Gemmill & Gould., 2008; Moschen *et al.,* 2012; Carey *et al*., 2013; Williams *et al*., 2013; Amato *et al*., 2014; David *et al*., 2014; Banskar *et al*., 2016; Sharp *et al*., 2017). Additionally, there appears to be a marked difference in microbial diversity and abundance between urban and rural environments, as also seen in Mueller *et al*. (2006). The results highlight the importance of feeding ecology and form baseline information for understanding the relationship between *Galago moholi* and gut microbes during seasonal fluctuations.

4.1 Effects of Seasonal Changes

The results revealed that seasonal changes play a fundamental role in shaping the gut microbial composition. Previous studies have shown a strong association of diet to the gut microbial community structure (Muegge *et al.* 2011; Wu *et al*. 2011). The 16S rRNA sequencing results have revealed that *G. moholi* had a marked increase in diversity when shifting to a winter diet. The changing seasonal dietary choices including a predominantly insectivorous diet in summer and largely gum exudates in winter can be seen in the varying composition of bacterial species identified in the individuals. The data generated in this study partially agreed with past studies regarding the high abundance of *Firmicutes* in primates. This was true for this study in winter as *Firmicutes* were the dominant phylum accounting for 33% of the total reads. However, in summer *Firmicutes* accounted for only 19% of the total

reads. *Bacteroidetes* accounted for the lowest portion of the total reads: only 19% in winter and 17% in summer. *Bacteroidetes* and *Firmicutes* have been shown to dominate the gut microbiomes of most primates studied (Eckburg *et al*. 2005; Turnbaugh *et al*. 2006; Uenishi *et al.* 2007; Ochman *et al*. 2010; Yildirim *et al*. 2010; Xu *et al.* 2015). These findings could be indicative of a diet containing little plant matter, as would be expected for this species.

It is necessary for insectivores to breakdown the hard, external skeleton of insects in order to achieve the necessary nutritional requirements, without excessive energy expenditure. Chitinase-producing bacteria, for instance members of *Bacillus* and *Serratia* genera, present in the gut of several insectivorous bat species are needed to assist in the digestion of the tough exoskeleton of insects (Whitaker Jr. *et al.,* 2004). In contrast to the study by Whitaker Jr. *et al.* (2004), these specific bacteria were found in very low abundance in the individuals of this study. Interestingly, Kay & Shein (1979) revealed that *Galago senegalensis* digested between 2% and 25% of chitin, most likely by chitin-producing bacteria. From the results of the present study, the possible use of other bacteria to assist in the digestion of insects and the fermentation of gum exudates is apparent is *G. moholi*. The phylum *Proteobacteria* comprised the vast majority of the phyla in each sample. Specifically, members of *Epsilonproteobacteria* were most common. In the summer season *Campylobacter* was the dominant genus, specifically *Campylobacter helveticus*, and was identified in all individuals. This could be indicative of an insectivorous diet as has been viewed in bat species (Jones *et al.*, 2013; Carillo-Araujo *et al*., 2015).

The *Bifidobacterium* phylotypes identified in populations increased from summer to winter. Such evidence could indicate their role in the digestion of arabinogalactan found in gum exudates. Similar results were found in a previous study by Grieshop *et al*. (2002) in which *Bifidobacterium* concentrations increased in dogs fed a diet rich in arabinogalactan. This pattern in species concentration shifts is also seen in genera *Sarcina*, *Sutterella*, *Streptococcus* and *Clostridium*. Previous studies have described the carbohydrate fermentation abilities necessary for the digestion of gum exudates in galagos (Crowther, 1971; Claus & Wilmanns, 1974; Nakamura *et al*., 2009; Lam-Himlin *et al*., 2011) in comparison to the largely insect diet in summer. This would also account for the drop in *Bacteroides* species populations in winter (Wu *et al*., 2011; Rothman *et al*., 2014). Additionally, previous studies have shown that species belonging to the genus *Bacteroides* occupy the surface of the gut lining preventing pathogenic species from adhering to the

mucosal lining and protecting the host from colitis (Mazmanian *et al*., 2005; 2008). Recently, a study suggested that members of the *Sutterella* genus were associated with commensalism in the mucosal epithelium of the human gut, and exhibited immunomodulatory abilities (Hiippala *et al.,* 2016). Members of the *Collinsella* genus were also found within the faeces of all individuals, where there was a marked decrease in abundance from summer to winter. This could be due to dietary shifts as previously suggested in studies conducted by Walker *et al.* (2011) focused on western lowland gorillas and Gomez-Arango *et al.* (2017) investigating irritable bowel syndrome in humans.

Bacteroides fragilis was found in all individual gut microbiomes. This organism been shown to assist in the breakdown of polysaccharides, such as those forming the exoskeleton of insects (Xu *et al*., 2003; Wexler, 2007; Coyne & Comstop, 2008). Our findings support this view, as the *B. fragilis* population decreased during winter when insect populations typically decrease. Moreover, *Helicobacter* populations, which thrive in acidic environments (Mori *et al*., 2007; Waldum *et al*., 2016), decreased during the winter period. Such acidity would be necessary in the digestion of insects. Interestingly, *Ruminococcus* constituted only a small percentage (0.01%; Appendix 14) of the total phylogenetic composition. Previous studies have suggested this species is important in the breakdown of fibre (Wu *et al*., 2011; David *et al*., 2014; Schnorr *et al.,* 2014).

4.2 Lifestyle Influence on Microbial Community Composition

The results revealed there that was a significant reduction in bacterial diversity and abundance of the microbes in the individuals situated at the RDNR when compared to the individuals of the NZG. A low bacterial diversity is often signified as a decreased health status (Fujimura *et al*., 2010). A recent study showed that isolated Yanomami Amerindian villager populations implementing a hunter-gatherer lifestyle have a more diverse gut microbiome compared to Northern Americans residing in a westernised environment (Clemente *et al*., 2015). However, the same is not apparent in this study. The limited sample size for the RDNR site restricts our analysis of this finding. Interestingly, *Bartonella*, an opportunistic pathogen (Chomel & Boulouis, 2005; Angelakis *et al*., 2010) was found in the RDNR galago population only. This finding could indicate an occurrence of the pathogen in this rural habitat and not in the urban environment and could suggest lowered health conditions of individuals in the rural galago population in winter. With this in mind, the

difference in bacterial diversity and abundance between the populations could suggest lifestyle plays an important role in the development of microbial composition.

4.3 Pathogenic Capabilities

There was a marked reduction in the body conditions of the individuals collected, as indicated by a reduction in body weight and a rise in dental illnesses, seen in winter (Appendix 2). These changes could be attributed to the poor nutritional food resources which would affect the feeding behaviour and general health of an individual. Members of the genus *Bifidobacterium* account for 20% of the total mapped bacteria. *Bifidobacterium* is considered beneficial to the health of the host gut and these organisms are considered to be probiotics which protect the gut mucosal barrier (Pinzone *et al*., 2012; Ghouri *et al*., 2014). *Bilophila wadsworthia, Salmonella enterica*, *Bacteroides fragilis*, *Escherichia coli*, *Shigella dysenteriae* are examples of potentially zoonotic pathogens (Wexler, 2007; Myhill & Robinson, 2015; Wattam *et al*., 2016) identified from various individuals within the study. However, due to the low abundance of these taxa it is unlikely infection is present.

4.4 Hypervariable Region Analyses

The efficiency of the hypervariable regions of the 16s rRNA gene was evaluated *ad hoc*. Studies have shown regions are best to identify specific species of bacteria (Bercovier & Vincent, 2001; Moreau *et al*., 2014; Ren *et al*., 2015; Barb *et al*., 2016; Clooney *et al*., 2016). From the data, it was apparent the hypervariable region V3 was most successful in identifying the most bacteria (Fig 16). Furthermore, in past literature, the V4 region has been a favoured region for amplification (Larsen *et al*., 2010; Moreau *et al*., 2014; McKenney *et al.*, 2015; Barb *et al*., 2016), however, in this study, this region was less informative than presumed as no unique bacterial phylotpyes were identified. In addition, noteworthy results have been observed when comparing the quality of the various Illumina platforms with the Ion Torrent Instruments (Salipante *et al*., 2014; Tyakht *et al*., 2014; Clooney *et al*., 2016).

There were several limitations identified during the course of the study. Firstly, the capture rate of *G. moholi* was too low to allow for a sufficient sample size. This predicament was only encountered in our rural site (Roodeplaat Dam Nature Reserve) where galagos have less interaction with humans. Once samples had been collected, only the DNA was extracted, whereas if the RNA were also extracted, amplified and sequenced, there would have been an

opportunity to identify active from inactive bacterial species. This would have improved our understanding of the bacterial functioning and whether any pathogenic species present were actively harmful. A longitudinal study could be conducted to ensure an efficient sample size for statistical analyses and hence be able to compare the gut microbiota of both urban and rural populations of *G. moholi*. It would then be viable to conduct an in-depth assessment of variations in the composition of gut bacteria between the different environments (rural versus urban).

4.5 Conclusions

Comparisons of the gut microbiomes of *G. moholi* in different periods of the year and inhabiting different environments has allowed us to examine the influences of diet and lifestyle on the microbial composition. Our results suggest a strong relationship between gut bacterial composition and dietary patterns. The clear abundance fluctuations of certain species, such as *Bifidobacterium callitrichos* and *Campylobacter helveticus*, from summer to winter are representative of the microbial responses to variations in food choices. The results regarding the different lifestyles of two populations could suggest that the gut microbiome of this primate species is capable of maintaining gut health allowing the species to survive in a variety of environments. Identification of the gut microbiome contents could provide a new tool for assessing the gut health in primates. Considering the critical role the gut microbiota play in the maintenance of health and digestive capabilities, it is important to understand the relationships between microbe and host as they may provide information to contribute to conservation efforts for endangered primate species.

Chapter 5

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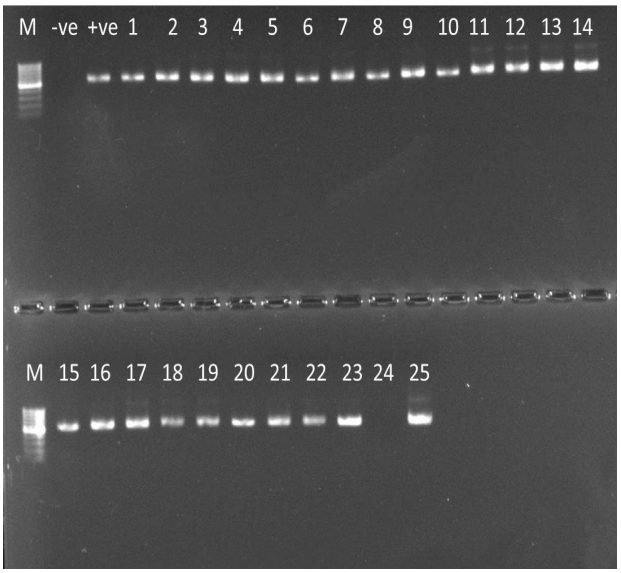
Appendices

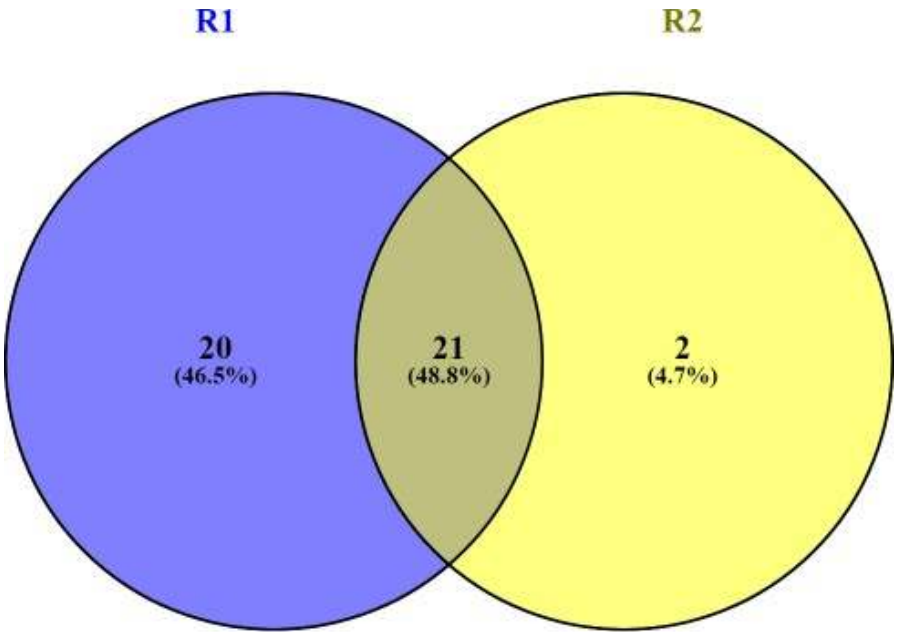


1 - RDNR W3
2 - RDNR W1
3 - RDNR W2
4 - NZG S5
5 - NZG S8
6 - NZG S4
7 - NZG S2
8 - NZG S7
9 - NZG S1
10 - NZG S6
11 - NZG W3
12 - NZG S3
13 - NZG W1
14 - NZG S10
15 - NZG W7
16 - NZG W9
17 - NZG W6
18 - NZG W5
19 - Positive
20 - NZG S9
21 - NZG W8
22 - NZG W2
23 - NZG W10
24 - Negative
25 - NZG W4

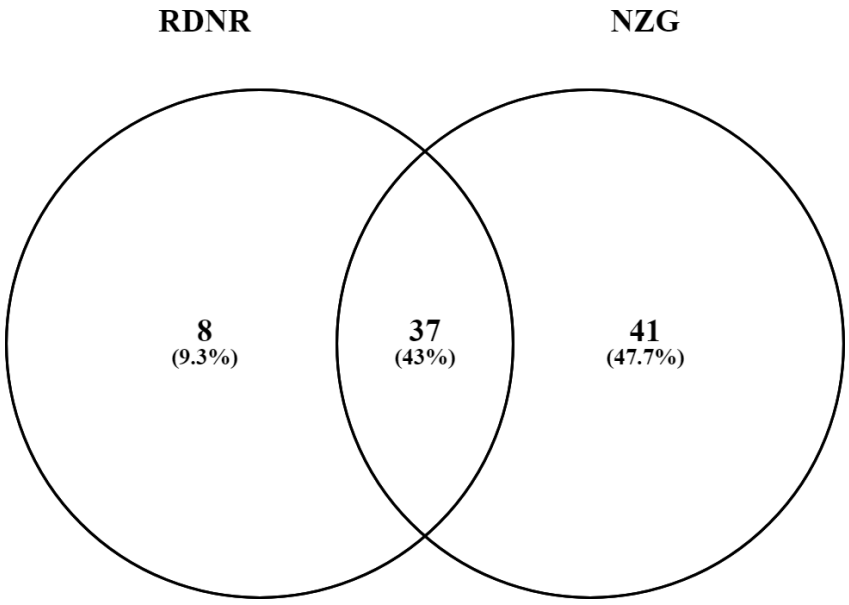
Appendix 1. 2% Agarose gel experiment. Numbers (1–25) and their corresponding notation indicate the sample number. RDNR – Roodeplaat Dam Nature Reserve; NZG – National Zoological Gardens, S –summer, W –winter.

Sample no	Date	Re-captured	Season	Site	Location	Age class	Sex	Body mass	Total body length	Tail length	Ear length		Pupil-to-pupil	Eye diameter		Hind foot		Canine length		Comments
											L	R		L	R	L	R	L	R	
S1	07/02/2017		S	NZG	Nyala Café	Sub-Adult	M	155.4	390	212	36.60	36.70	10.23	19.36	19.36	50.20	50.60	3.10	2.90	
S2	08/02/2017	DH	S	NZG	Director's House	Adult	M	223.2	420	222	39.15	39.40	12.11	23.20	23.20	52.20	52.28	2.06	2.32	Old (canine erosion); Nick in ear
S3	08/02/2017	DH	S	NZG	Nyala Café	Adult	F	192.2	405	225	38.91	38.93	18.98	11.00	11.00	48.50	48.90	2.67	2.66	
S4	09/02/2017		S	NZG	Director's House	Adult	F	227.4	445	254	40.80	40.80	19.78	11.86	11.86	51.99	52.39	2.40	1.32	Lactating; old (canine erosion)
RDNR 1	29/05/2017		W	RDNR	South	Adult	F	163.0	390	230	32.09	35.04	19.17	10.45	10.47	47.97	48.66	2.81	2.80	R thumb injury
RDNR 2	06/06/2017	Roadside	W	RDNR	Roadside	Juvenile	M	121.2	372	211	31.46	31.68	15.50	10.30	10.30	47.71	48.00	2.77	2.76	
W1	11/06/2017		W	NZG	Director's House	Adult	M	212.4	435	236	38.40	38.39	19.62	13.53	13.52	52.51	52.50	2.35	1.90	Canines broken
W2	26/06/2017	DH	W	NZG	Director's House	Adult	F	210.2	422	240	37.50	39.50	17.57	10.70	10.72	52.30	52.50	2.34	2.33	Sex uncertain; R canine tip broken; periodontal disease (loss of molar 1, premolar 1,2)
W3	27/06/2017		W	NZG	Director's House	Adult	M	223.0	424	245	39.84	38.33	18.55	11.5	11.67	51.63	53.1	2.90	2.90	
W4	03/07/2017		W	NZG	Director's House	Juvenile	F	130.2	351	195	32.77	37.01	15.89	9.34	9.33	44.58	44.75	2.46	2.11	

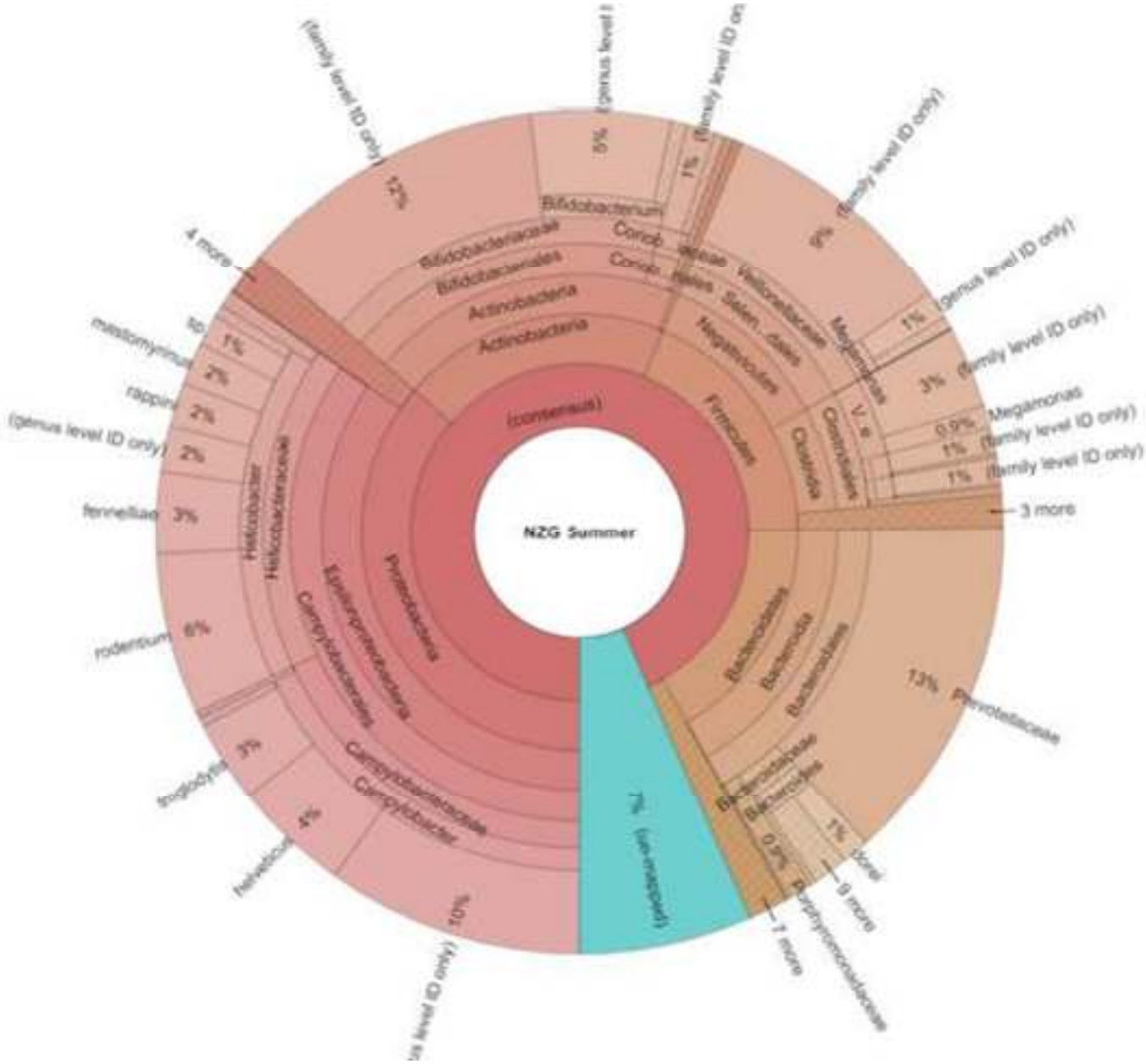
Appendix 2. Database of all samples, showing all details of each individual captured, the date of capture, site, age, gender, and various morphometric measurements. All measurements were taken with a certified veterinarianpresent.



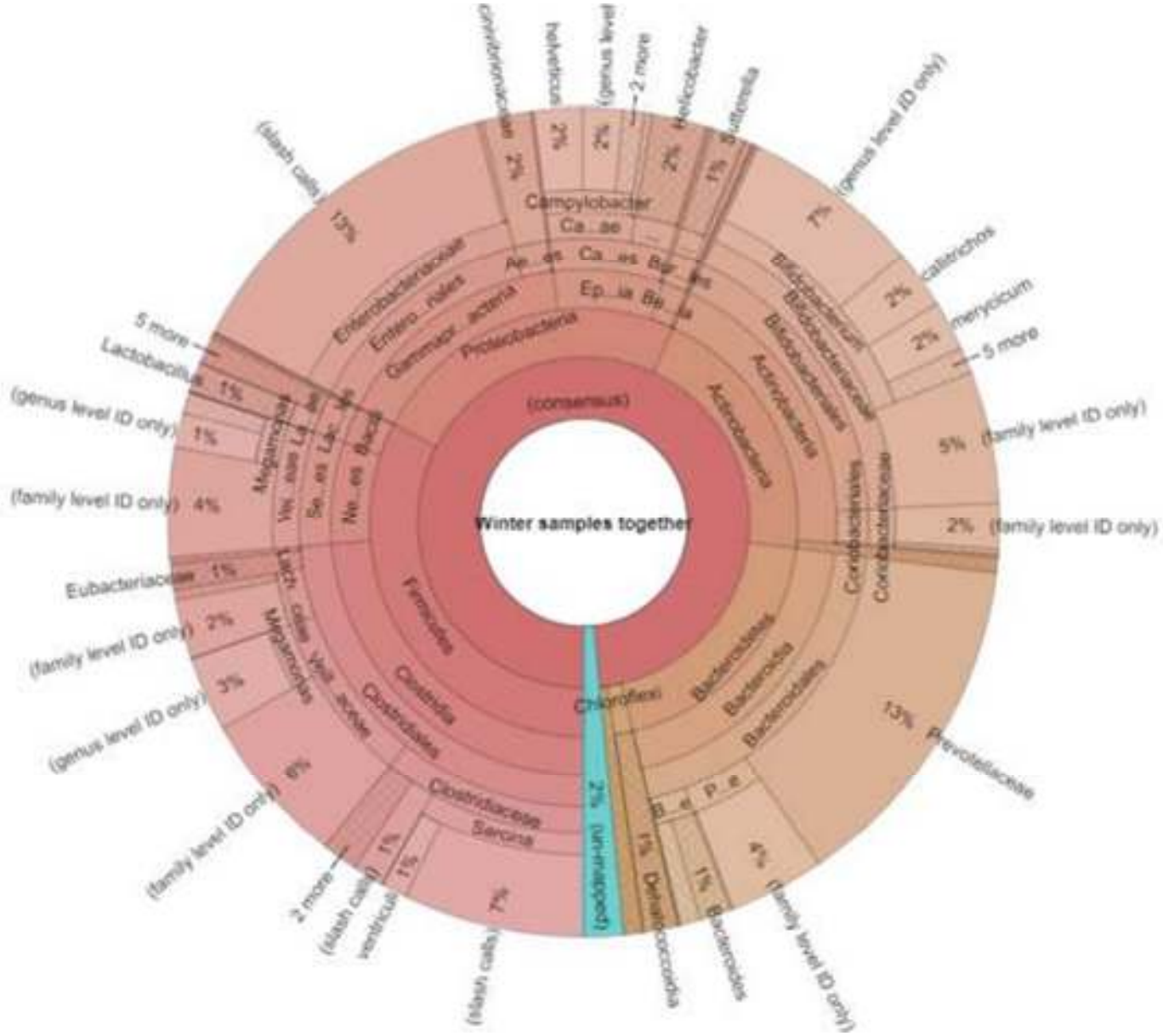
Appendix 3. A comparison of the Roodeplaat Dam Nature Reserve microbial composition between the two sites (R1 and R2). Note the vast difference between the number of genera represented in each sample. The numbers inside the diagram indicate the numbers of OTUs.



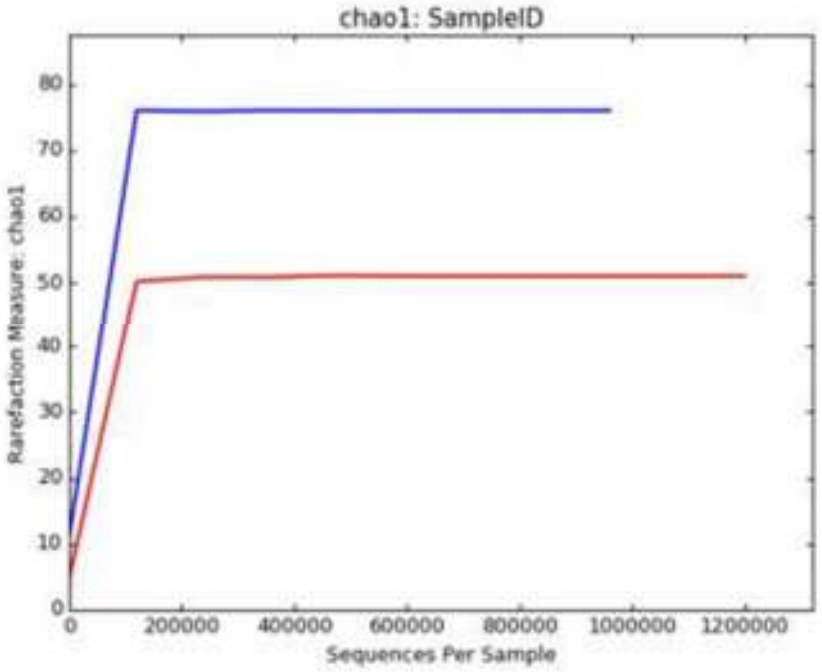
Appendix 4. A bacterial comparison between the winter samples collected from the NZG and Roodeplaat Dam. Note the difference in the number of unique genera present in the RDNR samples and those present in the NZG samples. Take note n = 2 at the RDNR site, while NZG n = 4. The numbers inside the diagram indicate the numbers of OTUs, and their respective percentages of the total winter composition.



Appendix 5. The total composition of bacteria present in the summer NZG samples (n=4). Note how *Proteobacteria* is most predominant, followed by *Actinobacteria*, then *Firmicutes* and *Bacteroides*.



Appendix 6. The total composition of bacteria present in the winter NZG samples. Note how *Firmicutes* is most prevalent, followed by *Proteobacteria*, then *Actinobacteria* and finally *Bacteroides* (n=4).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Rarefaction Measure: chao1
Sequences Per Sample
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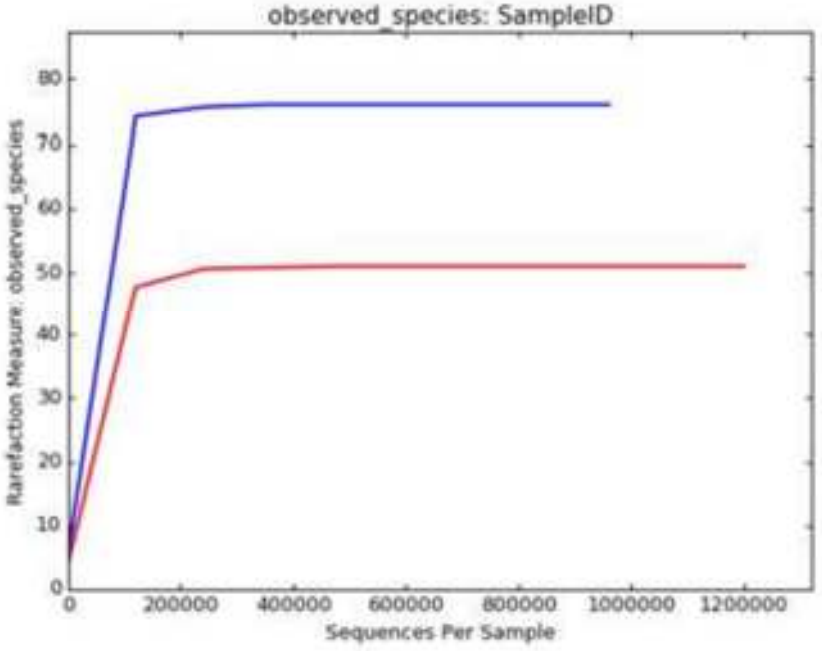
30
40
50
60
70
80
0
200000
400000
600000
800000
1000000
1200000

A


observed_species: SampleID
Rarefaction Measure: observed_species
Sequences Per Sample
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30
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70
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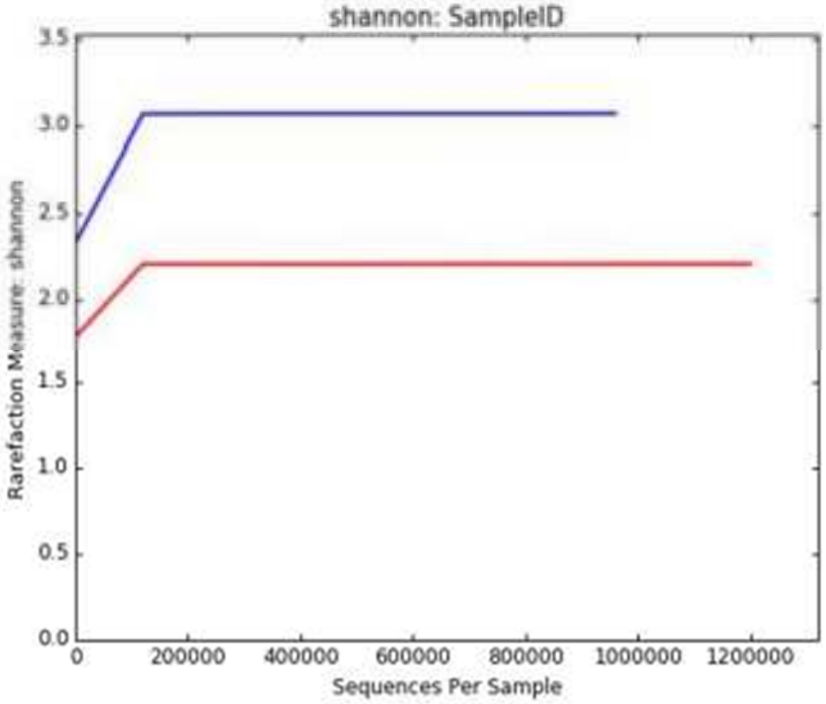
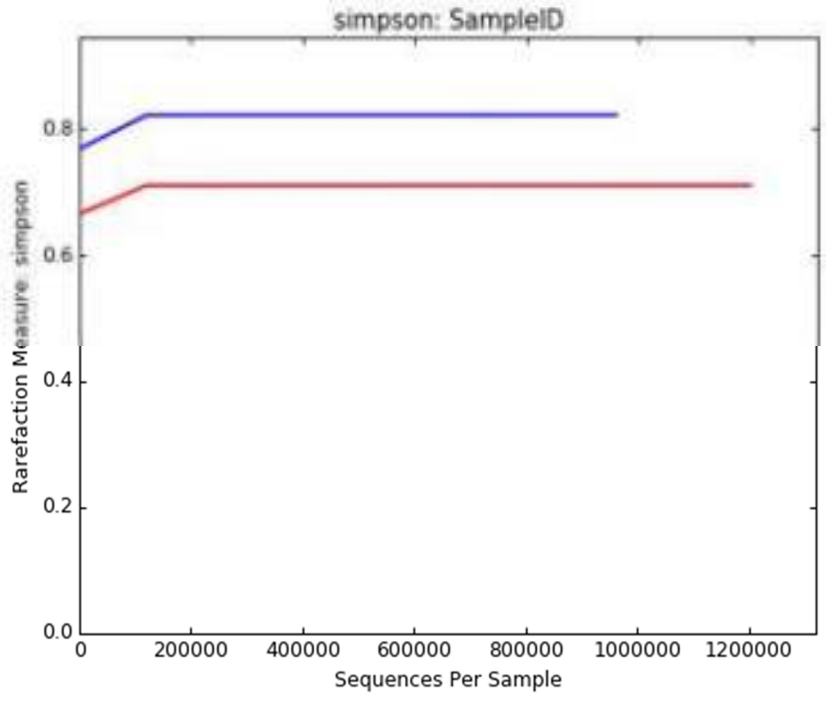
400000
600000
800000
1000000
1200000

B

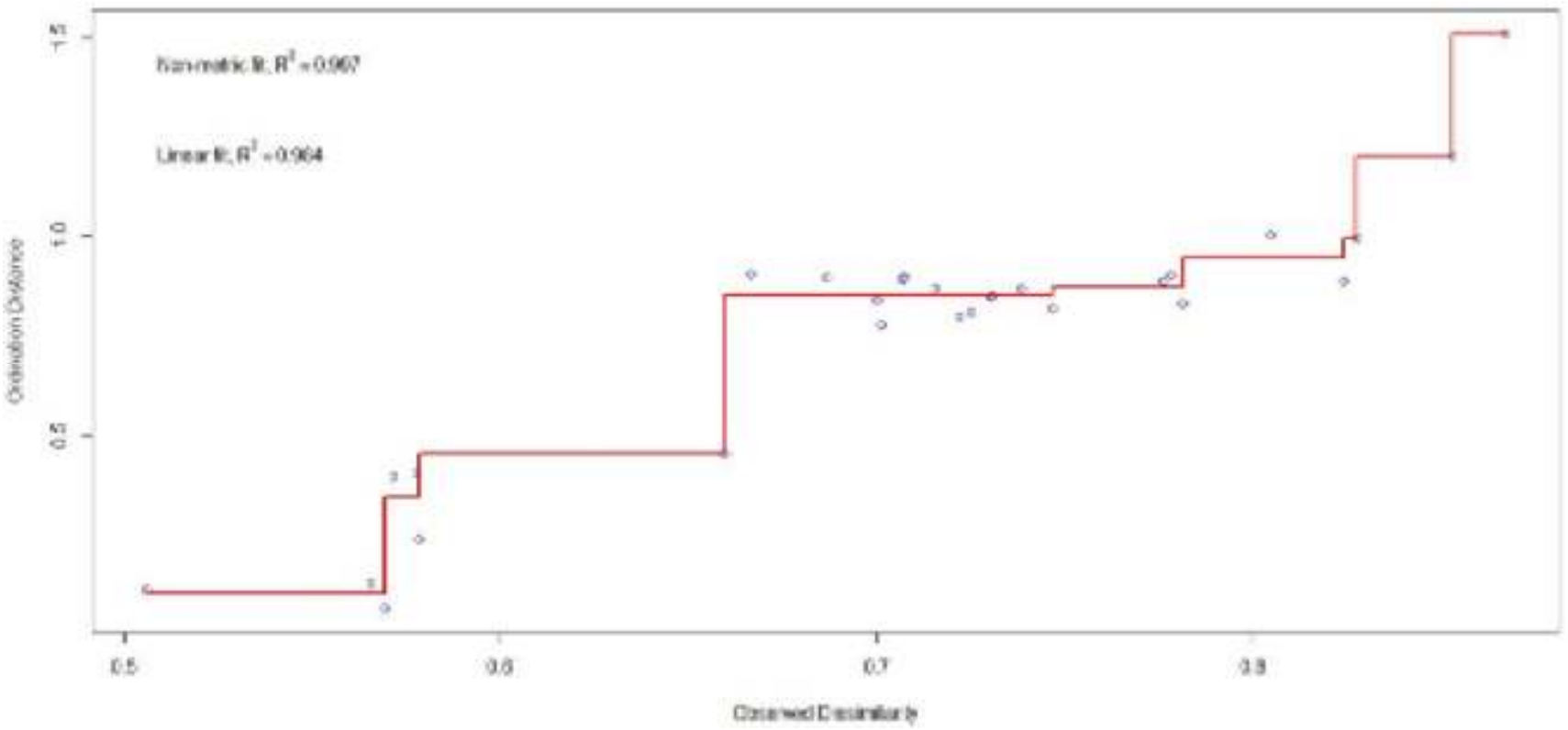


C

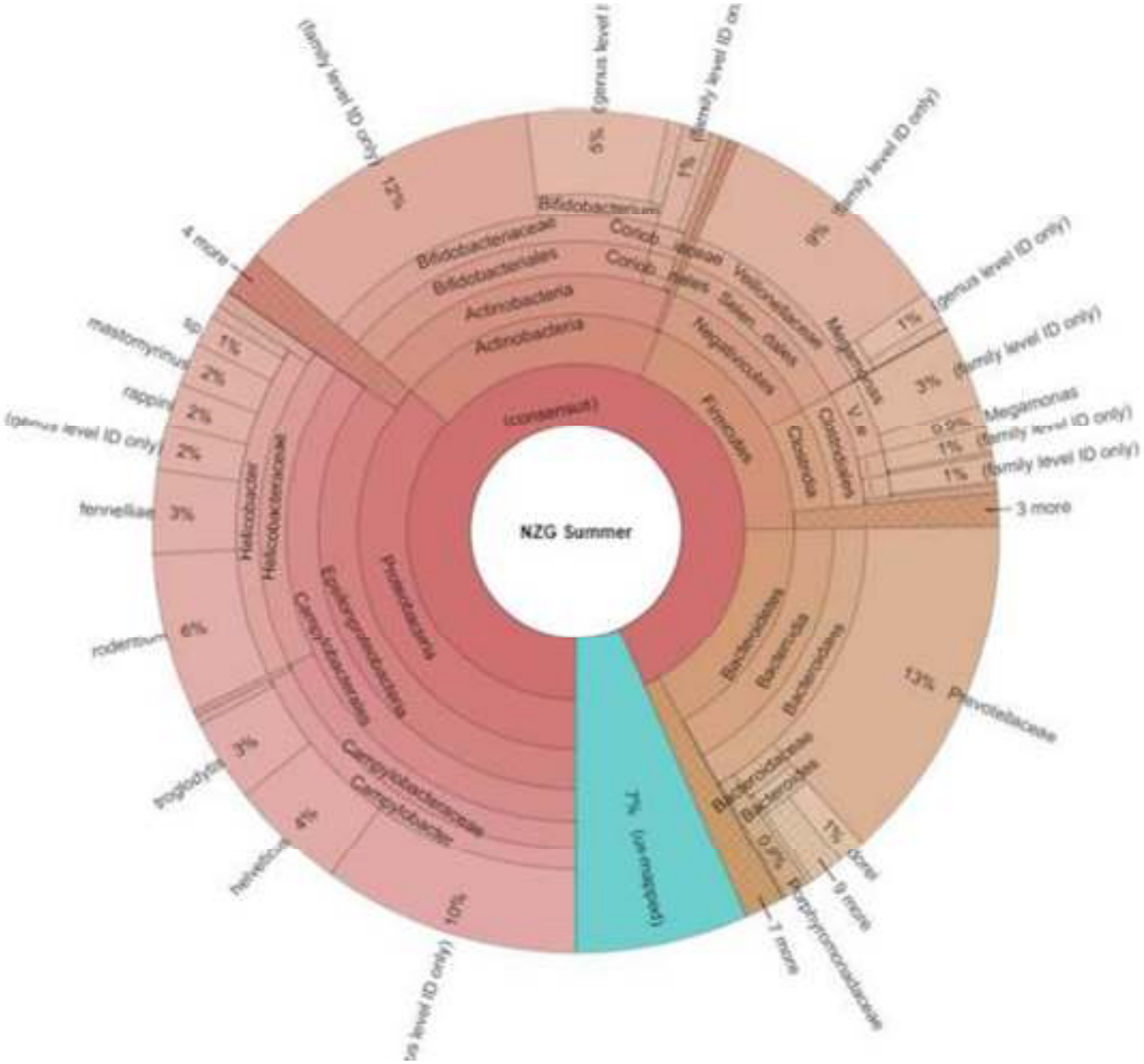


D

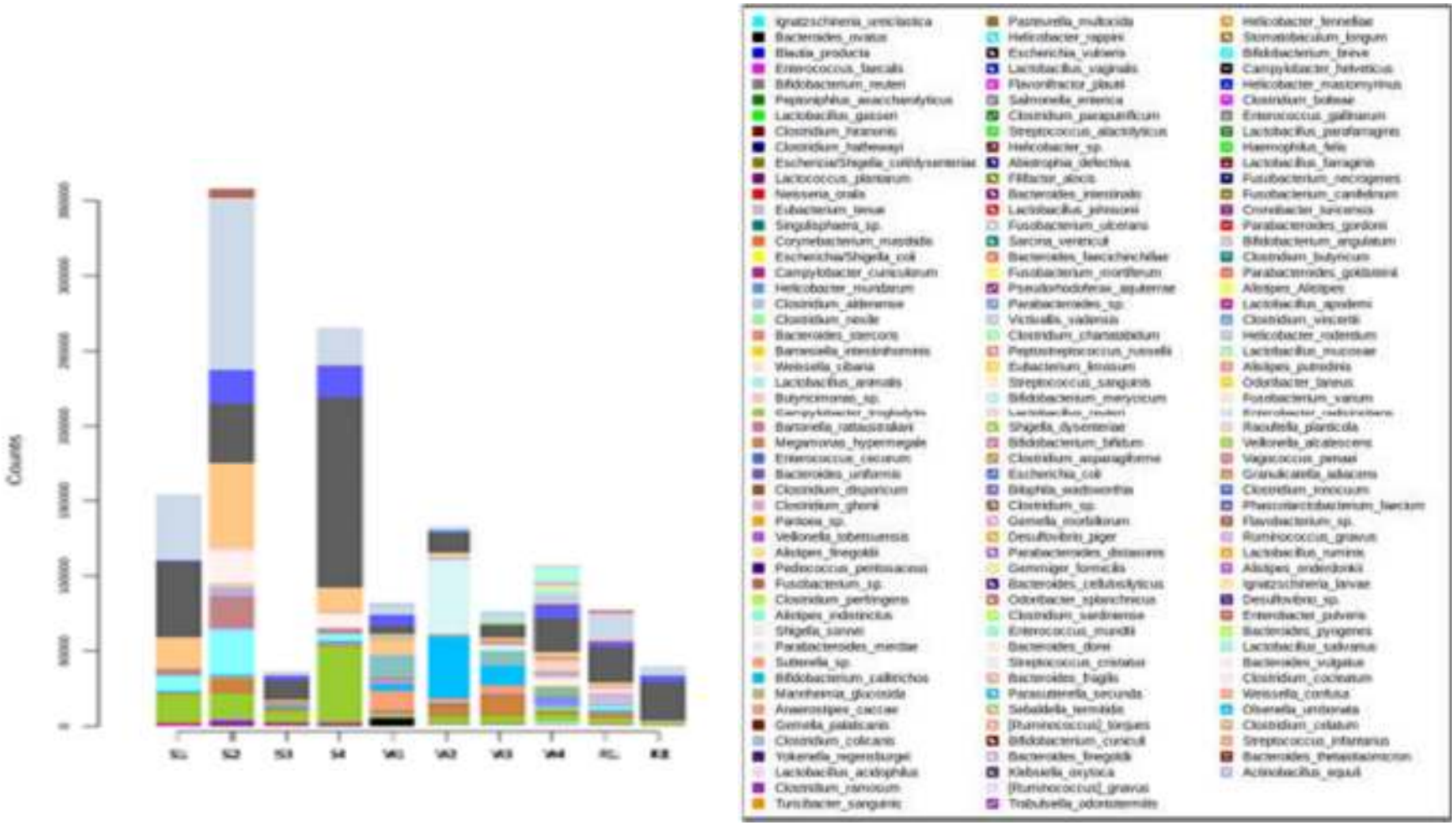
Appendix 7. Alpha diversity for both seasons. A) Chao1, B) Observed Species, C) Shannon Index, D) Simpson Index. Blue indicating winter, and red indicating summer.



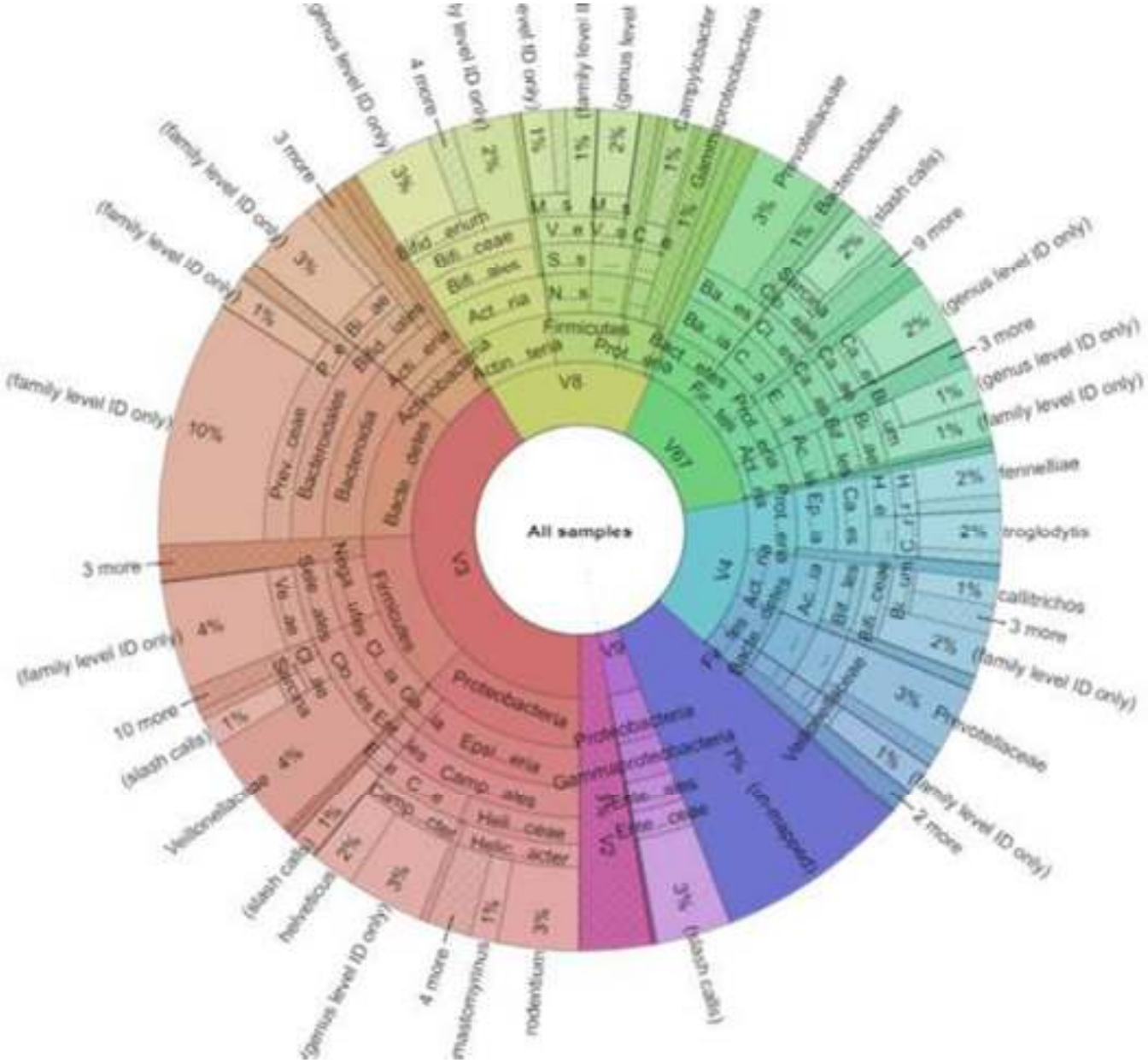
Appendix 8. Shepard plot indicating the stress values of all NZG samples (summer and winter) and the goodness of fit. Stress value = 0.13 was determined, indicating a good line of fit.



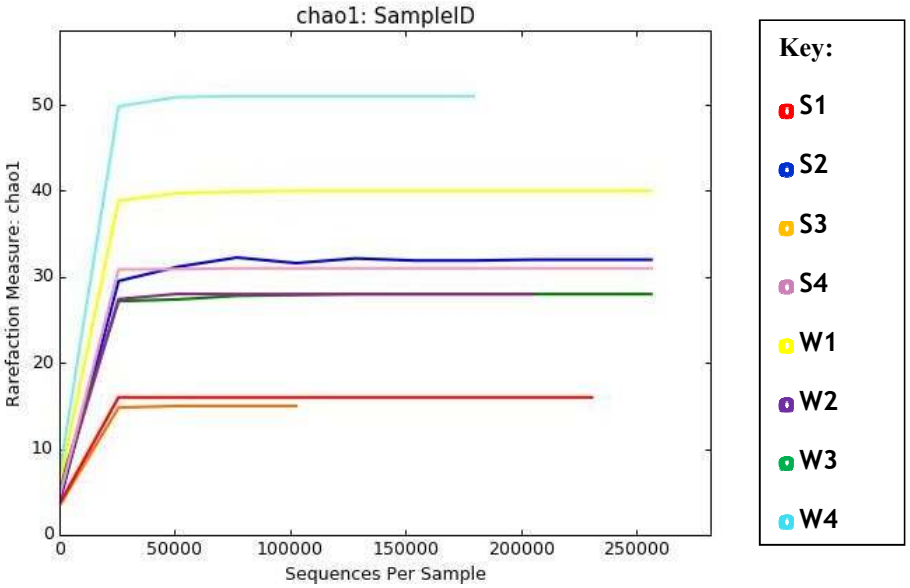
Appendix 9. The total composition of bacteria present in the winter RDNR samples (n=2). Note how *Proteobacteria* is most prevalent, followed by *Actinobacteria*, then *Firmicutes*, and finally *Bacteroides*.



Appendix 10. Bar graph demonstrating the OTUs at species level for each sample. S – summer samples (NZG); W – winter samples (NZG); R – RDNR. The legend on the right indicates the species and their corresponding shading colour.



Appendix 11. The total bacterial composition of *G. moholi* captured during summer and winter in both an urban and rural site in the Gauteng province. This pie chart indicates the bacterial genera found in each hypervariable region (V2, V3, V4, V6/7, V8, and V9). Note V3 consists of the greatest abundance of bacteria.


chao1: SampleID
Rarefaction Measure: chao1
Sequences Per Sample
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10
20
30
40
50
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150000
200000
250000
Key:
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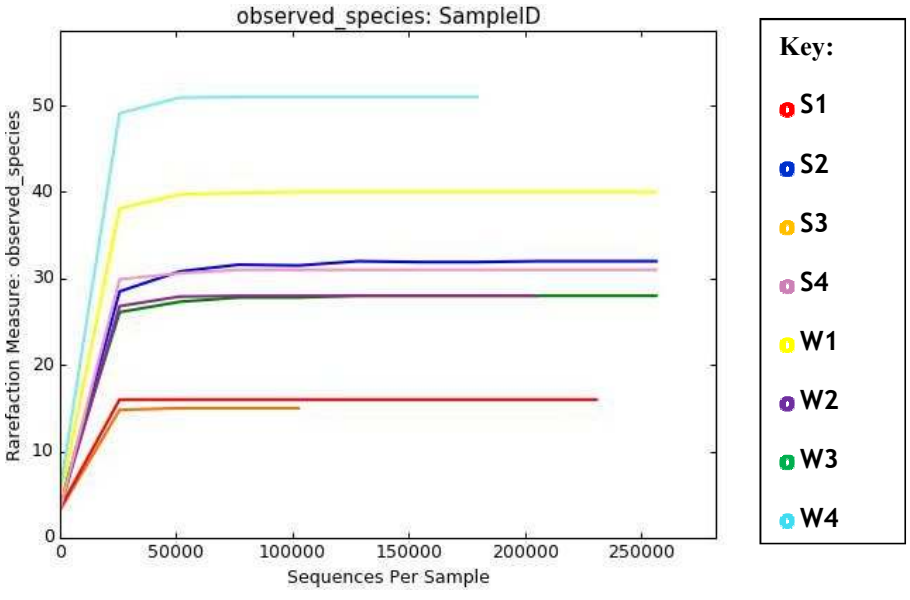
S2
S3
S4
W1
W2
W3
W4

A


observed_species: SampleID
Rarefaction Measure: observed_species
Sequences Per Sample
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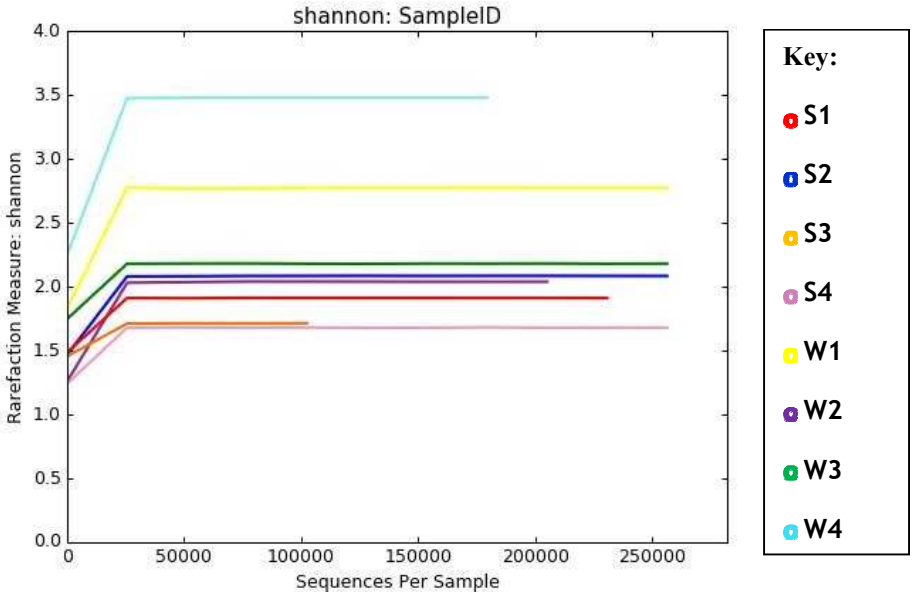
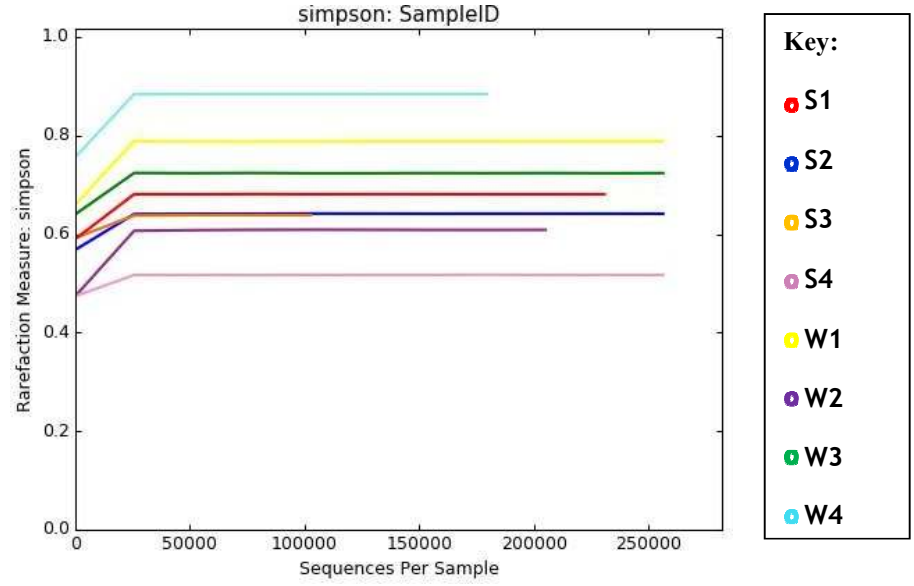
10
20
30
40
50
0
50000
100000
150000
200000
250000
Key:
S1
S2
S3
S4
W1
W2
W3
W4

B



C



D

Appendix 12. Rarefaction plot graphs for the winter (W1-W4) and summer (S1-S4) samples. A) Chao1 abundance, B) Observed Species, C) Shannon diversity, D) Simpson diversity.

	Chao1	Observed Species	Shannon	Simpson
RDNR1	35.36 ± 10.90	35.00 ± 11.48	2.56 ± 0.29	0.75 ± 0.04
RDNR2	16.48 ± 8.98	16.48 ± 8.98	1.66 ± 0.28	0.58 ± 0.06
W1	37.22 ± 9.38	36.65 ± 10.32	2.72 ± 0.17	0.61 ± 0.01
W2	25.75 ± 6.81	25.45 ± 7.19	2.00 ± 0.14	0.61 ± 0.01
W3	25.68 ± 6.95	25.49 ± 7.07	2.15 ± 0.09	0.72 ± 0.01
W4	47.07 ± 12.15	46.34 ± 13.94	3.39 ± 0.29	0.88 ± 0.02

Appendix 13. Alpha diversity for each sample in the winter sampling period from the RDNR (RDNR1 & RDNR2) and the NZG (W1–W4). Mean estimates for Chao1, Observed Species, Shannon Index, and Simpson Index were calculated.

Appendix 14. A table identifying the species found within all the samples sequenced and how many were present in each sample. The species are organised from highest abundance to least abundant. There is a total of 151 species identified.

Species	NZG S1	NZG S2	NZG S3	NZG S4	NZG W1	NZG W2	NZG W3	NZG W4	RD NR1	RD NR2	Total
Campylobacter helveticus	50172	39997	12286	126543	5825	12788	8724	22814	22782	25498	327429
Helicobacter rodentium	42751	113453	1180	24030	4725	2695	2298	4105	17062	5496	217795
Campylobacter troglodytis	18479	16252	7459	49739	2385	4451	4820	5282	3246	1204	113317
Helicobacter fennelliae	20299	55062	1002	16277	8501	2006	1751	3211	2238	625	110972
Helicobacter mastomyrinus	1251	21963	3387	20636	7185	792	314	7399	3966	5016	71909
Bifidobacterium callitrichos	1133	1517	1484	872	4680	41085	12926	958	592	130	65377
Bifidobacterium merycicum	68	0	133	105	27	48800	2947	0	131	10	52221
Helicobacter rappini	10503	30050	61	5102	505	208	209	485	3215	503	50841
Megamonas hypermegale	922	9970	2243	916	207	7396	14022	138	3207	519	39540
Bacteroides dorei	0	21638	10	8930	0	523	398	601	1519	72	33691
Helicobacter sp.	3804	20553	83	2847	1859	146	122	288	543	29	30274
Sarcina ventriculi	0	0	0	0	14887	483	9806	749	0	0	25925
Sutterella sp.	287	1326	311	1062	13035	2859	5562	287	196	16	24941
Bifidobacterium angulatum	1178	216	2392	722	128	242	4346	3493	504	136	13357
Bacteroides fragilis	0	3282	59	616	151	217	705	5979	1974	0	12983
Lactobacillus salivarius	0	0	0	0	0	0	0	9936	0	0	9936
Bifidobacterium cuniculi	466	124	2394	455	74	14	2430	1672	552	117	8298
Bacteroides thetaiotaomicron	0	6377	0	0	0	0	0	0	879	0	7256
Bilophila wadsworthia	0	5685	10	0	128	615	0	57	451	0	6946
Campylobacter cuniculorum	1346	2088	1593	905	0	0	0	0	149	0	6081
Bacteroides ovatus	137	21	0	572	4915	10	51	79	171	0	5956
Lactobacillus vaginalis	0	0	0	0	0	0	0	5847	0	0	5847
Parabacteroides distasonis	0	672	0	914	0	0	0	545	3319	0	5450
Streptococcus sanguinis	0	0	0	0	0	802	196	3816	0	17	4831
Bifidobacterium reuteri	333	228	695	224	1147	865	528	167	418	124	4729
Clostridium paraputrificum	0	0	0	0	11	0	0	4607	0	0	4618
Clostridium sp.	36	86	0	93	486	1278	159	2386	30	0	4554
Stomatobaculum longum	0	0	0	0	3726	11	0	0	12	17	3766
Parabacteroides sp.	0	0	0	0	28	0	14	290	3077	0	3409
Flavonifractor plautii	94	1388	0	284	1406	0	0	25	56	11	3264
Bacteroides uniformis	0	0	0	213	976	1004	601	28	281	0	3103
Desulfovibrio piger	0	1778	0	0	0	0	0	282	458	0	2518
Lactobacillus mucosae	0	0	0	0	0	0	0	2384	0	0	2384
Fusobacterium varium	0	765	26	0	0	26	29	1437	44	0	2327
Helicobacter muridarum	20	1515	0	413	114	68	18	56	13	0	2217

Bacteroides stercoris	617	0	0	691	45	19	142	276	359	0	2149
Cronobacter turicensis	0	0	0	205	520	0	0	1362	48	0	2135
Clostridium perfringens	0	0	0	0	0	532	90	1443	0	47	2112
Olsenella umbonata	0	0	0	0	603	10	1320	0	0	0	1933
Lactobacillus reuteri	0	0	0	0	0	0	0	1929	0	0	1929
Weissella confusa	0	0	0	0	953	0	0	0	602	0	1555
Escherichia coli	0	0	0	0	0	0	0	1516	0	0	1516
Alistipes onderdonkii	0	0	0	384	0	0	0	279	782	0	1445
Bacteroides vulgatus	0	0	0	0	372	953	0	0	0	0	1325
Lactobacillus animalis	0	0	0	0	0	0	0	1323	0	0	1323
Vagococcus penaei	0	0	0	0	0	0	0	1160	0	0	1160
Clostridium ramosum	0	0	0	442	24	56	0	227	356	0	1105
Clostridium colicanis	0	0	0	161	0	677	0	224	0	0	1062
Ruminococcus gnavus	0	0	0	0	0	0	0	825	232	0	1057
Streptococcus cristatus	0	0	0	0	418	290	0	160	120	0	988
Clostridium hathewayi	0	966	0	0	0	0	0	16	0	0	982
Clostridium disporicum	0	0	0	0	13	0	0	911	0	0	924
Clostridium cocleatum	0	0	0	326	50	29	12	169	36	0	622
Corynebacterium mastitidis	103	181	0	236	59	0	17	11	0	0	607
Enterobacter pulveris	0	0	0	0	0	0	0	0	604	0	604
Parabacteroides merdae	0	0	0	0	0	0	91	0	483	24	598
Weissella cibaria	0	0	0	0	414	0	0	0	168	0	582
Bifidobacterium breve	87	0	117	67	0	0	0	0	201	0	472
Odoribacter laneus	0	0	0	0	0	0	0	465	0	0	465
Lactobacillus acidophilus	0	0	0	0	0	0	0	419	0	0	419
Odoribacter splanchnicus	0	55	0	0	0	0	0	11	350	0	416
[Ruminococcus] gnavus	0	0	0	0	0	0	0	50	329	0	379
Mannheimia glucosida	0	0	0	0	55	154	162	0	0	0	371
Parabacteroides gordonii	0	0	0	62	0	0	0	0	304	0	366
Haemophilus felis	0	0	0	0	0	0	339	0	0	0	339
Lactobacillus johnsonii	0	0	0	0	0	0	0	320	0	0	320
Gemella palaticanis	0	0	0	0	297	12	0	10	0	0	319
Enterococcus gallinarum	0	0	0	299	0	0	0	10	0	0	309
Fusobacterium sp.	0	0	0	0	0	251	0	52	0	0	303
Clostridium ghonii	0	0	0	0	0	0	0	288	0	0	288
Clostridium vincentii	0	0	0	0	0	0	0	277	0	0	277
Bacteroides intestinalis	0	247	0	0	0	0	0	0	15	0	262
Fusobacterium necrogenes	0	0	0	0	0	0	0	252	0	0	252
Veillonella alcalescens	0	0	0	0	0	0	0	250	0	0	250
Neisseria oralis	0	0	0	0	0	22	208	0	0	0	230
Salmonella enterica	0	0	0	0	20	0	0	191	13	0	224
Clostridium innocuum	0	215	0	0	0	0	0	0	0	0	215
Peptoniphilus asaccharolyticus	0	0	0	210	0	0	0	0	0	0	210

Fusobacterium mortiferum	0	0	0	0	0	0	0	209	0	0	209
Clostridium aldenense	0	202	0	0	0	0	0	0	0	0	202
Ignatzschineria ureiclastica	0	0	0	0	0	0	0	198	0	0	198
Bifidobacterium bifidum	0	0	98	0	0	0	0	0	60	26	184
Streptococcus alactolyticus	0	0	0	0	0	91	0	87	0	0	178
Trabulsiella odontotermitis	0	0	0	0	31	0	0	101	26	0	158
Veillonella tobetsuensis	0	0	0	0	0	0	0	155	0	0	155
Peptostreptococcus russellii	0	0	0	0	0	0	0	131	0	0	131
Enterococcus faecalis	130	0	0	0	0	0	0	0	0	0	130
Filifactor alocis	0	0	0	0	0	124	0	0	0	0	124
Parabacteroides goldsteinii	0	0	0	0	0	0	0	10	109	0	119
Shigella coli	0	0	0	0	0	0	0	118	0	0	118
Butyricimonas sp.	0	109	0	0	0	0	0	0	0	0	109
Lactococcus plantarum	0	0	0	0	57	13	0	31	0	0	101
Bacteroides finegoldii	0	0	0	0	83	0	11	0	0	0	94
Escherichia vulneris	0	0	0	0	0	0	0	86	0	0	86
Clostridium butyricum	0	0	0	0	0	41	0	37	0	0	78
Barnesiella intestinihominis	0	0	0	0	0	0	0	30	45	0	75
Clostridium bolteae	0	72	0	0	0	0	0	0	0	0	72
Streptococcus infantarius	0	0	0	0	0	0	0	72	0	0	72
Bartonella rattaustraliani	0	0	0	0	0	0	0	0	15	55	70
Lactobacillus ruminis	0	0	0	0	0	0	0	69	0	0	69
Clostridium celatum	0	0	0	0	0	0	0	68	0	0	68
Fusobacterium ulcerans	0	68	0	0	0	0	0	0	0	0	68
Pasteurella multocida	0	0	0	0	0	66	0	0	0	0	66
Fusobacterium canifelinum	0	0	0	0	0	65	0	0	0	0	65
Abiotrophia defectiva	0	0	0	0	35	11	0	18	0	0	64
Pediococcus pentosaceus	0	0	0	0	0	0	0	0	0	56	56
Raoultella planticola	0	0	0	0	0	0	0	54	0	0	54
Lactobacillus apodemi	0	0	0	0	0	0	0	53	0	0	53
Desulfovibrio sp.	0	0	0	49	0	0	0	0	0	0	49
Flavobacterium sp.	0	0	0	47	0	0	0	0	0	0	47
Eubacterium limosum	0	0	0	0	46	0	0	0	0	0	46
Blautia producta	0	0	0	0	34	0	0	10	0	0	44
Alistipes sp.	0	0	0	0	0	0	0	0	42	0	42
Clostridium asparagiforme	0	42	0	0	0	0	0	0	0	0	42
Shigella sonnei	0	0	0	0	0	0	0	41	0	0	41
Lactobacillus farraginis	0	0	0	0	0	0	0	0	40	0	40
Lactobacillus gasseri	0	0	0	0	0	0	0	39	0	0	39
Bacteroides faecichinchillae	0	0	0	0	0	0	38	0	0	0	38
Gemmiger formicilis	0	38	0	0	0	0	0	0	0	0	38

Clostridium nexile	0	0	0	0	0	0	37	0	0	0	37
Lactobacillus parafarraginis	0	0	0	0	0	0	0	0	37	0	37
Enterococcus mundtii	36	0	0	0	0	0	0	0	0	0	36
Klebsiella oxytoca	0	0	0	0	0	0	0	35	0	0	35
Pseudorhodoferax aquiterrae	0	0	0	32	0	0	0	0	0	0	32
Bacteroides cellulosilyticus	0	31	0	0	0	0	0	0	0	0	31
Eubacterium tenue	0	0	0	0	0	0	0	29	0	0	29
Alistipes putredinis	0	0	0	0	28	0	0	0	0	0	28
Enterococcus cecorum	0	0	0	0	0	0	0	0	28	0	28
Eschericia dysenteriae	0	0	0	0	0	0	0	16	12	0	28
Gemella morbillorum	0	0	0	0	0	0	0	27	0	0	27
Victivallis vadensis	0	26	0	0	0	0	0	0	0	0	26
Shigella dysenteriae	0	0	0	0	0	0	0	25	0	0	25
Phascolarctobacterium faecium	0	0	0	0	0	0	0	23	0	0	23
Enterobacter radicincitans	0	0	0	0	0	0	0	0	20	0	20
Granulicatella adiacens	0	0	0	0	0	20	0	0	0	0	20
Pantoea sp.	0	0	0	0	0	0	0	0	19	0	19
Actinobacillus equuli	0	0	0	0	0	18	0	0	0	0	18
Turicibacter sanguinis	0	0	0	0	16	0	0	0	0	0	16
[Ruminococcus] torques	0	0	0	0	0	0	0	0	0	14	14
Alistipes finegoldii	0	0	0	0	14	0	0	0	0	0	14
Alistipes indistinctus	0	0	0	0	0	0	0	0	14	0	14
Bacteroides pyogenes	0	0	0	0	0	14	0	0	0	0	14
Ignatzschineria larvae	0	0	0	0	0	0	0	14	0	0	14
Sebaldella termitidis	0	0	14	0	0	0	0	0	0	0	14
Parasutterella secunda	0	0	0	0	0	0	0	0	13	0	13
Clostridium sardiniense	0	0	0	0	12	0	0	0	0	0	12
Clostridium hiranonis	0	0	0	0	0	0	0	11	0	0	11
Singulisphaera sp.	0	0	0	0	0	0	11	0	0	0	11
Yokenella regensburgei	0	0	0	0	0	0	0	11	0	0	11
Anaerostipes caccae	0	10	0	0	0	0	0	0	0	0	10
Clostridium chartatabidum	0	0	0	0	0	0	0	10	0	0	10