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List of Abbreviations.

ANOVA	Analysis of variance
DNA	Deoxyribonucleic acid
dsDNA	Double stranded deoxyribonucleic acid
EDTA	Ethylenediaminetetracetic acid.
IRT	Inhibitor Removal Technology
MAP	<i>Mycobacterium avium subspecies paratuberculosis</i>
MRI	Moredun Research Institute
NMDS	Non metric multidimensional scaling
OTU	Operational taxonomic units
PCR	Polymerase chain reaction
PERMANOVA	Permutational analysis of variance
PERMDISP	Permutational multivariate dispersion
PRIMER	Plymouth routines in multivariate ecological research
QIIME	Quantitative insight into microbial ecology

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1. Abstract

The gastrointestinal microbiome plays an invaluable role in the maintenance and wellbeing of their host. They are important in the development of host immunity and host digestion. Despite their vital importance, there is still much to be known about their role in the host and their diversity during bacterial and parasitic infections.

In the first part of this study, we examined the gut microbiome of 40 sheep (gimmers) infected only with gastrointestinal nematodes. Rectal faecal samples were taken before treatment and after treatment with anthelmintic. In the second part of the study, a total of 125 rectal faecal samples were collected from a sheep flock infected with *Mycobacterium avium* subspecies *paratuberculosis* and nematodes. Bacterial DNA was isolated from all rectal faecal samples using the MOBIO PowerFecal® DNA Isolation Kit.

The faecal samples acted as a surrogate to the gastrointestinal microbiota. Bacterial and archaeal ecosystems were examined by sequencing the 16sRNA gene V4 amplicons employing the Illumina sequencing platform. Raw sequenced data was then analysed by the use of QIIME (Quantitative Insight into Microbial Ecology) with the assigning of taxonomy to the raw sequenced data and the determination of diversity within the samples. Statistical analysis was carried out using PRIMER (Plymouth Routine into Microbial Ecological Research). PERMANOVA and PERMDISP statistical tools were used to analyse the multivariate data.

In the first part of the study with the nematode only infected sheep, we discovered that the gastrointestinal microbiome of sheep before and after treatment showed few differences ($P > 0.05$). This suggest that anthelmintic treatment did not have much effect on the bacterial and archaeal community in the gastrointestinal tract.

In the second part of the study with the MAP and helminth infected sheep, it was discovered that the Year 3 samples differ from the Year 2 and Year 1 samples with a P value of 0.001, suggesting that the progression of disease alters the gastrointestinal microbiome of sheep.

2. Introduction

2.1. *Mycobacterium avium subspecies paratuberculosis* (MAP).

Mycobacterium avium subspecies *paratuberculosis* is a member of the family *Mycobacteriaceae* which are gram positive, acid – fast organisms ((Harris, 2001)). Other members of significant pathogenic importance include *Mycobacterium tuberculosis* and *Mycobacterium leprae* (Wayne, and Kubica, 1986).

MAP is an obligate intracellular organism of about 0.5 µm by 1.2 µm in size ((El-Zaatari, 2004). It was first identified in Germany by Professor Johne and Dr Frothingham in 1894 as the causative agent of a severe gastroenteritis of ruminants. The disease was subsequently called Johne's disease after its founder ((Singh *et al.*, 2013). It has also been associated with Crohn's disease in humans making it a pathogen of zoonotic importance ((Grant, 2005).

MAP is a very slow growing bacteria that depends on the ferric iron extraction ability of mycobactin to grow on media due to its fastidious nutritional requirement (Sweeney, 1996). MAP has about 14 to 18 copies of IS900 inserted in its genome. This property is used to detect MAP by targeting the IS900 which results in more sensitive detection or diagnosis than targeting a single copy marker (Kim *et al.*, 2004). Research has shown that other mycobacteria also possess IS900 like sequences which means that, under certain circumstances, a more detailed check is needed to confirm the presence of MAP by molecular methods (Cousins *et al.*, 1999).

MAP is a difficult organism to isolate requiring months or years of incubation before they become visible to the eye, furthermore many strains cannot be grown at all (El-Zaatari, 2004). MAP can therefore be a difficult organism to culture under conventional laboratory conditions (El-Zaatari, 2004).

Polymerase chain reaction has been employed due to the limitations of traditional microbiology techniques in detection and diagnosis of MAP (Khare *et al.*, 2004). The possible drawback encountered with PCR includes excessive nonspecific DNA which can be derived from host or other organisms, presence of PCR inhibitors in samples and the quality of genomic DNA preparation (Khare *et al.*, 2004). Through the development of molecular biology the sensitivities of IS900 based PCR assays for the isolation of MAP from faecal and tissues samples have improved over the years enabling enhanced DNA extraction techniques to employ the use of qPCR assays using primers designed to avoid detection of environmental bacteria (Windsor, 2015). PCR targeting of IS900 is mostly used for the identification of MAP, providing the advantage of speed over mycobactin method of

identification (Cousins et al., 1999). MAP in liquid cultures from faecal origin and also in milk have been identified by IS900 PCR (Cousins et al., 1999).

MAP can survive for long periods in the environment ((Singh *et al.*, 2013). It is capable of slow movement in the soil remaining on grass and pasture resulting in infection when ingested by grazing animals (Salgado et al., 2011). MAP has an average survival rate of up to 152 to 246 days in the environment depending on favourable environmental conditions (Singh et al., 2013). It survives for longer periods in water with water reservoirs playing significant roles in MAP transmissions or infections on farms (Singh et al., 2013). MAP has also been cultured in milk of animals both clinically infected and sub - clinically infected (Grant, 2005). Milk contamination with MAP can be as a result of direct entry of MAP into the udder or as a result of contamination with infected faeces. Studies carried out on the effect of pasteurization of milk shows that MAP is more heat – resistant than other *Mycobacteria* which pasteurization targets (*Mycobacteria bovis*) with low amounts of viable MAP surviving pasteurization procedure ((Grant, 2005).

2.2. Johne's Disease

Johne's disease also known as Paratuberculosis is a disease of domestic and wild ruminants caused by *Mycobacterium avium* subspecies *paratuberculosis* characterized by chronic granulomatous gastroenteritis seen mainly in the ileum (Singh *et al.*, 2013). Since the identification of MAP as the causative agent of Johne's , the disease has spread to the entire world particularly in the dairy industries (Windsor, 2015). It is therefore a disease that has a global distribution, causing serious economic losses in the dairy industry due to falls in milk production and early culling of cows ((Harris, 2001). Johne's disease has been reported in almost every country involved in livestock production and has the laboratory capability to detect disease in livestock (Khare et al., 2004). It causes a drastic fall in production in all ruminants causing huge losses for the farmer (Singh *et al.*, 2013). Just like in cattle the distribution in other ruminants (deer, sheep and goats) is also worldwide (Windsor, 2015).

Johne's disease infection occurs most commonly during neonatal life via the oral route as a result of consuming infected material (soil, faeces, MAP infected milk or colostrum) or via oral contact with contaminated udder or surfaces with faeces ((Arsenault *et al.*, 2014). Following ingestion, the lymphoid tissue of the intestinal mucosa is the main target of MAP with the M cells of the Peyer's patches been the point of entry (Singh *et al.*, 2013). It then invades intestinal macrophages with the capability of resisting host defence mechanisms and undergoing multiplication within the macrophages as a result of its ability to prevent

activation of macrophages, prevent phagosome acidification and weakening of the presentation of antigens to the immune cells (Lamont *et al.*, 2012).

In sheep, 2 main pathological forms of the disease are described in animals manifesting clinical signs, namely (1) the Paucibacillary form which is associated with strong cell – mediated immunity with the inflammatory infiltrate made up of lymphocytes, small quantities of macrophages with a very low number of Mycobacteria, (2) Multibacillary form associated with weak cell – mediated immune response with inflammatory infiltrate comprising of macrophages packed with numerous Mycobacteria (Dennis, Reddacliff, and Whittington, 2011).

Gross pathology is observed in the intestine and mesenteric lymph nodes with the intestinal walls becoming thickened and oedematous with traverse folds seen in the mucosa. Lesions are also seen in the ileum but can also be observed on any part of the intestinal tract (Dennis, Reddacliff, and Whittington, 2011).

Clinical signs in cattle are observed 2 to 5 years after initial infection and include diarrhoea, progressive fall in body weight, general wasting and fall in milk production (Arsenault *et al.*, 2014). The diarrhoea seen in cattle is most of the time thick containing no blood, mucus or epithelial debris (Mohana *et al.*, 2015). Weeks after the onset of diarrhoea a swelling may occur below the jaw (bottle jaw) as a result of blood protein lost from blood stream to the digestive tract (Mohana *et al.*, 2015). Progression of the disease will lead to dehydration and severe cachexia (Mohana *et al.*, 2015).

In sheep the primary clinical symptoms are seen as a loss in body condition score (Weight lost), diarrhoea is only seen in few cases (Windsor, 2015). Anorexia, depression and diarrhoea may be seen in end stages of the disease in goats (Windsor, 2015).

In small ruminants the most definitive method of diagnosis of paratuberculosis is post mortem examination with histopathology confirmation, looking for pathological changes, fat reserve depletion, bowel wall thickening and enlargement of gut associated lymph nodes, and presence of lymphatic cords on serosa surfaces of ileum and caecum (Windsor, 2015).

Whole live – attenuated and killed MAP vaccines have been used in the past and are still in continuous use in many countries to control Johne's disease in livestock (Begg and Griffin, 2005). Existing vaccines decrease mortality and faecal shedding but do not prevent animals from getting infected (Windsor, 2006). Vaccination is a cost – efficient strategy which prevents the manifestation of clinical Johne's disease (Fridriksdottir *et al.*, 2000).

Vaccination has been used as a control strategy in many countries with good success (Fridriksdottir et al., 2000). The main disadvantage is that vaccines used do not differentiate infected from vaccinated animals thereby interfering with serological diagnosis of Johne's disease (Bastida and Juste, 2011). Because of this drawback MAP vaccination may not lead to the eradication of Johne's disease and can also interfere with tuberculosis eradication programs (Bastida and Juste, 2011). Vaccination again in sheep also produces a granulomatous lesion at the injection site due to the oil – based bacterin vaccines (Bastida and Juste, 2011).

2.3. Crohn's Disease.

Crohn's disease is a chronic inflammatory disorder of the gastrointestinal tract of humans affecting mainly the terminal ileum and the colon (El-Zaatari, 2004). The causative agent of Crohn's disease remain a subject of scientific debate with the general believe that the disease has a complex and multifactorial aetiology (Grant, 2005). Although the aetiological agent of Crohn's disease is still debatable, the majority of studies published since the year 2000 points to a higher detection rate of MAP by culture, IS900 PCR or MAP specific antibody response in patients suffering from Crohn's disease (Grant, 2005). It has been reported that around 13 out of 100,000 people in the United Kingdom may be afflicted with Crohn's disease with 3000 new cases of Crohn's disease diagnosed annually in the UK (Grant, 2005). If MAP plays a role in the pathogenesis of Crohn's disease then the most likely route of infection is either food or water born with the most likely culprits been milk (other dairy products), beef and water (Grant, 2005). Crohn's disease presents with loss of energy, loss of weight, night sweats, abdominal pain, and pain in the joints, in severe cases it might present as an abdominal emergency with peritonitis, perforation of terminal ileum or presenting as acute appendicitis ((El-Zaatari, 2004)).

2.4. Parasite vectors and MAP.

Nematode larvae can become contaminated with MAP serving as vectors for the transmission of Johne's disease (Whittington, Lloyd, & Reddacliff, 2001). Nematodes have a simple life cycle, the nematode egg hatches in faeces, it feeds on bacteria and undergo 2 moults becoming a third stage larvae enclosed within a sheath (Singh et al., 2013). The third stage larvae are negatively geotrophic but positively phototrophic thereby moving out of faeces and travelling up blades of vegetation where they are consumed by ruminants (Soulsby, 1968).

In farm conditions, sheep with the Multibacillary form of MAP infection excrete large amounts of MAP in their faeces and any nematode larvae developing from egg in the same

faeces will become contaminated with MAP (Whittington et al., 2001). Animals showing clinical signs of Johne's disease can shed 10^8 MAP / gram of faeces, the shedding of this large amount of MAP makes it highly probable that the surface of these nematode larvae can become contaminated with MAP (Whittington et al., 2001). The contamination of nematode larvae by MAP in a real farm environment is highly probable because the same environmental factors that favour survival of the 3 stage larvae also favour survival of MAP (Anderson, 1992).

The ability of the larva of *Haemonchus contortus*, *Ostertagia circumcincta* and *Trichostrongylus colubriformis* to take up MAP has been demonstrated (Whittington et al., 2001). Nematode larvae serve as a viable means or as mechanical vectors for the transmission of MAP (Singh et al., 2013). Ingestion of larvae contaminated with MAP will result in the release of MAP in the lumen of the intestine as the larvae gets rid of its sheath (Whittington et al., 2001). Moreover the ability of nematode larvae to penetrate mucosa of the gastrointestinal tract provides an additional route for the delivery of MAP to susceptible animal tissue which aids in the development of the disease (Whittington et al., 2001).

2.5. Gastrointestinal microbiome and the host.

Colonization of the host mammalian gastrointestinal tract begins soon after birth (Malmuthuge, Griebel, & Guan, 2015). Further exposure of the host to specific microbes will lead to further colonization of the gastrointestinal tract by more microbes during the animals life (Malmuthuge et al., 2015). The population or assemblage of these microbes within the gut and their collective genomes is known as the gastrointestinal microbiome ((McDermott & Huffnagle, 2014).

A symbiotic relationship exists between the host and the gastrointestinal microbiome where the host provides the microbes with nourishment and an ecosystem to live whereas the microbes aid in the development of the host gut mucosa enhancing immunity and also play a vital role in the digestion of complex plant materials (Leser & Mølbaek, 2009).

The immune system of the intestine is predominantly underdeveloped without microbial activities stimulating it to action (McDermott & Huffnagle, 2014). These microbes play significant roles in the normal development of the gut associated lymphoid tissues (Peyer's patches, crypt patches, and isolated lymphoid follicles), spread of gastrointestinal specific immune responses and prevention of pathogen colonization (Kamada et al., 2013). Numerous studies have shown that commensal bacteria can hinder pathogen colonization by directly competing for limited nutrients within the intestine thereby preventing pathogens from deriving nourishments (Kamada et al., 2013). A very good example of direct

competition for nourishment is seen where *Escherichia coli* competes with enterohaemorrhagic *E.coli* for organic acids, amino acids and other nutrients (Leatham *et al.*, 2009).

The rumen of ruminants is a complex ecosystem of beneficial microbes (Bacterial, Archaea, yeast, fungi and protozoa) that aid ruminants to digest plant material into utilizable energy (Sauer, Marx, & Mattanovich, 2012). The microbial population in the rumen play a very important role in the establishment and development of microbial fermentation beginning around 2 or 4 weeks as a result of solid feed consumption (Baldwin and Jesse, 1992). A complex process of digestion occurs in the rumen as a result of the presence of this vast assemblage of gastrointestinal microbes which makes it possible for ruminants to utilize cellulose and other structural and non-structural carbohydrates (Agrawal *et al.*, 2014). Enzymes needed for the degradation of complex plant materials (polysaccharides) are not produced by the ruminants themselves but are rather produced by microbes that live in the rumen ecosystem (Henderson *et al.*, 2015). The resultant fermentation process that occurs due to the activities of ruminal microbial organisms leads to the production of volatile fatty acids which serves as a major source of energy (Henderson *et al.*, 2015).

The population of ruminal microbes is usually affected by diet and feeding strategies, but despite these facts there is a similarity of rumen bacteria found to be abundant in different parts of the globe (Henderson *et al.*, 2015). The 7 most recognised abundant bacteria include *Prevotella*, *Butyrivibrio*, *Ruminococcus*, unclassified *Lachnospiraceae*, *Ruminococcaceae*, *Bacteroidales* and *clostridiales*. These group of bacteria can be regarded as the core rumen microbiome because of their presence in a large selection of ruminants (Henderson *et al.*, 2015). Another group of microbes found in the rumen are the archaea with the majority being methanogens and the dominant types were found to be similar in all regions of the world (Henderson *et al.*, 2015). For many rumen bacteria, diet plays a major role in determining their abundance. Bacteria populations from forage fed animals were discovered to be similar to each other while bacteria population from concentrate fed animals were also similar to each other (Henderson *et al.*, 2015).

The gastrointestinal microbiota of humans is composed of trillions of microbes most of which are non – pathogenic bacteria and viruses (Reyes *et al.*, 2010). The microbiota works in collaboration with the host immune system to protect the body against pathogen colonization and invasion. The microbiota also provides essential nutrients and vitamins and aids in extraction of short chain fatty acids and amino acids from diets. Disturbances in the microbiota can occur due to exposure to environmental factors such as diet, toxins, drugs and

pathogens. Enteric pathogens have the greatest probability to cause dysbiosis in the gut microbiota.

Crohn's disease is considered one of the prevalent forms of inflammatory bowel disease with a characteristic inflammation of the intestinal mucosa (Carding et al., 2015). The aetiology of Crohn's disease is still debatable but there is overwhelming evidence that intestinal microbial dysbiosis plays a major role in its pathogenesis (Baumgart & Carding, 2007). Ultimately patients suffering from Crohn's disease show a decrease in microbial population, functional diversity and stability of gut microbiota with specific decrease in *Firmicutes* and an accompanying increase in *Bacteroidetes* and facultative anaerobes such as *Enterobacteriaceae* (Hansen, Gulati, & Sartor, 2010).

2.6 Areas of further Study.

The need to understand the microbiome of sheep as it relates to Johne's disease is an area that will require more studies and research. Is dysbiosis in the gastrointestinal microbiome responsible for the clinical manifestation of the disease and if dysbiosis plays a role in the infection what are the factors that trigger this dysbiosis? Further understanding of the role of the gastrointestinal microbiome and its contribution to the development of the gastrointestinal tract immune system in ruminants is needed. Quite a number of bacteria, archaea are found in the rumen and other parts of the gastrointestinal tract of sheep but which ones are involved in the development of the gastrointestinal immune system and in the development of the gastrointestinal tract are yet to be identified.

Within the commercial farm environment, there is an association between animals that show clinical signs of Johne's disease and also carried high worm burdens. This association might imply a relationship in which the larvae of the worm act as either a vector carrying MAP on the surface of its body or inside its body. Further work is needed to establish an understanding of the relationship between nematodes infestation and MAP infection.

Johne's disease infection normally occurs early in life, at the neonatal stages in sheep with disease manifesting after 2 – 4 years, the susceptibility of age to infection is also an area that needs further investigation in order to establish what role the gastrointestinal microbiome plays in exposing young animals to infection with MAP and making adult animals more resistant to the disease.

In this particular project I intend to carry out an analytical study to understand the relationship between Johne's disease pathogenesis, gastrointestinal microbiome and gastrointestinal parasites. Initially, I will investigate the role of the gut microbiome and intestinal parasites. This will be followed by investigating the dual infection of intestinal parasites in association with the clinically affected Johne's diseased sheep from a

commercially run farm with a known history of MAP infection. By comparing these two studies, the unique bacterial flora that is associated with dual infections in sheep can be analysed.

The eventual aim of this project is to identify biomarkers that could be used in an improved diagnostic test and develop preventative control strategies that can be used to improve the wellbeing of livestock by manipulation of the gut flora using dietary supplements or probiotics to inhibit the colonisation of the gut with MAP. Probiotics can be used for regulating the equilibrium and activities of the gastrointestinal microbiome (Uyeno, Shigemori, and Shimosato, 2015)

3. Methodology

3.1 Rectal faecal sample collection

Rectal faecal samples of sheep (Scottish black face, gimmers) were collected as surrogate samples for the small intestine content from 9 commercial farms without a history of Johne's disease. They were divided into groups depending on which anthelmintic treatment they received: (following manufacturer's dose rate per kilogram body weight) as below:

Group 1 - Zolvix® (2.5mg kg bodyweight of Monenpantel)

Group 2 - Startect® (2mg Derquantel and 0.2mg Abamectin per kg bodyweight)

Group 3 – Zolvix® + Startect®

After 14 days faecal samples were taken from each sheep and frozen at -80°C

All the faecal samples taken from each sheep were frozen at -80°C.

This research was also part of a larger project to assess the impact of single or sequential administration of the two new anthelmintic compounds (Zolvix® and/or Startect®) and to determine if the sequential administration of the two new active drenches (Zolvix® and Startect®) has an additive/synergistic or antagonistic effect using animals sourced from a number of farms. Faecal samples obtained from Day 0 and Day 14 were stored at -80oC to determine if differences in treatment had an effect on the faecal microbiome of sheep in each treatment group pre and post treatment.

3.2 Collection and storage of sheep faecal samples

Ovine faecal samples were collected from 2 farms with Scottish black face breed of sheep with a history of Johne's disease and gastro-intestinal nematodes. Samples were collected once annually for a period of 3 years (Year 1 collection, Year 2 collection and Year 3 collection). The faecal samples were collected from the rectum and immediately packaged in plastic bags labelled with the sheep number, before being placed in a cold box and transported to the Moredun Research Institute where they were sorted and placed in the -80°C storage freezers.

Blood samples were also taken intravenously through the jugular vein. The blood samples were collected in vacutainer tubes and stored in cold boxes and then transported to the Moredun Research Institute for storage and subsequent forwarding to BioBest Laboratories for analysis for the presence of serum antibody using a commercial ELISA test (Appendix A).

3.3 Extraction of DNA from ovine faecal samples.

Microbial DNA was extracted from sheep faecal samples using MO BIO PowerFecal® DNA Isolation Kit by carefully following the manufacturer's extraction instructions. 0.25 grams of faecal sample were loaded into 2 ml tubes containing dry beads (provided in the kit). 750µl of Bead Solution was added to each tube. The faeces within the tubes were homogenized by vortexing for 10 seconds. 60µl of Solution C1 containing an anionic detergent (sodium dodecyl sulphate) was then added to each tube sample and vortexed for 10 seconds. The samples were then heated at 65°C for 10 minutes to further lyse the cells. The bead tubes were then secured in a horizontal MO BIO Vortex Adapter tube holder and vortex for 10 minutes at room temperature and afterwards centrifuged at 13000xg for 1 minute to pellet the cell and faecal debris. Between 400 - 500µl of the supernatant was then transferred from each tube to a clean 2ml collection tube. Non DNA organic and inorganic materials, cell debris and protein were precipitated from the supernatant by adding 250µl of solution C2 which is a patented Inhibitor Removal Technology ® (IRT); a reagent that precipitates non-DNA organic and inorganic material including polysaccharides, cell debris and proteins. The mixture was vortexed to mix for 10 seconds then incubated at 4°C for 5 minutes. Samples were again centrifuged at 13,000xg for 1 minute. About 600µl of the supernatant were then transferred to clean 2ml collection tubes, carefully avoiding the transfer of the pelleted material. 200µl Solution C3 was then added to the samples and vortexed before incubating again at 4°C for 5 minutes. The samples were then centrifuged at 13,000xg for 1 minute. Avoiding the pellet about 750µl of the supernatant was transferred to a clean 2ml collection tube. Solution C4 (a high salt concentrated solution) was mixed by shaking before adding 1200µl (C4) to each of the samples to aid in the binding of DNA to the silica within the spin filters columns provided by the manufacturer. A volume of 650µl of the mixture in each tube was then loaded into the spin filters and centrifuged at 13000xg for 1 minute. The flow through was discarded and the process repeated 3 times until all the sample supernatant was loaded into the spin filters. The spin filter columns were then washed twice with 500µl of an ethanol based solution C5 to further clean the silica bound DNA by removing residual salt and contaminants. The spin filters were centrifuged and the flow through discarded. After the second wash, the spin columns were then carefully placed in 2ml Eppendorf's (without covers) and centrifuged at 13,000xg for 5 minutes to further remove the ethanol from the spin filters. The spin filters were then carefully placed in clean 2ml collection tubes. 100µl of sterile elution buffer C6 (10mM Tris) was carefully added to the centre of the filter membrane and incubated for 1 minute to ensure the entire membrane was wet enough to enable a more efficient and complete release of the DNA from the silica filter membrane.

The samples were then centrifuged at 13,000xg for 1 minute. The spin filter columns were then discarded and the eluted DNA was collected and quantified before storage at -20°C.

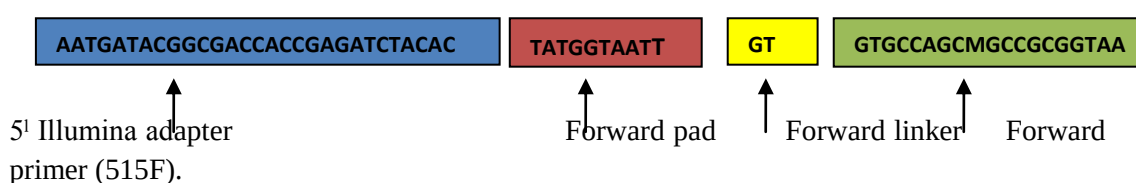
3.4 Quantification and quality control of DNA using Nano drop.

A Spectrophotometer was used to quantify the concentration of nucleic acid in all the samples extracted. This was done with the NanoDrop™ ND-1000 spectrophotometer machine (ThermoLabs) using 1.5µl of DNA sample and measuring the nucleic acid concentration and calculating the purity of the DNA by assessing the 260:280 and 260:230 ratios.

3.5 Bacterial and Archaeal amplification technique.

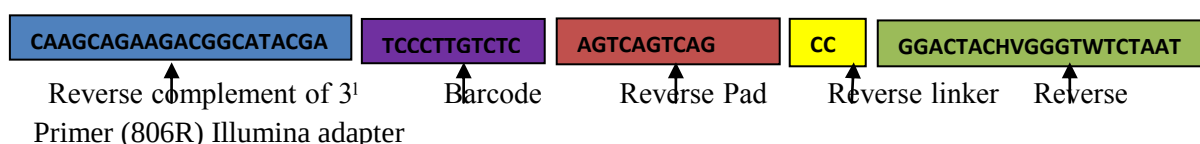
Bacterial and Archaeal DNA were amplified by Illumina bar coded polymerase chain reaction (PCR) following the method illustrated by Caporazo *et al.*, 2012. This was carried out in a DNA free PCR preparation room under sterile conditions using Taq DNA Polymerase dNTPack (ROCHE). A single reaction with a total volume of 25µl composed of 18.5µl nuclease free water, 2.5µl of 10 x PCR buffer, 1µl Magnesium chloride (25 mM), 0.5µl dNTPs (containing four deoxyribonucleotide triphosphate; adenine, guanine, thymine and cytosine), 0.25µl heat resistant Taq – polymerase (ROCHE) , 0.625µl 515F – forward 5' primer, 0.625µl reverse barcoded primer and 1µl of DNA template.

Figure 1: 515F primer sequence



The 515F primer is a short sequence of DNA that attaches to the 3' of the flanking region of the DNA strand .

Figure 2: 806R barcoded reverse primer



The reverse bar coded primer is a short sequence of DNA that attaches to the 3' end of the complementary DNA strand. Master mixes were transferred out of the DNA free room and 1µl of a DNA sample (extracted as described above) was added in each tube. 1µl of the

negative control DNA was also added to the bar code 0 which contained all mixtures but no faecal sample.

TABLE 1: Caporazo Illumina PCR Bar Coded reaction mixes

	1rtx (μ l)	12rtx (μ l)	25rtx (μ l)
Nuclease free water	18.5	222	462.5
10 x PCR buffer	2.5	30	62.5
MgCl ₂ (25 mM)	1	12	25
dNTPs	0.5	6	12.5
Taq-polymerase	0.25	3	6.25
515F - primer	0.625	7.5	15.625
Total volume aliquoted into each tube	23.375	280.5	584.375
Reverse Bar Coded primer	0.625	0.625	0.625
DNA template	1	1	1
Total volume per reaction/tube	25		

The process of PCR involved increasing the temperature to 94°C for 15 seconds to break the hydrogen bonds between the double stranded DNA strands. The solution was then cooled after DNA strand separation to 54°C to enable the DNA primers to bind to the homologous flanking regions of the DNA strands (the sequences of the DNA primers were complementary to the flanking sequence of the DNA strands, the 5' end of the primer bound to the complementary 3' end of the DNA to be replicated). The temperature was then increased to 72°C which is the optimal temperature of the heat resistant Taq – polymerase to bind to the DNA to begin adding the dNTPs (the elongation step). A thermal cycle of 94°C for 3 minutes, 94°C for 45 Seconds, 54°C for 60 seconds and 72°C for 90 seconds was repeated 25 times, Finally the PCR mix was heated to 72°C for 10minutes for final elongation, Samples were maintained at 20°C in the thermos-cycler.

3.6 Agarose gel electrophoresis.

A 1% agarose gel was prepared by mixing 1.30g of agarose with 130 ml of 1 x Tris Acetate – EDTA buffer (Sigma). The sample was microwaved on high for 60 seconds, it was taken out and swirled and then reheated in the microwave for another 30 seconds until all crystals

became clear. It was cooled under tap water until hand temperature. 10µl of gel red (GelRed™ Nucleic acid stain, 10,000x in water) was added and mixed. The mixture was then poured onto the tray, air bubbles removed; comb was placed and left to stand for about 30 minutes. After verifying that the gel was set, the agarose gel was carefully placed into electrophoresis machine ensuring that the 1x Tris Acetate - EDTA buffer was at a level just above the gel. The comb was then removed.

25µl PCR DNA samples were mixed with 5µl of 6x loading dye (Promega), then loaded into the agarose gel wells starting with the 100bp ladder (Bioline) and then the negative control. The amplified DNA in all the PCR samples were then separated by size through the process of electrophoresis. Electrophoresis was run at 100 volts. After 90 minutes it was turned off and the gel carefully lifted out of the machine. The DNA bands within the gel were visualised under ultraviolet light and the image of the gel was taken using Alphamager™ 2200 photographic machine.

The appropriately sized DNA band (400bp) on the gel were located and cut out under blue light using a size 15 scalpel. Each band was placed in a specific marked and identified collection tube ready for gel purification.

3.7 DNA Purification from gel.

DNA was purified from agarose using the Wizard® SV Gel PCR clean – up system, following the manufacturer's protocol (Promega). The gel sample in each collection tube was weighed and Membrane Binding solution was added at a ratio of 10µl per 100mg of agarose gel slice in each tube. The mixture was vortex and incubated at 65°C for 10 mins with vortex repeated every 2 minutes. The tubes were then spun in the centrifuge at 16,000xg for about 2 seconds to remove condensation from the lid of the tubes. The dissolved mixtures were then transferred to SV mini-columns that were placed in collection tubes and samples were incubated for one minute, at room temperature. The samples were then centrifuged at 16,000xg for 1 minute. The SV mini-columns were removed from each spin column assembly, the liquid was discarded, and the SV mini-column returned to the collection tube. The SV columns were washed by adding 700µl of membrane wash solution and centrifuged at 16,000xg for 1 minute. The flow through from each column was discarded, and another 700µl of membrane wash solution was again added and centrifuged and the flow through discarded. SV mini-columns were then placed in 1.5ml Eppendorf's with no lid and spun for 5 minutes at 16,000xg. The mini-columns were then carefully transferred to clean 1.5ml micro tube. 50µl of nuclease free water was added directly to the centre of the mini-columns,

incubated for 1 minute and then centrifuged for 1 minute at 16000xg. Mini-columns were then discarded and samples were stored at -20°C.

3.8 Quantifluor® One dsDNA System.

Quantitation of double stranded DNA was carried using Quantifluor® ONE dsDNA System (Promega). Quantifluor® system quantifies dsDNA by the use of a fluorescent double-stranded DNA-binding dye. The system operates in an “add and read” format that makes it possible to make sensitive quantitation of small concentrations of dsDNA.

The Promega GlowMax Multi+System fluorescence plate reader that is capable of measuring fluorescence at the appropriate wavelengths (490nm_{EX}/510-570nm_{EM}) was used for QuantiFluor® ONE dsDNA System.

Quantitation of unknown samples using QuantiFluor® was determined by comparison to a dilution series of dsDNA standards. Standards were prepared using 1:1 and 1:4 serial dilutions of QuantiFluor® ONE Lambda DNA (400ng/μl) as described in Table below.

Table 2: Quantifluor® serial dilution.

Standard	Volume of dsDNA Standard	Volume of 1xTE Buffer	Final dsDNA Concentration (ng/μl)
A	15μl of undiluted standard	0μl	400
B	10μl of Standard A	10μl	200
C	5μl of Standard B	15μl	50
D	5μl of Standard C	15μl	12.5
E	5μl of Standard D	15μl	3.1
F	5μl of Standard E	15μl	0.8
G	5μl of Standard F	15μl	0.2

199μl of QuantiFluor® ONE dsDNA was added to each well of a black 96 wells plate (Thermo scientific). 1μl of dsDNA standards (as described in the table above) was pipetted into rows A-G of the microplate in triplicate. For the blank 1μl of 1xTE buffer was pipetted into row H in triplicate. 1μl of each unknown DNA sample was pipetted to the desired number of wells on the microplate. A 200 multichannel pipette was set to 160μl to mix the

content of each well of the plate 3 times by pipetting and ejecting the volume very carefully and slowly (care was taken to avoid introducing air bubbles during mixing so as to avoid interference while reading the fluorescence in the GlowMax fluorimeter). The microplate plate (assay) was incubated for 5 minutes at room temperature protected from light. The multiwell plate was placed in the GlowMax fluorescence plate reader to measure the fluorescence ensuring that the Blue Fluorescence Optical Kit was inserted into the GloMax®. The dsDNA concentration was calculated by copying and pasting the raw fluorescence data into the Promega online tool: www.promega.com/resources/tools/quantifluordye-systems-data-analysis-workbook

3.9 Next Generation Sequencing (Illumina MiSeq).

PCR amplicons were pooled together, ensuring that all samples were equally represented. These pooled amplicon library was visualised by gel electrophoresis before being taken to Edinburgh Genomics (University of Edinburgh) for sequencing using the Illumina paired-end barcoded sequence to identify each sample in the pool. The Illumina MiSeq sequencing platform was used which employs the use of 3 separate sequencing primers. 2 of the primers are used to read sequences from the two different ends of the DNA and the third is to identify the Barcoded sequence unique to each sample.

Figure 3: Illumina MiSeq sequencing primers.

Caporaso Read 1 Primer: Which reads from the 5' end of the amplicon.

TATGGTAATT GT GTGCCAGCMGCCGCGGTAA

Caporaso Read 2 Primer: Which reads from the 3' end of the amplicon

AGTCAGTCAG CC GGACTACHVGGGTWTCTAAT

Indexing Primer: Reads the barcode sequence (Caporaso et al., 2010a)

ATTAGAWACCCBDGTAGTCC GG CTGACTGACT

3.9.1 QIIME

Quantitative insights into microbial ecology (QIIME) is an open source software pipeline that analyses and compares microbial community sequence data. QIIME supports a variety of microbial community analysis and visualization functions. By using QIIME pipeline, raw sequenced data can be analysed by operational taxonomic picking, taxonomic assignment, alpha diversity analysis, beta diversity analysis (Caporaso et al., 2010b).

Demultiplexed data (grouped into different samples based on barcoded primers) was obtained from Edinburgh Genomics of the University of Edinburgh. This data was then processed by using QIIME guard lines as follows:

Pairing of reads (forward and reverse reads) minimum of 200bp. Quality filter (reads shorter than 400bp of V4 region are filtered out as aborted reads) and combine the paired read files. The bacterial 16SrRNA V4 region is bigger than 250bp, therefore any short reads that falls below 250bp were filtered out with Python script. Python script is not part of the standard QIIME installation but was downloaded from Tony Walter's website of <https://gist.github.com/walters/7602058>. Robert Edgar's webpage was used to download the Usearch pipeline that was used for chimera sequences (DNA sequences made up of DNA from 2 or more parents) using the UCHIME function (Edgar et al., 2011). Approximately 3 – 5% of chimeric sequences were detected in the datasets. These chimeras (DNA sequences composed of DNA from two or more parents) were then filtered out. De novo operational taxonomic unit (OTU) picking was carried out clustering similar samples. Sequences that are at least 97% in resemblance were clustered together and taxonomic assignments of OTUs against GreenGenes (database for the 16SrRNA gene)13_8 was carried out by Uclust by clustering sequences that are similar. OTUs were summarized by taxonomic ranking. Taxonomic levels for the 16SrRNA gene datasets were Phylum, Class, Order, Family and Genus. Alpha rarefaction curves showing specie richness in each sample and Shannon diversity that shows specie richness and evenness of distribution of species in the samples were performed. Singletons OTUs that occur only once in the data set were removed.

3.9.2 Statistical analysis

PRIMER (Plymouth Routines in Multivariate Ecological Research) is a worldwide standard software tool used to analysed the QIIME output data. The OTU tables derived from QIIME were standardized by dividing each matrix entry by its column total and subsequently multiplied by 100 to form an impressive display or an orderly arrangement of relative abundance data. The relative abundance data were imported into Primer 6 version 6.1.12 (Prime – E, Ivybridge, UK).

Bray-Curtis coefficient similarity measure that is particularly common in ecological studies was used in PRIMER to examine resemblances between samples. A Bray-Curtis similarity of 100 represents 2 communities that are absolutely identical, while a zero Bray-Curtis similarity coefficient reveals no shared species between samples (Clarke & Warwick, 2001).

Multi-dimensional scaling (MDS) plots were generated from the Bray – Curtis similarity matrices. Lack of resemblance between samples is shown as distance between points in 2 dimensional plots (Clarke & Warwick, 2001). Kruskal stress value (Kruskal, 1964) was used to determine the precision of the MDS by the fitting of the various plots into the 2 dimensional plot.

Location and dispersion effects between multivariate samples were analysed by using PERMANOVA (permutational multivariate analysis of variance) and PERMDISP (Anderson et al., 2008) with the PERMANOVA + add on package for PRIMER 6. PERMANOVA engages distance based analysis of the multivariate data in response to analysis of variance (ANOVA). Permutational multivariate dispersion (PERMDISP) was used to test the homogeneity of the multivariate dispersions in comparing the beta-diversity of the samples.

4. Results

In this study, sheep from a variety of flocks (specified as flock number 1 to 9) were selected and purchased by Moredun Research Institute (MRI). They were moved to the MRI Firth Mains farm where they were quarantined. The gimmers (young female sheep) were individually weighed and the flock split into 3 groups based on the anthelmintic agent used (Table 1).

Seven days after arrival to Firth Mains, rectal faecal samples were taken from each sheep and a faecal egg count was carried out to identify the level of worm burdens in the individual gimmers. The faecal samples were labelled in a bag with the sheep number and date of collection (07/09/15) and frozen at -80°C. The day of this faecal collection which was also the day of anthelmintic administration after faecal collection was identified as Day 0.

Table 1: Grouping of gimmers based on anthelmintic treatment after faecal collection.

Sheep Group	Anthelmintic
Group 1	Zolvix® (2.5mg per kg bodyweight of monepantel)
Group 2	Startect®(2mg derquantel and 0.2mg abamectin per kg bodyweight).
Group 3	As above in groups 1 and 2 administered sequentially.

Day 14:

Fourteen days post treatment, rectal faecal samples were again collected par rectum from each sheep (gimmer) and frozen at -80°C. Faecal egg counts were carried out in all the samples to determine the efficacy of the anthelmintic used.

4.1 DNA Extraction

DNA was extracted from the stored faecal samples (-80°C) using the MOBIO PowerFecal® DNA Isolation kit. The quantity of DNA from each faecal sample collected from both Day 0 and Day 14 was determined (Table 2).

Table 2: Set of DNA samples extracted from rectal samples taken on Day 0 and Day 14

Animal	Flock	Day 0	Day 14
--------	-------	-------	--------

ID	Number ¹	DNA ID	DNA concentration (ng/μl)	DNA ID	DNA concentration (ng/μl)
6283	1	DNA 1A7	28.77	DNA 1A21	99.44
6285	1	DNA 1C7	48.96	DNA 1C21	142.92
6289	1	DNA 1D7	25.13	DNA 1D21	112.96
6291	1	DNA 1E7	66.3	DNA 1E21	106.25
6293	1	DNA 1F7	27.8	DNA 1F21	71.88
6295	1	DNA 1G7	56.66	DNA 1G21	81.07
6332	1	DNA 1H7	23.89	DNA 1H21	Not selected
6350	1	DNA 1I7	76.62	DNA 1I21	89.76
6357	1	DNA 1J7	78.42	DNA 1J21	Not selected
6354	1	DNA 1K7	43.4	DNA 1K21	Not selected
334	2	DNA 2A7	109.45	DNA 2A21	Not selected
336	2	DNA 2B7	112.81	DNA 2B21	Not selected
2279	2	DNA 2C7	70.67	DNA 2C21	89.93
2283	2	DNA 2D7	8.81	DNA 2D21	Not selected
2286	2	DNA 2E7	91.94	DNA 2E21	148.53
2287	2	DNA 2F7	100.13	DNA 2F21	91.25
2294	2	DNA 2G 7	113.69	DNA 2G21	98.70
2296	2	DNA 2H7	17.92	DNA 2H21	Not selected
2305	2	DNA 2I7	93.32	DNA 2I21	69.23
2362	2	DNA 2J7	125.29	DNA 2J21	100.76
586	4	DNA 4A7	49.66	DNA 4A21	147.76
587	4	DNA 4B7	56.47	DNA 4B21	104.95
588	4	DNA 4C7	99.5	DNA 4C21	84.90
597	4	DNA 4D7	75.46	DNA 4D21	Not selected

605	4	DNA 4E7	47.77	DNA 4E21	39.75
609	4	DNA 4F7	34.29	DNA 4F21	82.41
610	4	DNA 4G7	99.57	DNA 4G21	10.74
613	4	DNA 4H7	48.49	DNA 4H21	113.94
615	4	DNA 4I7	73.5	DNA 4I21	82.05
625	4	DNA 4K7	46.2	DNA 4K21	71.42
4687	6	DNA 6A7	88.98	DNA 6A21	81.18
4689	6	DNA 6B7	98.59	DNA 6B21	42.55
4720	6	DNA 6C7	60.71	DNA 6C21	70.68
5712	6	DNA 6D7	18.86	DNA 6D21	Not selected
5714	6	DNA 6E7	72.55	DNA 6E21	88.20
5790	6	DNA 6F7	6.64	DNA 6F21	55.55
5798	6	DNA 6G7	14.75	DNA 6G21	Not selected
5908	6	DNA 6I7	90.38	DNA 6I21	Not selected
6005	6	DNA 6J7	86.83	DNA 6J21	Not selected
6011	6	DNA 6K7	49.85	DNA 6K21	Not selected
6014	6	DNA 6L7	72.4	DNA 6L21	Not selected
5323	7	DNA 7A7	114.66	DNA 7A21	Not selected
9715	7	DNA 7B7	28.01	DNA 7B21	Not selected
9718	7	DNA 7C7	10.24	DNA 7C21	Not selected
9719	7	DNA 7D7	122.73	DNA 7D21	Not selected
13813	7	DNA 7E7	85.24	DNA 7E21	Not selected
13816	7	DNA 7F7	107.16	DNA 7F21	75.29
13818	7	DNA 7G7	113.44	DNA 7G21	Not selected
13819	7	DNA 7H7	92.23	DNA 7H21	125.12
13820	7	DNA 7I7	116.58	DNA 7I21	98.27

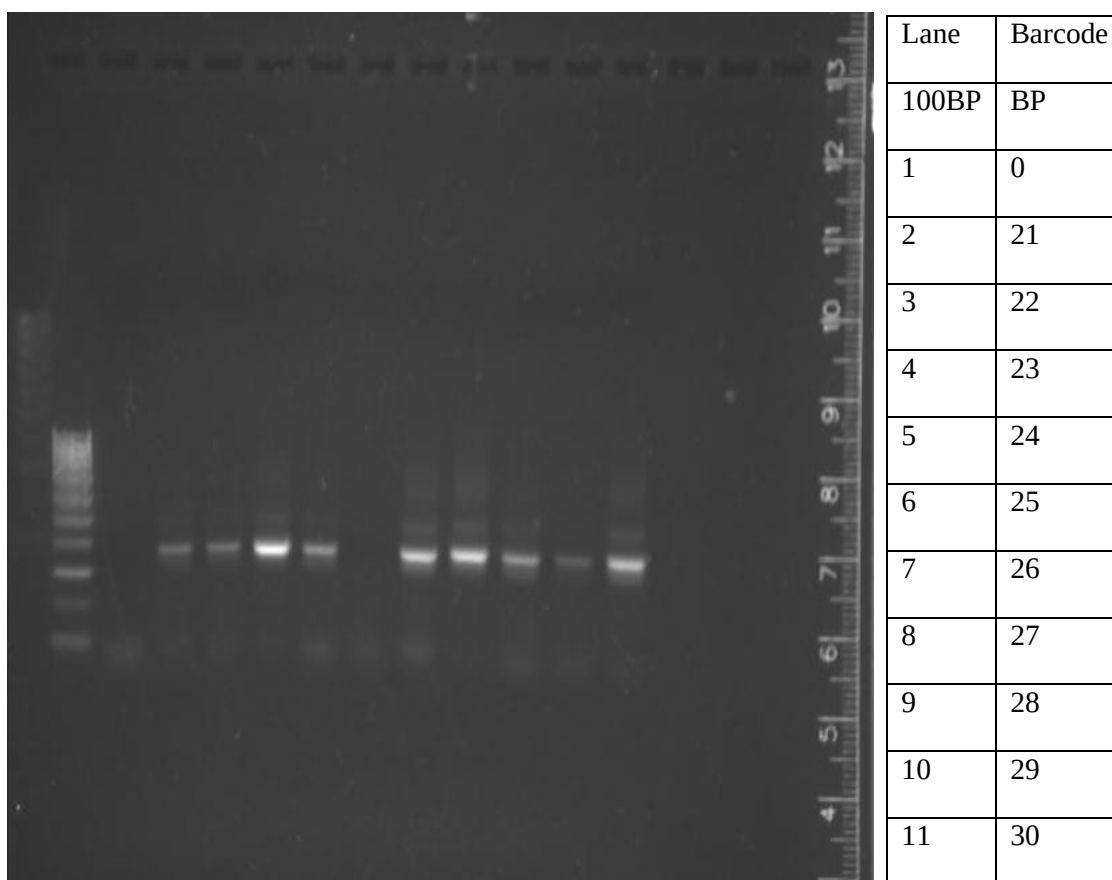
13828	7	DNA 7J7	58.29	DNA 7J21	108.53
2472	8	DNA 8A7	131.99	DNA 8A21	140.66
2474	8	DNA 8B7	116.62	DNA 8B21	116.94
2489	8	DNA 8C7	148.88	DNA 8C21	192.55
3351	8	DNA 8D7	177.36	DNA 8D21	99.62
3494	8	DNA 8E7	112.39	DNA 8E21	217.08
3574	8	DNA 8F7	114.16	DNA 8F21	Not selected
3575	8	DNA 8G7	126.95	DNA 8G21	91.84
3578	8	DNA 8H7	134.82	DNA 8H21	102.51
3587	8	DNA 8I7	19.26	DNA 8I21	156.34
3641	8	DNA 8J7	129.93	DNA 8J21	26.86

¹ flock of origin, before transport to MRI Firth Mains Farm

4.2 Polymerase Chain Reaction (PCR).

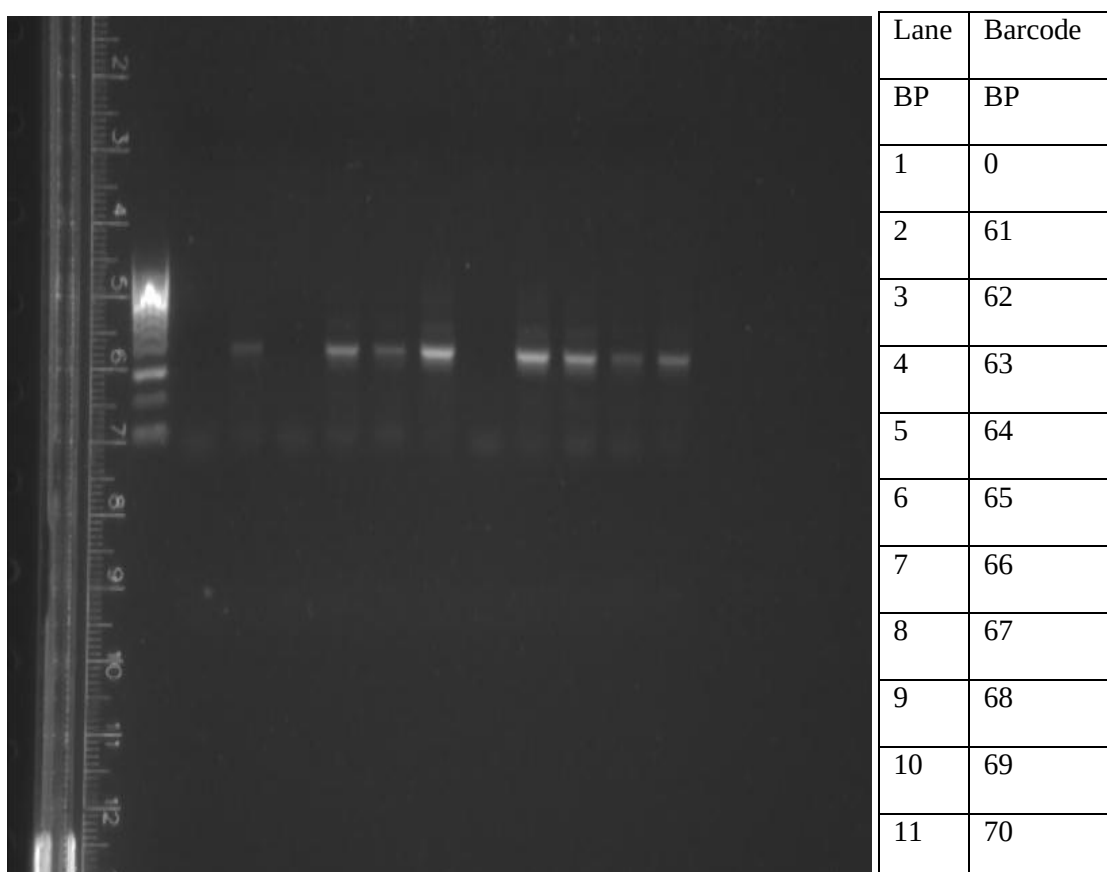
Illumina bar coded PCR (Caporazo et al., 2012) was carried out in pre - treatment and post treated DNA extracted samples. Pre-treatment DNA samples were assigned barcodes 21 to 60 while post-treated DNA samples were assigned bar codes 61 to 99. Negative control that is the kit control that had no faecal sample but went through the same process of extraction and PCR like other samples was assigned a bar code of 0.

Figure 1: A representative ultraviolet Image of the PCR results for pre – treatment samples:



From figure 1, it can be seen that there are no bands in lane 1 which is expected because it is the well with the negative control BC 0 sample which does not contain any faecal sample. DNA bands can be seen in all the other lanes except lane 6 which represents the bar coded PCR product BC 25 from DNA ID 1F7, identified as animal ID 6293. The PCR reaction was repeated for sample BC 25 at a template concentration of 1:10 which subsequently worked for this sample

Figure 2: A representative ultraviolet image of the PCR results for post-treated.



From the above picture there is no DNA band in lane 1 which contains the negative control 0 with no faecal sample. There are no bands in lane 3 and lane 7 which contain DNA amplicons BC 62 and BC 67 respectively. Sample BC 62 and BC 67 were repeated at a template concentration of 1:10 which subsequently worked for these samples.

4.2.1 Gel purified PCR products (DNA) ready for sequencing.

After DNA extraction from the pre-treatment and post treated samples and the amplification of the 16SrRNA gene V4 using PCR, a total of 38 pre-treated samples and 37 post- treated samples were gel purified and selected for sequencing. Pre-treatment samples BC46 and

BC57 failed after PCR which means their corresponding post-treated samples BC86 and BC97 were also not selected. Pre-treatment sample BC60 does not have a corresponding post-treated sample.

Table 3: Pre-treatment and Post treated samples selected for sequencing.

Animal ID	Flock ID	DNA ID	DNA ng/µl	Bar Code	DATE Sample collected	Additional Information
6283	1	1A7	28.77	21	07/09/2015	Pre-treatment
6285	1	1C7	48.96	22	07/09/2015	Pre-treatment
6289	1	1D7	25.13	23	07/09/2015	Pre-treatment
6291	1	1E7	66.3	24	07/09/2015	Pre-treatment
6293	1	1F7	27.8	25	07/09/2015	Pre-treatment
6295	1	1G7	56.66	26	07/09/2015	Pre-treatment
6350	1	1I7	76.62	27	07/09/2015	Pre-treatment
2279	3	2C7	70.67	28	07/09/2015	Pre-treatment
2286	3	2E7	91.94	29	07/09/2015	Pre-treatment
2287	3	2F7	100.13	30	07/09/2015	Pre-treatment
2294	3	2G7	113.69	31	07/09/2015	Pre-treatment
2305	3	2I7	93.32	32	07/09/2015	Pre-treatment
2362	3	2J7	125.29	33	07/09/2015	Pre-treatment
586	4	4A7	49.66	34	07/09/2015	Pre-treatment
587	4	4B7	56.47	35	07/09/2015	Pre-treatment
588	4	4C7	99.5	36	07/09/2015	Pre-treatment
605	4	4E7	47.77	37	07/09/2015	Pre-treatment
609	4	4F7	34.29	38	07/09/2015	Pre-treatment
610	4	4G7	99.57	39	07/09/2015	Pre-treatment
613	4	4H7	48.49	40	07/09/2015	Pre-treatment

615	4	4I7	73.5	41	07/09/2015	Pre-treatment
625	4	4k7	46.2	42	07/09/2015	Pre-treatment
4687	6	6A7	88.98	43	07/09/2015	Pre-treatment
4689	6	6B7	98.59	44	07/09/2015	Pre-treatment
4720	6	6C7	60.71	45	07/09/2015	Pre-treatment
5790	6	6F7	36.63	47	07/09/2015	Pre-treatment
13813	7	7E7	85.24	48	07/09/2015	Pre-treatment
13816	7	7F7	107.16	49	07/09/2015	Pre-treatment
13819	7	7H7	92.23	50	07/09/2015	Pre-treatment
13820	7	7I7	116.58	51	07/09/2015	Pre-treatment
13828	7	7J7	58.29	52	07/09/2015	Pre-treatment
2472	8	8A7	131.99	53	07/09/2015	Pre-treatment
2474	8	8B7	116.62	54	07/09/2015	Pre-treatment
2489	8	8C7	148.88	55	07/09/2015	Pre-treatment
3351	8	8D7	177.36	56	07/09/2015	Pre-treatment
3575	8	8G7	126.95	58	07/09/2015	Pre-treatment
3578	8	8H7	134.82	59	07/09/2015	Pre-treatment
3641	8	8J7	129.93	60	07/09/2015	Pre-treated. No sample 100 to compare with.
6283	1	1A21	99.4	61	21/09/2015	Startect®
6285	1	1C21	142.92	62	21/09/2015	Zolvix®+ Startect®
6289	1	1D21	112.96	63	21/09/2015	Startect®
6291	1	1E21	106.25	64.	21/09/2015	Zolvix®+ Startect®

6293	1	1F21	71.88	65	21/09/2015	Startect®
6295	1	1G21	81.07	66	21/09/2015	Zolvix®
6350	1	1I21	89.76	67	21/09/2015	Startect®
2279	3	2C21	89.93	68	21/09/2015	Zolvix®
2286	3	2E21	148.53	69	21/09/2015	Zolvix®
2287	3	2F21	91.25	70	21/09/2015	Zolvix®
2294	3	2G21	98.7	71	21/09/2015	Zolvix®
2305	3	2I21	69.23	72	21/09/2015	Zolvix®+ Startect®
2362	3	2J21	100.76	73	21/09/2015	Startect®
586	4	4A21	147.76	74	21/09/2015	Startect®
587	4	4B21	104.95	75	21/09/2015	Zolvix®
588	4	4C21	84.9	76	21/09/2015	Startect®
605	4	4E21	39.75	77	21/09/2015	Zolvix®
609	4	4F21	82.41	78	21/09/2015	Startect®
610	4	4G21	10.74	79	21/09/2015	Zolvix®
613	4	4H21	113.94	80	21/09/2015	Zolvix®+ Startect®
615	4	4I21	82.05	81	21/09/2015	Zolvix®
625	4	4k21	71.42	82	21/09/2015	Zolvix®
4687	6	6A21	81.18	83	21/09/2015	Startect®

4689	6	6B21	42.55	84	21/09/2015	Zolvix®
4720	6	6C21	70.68	85	21/09/2015	Zolvix®
5790	6	6F21	55.55	87	21/09/2015	Zolvix®+ Startect®
13813	7	7E21	47.8	88	21/09/2015	Zolvix®
13816	7	7F21	75.29	89	21/09/2015	Zolvix®
13819	7	7H21	125.12	90	21/09/2015	Startect®
13820	7	7I21	98.27	91	21/09/2015	Zolvix®+ Startect®
13828	7	7J21	108.53	92	21/09/2015	Zolvix®+ Startect®
2472	8	8A21	140.66	93	21/09/2015	Zolvix®+ Startect®
2474	8	8B21	116.94	94	21/09/2015	Zolvix®
2489	8	8C21	192.55	95	21/09/2015	Zolvix®+ Startect®
3351	8	8D21	99.62	96	21/09/2015	Startect®
3575	8	8G21	91.84	98	21/09/2015	Startect®
3578	8	8H21	102.51	99	21/09/2015	Zolvix®+ Startect®

4.3 Analysis of Bacterial and Archaeal community.

All the samples were pooled together to form an amplicon library. The bacterial 16SrRNA gene V4 region amplicons were sequenced by the use of the Illumina barcoded MiSeq platform. Sequences were separated into different samples based on their respective barcodes

(demultiplexed) by the Edinburgh Genomics at the University of Edinburgh. Forward and reverse reads were paired and reads that were shorter than the expected 400bp PCR product of the V4 region were filtered out as unsuccessful reads. DNA sequences composing of DNA from two or more parents (Chimeras) were removed by the use of UCHIME. Denovo OTU (operational Taxonomic Unit) picking was carried out using QIIME. PyNast (Python Nearest alignment Space Termination) failures were removed. OTUs that only contain one sequence (singletons) across the entire database were also remove.

4.4 QIIME Taxonomy Results.

Raw sequenced data, obtained from Edinburgh Genomics, for 38 pre-treatment (BC21-BC60) and the 37 post-treated (BC61-BC99) PCR amplicons, was analysed using the QIIME pipeline. About 10 million raw data sequences were analysed. However, approximately 36,500 sequences that were less than 240bp were filtered out. Usearch chimeric checking was performed and 443,328 chimeric sequences were detected and filtered out. Complete QIIME pipeline analysis was carried out including PyNast (python nearest alignment space termination) with Greengenes 13_8 been the default database. OTUs were assigned. An OTU table was made excluding the PyNast failures (11 OTUs). Singletons (227,927 OTUs) were then removed from the filtered OTU table.

Table 4: OTU Sequence Counts for the Pre-treatment samples (BC21-BC60) and post-treated samples (BC61-BC99).

Pre-treatment		Post-treatment	
Barcodes	Counts	Barcodes	Counts
BC 21	79338.0	BC 61	61025.0
BC 22	125786.0	BC 62	110186.0
BC 23	101648.0	BC 63	84886.0
BC 24	89120.0	BC 64	71072.0
BC 25	115771.0	BC 65	74742.0
BC 26	109169.0	BC 66	98736.0
BC 27	89661.0	BC 67	96784.0
BC 28	86304.0	BC 68	80420.0
BC 29	79240.0	BC 69	76100.0

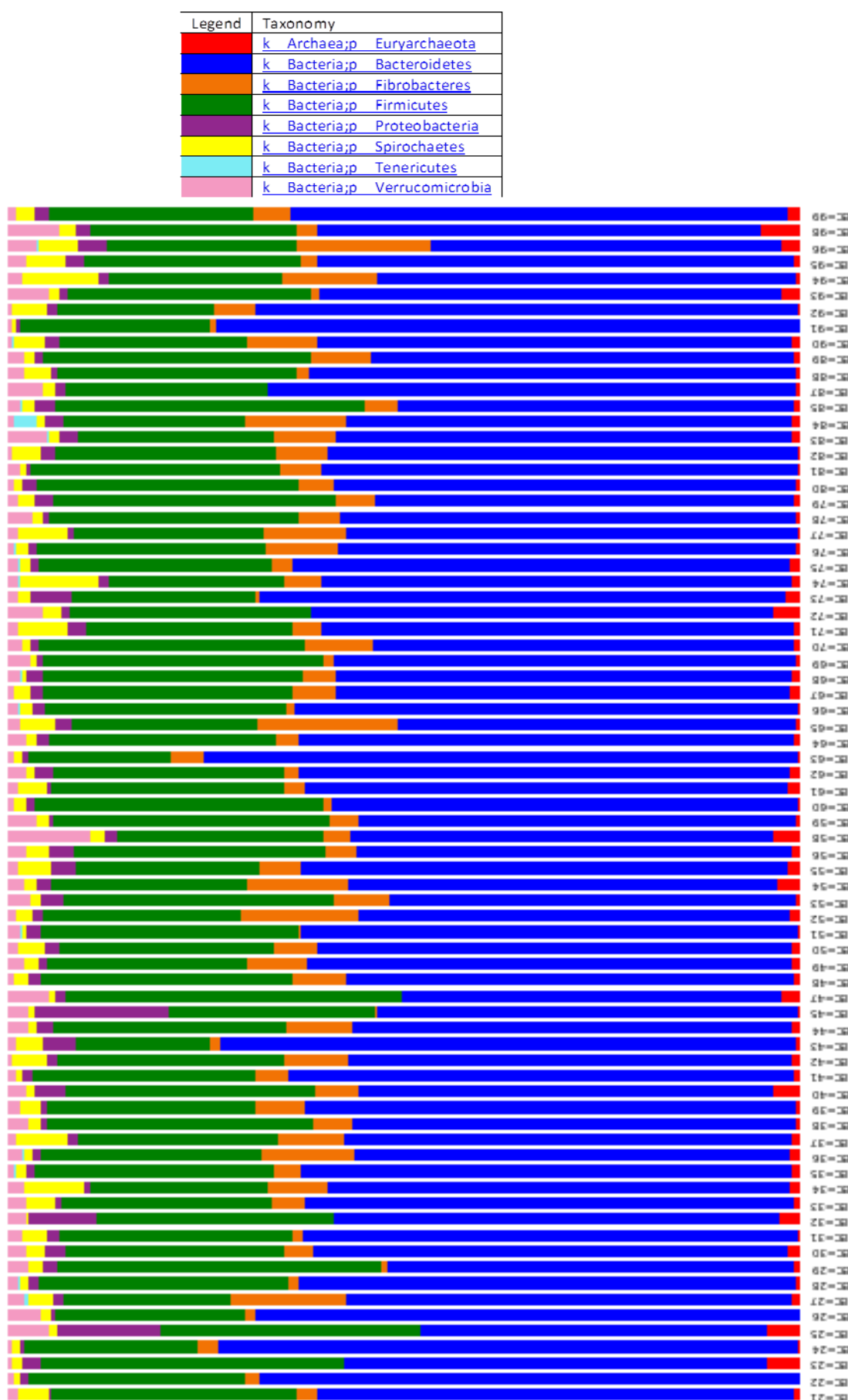
BC 30	95668.0	BC 70	81729.0
BC 31	101178.0	BC 71	110246.0
BC 32	117126.0	BC 72	122820.0
BC 33	83029.0	BC 73	101218.0
BC 34	114970.0	BC 74	118781.0
BC 35	59547.0	BC 75	142518.0
BC 36	78165.0	BC 76	69503.0
BC 37	128597.0	BC 77	108117.0
BC 38	87923.0	BC 78	79144.0
BC 39	86599.0	BC 79	168570.0
BC 40	124386.0	BC 80	86027.0
BC=41	68521.0	BC 81	135880.0
BC 42	82778.0	BC 82	75120.0
BC 43	70031.0	BC 83	68215.0
BC 44	70180.0	BC 84	75456.0
BC 45	87470.0	BC 85	76631.0
BC 47	75934.0	BC 86	93907.0
BC 48	28630.0	BC 87	87243.0
BC 49	76263.0	BC 88	68385.0
BC 50	71172.0	BC 89	91239.0
BC 51	115629.0	BC 90	112459.0
BC 52	96081.0	BC 91	67955.0
BC 53	101980.0	BC 92	103809.0
BC 54	130344.0	BC 93	129178.0
BC 55	111827.0	BC=94	77341.0
BC 56	564440.0	BC=95	83735.0

BC 58	114452.0	BC 96	81117.0
BC 59	91743.0	BC 98	98168.0
BC 60	114526.0	BC 99	66240.0

As can be seen from table 4, BC 48 had the lowest sequence count of 28,630 while BC 56 had the highest sequence counts of 564,440 sequences from the pre-treatment samples. The average sequence count for the pre-treated samples was 105,927. For the Post-treated samples, BC 61 had the lowest sequence count of 61,025 while BC 79 had the highest sequence count of 168,570. The average sequence count for the post-treated samples was 93,018.

4.4.1 Taxonomy summary (Phylum level).

Figure 3: Percentage Abundance plot at the phylum level of taxonomy.

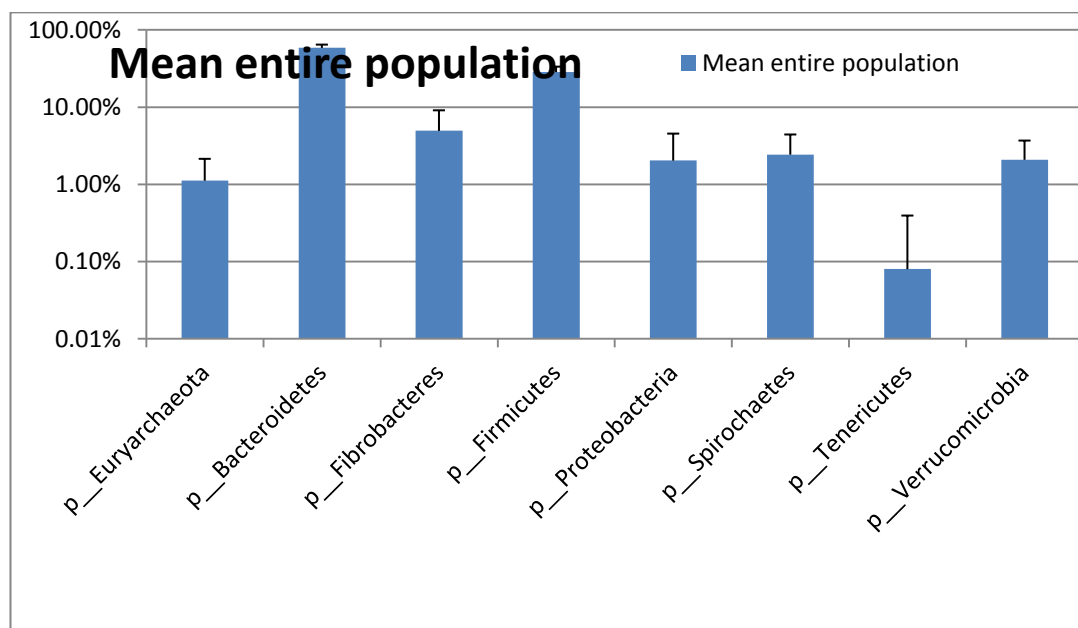


At the Phylum level of taxonomy, *Bacteroidetes* made up 58.76% relative abundance in the entire microbial population (pre-treatment + post treatment samples). *Firmicutes* (gram positive bacteria) made up 28.53% relative abundance of the entire microbial community. *Proteobacteria* had a relative abundance of 2.04%. *Spirochaetes* were 2.44% in relative abundance, *Tenericutes* had a relative abundance of 0.08% while *Verrucomicrobia* are 2.09% in relative abundance and *Euryarchaeota* had a relative abundance of 1.12% (Table 5, Figure 4 and Figure 3).

Table 5: Percentage mean of relative abundance at Phylum level of all samples (Pre-treatment + Post-treated).

Phylum	Mean	Standard deviation
<i>Euryarchaeota</i>	1.12%	1.03%
<i>Bacteroidetes</i>	58.76%	6.09%
<i>Fibrobacteres</i>	4.95%	4.19%
<i>Firmicutes</i>	28.53%	5.09%
<i>Proteobacteria</i>	2.04%	2.53%
<i>Spirochaetes</i>	2.44%	2.01%
<i>Tenericutes</i>	0.08%	0.32%
<i>Verrucomicrobia</i>	2.09%	1.62%

Figure 4: Graph showing the percentage relative abundance at Phylum level in the entire microbial population (pre-treatment plus post-treated). Graph plotted in logarithm scale base 10.



At the Phylum level of taxonomy, *Bacteroidetes* and *Firmicutes* were the two dominant Phyla making up to 87.31% mean percentage relative abundance in pre-treatment samples. The Phylum *Tenericutes* had the lowest mean percentage relative abundance in the pre-treatment samples with a mean of 0.04%.

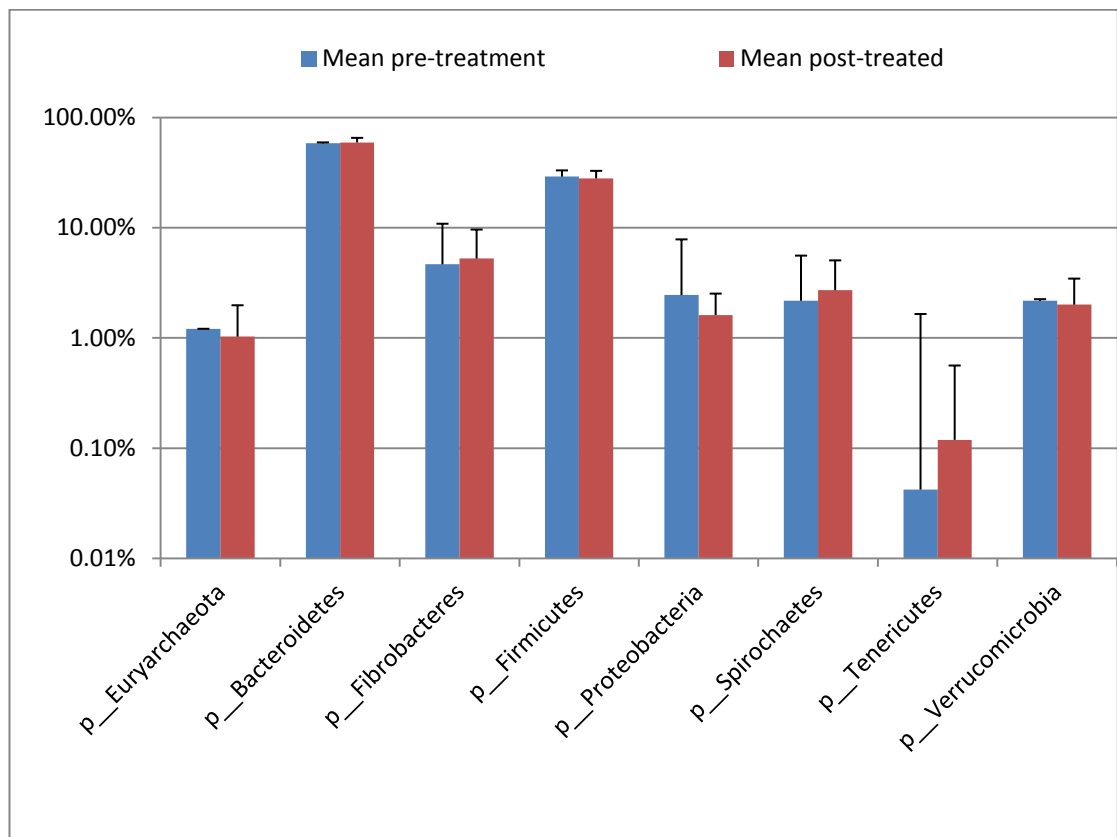
In the post treated samples the Phylum *Bacteroidetes* was the most dominant with a percentage mean relative abundance of 59.26% while *Firmicutes* was a distant second with a mean percentage relative abundance of 27.99%. The lowest mean percentage relative abundance for the post treated samples at the Phylum level was also recorded in *Tenericutes* with a mean of 0.12% (Table 6 and Figure 5).

Table 6: Percentage mean and standard deviation of relative abundance in Pre-treatment and Post-treated samples at phylum level.

Phylum	Mean Percentage Relative Abundance in Pre-Treatment samples (21-60)	Standard deviation Pre-treated samples (21-60).	Mean Percentage Relative Abundance in Post-Treated samples (61-99).	Standard deviation Post-treated samples (61-99).

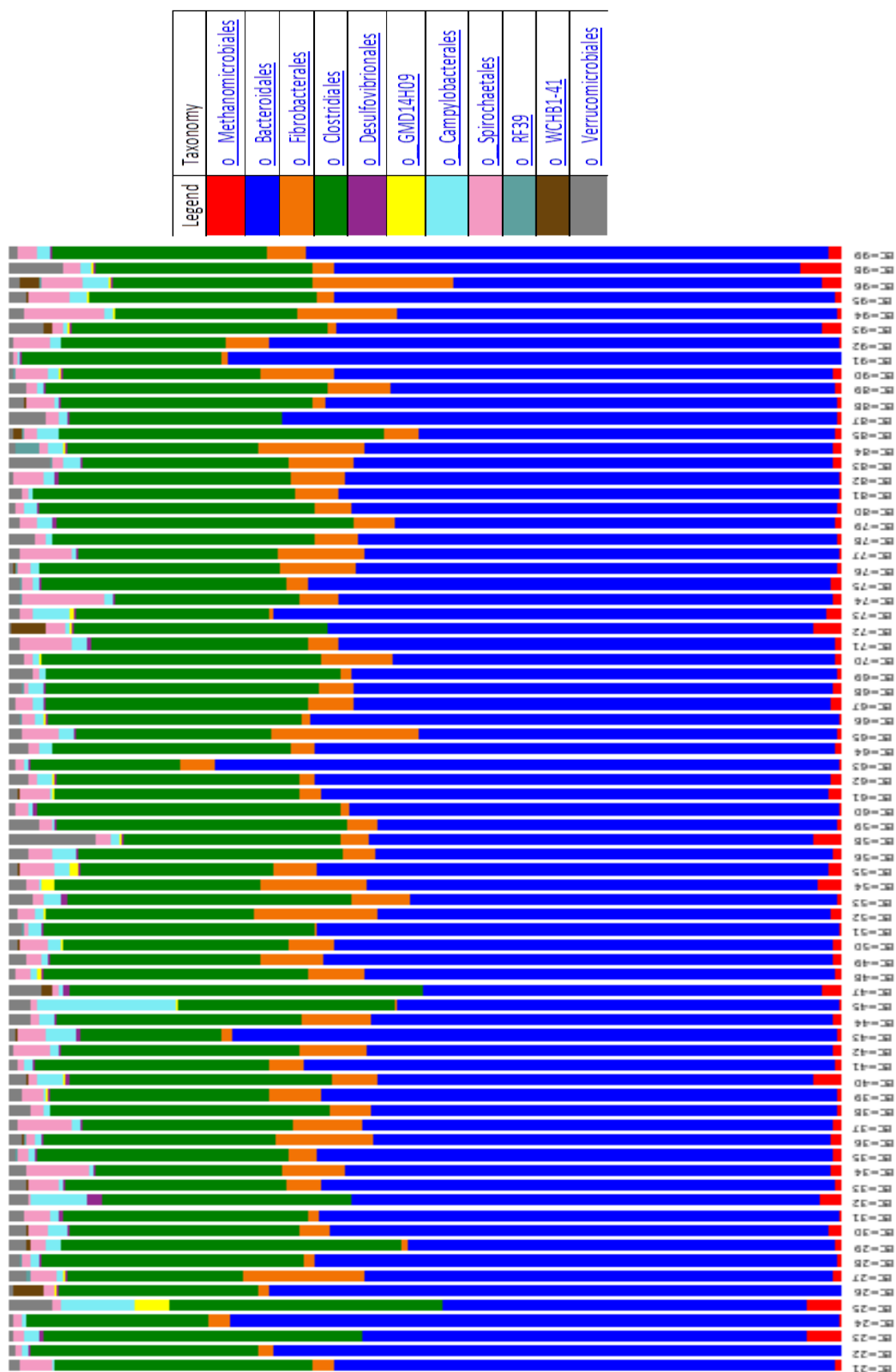
<i>Euryarchaeota</i>	1.21%	1.11%	1.03%	0.95%
<i>Bacteroidetes</i>	58.26%	6.21%	59.26%	6.01%
<i>Fibrobacteres</i>	4.65%	4.05%	5.25%	4.37%
<i>Firmicutes</i>	29.05%	5.39%	27.99%	4.78%
<i>Proteobacteria</i>	2.45%	3.41%	1.61%	0.91%
<i>Spirochaetes</i>	2.17%	1.61%	2.71%	2.35%
<i>Tenericutes</i>	0.04%	0.08%	0.12%	0.44%
<i>Verrucomicrobial</i>	2.17%	1.79%	2.00%	1.45%

Figure 5: Graph comparing percentage relative abundance at the Phylum level between pre-treatment (21-60) and post-treated(61-99) samples. Graph plotted in a logarithm scale base 10.



4.4.2 Taxonomy Summary (Order level).

Figure 6: Percentage Abundance Plot at Order level of Taxonomy.

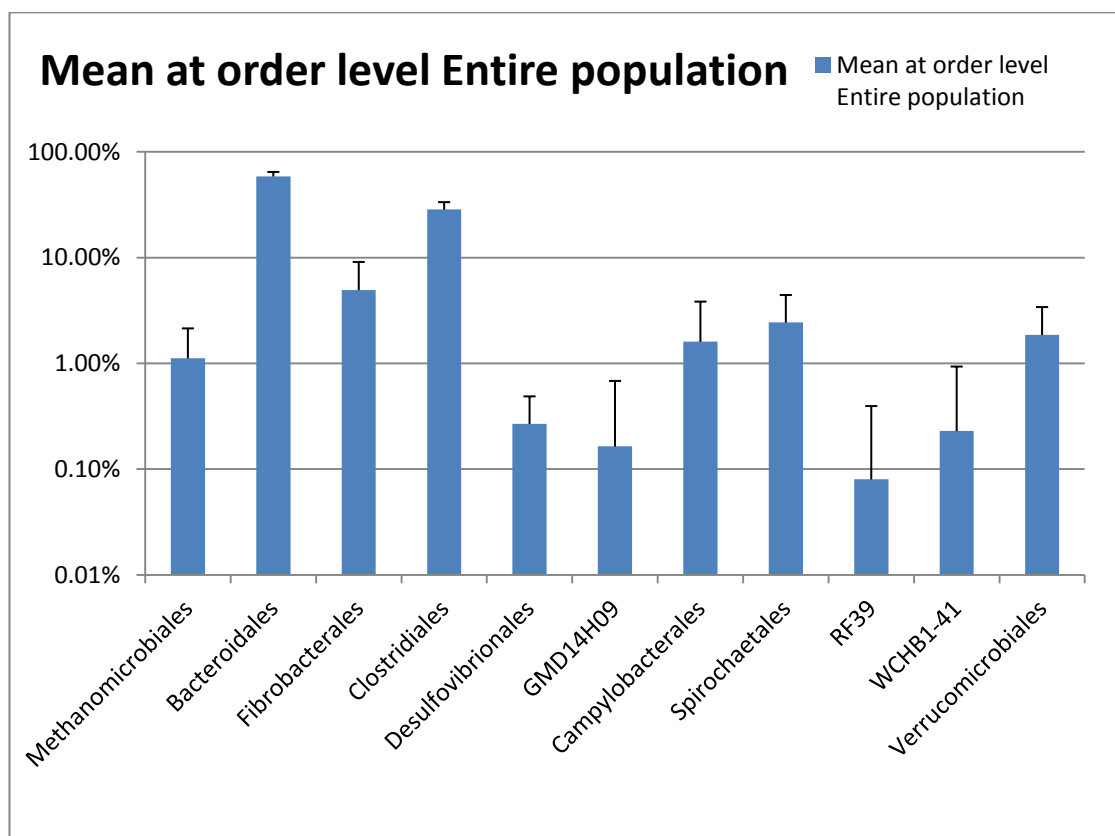


At the Order level of taxonomy, *Bacteroidales* and *Clostridiales* made up to 87.30% of the mean percentage relative abundance of the entire population (pre-treatment + post treatment). The lowest percentage mean relative abundance at the order level of taxonomy was seen in the uncultured Order RF39 from the Class *Mollicutes* which stood at 0.10% (Table 7, Figure 7 and Figure 6).

Table 7: Combine percentage mean of relative abundance at order level of all samples (Pre-treatment + Post-treated).

Order	Mean percentage relative abundance (Pre-treatment + Post treated.	Standard deviation entire population (Pre-treatment + Post-treatment)
<i>Methanomicrobiales</i>	1.10%	1.03%
<i>Bacteroidales</i>	58.80%	6.09%
<i>Fibrobacterales</i>	4.90%	4.19%
<i>Clostridiales</i>	28.50%	5.09%
<i>Desulfovibrionales</i>	0.30%	0.22%
<i>GMD14H09</i>	0.20%	0.52%
<i>Campylobacterales</i>	1.60%	2.25%
<i>Spirochaetales</i>	2.40%	2.01%
Uncultured, Order RF39, <i>Mollicutes</i>	0.10%	0.32%
Uncultured, Genus WCHB1-41, Class Verruco-5	0.20%	0.71%
<i>Verrucomicrobiales</i>	1.90%	1.56%

Figure 7: Graph showing the percentage relative abundance at Order level in the entire microbial population (pre-treatment + post-treated). Graph plotted in logarithm scale base 10.



Bacteroidales and *Clostridiales* were the dominant Orders in the pre-treatment samples with relative abundance of 58.26% and 29.05% respectively. The least dominant order in the pre-treatment sample was recorded in the uncultured Order RF39 from the Class *Mollicutes* with a mean percentage relative abundance of 0.04% (Table 8, figure 8).

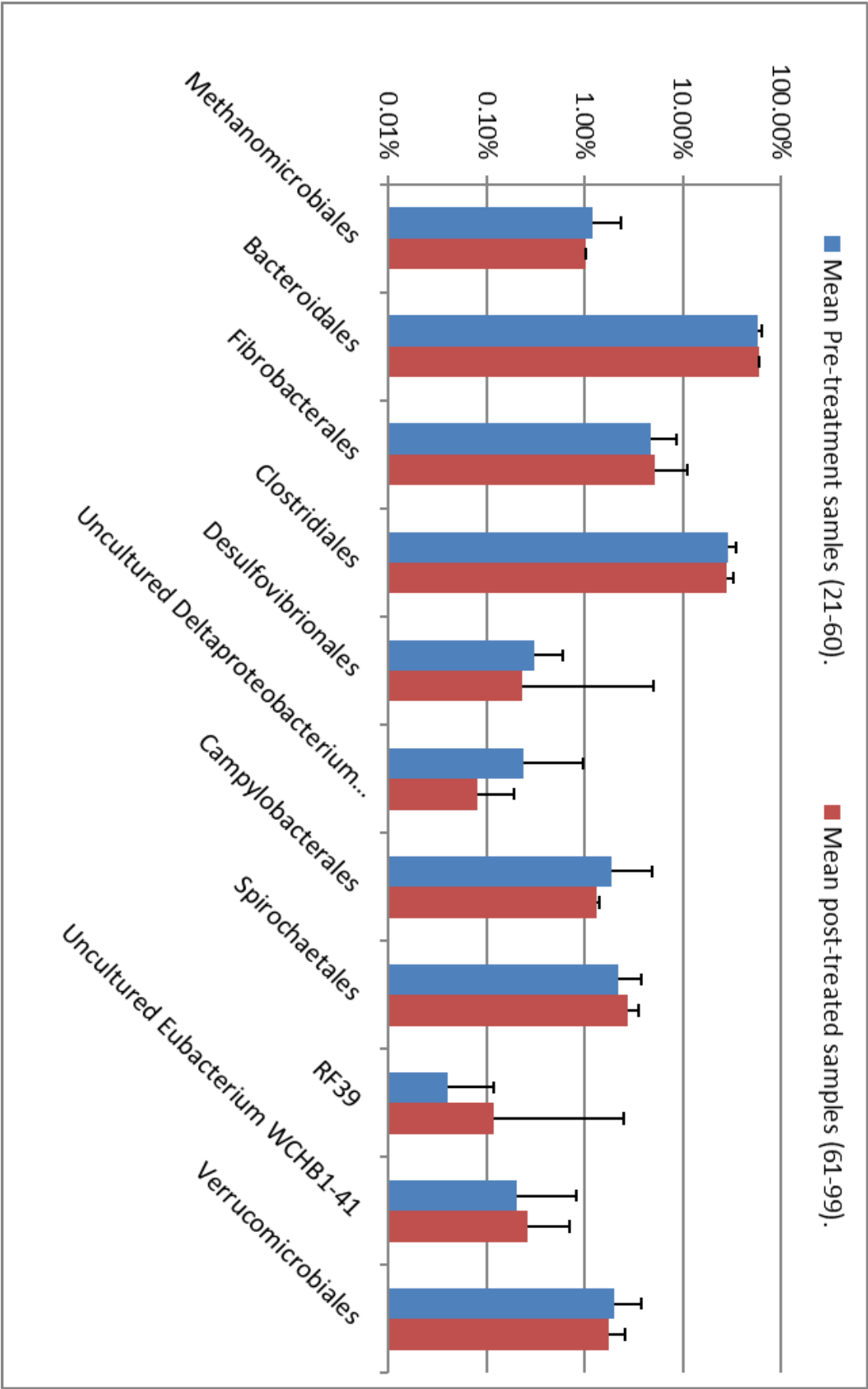
For the post-treated samples (61-99) at the Order level of taxonomy, *Bacteroidales* was again dominant with a mean percentage relative abundance of 59.26% while the lowest mean was seen in the uncultured Order Delta GMD14H09 (Table 8 and Figure 8).

Table 8: Percentage mean and standard deviation of relative abundance in Pre-treatment and Post-treated samples at Order level of taxonomy.

Order	Mean Pre-treatment samples (21-60).	Standard deviation Pre-treatment samples	Mean post-treated samples (61-99).	Standard deviation Post-Treated samples (61-99).

		(21-60).		
<i>Methanomicrobiales</i>	1.21%	1.11%	1.03%	0.95%
<i>Bacteroidales</i>	58.26%	6.21%	59.26%	6.01%
<i>Fibrobacterales</i>	4.65%	4.05%	5.25%	4.37%
<i>Clostridiales</i>	29.05%	5.39%	27.99%	4.78%
<i>Desulfovibrionales</i>	0.31%	0.29%	0.23%	0.11%
<i>Uncultured Deltaproteobacterium GMD14H09</i>	0.24%	0.72%	0.08%	0.11%
<i>Campylobacterales</i>	1.89%	3.04%	1.31%	0.84%
<i>Spirochaetales</i>	2.17%	1.61%	2.71%	2.35%
<i>Uncultured, Genus RF39, Class Mollicutes</i>	0.04%	0.08%	0.12%	0.44%
<i>Uncultured, Genus WCHB1-41, Class Verruco-5</i>	0.20%	0.61%	0.26%	0.81%
<i>Verrucomicrobiales</i>	1.97%	1.75%	1.74%	1.36%

Figure 8: Graph of mean relative abundance in pre-treatment samples (21-60) and post treated samples (61-99) at Order level of Taxonomy.



4.4.3 Taxonomy summary of different groups based on anthelmintic drug used for Treatment.

Gimmers were divided into 3 different groups based on the anthelmintic therapy instituted. The tables below gives the identification of the different animals in the 3 different groups followed by a description of relative abundance in the 3 different groups. Figures 9 to 11 illustrate the relative abundance at the Order level of taxonomy under the three different treatment groups.

Table 9: Identity of Group 1 Animals with their bar codes (Zolvix® 2.5mg per kg bodyweight of monepantel). n = 15.

Animal ID	DNA ID	Barcode
6295	1G21	66
2279	2C21	68
2286	2E21	69
2287	2F21	70
2294	2G21	71
587	4B21	75
605	4E21	77
610	4G21	79
615	4I21	81
625	4K21	82
4689	6B21	84
4720	6C21	85
13813	7E21	88
13816	7F21	89
2474	8B21	94

In Group 1 (gimmers treated with Zolvix®) at the Order level *Bacteroidales* and *Clostridiales* had a combined mean percentage relative abundance of 87.59%. The least

dominant in mean percentage relative abundance was the uncultured Order *GMD14H09* from the Class *Deltaproteobacteria* with a mean percentage relative abundance of 0.07% (Table 10, Figure 9).

Table 10. Percentage mean and standard deviation of relative abundance in group 1 (treated with Zolvix®) (2.5mg per kg bodyweight of Monepantel). **n = 15**

Order	Mean	Standard deviation.
<i>Methanomicrobiales</i>	0.63%	0.34%
<i>Bacteroidales</i>	57.25%	4.04%
<i>Fibrobacterales</i>	5.73%	3.79%
<i>Clostridiales</i>	30.34%	4.98%
<i>Desulfovibrionales</i>	0.25%	0.11%
Uncultured Order <i>GMD14H09</i> , Class <i>Deltaproteobacteria</i>	0.07%	0.09%
<i>Campylobacterales</i>	1.17%	0.61%
<i>Spirochaetales</i>	2.75%	2.65%
Uncultured Order <i>RF39</i> Class <i>Mollicutes</i>	0.25%	0.68%
Uncultured Order <i>WCHB1-41</i> Class <i>Verruco-5</i>	0.11%	0.33%
<i>Verrucomicrobiales</i>	1.46%	0.60%

Figure 9: Graph of relative abundance in Group 1 (Zolvix® treated samples) at Order level taxonomy.

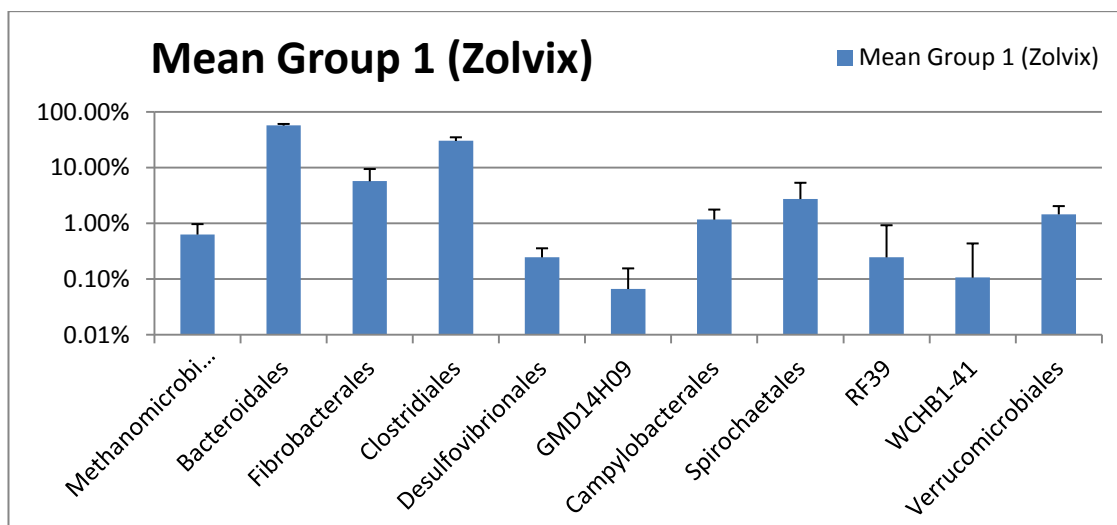


Table 11: Identity of Group 2 Animals (treated with anthelmintic Startect®) (2mg derquantel and 0.2mg abamectin per kg bodyweight. n = 12.

Animal ID	DNA ID	Barcode
6283	1A21	61
6289	1D21	63
6293	1F21	65
6350	1I21	67
2362	2J21	73
586	4A21	74
588	4C21	76
609	4F21	78
4687	6A21	83
13819	7H21	90
3351	8D21	96
3575	8G21	98

In Group 2 (animals treated with Startect®) the Order *Bacteroidales* had a mean percentage relative abundance of 58.56% while *Clostridiales* had a mean percentage relative abundance of 25.53%. Uncultured Order RF39 from the Class *Mollicutes* was the least dominant with a mean percentage relative abundance of 0.05% in group 2 samples (Table 12, Figure 10).

Table 12: Percentage mean and standard deviation of relative abundance in group 2 :Startect® (2mg derquantel and 0.2mg abamectin per kg bodyweight). n = 12

Order	Mean	Standard deviation
<i>Methanomicrobiales</i>	1.42%	1.26%
<i>Bacteroidales</i>	58.56%	7.55%
<i>Fibrobacterales</i>	7.12%	5.38%
<i>Clostridiales</i>	25.53%	4.07%
<i>Desulfovibrionales</i>	0.22%	0.10%
Uncultured, Order GMD14H09, Class Deltaproteobacteria	0.12%	0.14%
<i>Campylobacterales</i>	1.57%	1.19%
<i>Spirochaetales</i>	3.20%	2.58%
Uncultured Order RF39, <i>Mollicutes</i>	0.05%	0.09%
Uncultured Order WCHB1-41 Class Verruco-5	0.22%	0.69%
<i>Verrucomicrobiales</i>	1.99%	1.89%

Figure 10: Graph of relative abundance in Group 2 (Startect®) treated samples at Order level of Taxonomy.

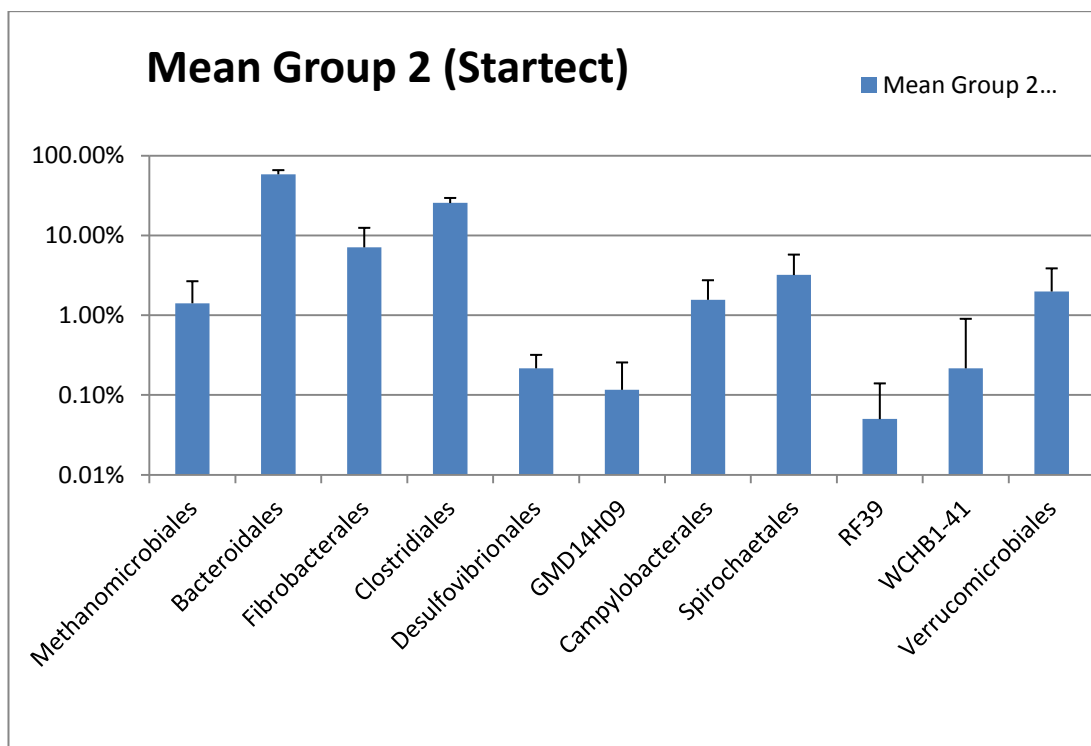


Table 13: Identity of Group 3 of Animals treated with combination of Zolvix® and Startect®. n = 10

Animal ID	DNA ID	Barcode
6285	1C21	62
6291	1E21	64
2305	2I21	72
613	4H21	80
5790	6F21	87
13820	7I21	91
13828	7J21	92
2472	8A21	93
2489	8C21	95

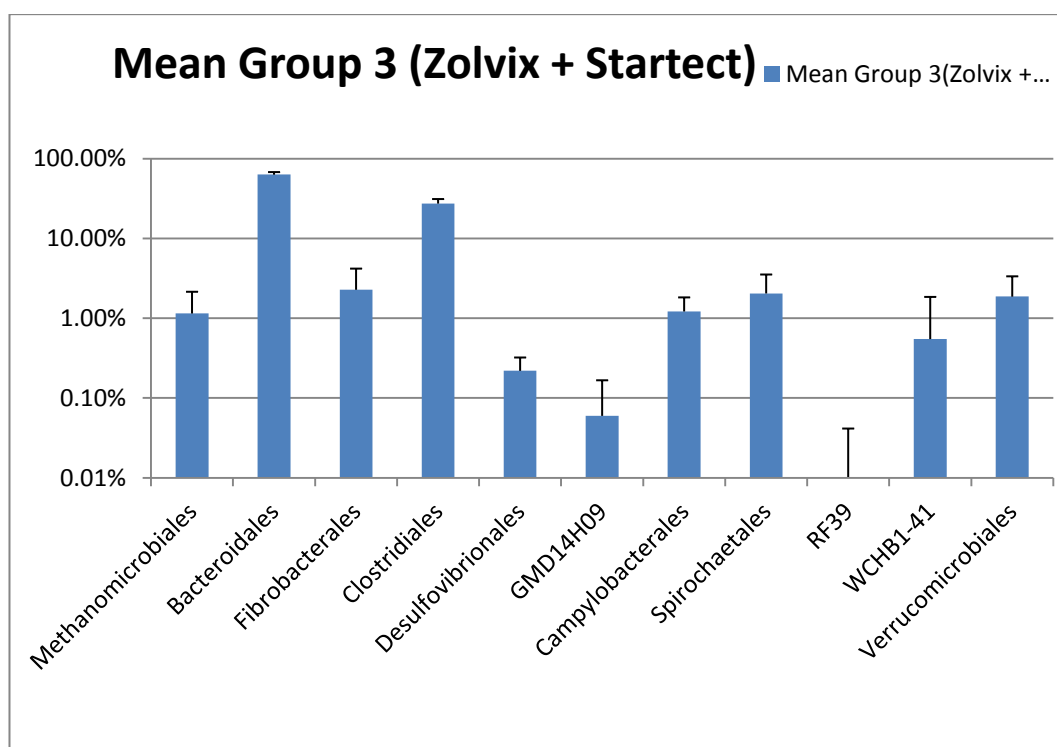
3578	8H21	99
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The highest average relative abundance in Group 3 (gimmers treated with a combination of Zolvix® and Startect®) was seen in the order *Bacteroidales* at 63.13%. *Clostridiales* was a distant second with a mean of 27.43%. The uncultured Order RF39 from the Class *Mollicutes* was also the least in relative abundance in this group with a mean of 0.01% (Table 14, Figure 11).

Table 14: Percentage mean and standard deviation of relative abundance in group 3. Combination of Zolvix® (2.5mg per kg bodyweight of monepantel) and Startect® (2mg derquantel and 0.2mg abamectin per kg bodyweight). n = 10.

Order level of Taxonomy	Mean	Standard deviation.
<i>Methanomicrobiales</i>	1.15%	1.00%
<i>Bacteroidales</i>	63.13%	5.02%
<i>Fibrobacterales</i>	2.28%	1.92%
<i>Clostridiales</i>	27.43%	3.87%
<i>Desulfovibrionales</i>	0.22%	0.10%
Uncultured, Order GMD14H09, Class Deltaproteobacteria	0.06%	0.11%
<i>Campylobacterales</i>	1.22%	0.61%
<i>Spirochaetales</i>	2.05%	1.49%
Uncultured Order RF39, Class <i>Mollicutes</i>	0.01%	0.03%
Uncultured Order WCHB1- 41, Class <i>Verruco-5</i> .	0.55%	1.31%
<i>Verrucomicrobiales</i>	1.87%	1.49%

Figure 11: Graph of relative abundance in Zolvix® + Startect® treated samples to the Taxonomic level of Order.



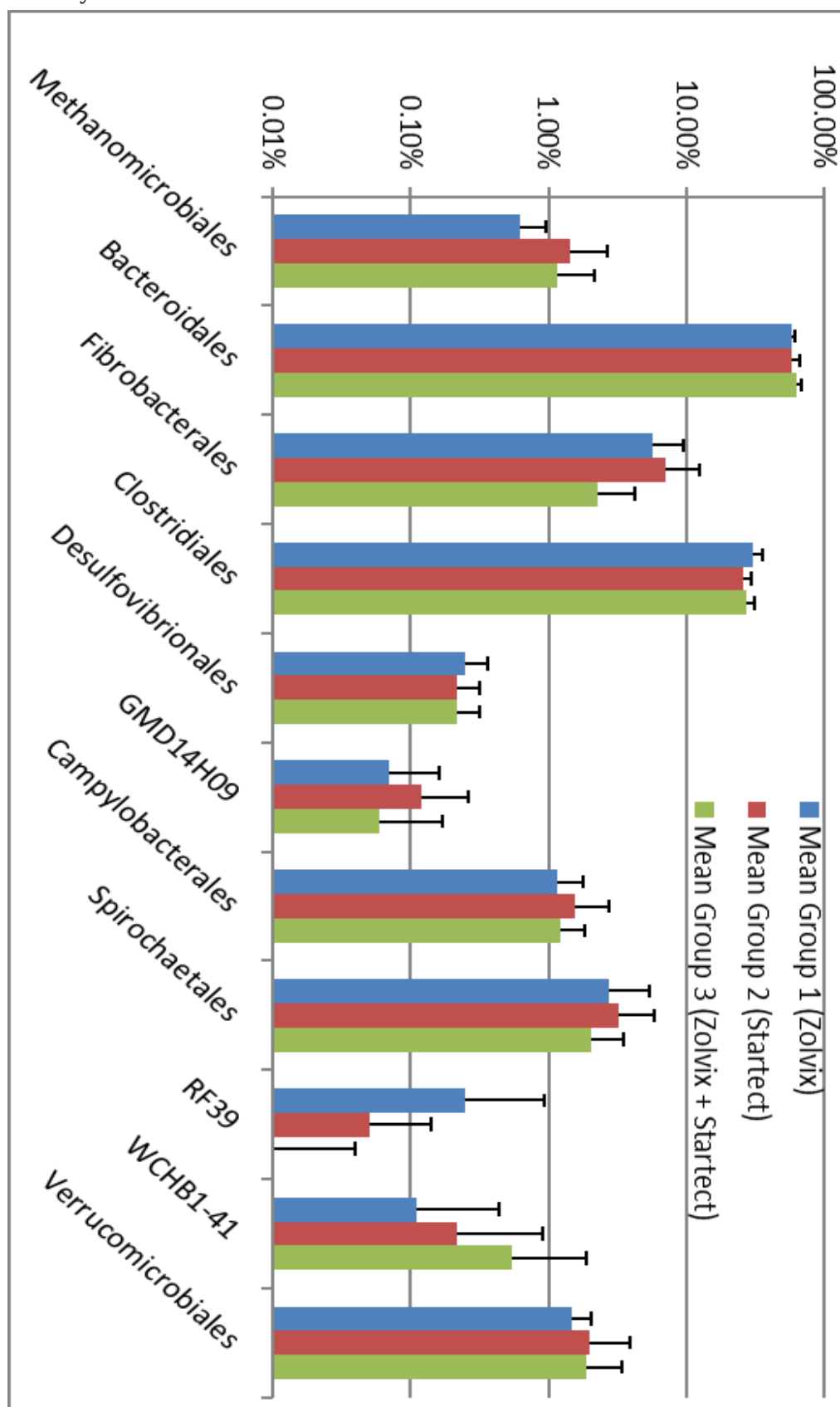
The mean percentage relative abundance in each of the post treated groups was compared. *Bacteroidales* had the highest mean percentage relative abundance of 63.13% in the group 3, it stood at 58.56% in group 2 samples and 57.25% in group 1 samples. *Clostridiales* recorded the highest mean percentage relative abundance of 30.34% in group 1, closely followed by a mean percentage relative abundance of 27.43% in group 3 samples. Uncultured Order RF39 from the Class Mollicutes had a mean percentage relative abundance of 0.25% in group 1, 0.05% in group 2 and 0.01% in group 3 (Table 15, Figure 12).

Table 15: Percentage mean of relative abundance in group 1, group 2, and group 3

Order	Mean Group 1 (Zolvix®)	Mean Group 2 (Startect®)	Mean Group 3 (Zolvix® + Startect®)
<i>Methanomicrobiales</i>	0.63%	1.42%	1.15%
<i>Bacteroidales</i>	57.25%	58.56%	63.13%
<i>Fibrobacterales</i>	5.73%	7.12%	2.28%

<i>Clostridiales</i>	30.34%	25.53%	27.43%
<i>Desulfovibrionales</i>	0.25%	0.22%	0.22%
<i>Uncultured, Order GMD14H09, Class Deltaproteobacteria</i>	0.07%	0.12%	0.06%
<i>Campylobacterales</i>	1.17%	1.57%	1.22%
<i>Spirochaetales</i>	2.75%	3.20%	2.05%
<i>Uncultured, Order RF39, Class Mollicutes</i>	0.25%	0.05%	0.01%
<i>Uncultured Order WCHB1-41 Class Verruco-5</i>	0.11%	0.22%	0.55%
<i>Verrucomicrobiales</i>	1.46%	1.99%	1.87%

Figure 12: Graph of mean relative abundance in Group 1, Group 2 and Group 3 Order level of taxonomy.



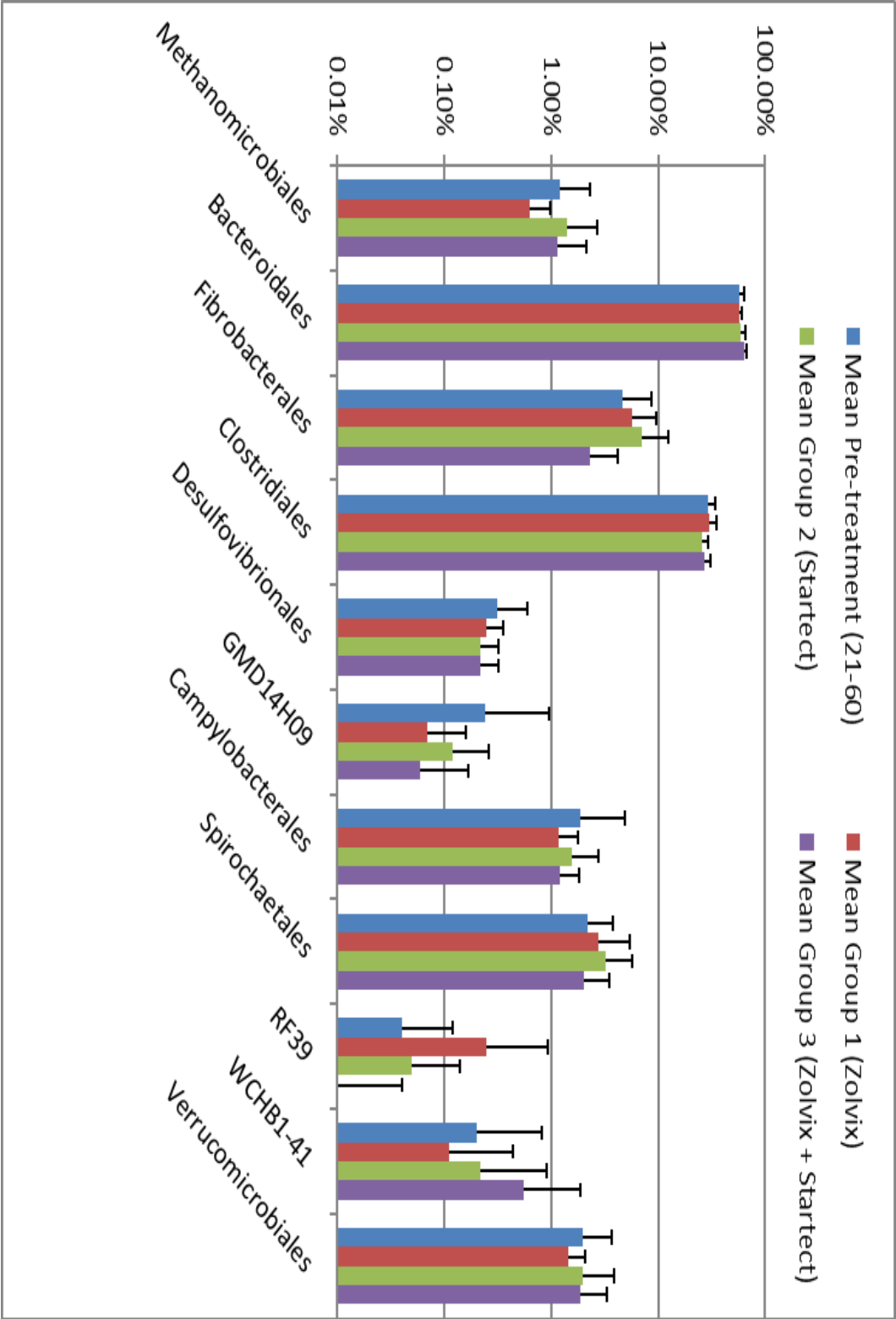
The mean percentage relative abundance in the pre – treatment samples was compared with the mean percentage relative abundance in different groups of the post – treated samples (group 1, group 2 and group 3). The Order *Bacteroidales* had a mean percentage relative abundance of 58.26% in the pre – treated samples, 63.13% in group 3, 58.56% in group 2, 57.25% in group 1. The order *Clostridiales* had a mean percentage relative abundance of 29.05% in the pre-treatment samples and a mean of 30.34% in group 1. *Clostridiales* also recorded a mean of 25.53% in group 2, and 27.43% in group 3. *Campylobacterales* had a mean percentage relative abundance of 1.89% in the pre-treatment samples. In group 1 *Campylobacterales* had a mean of 1.17%, a mean of 1.57% in group 2 and a mean of 1.22% in the group 3 samples (Table 16, Figure 13).

Table 16: Percentage mean relative abundance in Pre-treated, Group 1, Group 2, Group 3 at Order level of Taxonomy.

Order level of Taxonomy	Mean Pre-treatment (21-60)	Mean Group 1 (Zolvix®)	Mean Group 2 (Startect®)	Mean Group 3 (Zolvix® + Startect®)
Methanomicrobiales	1.21%	0.63%	1.42%	1.15%
Bacteroidales	58.26%	57.25%	58.56%	63.13%
Fibrobacterales	4.65%	5.73%	7.12%	2.28%
Clostridiales	29.05%	30.34%	25.53%	27.43%
Desulfovibrionales	0.31%	0.25%	0.22%	0.22%
Uncultured Order GMD14H09, Class Deltaproteobacteria	0.24%	0.07%	0.12%	0.06%
Campylobacterales	1.89%	1.17%	1.57%	1.22%
Spirochaetales	2.17%	2.75%	3.20%	2.05%
Uncultured Order, RF39, Class Mollicutes	0.04%	0.25%	0.05%	0.01%
Uncultured Order WCHB1-41, Class Verruco-5	0.20%	0.11%	0.22%	0.55%

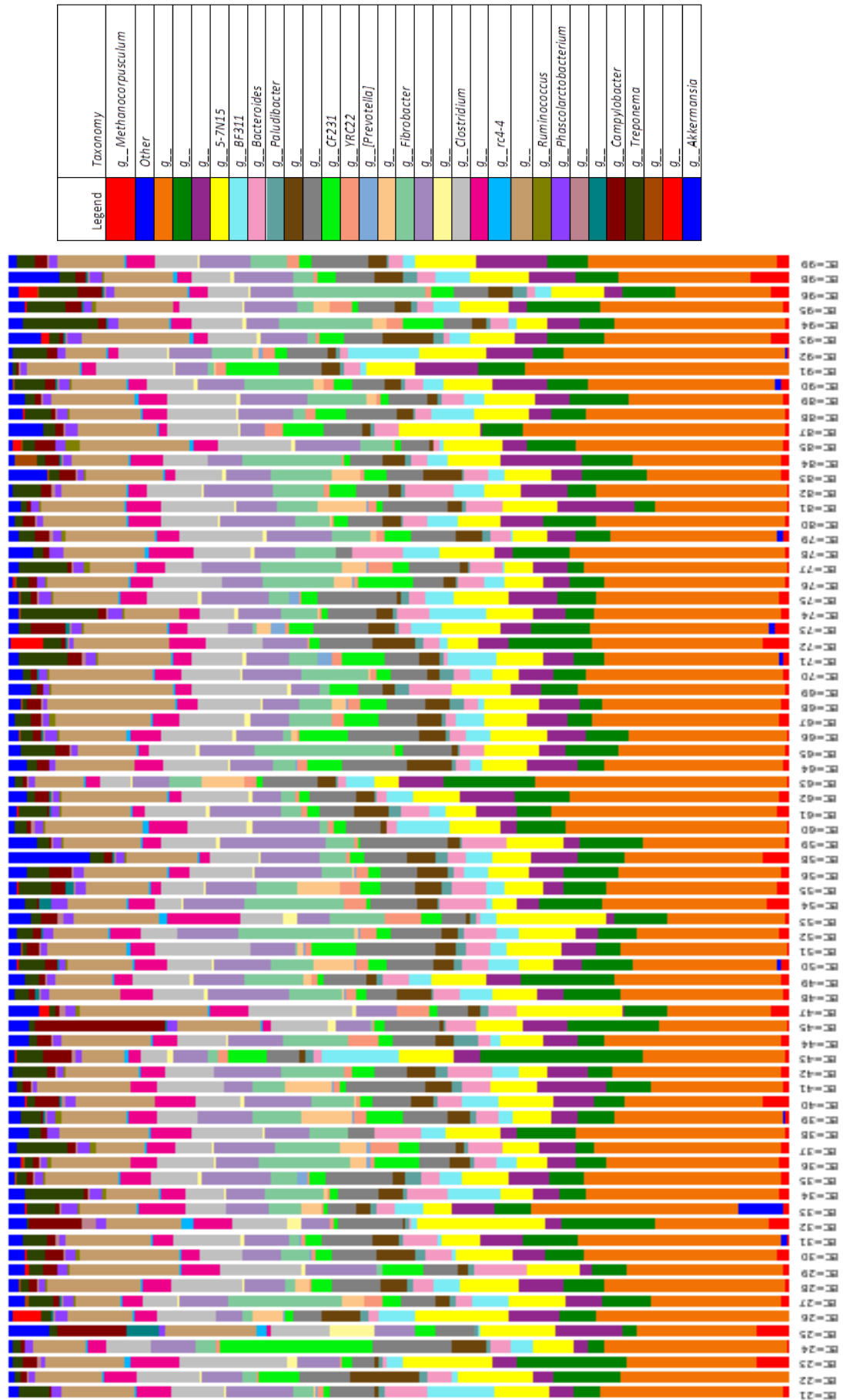
Verrucomicrobiales	1.97%	1.46%	1.99%	1.87%
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Figure 13: Graph of mean relative abundance in pre-treatment samples, Group1, Group2, and Group3 samples.



4.4.4 Taxonomy Summary Genus level.

Figure 14: Bar chart Taxonomy summary at Genus level.



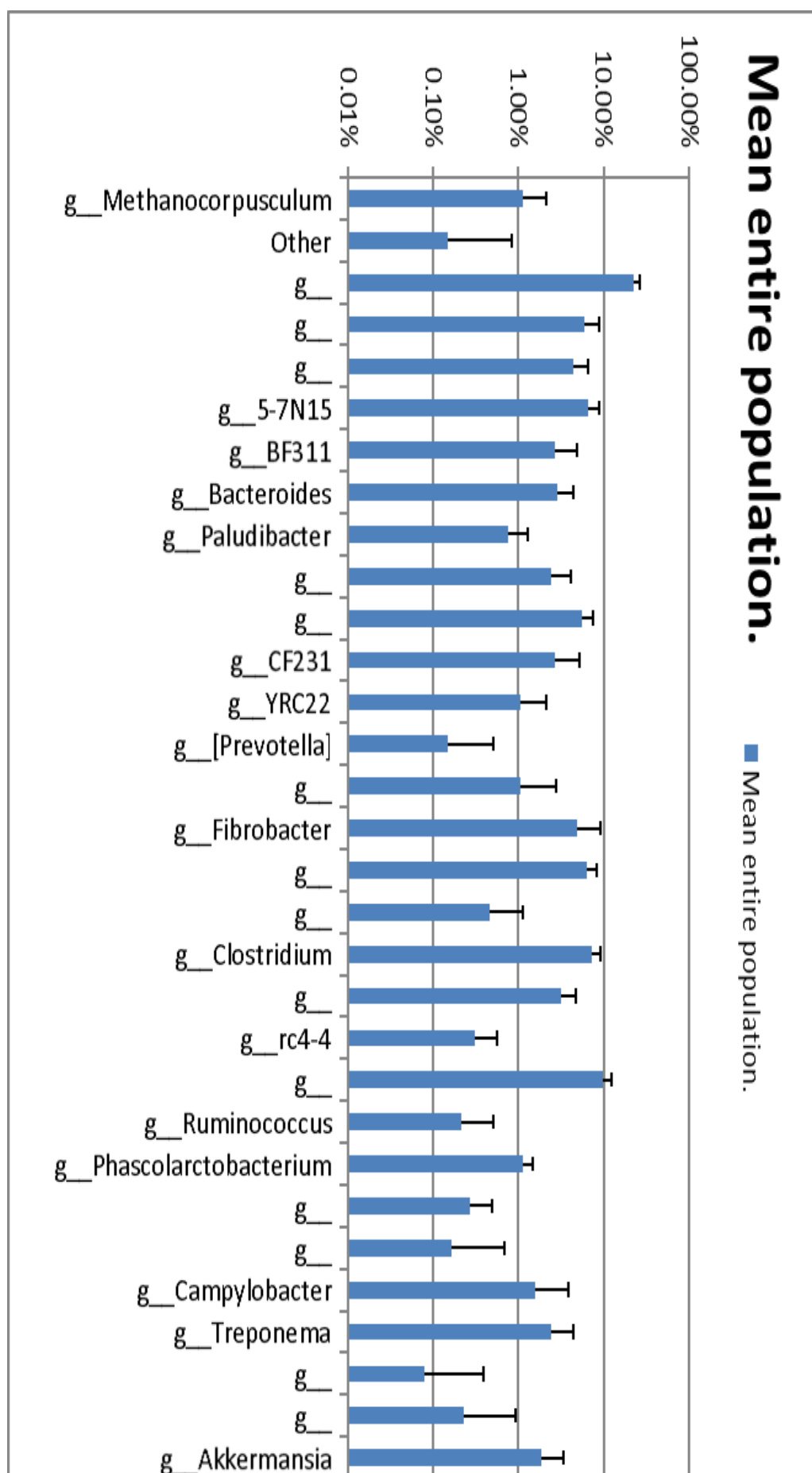
At the Genus level of Taxonomy, an uncultured genus from the Order *Bacteroidales* was the most dominant Genus with a percentage mean relative abundance of 22.27% and a standard deviation of 4.35% in the entire population. An uncultured Genus from the Family *Ruminococcaceae* was a distant second in dominance with a mean of 9.73% closely followed by the Genus *Clostridium* which had a mean of 7.19%. The lowest mean percentage relative abundance at the Genus level of 0.08% was recorded in an uncultured Genus from the uncultured Order RF39 from the Class *Mollicutes* (Table 17, Figure 15 and Figure 14).

Table 17: Percentage mean relative abundance at Genus level in all samples.

Genus	Mean	Standard deviation
<i>Methanocorpusculum</i>	1.12%	1.03%
Uncultured Genus, Order <i>Bacteroidales</i>	0.15%	0.69%
Uncultured Genus, Order <i>Bacteroidales</i>	22.27%	4.35%
Uncultured Genus, Order <i>Bacteroidales</i>	5.97%	3.05%
Uncultured Genus, Family <i>Bacteroidaceae</i>	4.43%	2.07%
Uncultured Genus 5-7N15, Family <i>Bacteroidaceae</i>	6.58%	2.42%
Uncultured Genus BF311, Family <i>Bacteroidaceae</i>	2.71%	2.24%
<i>Bacteroides</i>	2.88%	1.61%
<i>Paludibacter</i>	0.76%	0.56%
Uncultured Genus, Order <i>Bacteroidales</i>	2.46%	1.61%
Uncultured Genus, Family <i>Rikenellaceae</i>	5.57%	1.88%
Uncultured Genus CF231, Family <i>Paraprevotellaceae</i>	2.70%	2.61%
Uncultured Genus YRC22, Family <i>Paraprevotellaceae</i>	1.05%	1.04%

<i>[Prevotella]</i>	0.15%	0.36%
<i>Uncultured Genus, Order Bacteroidales</i>	1.06%	1.76%
<i>Fibrobacter</i>	4.95%	4.19%
<i>Uncultured Genus, Order Clostridiales</i>	6.32%	1.93%
<i>Uncultured Genus, Family Christensenellaceae</i>	0.46%	0.69%
<i>Clostridium</i>	7.19%	2.11%
<i>Uncultured Genus, Family Lachnospiraceae</i>	3.17%	1.48%
<i>Uncultured Genus rc4-4, Family Peptococcaceae</i>	0.31%	0.25%
<i>Uncultured Genus, Family Ruminococcaceae</i>	9.73%	2.50%
<i>Ruminococcus</i>	0.22%	0.29%
<i>Phascolarctobacterium</i>	1.13%	0.36%
<i>Uncultured Genus, Family Desulfovibrionaceae</i>	0.27%	0.22%
<i>Uncultured Genus, Class Deltaproteobacteria</i>	0.16%	0.52%
<i>Campylobacter</i>	1.60%	2.25%
<i>Treponema</i>	2.44%	2.01%
<i>Uncultured Genus, Class Mollicutes</i>	0.08%	0.32%
<i>Uncultured Genus, Class Verruco-5</i>	0.23%	0.71%
<i>Akkermansia</i>	1.86%	1.56%

Figure 15: Graph of mean percentage relative abundance at Genus level in entire population.



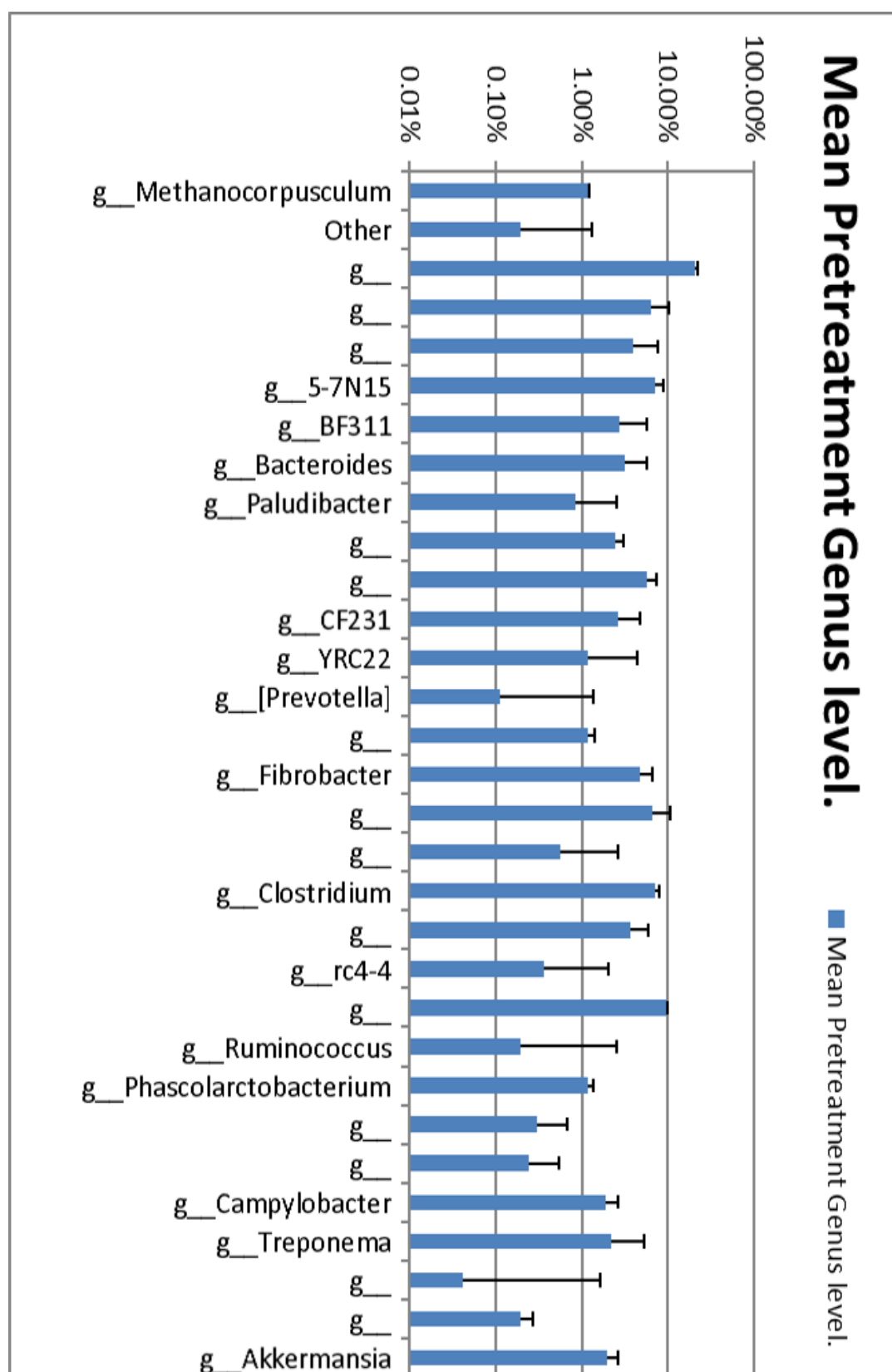
In the pre-treatment samples, an uncultured Genus from the Order *Bacteroidales* was the most dominant with a mean percentage relative abundance of 20.85% and a standard deviation of 3.78%. An uncultured Genus from the Family *Ruminococcaceae* had a mean of 9.60% with a standard deviation of 2.32% making it second in dominance. The uncultured Genus from the Uncultured Order RF39 from the Class *Mollicutes* was the least dominant with a mean of 0.04% (Table 18, Figure 16).

Table 18: Mean percentage relative abundance at Genus level in Pre-treatment samples.

Taxonomy	Mean Pre-treatment Genus level.	Standard deviation Pre-treatment
Methanocorpusculum	1.21%	1.11%
Uncultured Genus, Order <i>Bacteroidales</i>	0.20%	0.94%
Uncultured Genus, Order <i>Bacteroidales</i>	20.85%	3.78%
Uncultured Genus, Order <i>Bacteroidales</i>	6.35%	3.71%
Uncultured Genus, Family <i>Bacteroidaceae</i>	3.94%	1.89%
Uncultured Genus 5-7N15, Family <i>Bacteroidaceae</i>	7.00%	2.99%
Uncultured Genus BF311 Family <i>Bacteroidaceae</i>	2.73%	2.38%
<i>Bacteroides</i>	3.19%	1.72%
<i>Paludibacter</i>	0.83%	0.58%
Uncultured Genus, Order <i>Bacteroidales</i>	2.42%	1.75%
Uncultured Genus, Family <i>Rikenellaceae</i>	5.65%	2.08%
Uncultured Genus CF231, Family <i>Paraprevotellaceae</i>	2.65%	3.20%

<i>Uncultured Genus YRC22</i> <i>Family Paraprevotellaceae</i>	1.16%	1.25%
<i>[Prevotella]</i>	0.11%	0.23%
<i>Uncultured Genus, Order</i> <i>Bacteroidales</i>	1.17%	1.96%
<i>Fibrobacter</i>	4.65%	4.05%
<i>Uncultured Genus, Order</i> <i>Clostridiales</i>	6.57%	2.09%
<i>Uncultured Genus, Family</i> <i>Christensenellaceae</i>	0.57%	0.95%
<i>Clostridium</i>	7.01%	2.34%
<i>Uncultured Genus, Family</i> <i>Lachnospiraceae</i>	3.59%	1.65%
<i>Uncultured Genus rc4-4,</i> <i>Family Peptococcaceae</i>	0.36%	0.33%
<i>Uncultured Genus, Family</i> <i>Ruminococcaceae</i>	9.60%	2.32%
<i>Ruminococcus</i>	0.19%	0.21%
<i>Phascolarctobacterium</i>	1.15%	0.37%
<i>Uncultured Genus, Family</i> <i>Desulfovibrionaceae</i>	0.31%	0.29%
<i>Uncultured Genus, Class</i> <i>Deltaproteobacteria</i>	0.24%	0.72%
<i>Campylobacter</i>	1.89%	3.04%
<i>Treponema</i>	2.17%	1.61%
<i>Uncultured Genus, Class</i> <i>Mollicutes</i>	0.04%	0.08%
<i>Uncultured Genus, Class</i> <i>Verruco-5</i>	0.20%	0.61%
<i>Akkermansia</i>	1.97%	1.75%

Figure 16: Graph of mean percentage relative abundance at Genus level in Pre-treatment samples.



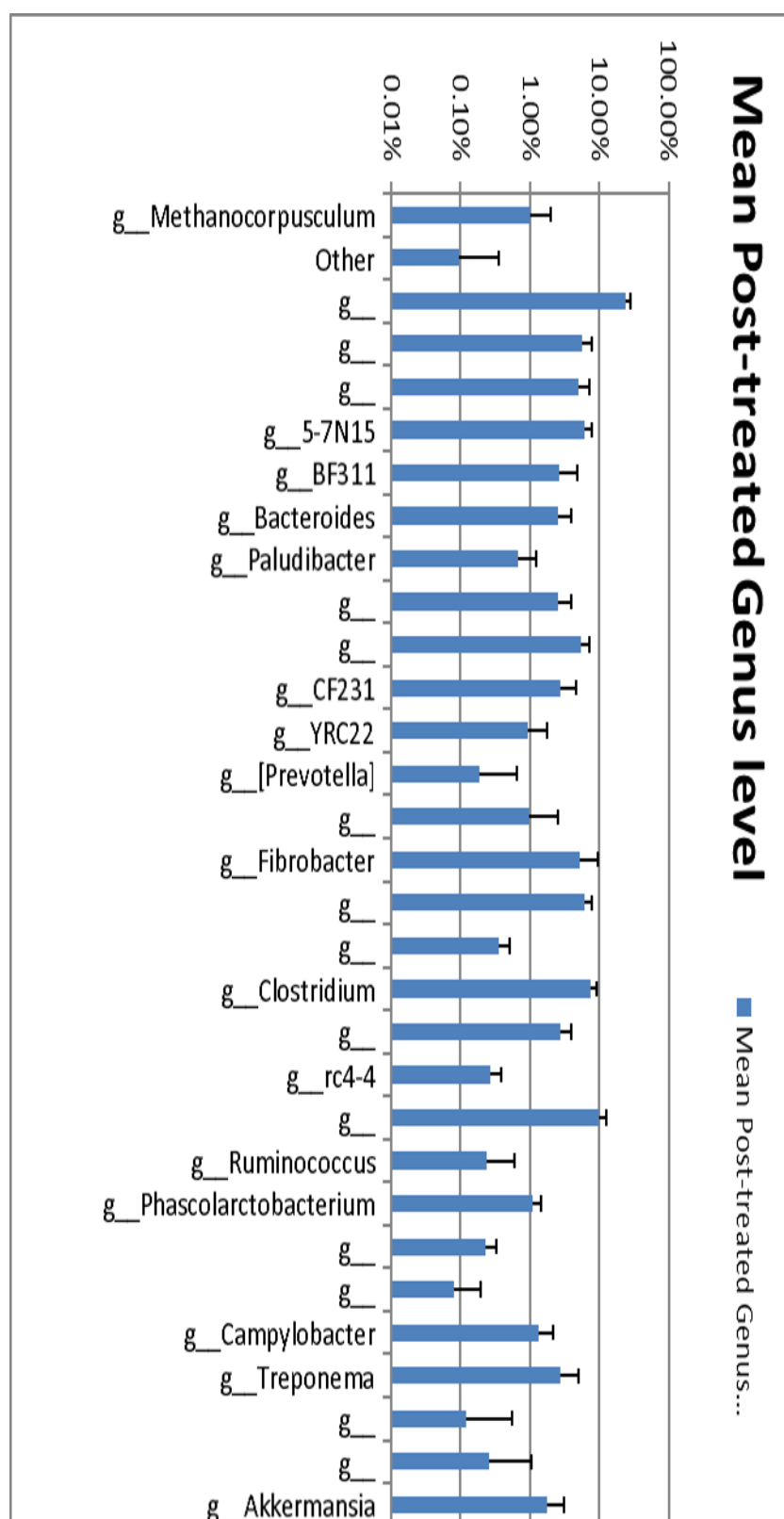
The Genus that was dominant in the post treated sample was again the uncultured Genus from the Order *Bacteroidales* with a mean percentage relative abundance of 23.73% and a standard deviation of 4.46%. An uncultured Genus from the Family *Ruminococcaceae* with a mean of 9.86% and a standard deviation of 2.70% was second in dominance. The least with mean relative abundance of 0.08% was the uncultured Genus from the uncultured Order GMD14H09 from the class *Deltaproteobacteria* (Table 19, Figure 17).

Table 19: Mean percentage relative abundance at Genus level in Post treated samples.

Taxonomy	Mean Post-treatment Genus level	Standard deviation Post-treated Genus level.
<i>Methanocorpusculum</i>	1.03%	0.95%
Uncultured Genus, Order <i>Bacteroidales</i>	0.09%	0.26%
Uncultured Genus, Order <i>Bacteroidales</i>	23.73%	4.46%
Uncultured Genus, Order <i>Bacteroidales</i>	5.59%	2.17%
Uncultured Genus, Family <i>Bacteroidaceae</i>	4.93%	2.15%
Uncultured Genus 5-7N15, Family <i>Bacteroidaceae</i>	6.15%	1.58%
Uncultured Genus BF311 Family <i>Bacteroidaceae</i>	2.69%	2.12%
<i>Bacteroides</i>	2.57%	1.43%
<i>Paludibacter</i>	0.68%	0.53%
Uncultured Genus, Order <i>Bacteroidales</i>	2.50%	1.48%
Uncultured Genus, Family <i>Rikenellaceae</i>	5.48%	1.66%
Uncultured Genus CF231, Family <i>Paraprevotellaceae</i>	2.74%	1.86%
Uncultured Genus YRC22	0.95%	0.79%

<i>Family Paraprevotellaceae</i>		
<i>[Prevotella]</i>	0.19%	0.46%
<i>Uncultured Genus, Order Bacteroidales</i>	0.95%	1.56%
<i>Fibrobacter</i>	5.25%	4.37%
<i>Uncultured Genus, Order Clostridiales</i>	6.06%	1.75%
<i>Uncultured Genus, Family Christensenellaceae</i>	0.35%	0.17%
<i>Clostridium</i>	7.37%	1.85%
<i>Uncultured Genus, Family Lachnospiraceae</i>	2.74%	1.14%
<i>Uncultured Genus rc4-4, Family Peptococcaceae</i>	0.26%	0.12%
<i>Uncultured Genus, Family Ruminococcaceae</i>	9.86%	2.70%
<i>Ruminococcus</i>	0.24%	0.35%
<i>Phascolarctobacterium</i>	1.11%	0.36%
<i>Uncultured Genus, Family Desulfovibrionaceae</i>	0.23%	0.11%
<i>Uncultured Genus GMD14H09, Class Deltaproteobacteria</i>	0.08%	0.11%
<i>Campylobacter</i>	1.31%	0.84%
<i>Treponema</i>	2.71%	2.35%
<i>Uncultured Genus, Class Mollicutes</i>	0.12%	0.44%
<i>Uncultured Genus, Class Verruco-5</i>	0.26%	0.81%
<i>Akkermansia</i>	1.74%	1.36%

Figure 17: Graph of mean percentage relative abundance at Genus level in Post treated samples.



The uncultured Genus from the Order *Bacteroidales* was the most dominant in both pre-treatment and post-treated samples while the second most dominant in both pre-treatment and post treated was the uncultured Genus from the Family *Ruminococcaceae* .In the pre-treatment samples the least dominant was the uncultured Genus from the uncultured Order RF39 from the Class *Mollicutes* while in the post treated samples the least dominant was the uncultured Genus from an uncultured Order GMD14H09 from the Class *Deltaproteobacteria* (Table 20, Figure 18).

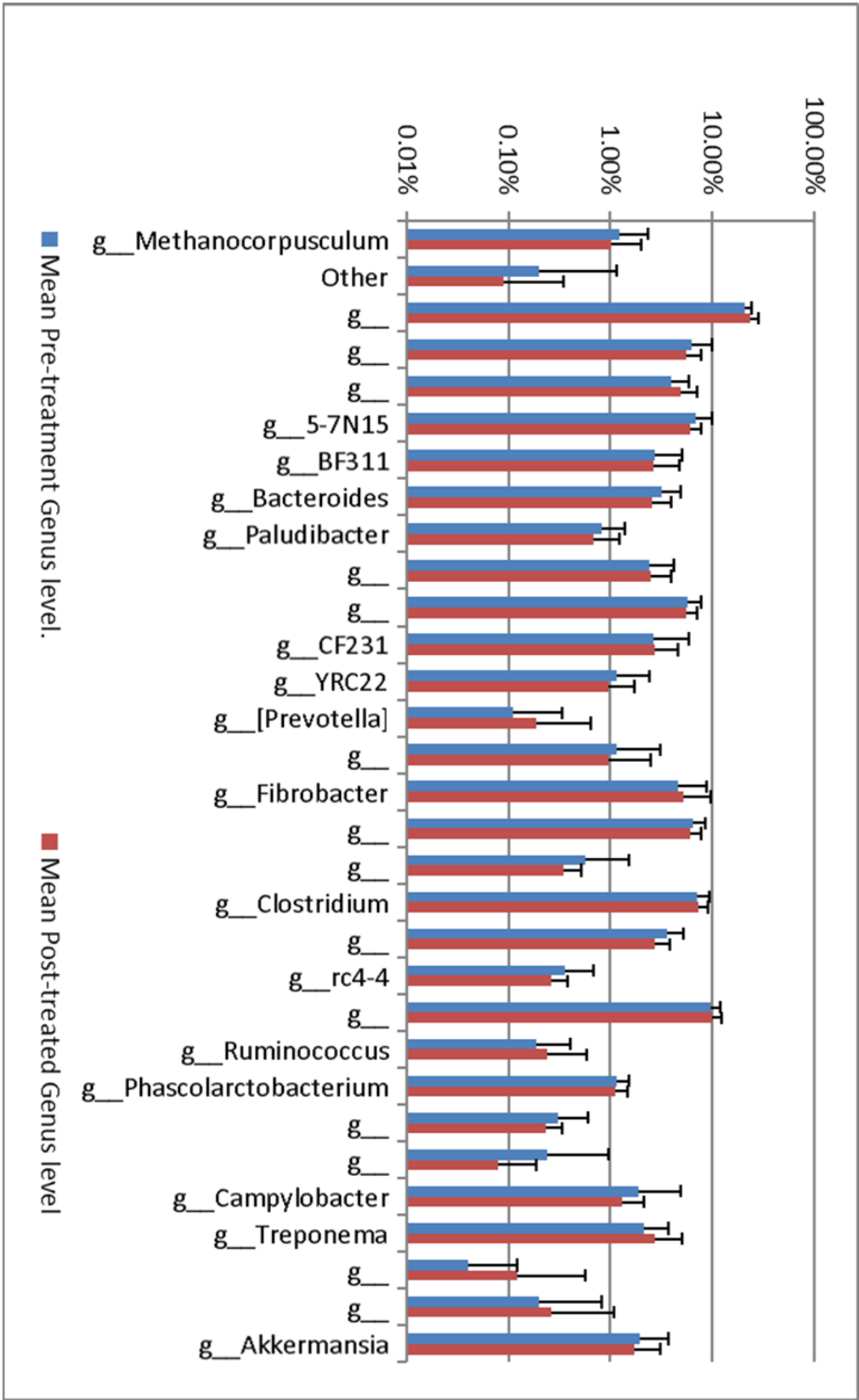
Table 20: Comparing Mean percentage relative abundance at Genus level in Pre-treatment samples and Post treated samples.

Taxonomy	Mean Pre-treatment Genus level.	Standard deviation Pre-treatment	Mean Post-treated Genus level	Standard deviation Post treated Genus level.
<i>Methanocorpusculum</i>	1.21%	1.11%	1.03%	0.95%
<i>Uncultured Genus, Order Bacteroidales</i>	0.20%	0.94%	0.09%	0.26%
<i>Uncultured Genus, Order Bacteroidales</i>	20.85%	3.78%	23.73%	4.46%
<i>Uncultured Genus, Order Bacteroidales</i>	6.35%	3.71%	5.59%	2.17%
<i>Uncultured Genus, Family Bacteroidaceae</i>	3.94%	1.89%	4.93%	2.15%
<i>Uncultured Genus 5-7N15, Family Bacteroidaceae</i>	7.00%	2.99%	6.15%	1.58%
<i>Uncultured Genus BF311 Family Bacteroidaceae</i>	2.73%	2.38%	2.69%	2.12%
<i>Bacteroides</i>	3.19%	1.72%	2.57%	1.43%
<i>Paludibacter</i>	0.83%	0.58%	0.68%	0.53%
<i>Uncultured Genus, Order Bacteroidales</i>	2.42%	1.75%	2.50%	1.48%

<i>Uncultured Genus, Family Rikenellaceae</i>	5.65%	2.08%	5.48%	1.66%
<i>Uncultured Genus CF231, Family Paraprevotellaceae</i>	2.65%	3.20%	2.74%	1.86%
<i>Uncultured Genus YRC22 Family Paraprevotellaceae</i>	1.16%	1.25%	0.95%	0.79%
<i>[Prevotella]</i>	0.11%	0.23%	0.19%	0.46%
<i>Uncultured Genus, Order Bacteroidales</i>	1.17%	1.96%	0.95%	1.56%
<i>Fibrobacter</i>	4.65%	4.05%	5.25%	4.37%
<i>Uncultured Genus, Order Clostridiales</i>	6.57%	2.09%	6.06%	1.75%
<i>Uncultured Genus, Family Christensenellaceae</i>	0.57%	0.95%	0.35%	0.17%
<i>Clostridium</i>	7.01%	2.34%	7.37%	1.85%
<i>Uncultured Genus, Family Lachnospiraceae</i>	3.59%	1.65%	2.74%	1.14%
<i>Uncultured Genus rc4-4, Family Peptococcaceae</i>	0.36%	0.33%	0.26%	0.12%
<i>Uncultured Genus, Family Ruminococcaceae</i>	9.60%	2.32%	9.86%	2.70%
<i>Ruminococcus</i>	0.19%	0.21%	0.24%	0.35%
<i>Phascolarctobacterium</i>	1.15%	0.37%	1.11%	0.36%
<i>Uncultured Genus, Family Desulfovibrionaceae</i>	0.31%	0.29%	0.23%	0.11%
<i>Uncultured Genus, Class Deltaproteobacteria</i>	0.24%	0.72%	0.08%	0.11%
<i>Campylobacter</i>	1.89%	3.04%	1.31%	0.84%
<i>Treponema</i>	2.17%	1.61%	2.71%	2.35%
<i>Uncultured Genus, Class</i>	0.04%	0.08%	0.12%	0.44%

<i>Mollicutes</i>				
Uncultured Genus,Class Verruco-5	0.20%	0.61%	0.26%	0.81%
<i>Akkermansia</i>	1.97%	1.75%	1.74%	1.36%

Figure 18: Graph comparing the mean percentage relative abundance at Genus level pre-treatment and post treated samples.



In group 1 (Zolvix®), the uncultured Genus from the Order *Bacteroidales* was the most dominant with a mean percentage relative abundance of 22.86% and a standard deviation of 2.96%. The uncultured Genus from the Family *Ruminococcaceae* was a distant second with a mean of 10.34% closely followed by the Genus *Clostridium* with a mean of 8.09%. The lowest mean percentage relative abundance of 0.07% was observed in the uncultured Genus from the uncultured Order GMD14H09 from the Class *Deltaproteobacteria* (Table 21, Figure 19).

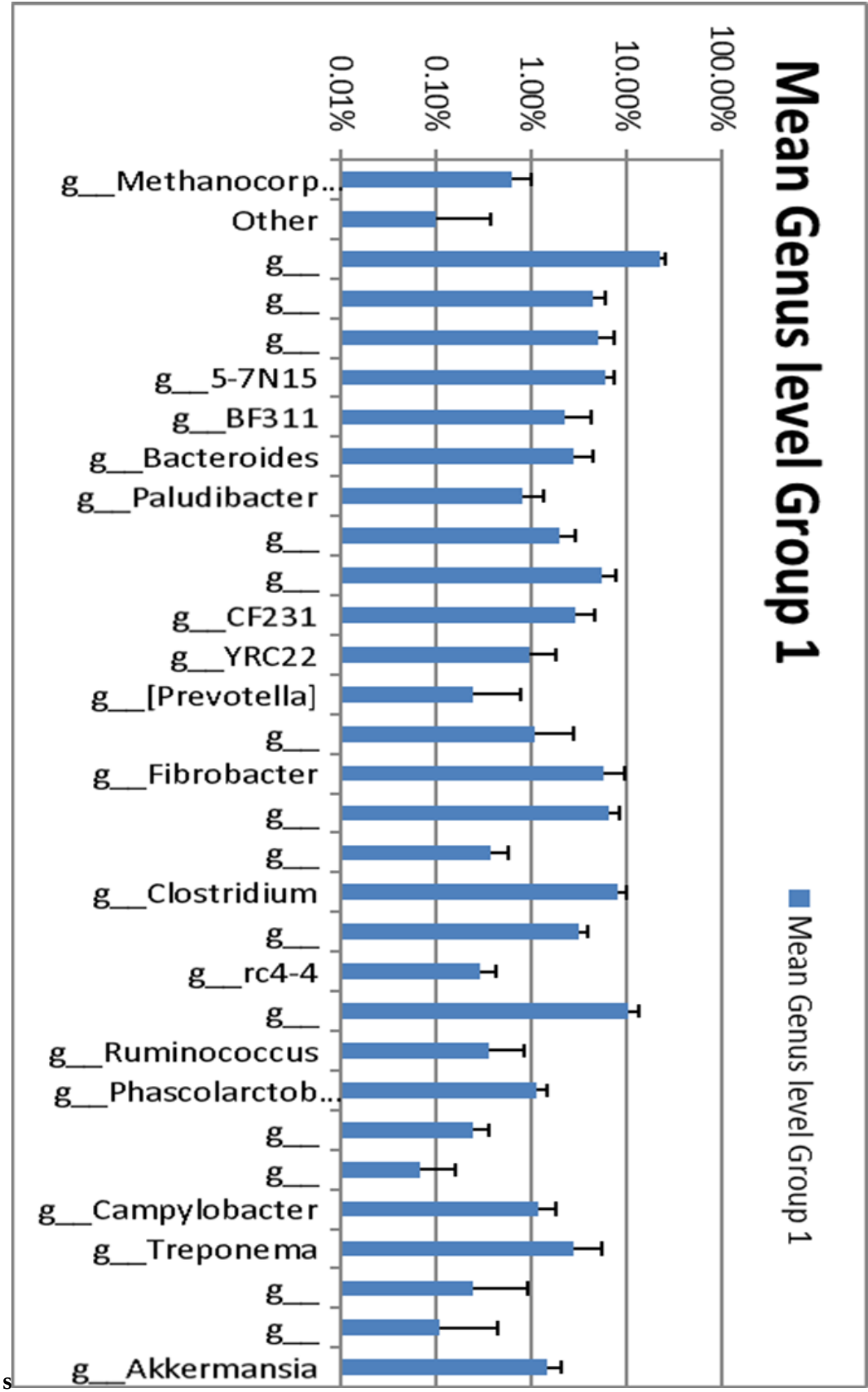
Table 21: Mean percentage relative abundance at Genus level in Group 1 (Zolvix®).

Taxonomy	Mean Genus level Group 1	Standard deviation Genus level Group 1
<i>Methanocorpusculum</i>	0.63%	0.34%
Uncultured Genus, Order <i>Bacteroidales</i>	0.10%	0.27%
Uncultured Genus, Order <i>Bacteroidales</i>	22.86%	2.96%
Uncultured Genus, Order <i>Bacteroidales</i>	4.53%	1.52%
Uncultured Genus, Family <i>Bacteroidaceae</i>	5.13%	2.25%
Uncultured Genus 5-7N15, Family <i>Bacteroidaceae</i>	5.99%	1.56%
Uncultured Genus BF311 Family <i>Bacteroidaceae</i>	2.23%	2.02%
<i>Bacteroides</i>	2.79%	1.60%
<i>Paludibacter</i>	0.79%	0.57%
Uncultured Genus, Order <i>Bacteroidales</i>	1.97%	0.95%
Uncultured Genus, Family	5.61%	2.08%

<i>Rikenellaceae</i>		
<i>Uncultured Genus CF231, Family Paraprevotellaceae</i>	2.94%	1.80%
<i>Uncultured Genus YRC22 Family Paraprevotellaceae</i>	0.97%	0.83%
<i>[Prevotella]</i>	0.25%	0.53%
<i>Uncultured Genus, Order Bacteroidales</i>	1.08%	1.74%
<i>Fibrobacter</i>	5.73%	3.79%
<i>Uncultured Genus, Order Clostridiales</i>	6.56%	1.81%
<i>Uncultured Genus, Family Christensenellaceae</i>	0.38%	0.18%
<i>Clostridium</i>	8.09%	1.96%
<i>Uncultured Genus, Family Lachnospiraceae</i>	3.18%	0.67%
<i>Uncultured Genus rc4-4, Family Peptococcaceae</i>	0.29%	0.13%
<i>Uncultured Genus, Family Ruminococcaceae</i>	10.34%	3.23%
<i>Ruminococcus</i>	0.35%	0.47%
<i>Phascolarctobacterium</i>	1.14%	0.29%
<i>Uncultured Genus, Family Desulfovibrionaceae</i>	0.25%	0.11%
<i>Uncultured Genus, Class Deltaproteobacteria</i>	0.07%	0.09%
<i>Campylobacter</i>	1.17%	0.61%
<i>Treponema</i>	2.75%	2.65%
<i>Uncultured Genus, Class Mollicutes</i>	0.25%	0.68%

<i>Uncultured</i> Genus,Class,Verruco-5	0.11%	0.33%
<i>Akkermansia</i>	1.46%	0.60%

Figure 19: Graph showing percentage relative abundance at Genus in Group 1 (Zolvix®).



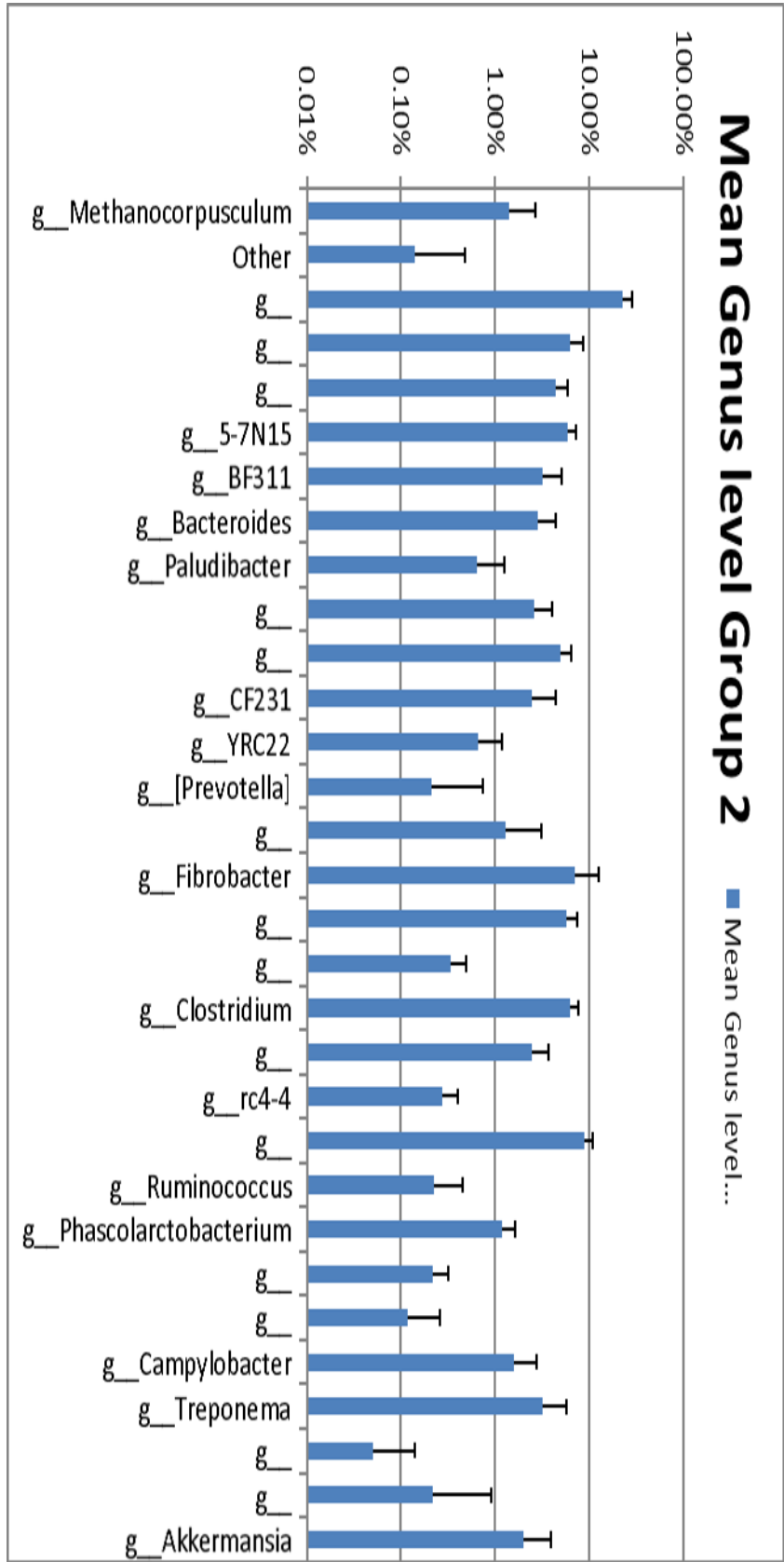
In the group 2 (Startect®), the uncultured Genus from the Order *Bacteroidales* was dominant with a mean percentage relative abundance of 22.87%. A distant second was an uncultured Genus from the Family *Ruminococcaceae* with a mean percentage relative abundance of 9.03%. *Fibrobacter* had a mean percentage relative abundance of 7.12% which was closely followed by *Clostridium* that recorded a mean of 6.21%. The lowest mean percentage relative abundance was recorded in the uncultured Genus from the uncultured Order RF39 from the Class *Mollicutes* (Table 22, figure20).

Table 22: Mean percentage relative abundance at Genus level in Group 2 (Startect®).

Genus	Mean Genus level Group 2	Standard deviation Genus level Group 2
<i>Methanocorpusculum</i>	1.42%	1.26%
<i>Uncultured Genus, Order Bacteroidales</i>	0.14%	0.33%
<i>Uncultured Genus, Order Bacteroidales</i>	22.87%	5.54%
<i>Uncultured Genus, Order Bacteroidales</i>	6.19%	2.41%
<i>Uncultured Genus, Family Bacteroidaceae</i>	4.38%	1.51%
<i>Uncultured Genus 5-7N15, Family Bacteroidaceae</i>	5.98%	1.37%
<i>Uncultured Genus BF311 Family Bacteroidaceae</i>	3.24%	1.81%
<i>Bacteroides</i>	2.83%	1.59%
<i>Paludibacter</i>	0.64%	0.63%
<i>Uncultured Genus, Order Bacteroidales</i>	2.64%	1.42%
<i>Uncultured Genus, Family Rikenellaceae</i>	5.01%	1.37%
<i>Uncultured Genus CF231, Family Paraprevotellaceae</i>	2.46%	1.90%

<i>Uncultured Genus YRC22</i> <i>Family Paraprevotellaceae</i>	0.67%	0.53%
<i>[Prevotella]</i>	0.21%	0.54%
<i>Uncultured Genus, Order</i> <i>Bacteroidales</i>	1.28%	1.81%
<i>Fibrobacter</i>	7.12%	5.38%
<i>Uncultured Genus, Order</i> <i>Clostridiales</i>	5.80%	1.59%
<i>Uncultured Genus, Family</i> <i>Christensenellaceae</i>	0.33%	0.16%
<i>Clostridium</i>	6.21%	1.58%
<i>Uncultured Genus, Family</i> <i>Lachnospiraceae</i>	2.47%	1.22%
<i>Uncultured Genus rc4-4,</i> <i>Family Peptococcaceae</i>	0.28%	0.12%
<i>Uncultured Genus, Family</i> <i>Ruminococcaceae</i>	9.03%	1.82%
<i>Ruminococcus</i>	0.23%	0.23%
<i>Phascolarctobacterium</i>	1.20%	0.43%
<i>Uncultured Genus, Family</i> <i>Desulfovibrionaceae</i>	0.22%	0.10%
<i>Uncultured Genus, Class</i> <i>Deltaproteobacteria</i>	0.12%	0.14%
<i>Campylobacter</i>	1.57%	1.19%
<i>Treponema</i>	3.20%	2.58%
<i>Uncultured Genus, Class</i> <i>Mollicutes</i>	0.05%	0.09%
<i>Uncultured Genus, Class</i> <i>Veruco-5</i>	0.22%	0.69%
<i>Akkermansia</i>	1.99%	1.89%

Figure 20: Graph showing percentage relative abundance at Genus level in Group 2 (Startect®).



In Group 3 (Zolvix® + Startect®), the uncultured Genus from the Order *Bacteroidales* recorded the highest mean percentage relative abundance 26.07%. An Uncultured Genus from the Family *Ruminococcaceae* was second with a mean of 10.14%. The lowest mean was observed in an uncultured Genus from the uncultured Order RF39 from the Class *Mollicutes* (Table 23, Figure 21).

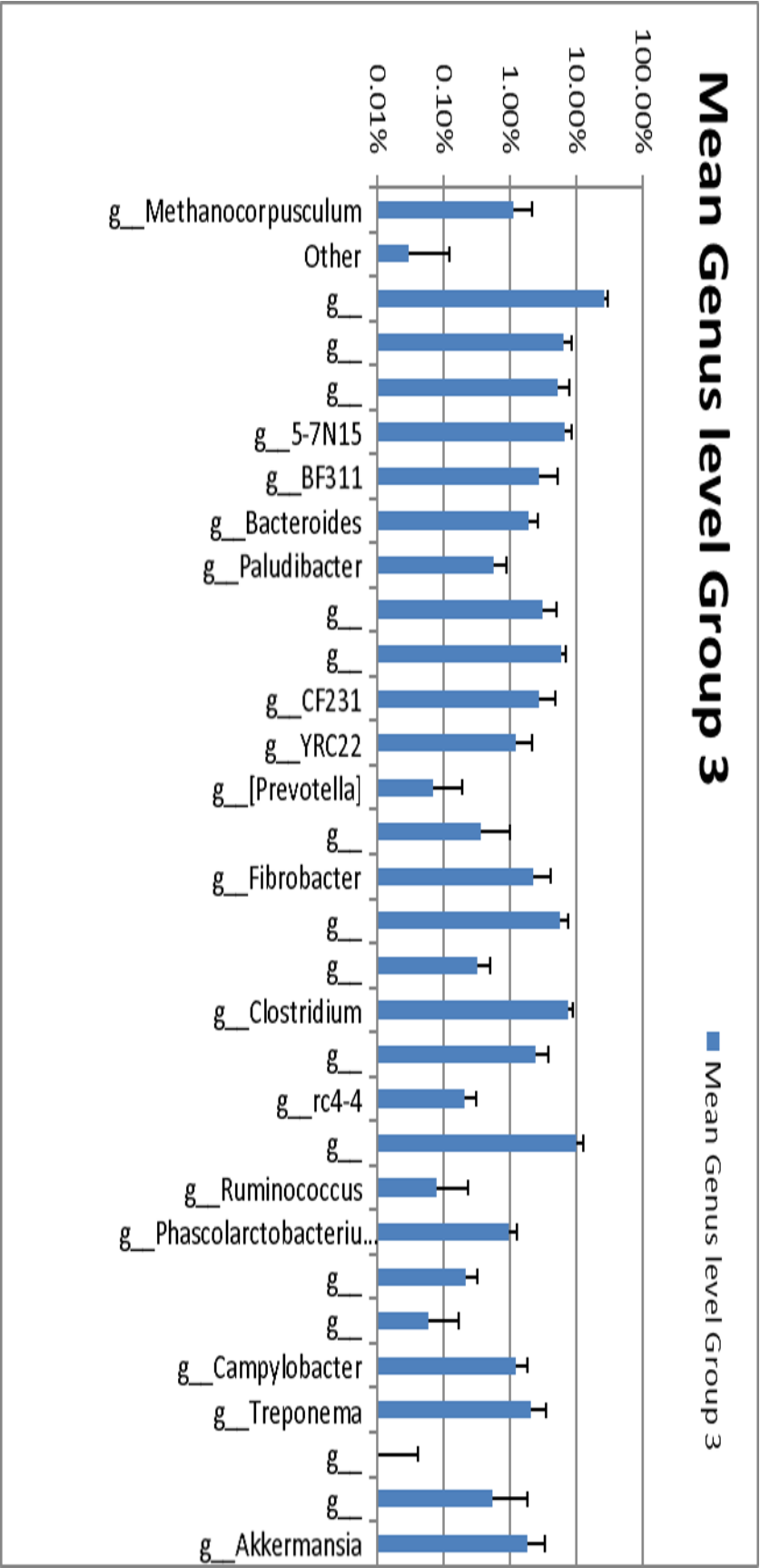
Table 23: Mean percentage relative abundance at Genus level in Group 3

Taxonomy	Mean Genus level Group 3	Standard deviation Genus level Group 3
<i>Methanocorpusculum</i>	1.15%	1.00%
<i>Uncultured Genus, Order Bacteroidales</i>	0.03%	0.09%
<i>Uncultured Genus, Order Bacteroidales</i>	26.07%	4.49%
<i>Uncultured Genus, Order Bacteroidales</i>	6.46%	2.23%
<i>Uncultured Genus, Family Bacteroidaceae</i>	5.28%	2.69%
<i>Uncultured Genus 5-7N15, Family Bacteroidaceae</i>	6.61%	1.87%
<i>Uncultured Genus BF311 Family Bacteroidaceae</i>	2.71%	2.62%
<i>Bacteroides</i>	1.94%	0.73%
<i>Paludibacter</i>	0.56%	0.32%
<i>Uncultured Genus, Order Bacteroidales</i>	3.13%	1.99%
<i>Uncultured Genus, Family Rikenellaceae</i>	5.85%	1.26%
<i>Uncultured Genus CF231, Family Paraprevotellaceae</i>	2.79%	2.04%

<i>Uncultured Genus YRC22</i> <i>Family</i> <i>Paraprevotellaceae</i>	1.25%	0.92%
<i>[Prevotella]</i>	0.07%	0.13%
<i>Uncultured Genus, Order</i> <i>Bacteroidales</i>	0.36%	0.63%
<i>Fibrobacter</i>	2.28%	1.92%
<i>Uncultured Genus, Order</i> <i>Clostridiales</i>	5.62%	1.83%
<i>Uncultured Genus,</i> <i>Family</i> <i>Christensenellaceae</i>	0.33%	0.17%
<i>Clostridium</i>	7.67%	1.36%
<i>Uncultured Genus,</i> <i>Family Lachnospiraceae</i>	2.42%	1.45%
<i>Uncultured Genus rc4-4,</i> <i>Family Peptococcaceae</i>	0.21%	0.10%
<i>Uncultured Genus,</i> <i>Family</i> <i>Ruminococcaceae</i>	10.14%	2.73%
<i>Ruminococcus</i>	0.08%	0.15%
<i>Phascolarctobacterium</i>	0.96%	0.33%
<i>Uncultured Genus,</i> <i>Family</i> <i>Desulfovibrionaceae</i>	0.22%	0.10%
<i>Uncultured Genus, Class</i> <i>Deltaproteobacteria</i>	0.06%	0.11%
<i>Campylobacter</i>	1.22%	0.61%
<i>Treponema</i>	2.05%	1.49%
<i>Uncultured Genus, Class</i> <i>Mollicutes</i>	0.01%	0.03%
<i>Uncultured Genus,Class</i>	0.55%	1.31%

<i>Verruco-5</i>		
<i>Akkermansia</i>	1.87%	1.49%

Figure 21: Graph showing relative abundance at Genus level in Group 3 (Zolvix® + Startect®).



The uncultured Genus from the Order *Bacteroidales* maintained its dominance in all the groups. The uncultured Genus from the Family *Ruminococcaceae* was a distant second in all the 3 groups closely followed by the Genus *Clostridium*. (Table 24, Figure 22).

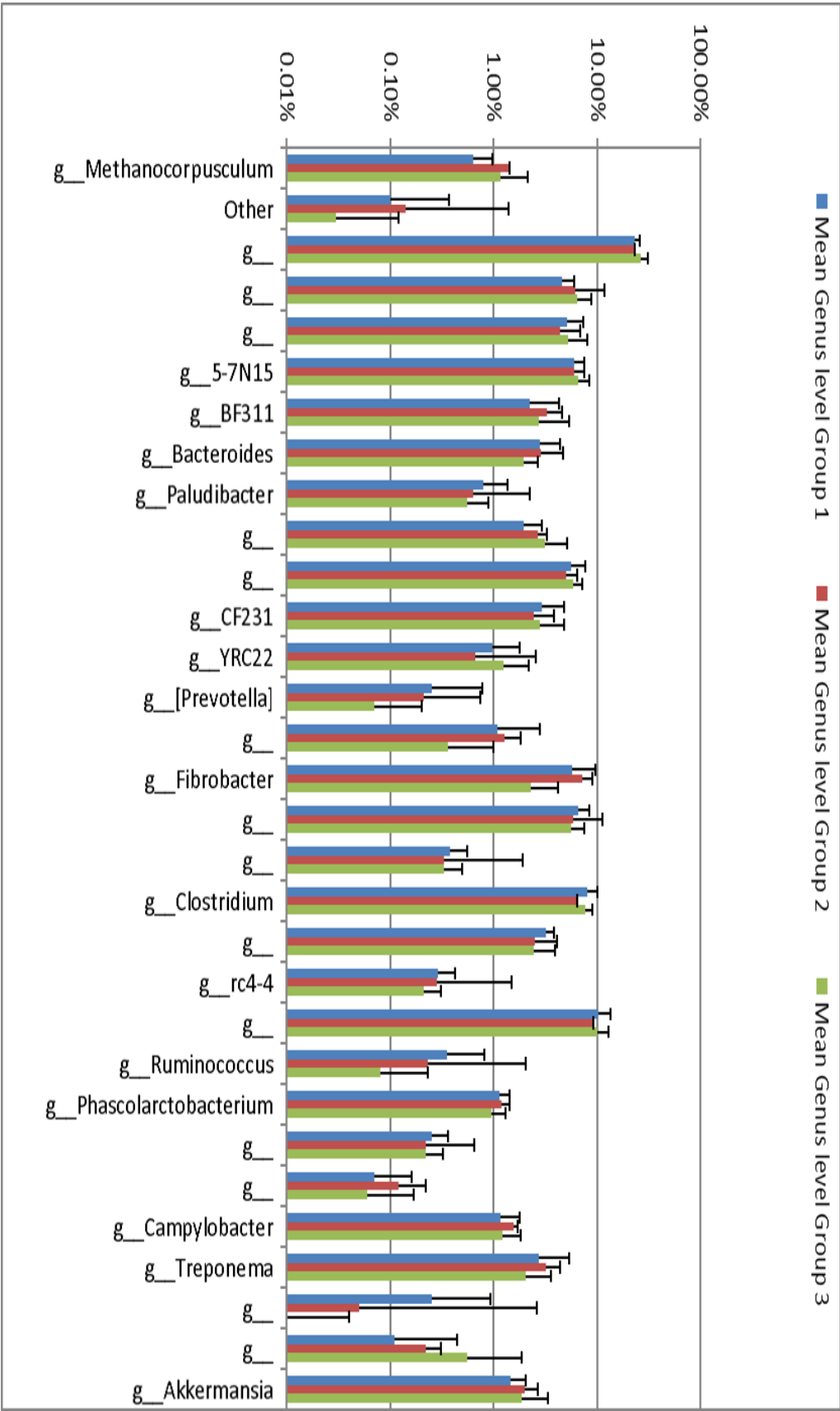
Table 24: Table comparing the mean percentage relative abundance in Group 1, Group 2, Group 3.

Taxonomy	Mean Genus level Group 1	Standard deviation Genus level Group 1	Mean Genus level Group 2	Standard deviation Genus level Group 2	Mean Genus level Group 3	Standard deviation Genus level Group 3
<i>Methanocorpusculum</i>	0.63%	0.34%	1.42%	1.26%	1.15%	1.00%
<i>Uncultured Genus, Order Bacteroidales</i>	0.10%	0.27%	0.14%	0.33%	0.03%	0.09%
<i>Uncultured Genus, Order Bacteroidales</i>	22.86 %	2.96%	22.87 %	5.54%	26.07 %	4.49%
<i>Uncultured Genus, Order Bacteroidales</i>	4.53%	1.52%	6.19%	2.41%	6.46%	2.23%
<i>Uncultured Genus, Family Bacteroidaceae</i>	5.13%	2.25%	4.38%	1.51%	5.28%	2.69%
<i>Uncultured Genus 5-7N15, Family Bacteroidaceae</i>	5.99%	1.56%	5.98%	1.37%	6.61%	1.87%
<i>Uncultured Genus BF311 Family Bacteroidaceae</i>	2.23%	2.02%	3.24%	1.81%	2.71%	2.62%
<i>Bacteroides</i>	2.79%	1.60%	2.83%	1.59%	1.94%	0.73%
<i>Paludibacter</i>	0.79%	0.57%	0.64%	0.63%	0.56%	0.32%
<i>Uncultured Genus, Order Bacteroidales</i>	1.97%	0.95%	2.64%	1.42%	3.13%	1.99%
<i>Uncultured Genus, Family Rikenellaceae</i>	5.61%	2.08%	5.01%	1.37%	5.85%	1.26%
<i>Uncultured Genus CF231, Family</i>	2.94%	1.80%	2.46%	1.90%	2.79%	2.04%

<i>Paraprevotellaceae</i>						
<i>Uncultured Genus YRC22 Family Paraprevotellaceae</i>	0.97%	0.83%	0.67%	0.53%	1.25%	0.92%
<i>[Prevotella]</i>	0.25%	0.53%	0.21%	0.54%	0.07%	0.13%
<i>Uncultured Genus, Order Bacteroidales</i>	1.08%	1.74%	1.28%	1.81%	0.36%	0.63%
<i>Fibrobacter</i>	5.73%	3.79%	7.12%	5.38%	2.28%	1.92%
<i>Uncultured Genus, Order Clostridiales</i>	6.56%	1.81%	5.80%	1.59%	5.62%	1.83%
<i>Uncultured Genus, Family Christensenellaceae</i>	0.38%	0.18%	0.33%	0.16%	0.33%	0.17%
<i>Clostridium</i>	8.09%	1.96%	6.21%	1.58%	7.67%	1.36%
<i>Uncultured Genus, Family Lachnospiraceae</i>	3.18%	0.67%	2.47%	1.22%	2.42%	1.45%
<i>Uncultured Genus rc4-4, Family Peptococcaceae</i>	0.29%	0.13%	0.28%	0.12%	0.21%	0.10%
<i>Uncultured Genus, Family Ruminococcaceae</i>	10.34%	3.23%	9.03%	1.82%	10.14%	2.73%
<i>Ruminococcus</i>	0.35%	0.47%	0.23%	0.23%	0.08%	0.15%
<i>Phascolarctobacterium</i>	1.14%	0.29%	1.20%	0.43%	0.96%	0.33%
<i>Uncultured Genus, Family Desulfovibrionaceae</i>	0.25%	0.11%	0.22%	0.10%	0.22%	0.10%
<i>Uncultured Genus, Class Deltaproteobacteria</i>	0.07%	0.09%	0.12%	0.14%	0.06%	0.11%
<i>Campylobacter</i>	1.17%	0.61%	1.57%	1.19%	1.22%	0.61%

<i>Treponema</i>	2.75%	2.65%	3.20%	2.58%	2.05%	1.49%
<i>Uncultured Genus, Class Mollicutes</i>	0.25%	0.68%	0.05%	0.09%	0.01%	0.03%
<i>Uncultured Genus,Class Verruco5</i>	0.11%	0.33%	0.22%	0.69%	0.55%	1.31%
<i>Akkermansia</i>	1.46%	0.60%	1.99%	1.89%	1.87%	1.49%

Figure 22: Graph comparing mean percentage relative abundance at Genus in group 1, group 2 and group 3.



The uncultured Genus from the Order *Bacteroidales* recorded its highest mean percentage relative abundance in group 3 with 26.07%. Its least in relative abundance was recorded in the pre-treatment sample with a mean of 20.85%. The uncultured Genus from the Family *Ruminococcaceae* recorded its highest mean of 10.34% in group 1 while its lowest mean was recorded in the pre-treatment samples as well. The least dominant Genus in the pre-treatment samples with a mean of 0.04% is an uncultured Genus from the uncultured Order RF39 from the Class *Mollicutes*. This same Genus was also the lowest in mean percentage relative abundance in group 2 and 3. The least dominant relative abundance in group 1 was recorded in the uncultured Genus from the uncultured Order GMD14H09 from the Class *Deltaproteobacteria* (table 25, figure 23).

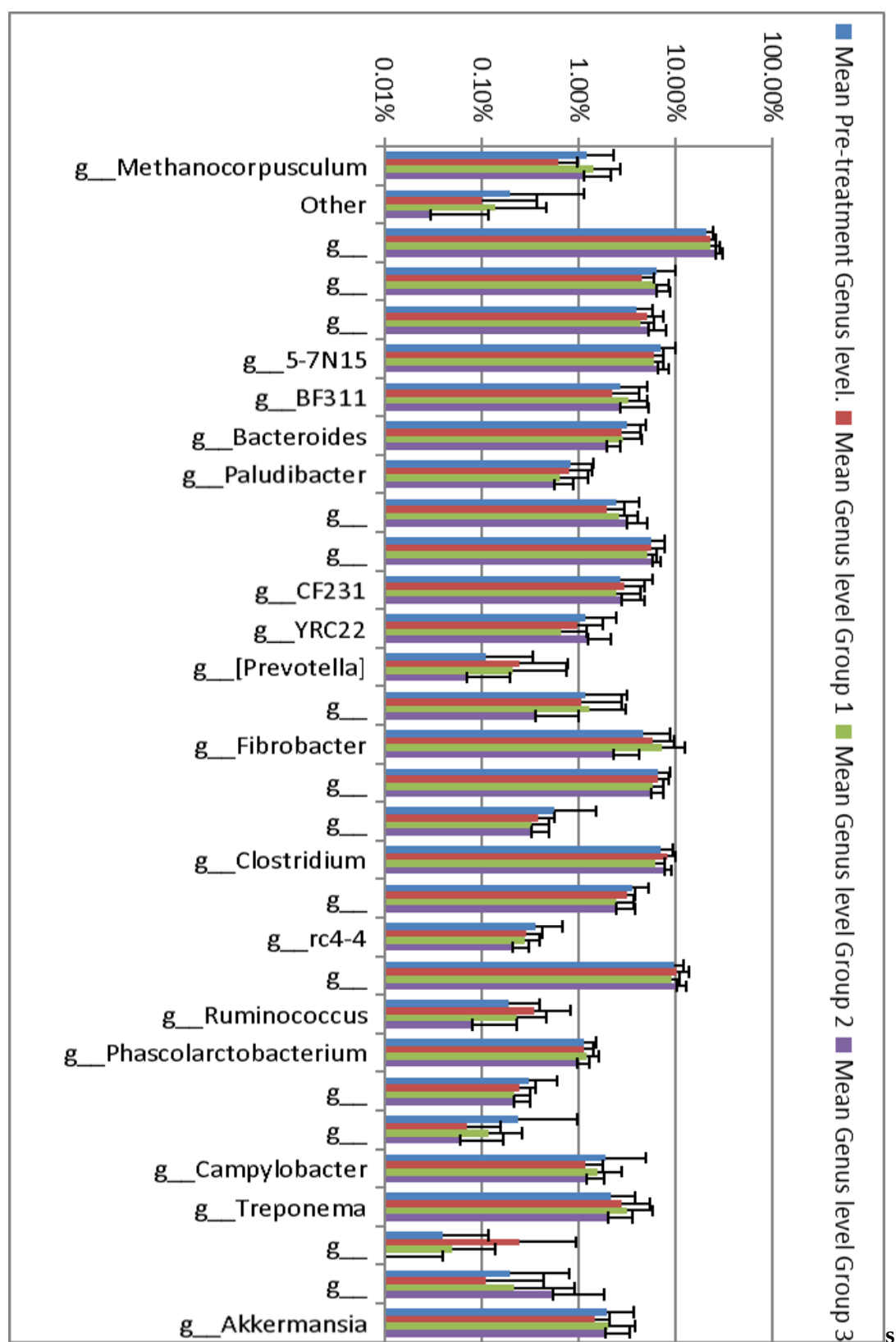
Table 25: Table comparing the mean percentage relative abundance in the pre-treatment, group 1, group 2, group 3.

Taxonomy	Mean Pre-treatment Genus level.	Mean Genus level Group 1	Mean Genus level Group 2	Mean Genus level Group 3
<i>Methanocorpusculum</i>	1.21%	0.63%	1.42%	1.15%
<i>Uncultured Genus, Order Bacteroidales</i>	0.20%	0.10%	0.14%	0.03%
<i>Uncultured Genus, Order Bacteroidales</i>	20.85%	22.86%	22.87%	26.07%
<i>Uncultured Genus, Order Bacteroidales</i>	6.35%	4.53%	6.19%	6.46%
Uncultured Genus, Family Bacteroidaceae	3.94%	5.13%	4.38%	5.28%
<i>Uncultured Genus 5-7N15, Family Bacteroidaceae</i>	7.00%	5.99%	5.98%	6.61%
<i>Uncultured Genus BF311 Family Bacteroidaceae</i>	2.73%	2.23%	3.24%	2.71%
<i>Bacteroides</i>	3.19%	2.79%	2.83%	1.94%
<i>Paludibacter</i>	0.83%	0.79%	0.64%	0.56%
<i>Uncultured Genus, Order Bacteroidales</i>	2.42%	1.97%	2.64%	3.13%

<i>Uncultured Genus, Family Rikenellaceae</i>	5.65%	5.61%	5.01%	5.85%
<i>Uncultured Genus CF231, Family Paraprevotellaceae</i>	2.65%	2.94%	2.46%	2.79%
<i>Uncultured Genus YRC22 Family Paraprevotellaceae</i>	1.16%	0.97%	0.67%	1.25%
<i>[Prevotella]</i>	0.11%	0.25%	0.21%	0.07%
<i>Uncultured Genus, Order Bacteroidales</i>	1.17%	1.08%	1.28%	0.36%
<i>Fibrobacter</i>	4.65%	5.73%	7.12%	2.28%
<i>Uncultured Genus, Order Clostridiales</i>	6.57%	6.56%	5.80%	5.62%
<i>Uncultured Genus, Family Christensenellaceae</i>	0.57%	0.38%	0.33%	0.33%
<i>Clostridium</i>	7.01%	8.09%	6.21%	7.67%
<i>Uncultured Genus, Family Lachnospiraceae</i>	3.59%	3.18%	2.47%	2.42%
<i>Uncultured Genus rc4- 4,Family Peptococcaceae</i>	0.36%	0.29%	0.28%	0.21%
<i>Uncultured Genus, Family Ruminococcaceae</i>	9.60%	10.34%	9.03%	10.14%
<i>Ruminococcus</i>	0.19%	0.35%	0.23%	0.08%
<i>Phascolarctobacterium</i>	1.15%	1.14%	1.20%	0.96%
<i>Uncultured Genus, Family Desulfovibrionaceae</i>	0.31%	0.25%	0.22%	0.22%
<i>Uncultured Genus, Class Deltaproteobacteria</i>	0.24%	0.07%	0.12%	0.06%

<i>Campylobacter</i>	1.89%	1.17%	1.57%	1.22%
<i>Treponema</i>	2.17%	2.75%	3.20%	2.05%
<i>Uncultured Genus, Class Mollicutes</i>	0.04%	0.25%	0.05%	0.01%
<i>Uncultured Genus,Class Verruco-5</i>	0.20%	0.11%	0.22%	0.55%
<i>Akkermansia</i>	1.97%	1.46%	1.99%	1.87%

Figure 23: Graph comparing mean percentage relative abundance at Genus in pre-treatment, group 1, group 2 and group 3.



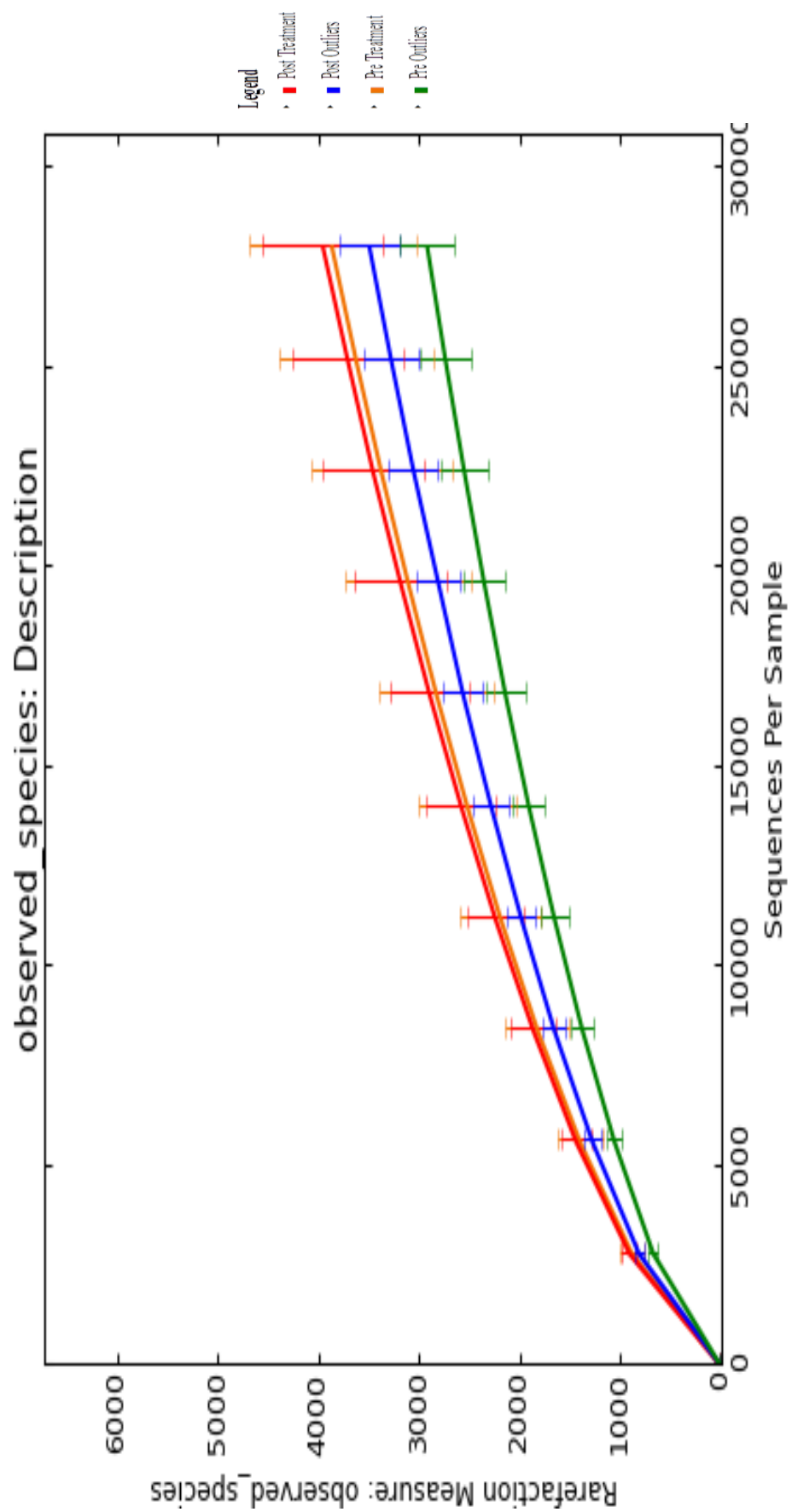
4.5 Rarefaction Curves.

Species richness (α -diversity) in both pre-treatment and post-treated samples was measured by the use of rarefaction curve. A rarefaction curve is a plot of the number of species as a function of the number of samples. It is determined by scaling down the number of sequences to the same number in all the samples and determining the number of species in the standardized sequences within each sample. Rarefaction is expressed statistically as $E(S_n)$ where n is the expected number of species in a standardized sequence from a larger sample sequence N . Alpha rarefaction was performed using the QIIME pipeline.

The gradient of a rarefaction curve determines the species richness of a sample. The steeper the gradient, the greater the species richness (alpha-diversity) in the sample. Figure 24 represents the rarefaction curve of the pre-treatment samples and the post-treated samples with pre-treatment outliers and post treated outliers performed with QIIME. The post treated samples (red curve) have a slightly higher gradient than the pre-treatment samples (orange) indicating that they might have a slightly higher species richness than the pre-treated samples. The post-treated outliers (blue) have a higher gradient than the pre-treated outliers (green) indicating there is more alpha diversity in the post-treated outliers than the pre-treated outliers.

There was no significant difference between the post treated group (red) and the pre-treatment group (orange) with $P = 1$ and a t value of -1.33 . The post treated group (Red) and the post treated outliers (blue) were significantly different with $P = 0.006$ and a t value of 3.10 . The post treated group (red) and the pre-treatment outliers (green) were also significantly different with a P value of 0.006 and a t value of 8.81 . The pre-treatment group (orange) and the post treated outliers (blue) were not significantly different with a $P = 0.672$ and $t = 1.57$. The pre-treatment group (orange) and the pre-treatment outliers were significantly different with a P value of 0.006 a t value of 5.50 . The pre-treatment outliers (green) and the post treated outliers (blue) were also significantly different with $P = 0.01$ and $t = 3.96$.

Figure 24: Rarefaction curve of pre-treatment, post-treated samples with pre-treated and post-treated outliers.

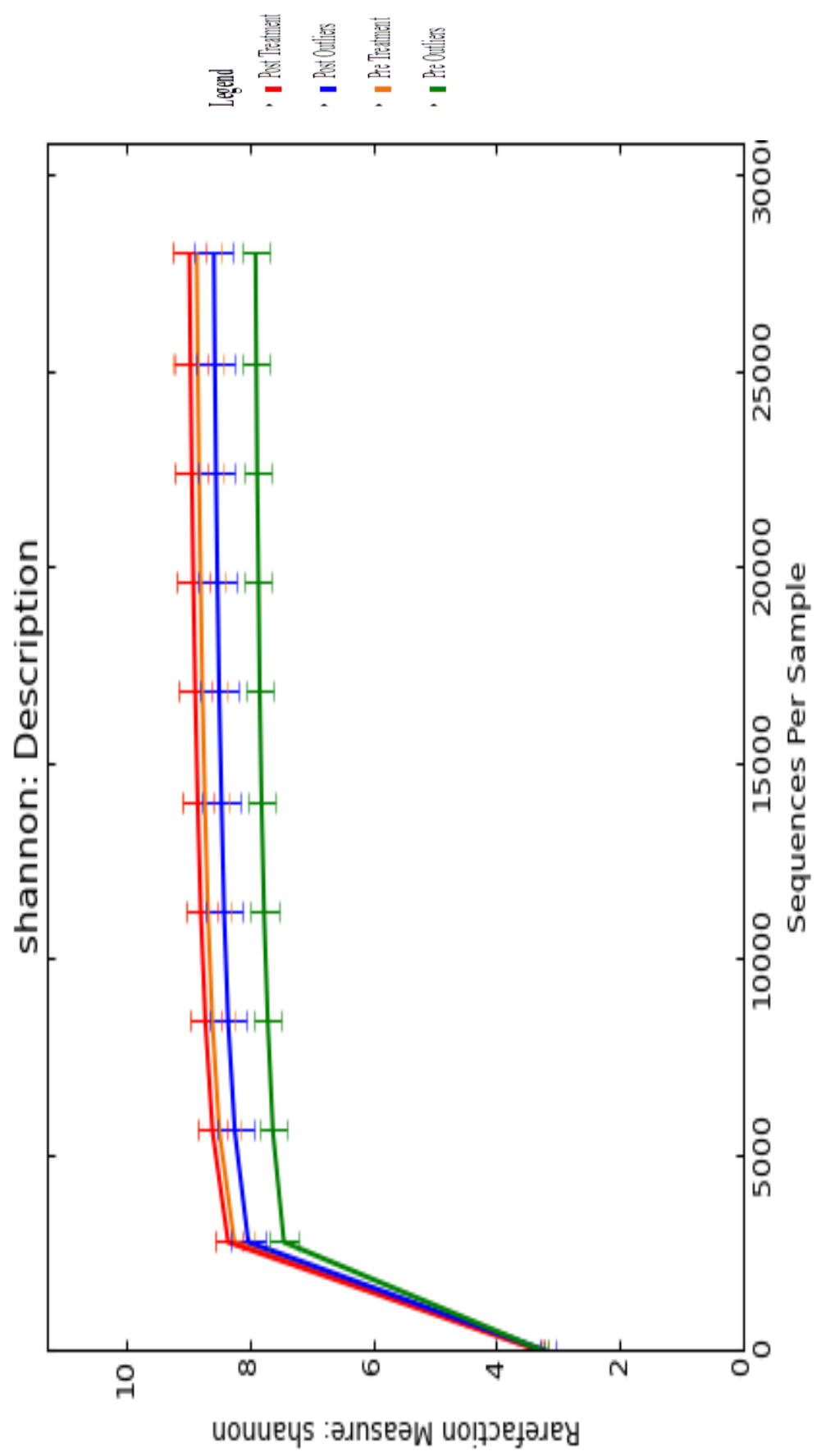


4.6 Shannon – diversity Index

Shannon diversity is a statistical tool that measures alpha diversity and the proportion of the distribution of each species within the sample. Samples high in species richness that are more evenly distributed will have a higher Shannon diversity index than samples with low species richness being less evenly distributed.

Figure 25 represents the Shannon diversity index of the pre-treatment and post treated samples and their outliers. The post-treated sample has a higher Shannon diversity index which means it is higher in species richness with the species more evenly distributed than the other samples. The pre-treated sample outlier is the lowest in Shannon diversity index which means it has the lowest species richness that are less evenly distributed.

Figure 25: Shannon diversity of pre-treated and post-treated samples with pre-treated and post treated outliers



4.7 Metric Multidimensional Scaling Analysis.

Similarity or resemblance between the pre-treatment and post-treated samples was measured using the Bray – Curtis statistical tool based on the relative abundance of the OTUs in each of the samples. Bray – Curtis similarity matrices were used to generate non – metric multidimensional plots that show similarity or resemblance between the pre-treatment and post-treated samples in a 2dimensional view. Factors namely Pre-treatment, S (Startect®), same as group 2), Z+S (Zolvix® + Startect®), same as group 3), Z (Zolvix® same as group 1) was used to generate the Bray - Curtis similarity curve. The Pre-treatment factor colour coded green triangle represents barcodes 21-60 (38 samples), the Factor S colour coded blue inverted triangle represents all the samples treated with Startect® that is group 2 (12 samples), the factor Z colour coded red rhombus represents samples treated with Zolvix® that is group 1 (15 samples), while factor Z+S colour coded blue square represents samples treated with a combination of Zolvix® plus Startect® that is group 3 (10 samples). The Kruskal stress value which determines the fitness of the plot in the 2dimensional view was 0.18 which is within the acceptable range (stress < 0.2, if > 0.2 the plot is distorted).

Samples that resemble each other appear close together in the plot while samples that are not similar are further apart from each other. There is a clustering together of most pre-treatment samples with the post-treated samples. There is a clustering of post-treated samples (Z, S, Z+S, that is group 1, 2 and 3). Most of the outliers appear in the pre-treated samples (23, 25, 32, 43, 45, 47, 53).

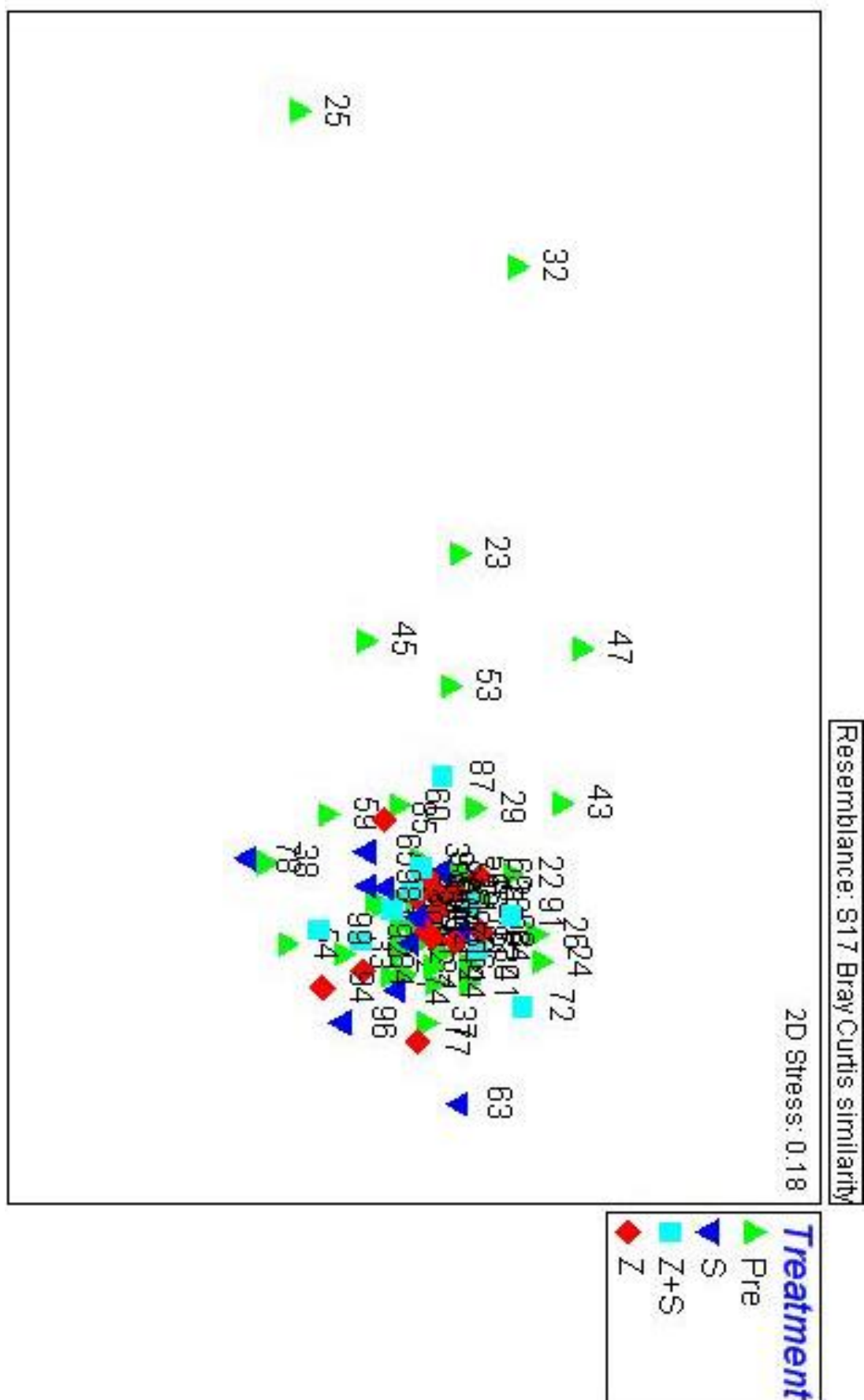
From the statistical analysis by PERMANOVA, there was no significant difference between the pre-treatment samples and the group 2 (Startect®) where $P = 0.166$ with a t value of 1.084. PERMANOVA revealed a marginal significance in differences between the pre-treatment samples and the group 3 samples (Zolvix® + Startect®) with $P = 0.052$ and a t value of 1.159. There was no significant difference between the pre-treatment samples and group 1 (Zolvix®) with a P value of 0.06 and a t value of 1.156. The difference in the group 2 (Startect®) and group 3 was also not significant with $P = 0.206$ and $t = 1.0535$. Again PERMANOVA also revealed no difference between the group 1 (Zolvix®) and the group 2 (Startect®) with P value of 0.161 and a t value of 1.0593. There was a significant difference

between the group 3 (Zolvix® + Startect®) and the group 1 (Zolvix®) with $P = 0.01$ and $t = 1.169$ (table 26a).

Table 26a: PERMANOVA results of pre-treatment and post treatment pairwise with outliers.

Groups	t value	P (perm)	Unique perms
Pre-treatment, group 2 (Startect®)	1.0841	0.166	997
Pre-treatment, group 3 (Zolvix®+Startect®)	1.1599	0.052	998
Pre-treatment, group 1 (Zolvix®)	1.1564	0.06	994
Group2 (Startect®), group3 (Zolvix®+Startect®)	1.0535	0.206	996
Group2 (Startect®), Group1 (Zolvix®)	1.0593	0.161	997
Group3 (Zolvix®+Startect®), Group 1 (Zolvix®).	1.1686	0.01	996

Figure 26a: Bray – Curtis MDS plot based on relative abundance of OTUs for pre-treatment and post-treated samples (S, Z+S, Z) revealing outliers.



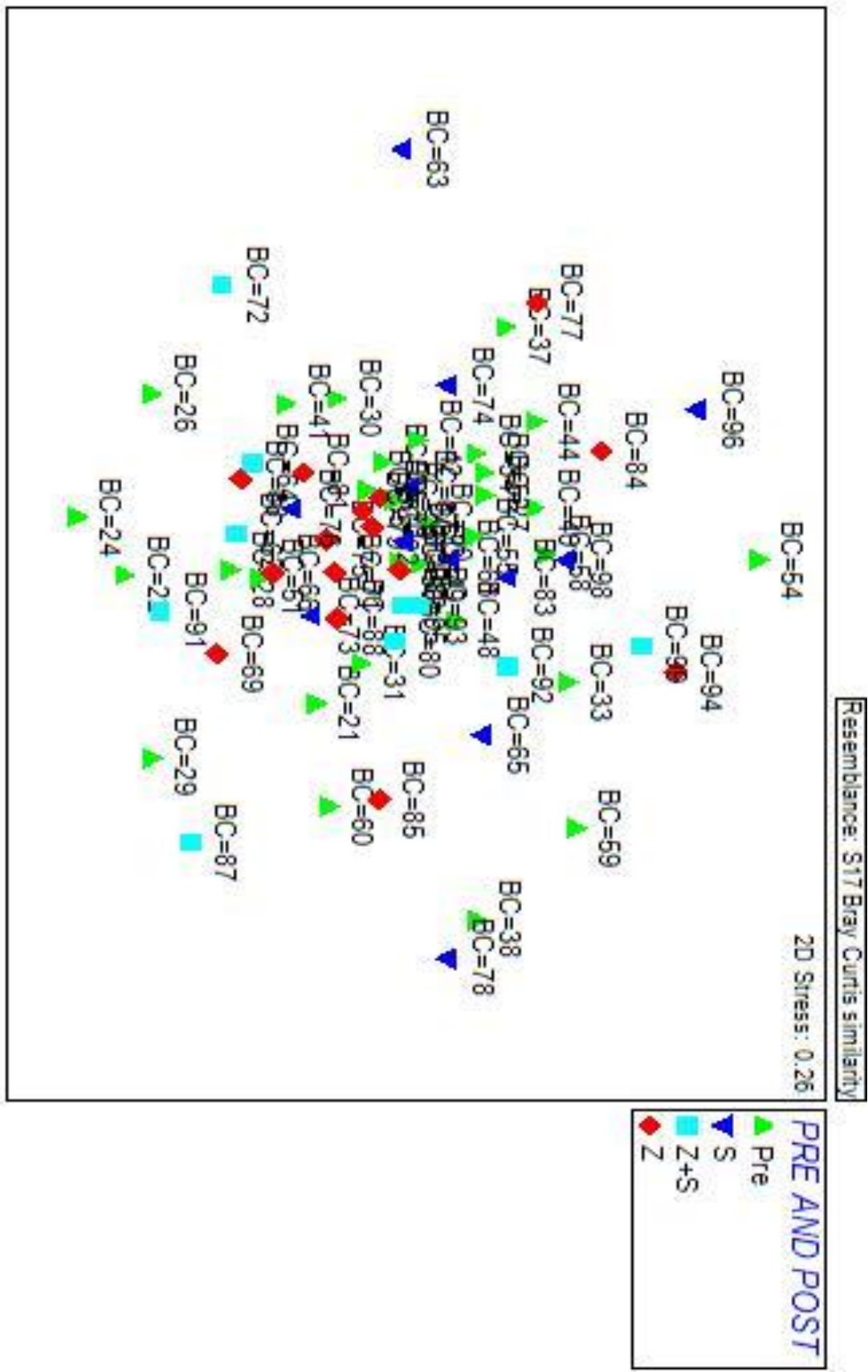
An MDS was again plotted to further view the relationship of the different groups without the pre-treatment outliers. This was performed so as to view with a bit of details the Bray-Curtis similarity curve (Figure 26b). PERMANOVA pair wise test revealed without the outliers revealed no significant difference between the clustering of the pre-treatment samples and the group 2 (Startect®),) with a P value of 0.221 and a t value of 1.046. It revealed a significant difference between the clustering of pre-treatment samples and group 3 (Zolvix®), plus Startect®),) with P = 0.01 and t = 1.210. There was no significant difference between the clustering of pre-treatment samples and group 1 (Zolvix®),) with P = 0.111 and t = 1.098. It also showed no significant difference between the clustering of group 2 (Startect®),) and group 3 (Zolvix®), plus Startect®),) P value of 0.167 t value of 1.053. Again there was no significant difference between the clustering of group 2 (Startect®),) and group 1 (Zolvix®),) P = 0.161 and t = 1.059. But it revealed a significant difference between the clustering of Group 1(Zolvix®),) and Group 3 (Zolvix®), plus Startect®) with a P value of 0.012 and a t value of 1.168 (table 26b).

Table 26b: PERMANOVA results of pre-treatment and post treatment pairwise without outliers.

Groups	t value	P (perm)	Unique perms
Pre-treatment, group 2 (Startect®)	1.046	0.221	995
Pre-treatment, group 3 (Zolvix+Startect®)	1.210	0.01	997
Pre-treatment group 1 (Zolvix®)	1.098	0.111	996
Group 2 (Startect®), group 3 (Zolvix®),+Startect®)	1.053	0.167	995
Group 2 (Startect®), Group 3 (Zolvix®), plus Startect®)	1.059	0.161	996

1 (Zolvix®)			
Group 3 (Zolvix®)+Startect®),Group 1 (Zolvix®).	1.168	0.012	996

Figure 26b: Bray – Curtis MDS plot based on relative abundance of OTUs for pre-treatment and post-treated samples (S, Z+S, Z) without outliers. Annotation of data is the sample Barcode.



4.7.1 Statistical View of Outliers at Order level.

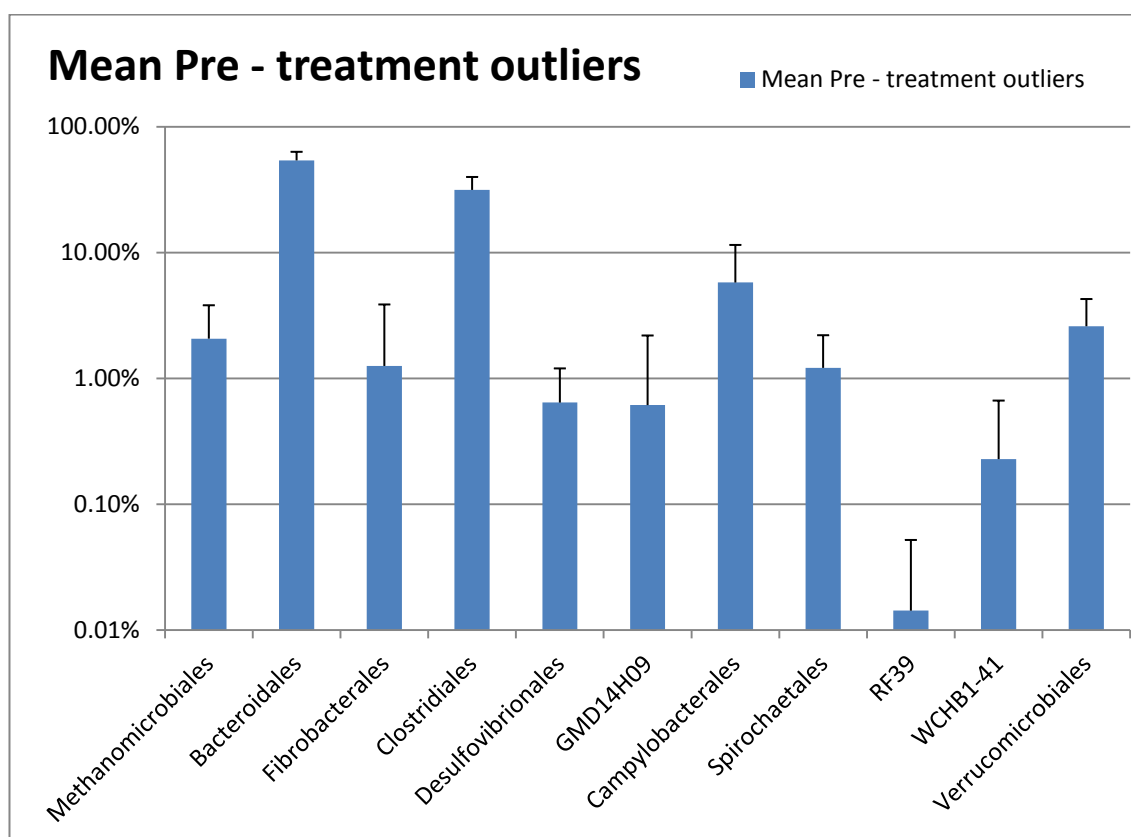
From the MDS plot in Figure 26a, it is observed that some of the pre – treatment samples appeared as outliers. The barcodes that appear as outliers include BC25, BC32, BC23, BC45, BC53, BC47, and BC43.

Methanomicrobiales had a mean percentage relative of 2.07% in the pre – treatment outliers. *Bacteroidales* which was the most dominant had a mean percentage relative abundance of 54.06% followed by the Order *Clostridiales* with a mean of 31.51%. *Campylobacterales* recorded a mean percentage relative abundance of 5.79%. The least dominant was the uncultured Order RF39 from the Class *Mollicutes* with a mean of 0.01%.

Table 27: Mean percentage relative abundance in Pre – treatment outliers.

Taxonomy	Mean Pre - treatment outliers	Standard deviation outliers.
Methanomicrobiales	2.07%	1.75%
Bacteroidales	54.06%	9.20%
Fibrobacterales	1.26%	2.62%
Clostridiales	31.51%	8.42%
Desulfovibrionales	0.64%	0.56%
GMD14H09	0.61%	1.58%
Campylobacterales	5.79%	5.72%
Spirochaetales	1.21%	0.99%
Uncultured Order RF39, Class <i>Mollicutes</i>	0.01%	0.04%
Uncultured Order WCHB1-41, Class <i>Veruoco-5</i>	0.23%	0.44%
<i>Verrucomicrobiales</i>	2.60%	1.68%

Figure 27: Graph representing the mean percentage relative abundance in the pre – treatment outliers at Order level of Taxonomy.



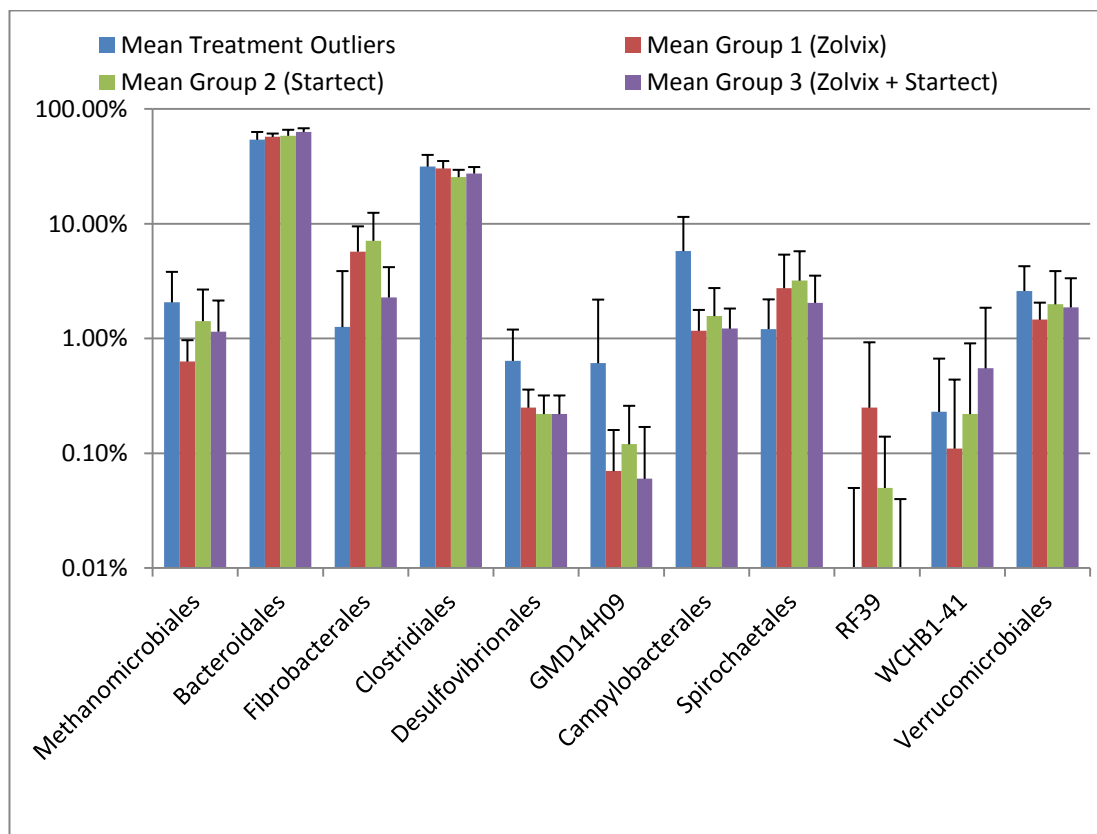
The Order *Bacteroidales* was most dominant in group 3. The Order *Clostridiales* were most dominant in the outliers with a mean of 31.51%. The uncultured Order RF39 from the Class *Mollicutes* was least in both the pre-treatment outliers and Group 3 (Table 28, Figure 28).

Table 28: Mean percentage relative abundance in pre – treatment outliers, Group 1 (Zolvix®), Group 2 (Startect®), Group 3 (Zolvix® + Startect®).

Taxonomy	Mean Pre - treatment outliers	Mean Group 1	Mean Group 2	Mean Group 3
<i>Methanomicrobiales</i>	2.07%	0.63%	1.42%	1.15%
<i>Bacteroidales</i>	54.06%	57.25%	58.56%	63.13%
<i>Fibrobacterales</i>	1.26%	5.73%	7.12%	2.28%
<i>Clostridiales</i>	31.51%	30.34%	25.53%	27.43%
<i>Desulfovibrionales</i>	0.64%	0.25%	0.22%	0.22%

<i>Uncultured Order, GMD14H09 Class Deltaproteobacteria</i>	0.61%	0.07%	0.12%	0.06%
<i>Campylobacteriales</i>	5.79%	1.17%	1.57%	1.22%
<i>Spirochaetales</i>	1.21%	2.75%	3.20%	2.05%
<i>Uncultured Order RF39, Class Mollicutes</i>	0.01%	0.25%	0.05%	0.01%
<i>Uncultured Order WCHB1-41, Class Verruco-5</i>	0.23%	0.11%	0.22%	0.55%
<i>Verrucomicrobiales</i>	2.60%	1.46%	1.99%	1.87%

Figure 28: Graph comparing mean percentage relative abundance in pre – treatment outliers, group1, group 2 and group 3 at Order level of Taxonomy.



4.7.2 Statistical view of outliers at Genus level of Taxonomy.

At the Genus level of Taxonomy amongst the outliers, the uncultured Genus from the Order *Bacteroidales* was the most dominant with a mean percentage relative abundance of 15.67% and a standard deviation of 1.64%. The second most dominant in the outliers was another uncultured Genus 5-7N15 from the Family *Bacteroidaceae* with a mean percentage relative abundance of 11.13% and a standard deviation of 3.81%. A mean percentage relative abundance of 0.00% were recorded for 3 Genera amongst the outliers (table 29, figure 29).

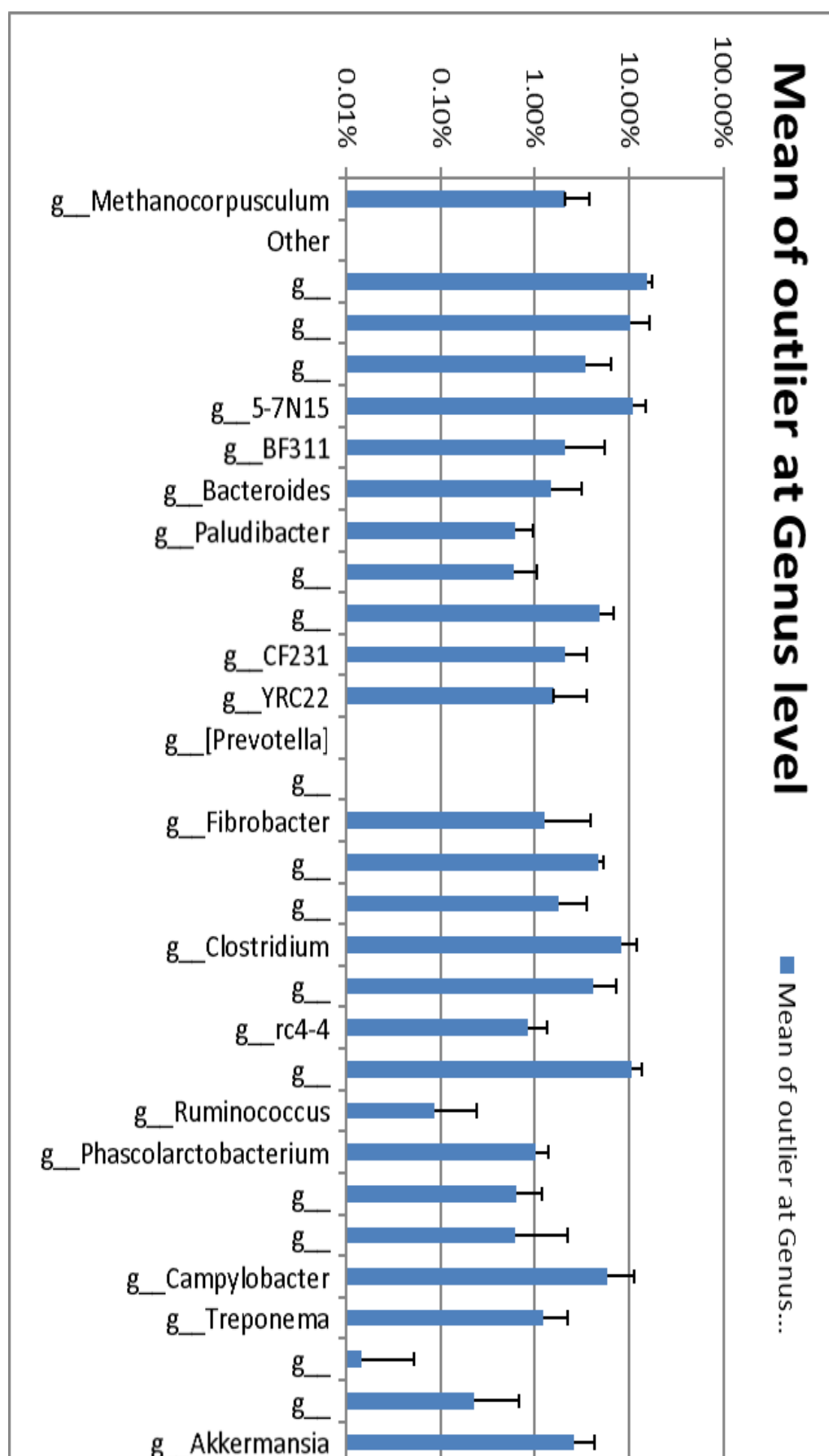
Table 29: Mean percentage relative abundance in outliers at Genus level (BC23, BC25, BC32, BC43, BC45, BC47, BC53 all members of the pre-treatment sample).

Taxonomy	Mean of outlier at Genus level	Standard deviation outliers at Genus level
<i>Methanocorpusculum</i>	2.07%	1.75%
<i>Uncultured Genus, Order Bacteroidales</i>	0.00%	0.00%
<i>Uncultured Genus, Order Bacteroidales</i>	15.67%	1.64%
<i>Uncultured Genus, Order Bacteroidales</i>	10.43%	6.20%
<i>Uncultured Genus, Family Bacteroidaceae</i>	3.46%	2.95%
<i>Uncultured Genus 5-7N15, Family Bacteroidaceae</i>	11.13%	3.81%
<i>Uncultured Genus BF311 Family Bacteroidaceae</i>	2.10%	3.50%
<i>Bacteroides</i>	1.49%	1.65%
<i>Paludibacter</i>	0.61%	0.33%
<i>Uncultured Genus, Order Bacteroidales</i>	0.60%	0.45%
<i>Uncultured Genus, Family Rikenellaceae</i>	4.87%	2.06%

<i>Uncultured Genus</i> <i>CF231, Family</i> <i>Paraprevotellaceae</i>	2.07%	1.49%
<i>Uncultured Genus</i> <i>YRC22 Family</i> <i>Paraprevotellaceae</i>	1.60%	1.97%
<i>[Prevotella]</i>	0.00%	0.00%
<i>Uncultured Genus,</i> <i>Order Bacteroidales</i>	0.00%	0.00%
<i>Fibrobacter</i>	1.26%	2.62%
<i>Uncultured Genus,</i> <i>Order Clostridiales</i>	4.71%	0.63%
<i>Uncultured Genus,</i> <i>Family</i> <i>Christensenellaceae</i>	1.81%	1.75%
<i>Clostridium</i>	8.23%	3.80%
<i>Uncultured Genus,</i> <i>Family</i> <i>Lachnospiraceae</i>	4.11%	3.23%
<i>Uncultured Genus rc4-</i> <i>4, Family</i> <i>Peptococcaceae</i>	0.84%	0.52%
<i>Uncultured Genus,</i> <i>Family</i> <i>Ruminococcaceae</i>	10.67%	3.16%
<i>Ruminococcus</i>	0.09%	0.16%
<i>Phascolarctobacterium</i>	1.01%	0.38%
<i>Uncultured Genus,</i> <i>Family</i> <i>Desulfovibrionaceae</i>	0.64%	0.56%
<i>Uncultured Genus,</i> <i>Class</i> <i>Deltaproteobacteria</i>	0.61%	1.58%

<i>Campylobacter</i>	5.79%	5.72%
<i>Treponema</i>	1.21%	0.99%
<i>Uncultured Genus, Class Mollicutes</i>	0.01%	0.04%
<i>Uncultured Genus,Class Verruco-5</i>	0.23%	0.44%
<i>Akkermansia</i>	2.60%	1.68%

Figure 29: Graph of mean percentage relative abundance at Genus level in pre-treatment outliers.



The uncultured Genus from the Order *Bacteroidales* recorded the highest mean percentage relative abundance of 26.07% in group 3 while its lowest was recorded in the pre-treatment outliers. A mean percentage relative abundance of 0.00% was seen in 3 Genera in the pre-treatment outliers. Another uncultured Genus 5-7N15 from the Family *Bacteroidaceae* had a mean percentage relative abundance of 11.13% in the pre-treatment outliers which was higher than what it recorded in all the other groups. *Campylobacter* recorded a mean percentage relative abundance of 5.79% in the pre-treatment outliers which was higher than what it recorded in the other samples (table 30, figure 30).

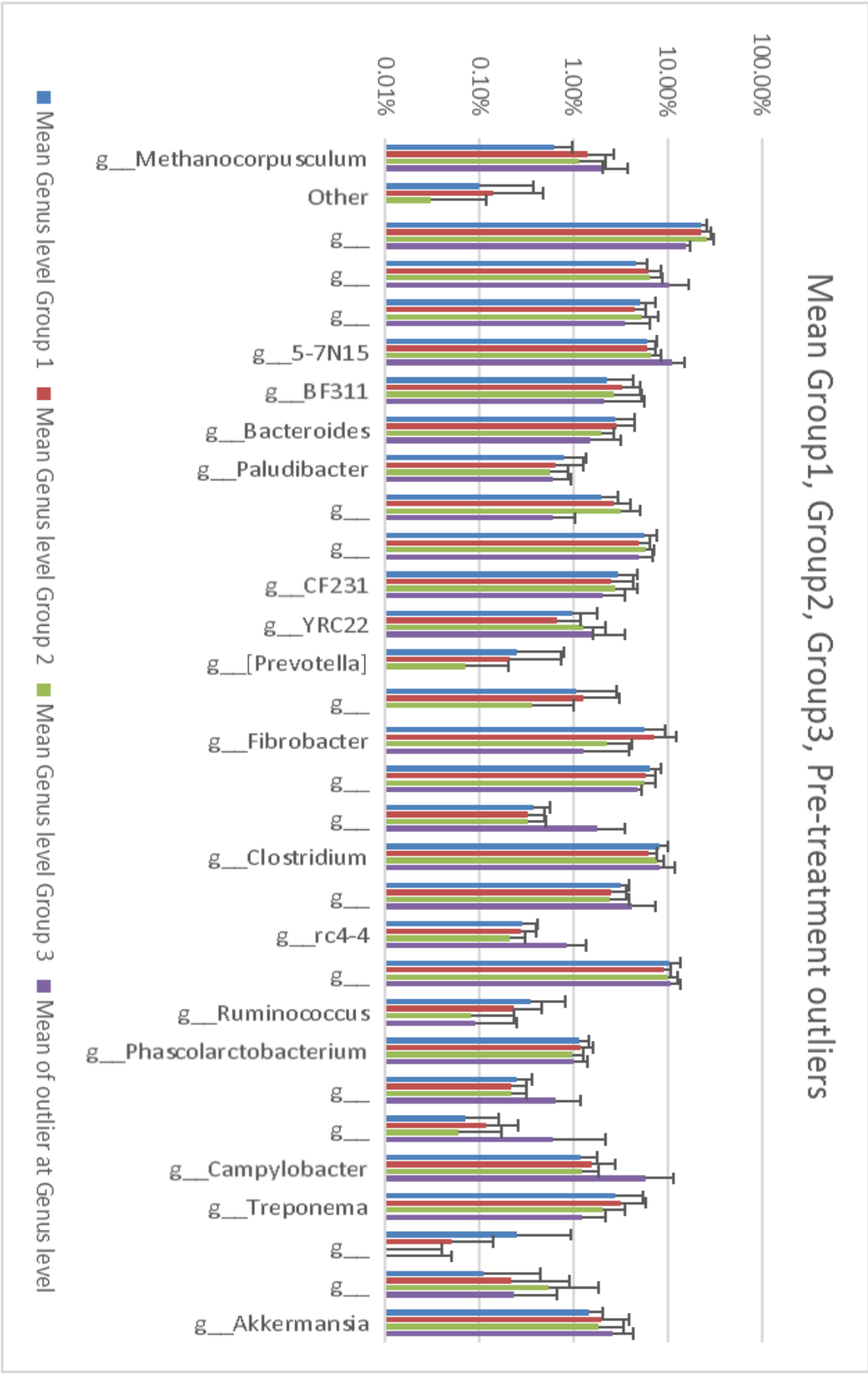
Table 30: Table comparing mean percentage relative abundance at Genus level in Group1 (Zolvix®), Group2 (Startect®), Group3 (Zolvix® + Startect®) and Pre-treatment outliers.

Taxonomy	Mean Genus level Group 1	Mean Genus level Group 2	Mean Genus level Group 3	Mean of outlier at Genus level
<i>Methanocorpusculum</i>	0.63%	1.42%	1.15%	2.07%
<i>Uncultured Genus, Order Bacteroidales</i>	0.10%	0.14%	0.03%	0.00%
<i>Uncultured Genus, Order Bacteroidales</i>	22.86%	22.87%	26.07%	15.67%
<i>Uncultured Genus, Order Bacteroidales</i>	4.53%	6.19%	6.46%	10.43%
<i>Uncultured Genus, Family Bacteroidaceae</i>	5.13%	4.38%	5.28%	3.46%
<i>Uncultured Genus 5-7N15, Family Bacteroidaceae</i>	5.99%	5.98%	6.61%	11.13%
<i>Uncultured Genus BF311, Family Bacteroidaceae</i>	2.23%	3.24%	2.71%	2.10%
<i>Bacteroides</i>	2.79%	2.83%	1.94%	1.49%
<i>Paludibacter</i>	0.79%	0.64%	0.56%	0.61%
<i>Uncultured Genus,</i>	1.97%	2.64%	3.13%	0.60%

<i>Order Bacteroidales</i>				
<i>Uncultured Genus, Family Rikenellaceae</i>	5.61%	5.01%	5.85%	4.87%
<i>Uncultured Genus CF231, Family Paraprevotellaceae</i>	2.94%	2.46%	2.79%	2.07%
<i>Uncultured Genus YRC22 Family Paraprevotellaceae</i>	0.97%	0.67%	1.25%	1.60%
<i>[Prevotella]</i>	0.25%	0.21%	0.07%	0.00%
<i>Uncultured Genus, Order Bacteroidales</i>	1.08%	1.28%	0.36%	0.00%
<i>Fibrobacter</i>	5.73%	7.12%	2.28%	1.26%
<i>Uncultured Genus, Order Clostridiales</i>	6.56%	5.80%	5.62%	4.71%
<i>Uncultured Genus, Family Christensenellaceae</i>	0.38%	0.33%	0.33%	1.81%
<i>Clostridium</i>	8.09%	6.21%	7.67%	8.23%
<i>Uncultured Genus, Family Lachnospiraceae</i>	3.18%	2.47%	2.42%	4.11%
<i>Uncultured Genus rc4-4, Family Peptococcaceae</i>	0.29%	0.28%	0.21%	0.84%
<i>Uncultured Genus, Family Ruminococcaceae</i>	10.34%	9.03%	10.14%	10.67%
<i>Ruminococcus</i>	0.35%	0.23%	0.08%	0.09%
<i>Phascolarctobacterium</i>	1.14%	1.20%	0.96%	1.01%
<i>Uncultured Genus, Family Desulfovibrionaceae</i>	0.25%	0.22%	0.22%	0.64%

<i>Uncultured Genus, Class Deltaproteobacteria</i>	0.07%	0.12%	0.06%	0.61%
<i>Campylobacter</i>	1.17%	1.57%	1.22%	5.79%
<i>Treponema</i>	2.75%	3.20%	2.05%	1.21%
<i>Uncultured Genus, Class Mollicutes</i>	0.25%	0.05%	0.01%	0.01%
<i>Uncultured Genus,Class Verruco-5</i>	0.11%	0.22%	0.55%	0.23%
<i>Akkermansia</i>	1.46%	1.99%	1.87%	2.60%

Figure 30: Graph comparing mean percentage relative abundance in Group1, Group2, Group3 and Pre-treatment outliers at Genus level of Taxonomy.



4.8 MAP and round worm dual infected group (Year 1 collection, Year 2 collection and Year 3 collection).

Rectal faecal samples were collected from blackface breed of sheep, annually for a period of 3 years from a known Johne's disease infected commercial sheep farm. The samples were designated First collection (faecal samples collected in Year 1), second collection (faecal samples collected in Year 2) and third collections (faecal samples collected in Year 3).

DNA was extracted from all the samples (Year 1 collection, Year 2 collection and Year 3 collection) using the MOBIO PowerFecal® DNA Isolation kit. Extracted DNA was quantified using Nanodrop spectrophotometry. Illumina bar coded PCR (Caporazo et al., 2012) was carried out in all the extracted samples.

Table 31: Identity of samples in Years 1, 2 and 3 collections with their barcodes.

Total number of samples were: Year 1: n = 29, Year 2: n = 40, Year 3: n = 56.

Year 1 ¹		Year 2 ¹		Year 3 ²	
Animal ID	Barcode	Animal ID	Barcode	Animal ID	Barcode
1377	JF-2	C-823	JF-22	810	BC1
200043	JF-3	C-830	JF-23	56	BC2
200057	JF-4	C-833	JF-24	61	BC3
200154	JF-5	C-835	JF-25	67	BC4
200823	JF-6	C-1446	JF-26	848	BC5
200830	JF-7	C-1542	JF-27	818	BC6
200835	JF-8	C-2048	JF-28	934	BC7
200890	JF-9	LK-211	JF-29	84	BC8
200945	JF-10	LK-764	JF-30	176	BC9
1418	JF-11	LK-843	JF-31	833	BC10
200980	JF-71	LK-1723	JF-32	1532	BC11
67	JF-72	LK-33	JF-33	1438	BC12
1438	JF-73	MRI-1723	JF-34	2294	BC13

200948	JF-74	MRI-747	JF-35	130	BC14
1542	JF-75	LK-1437	JF-36	219	BC15
1351	JF-76	LK-779	JF-37	1542	BC16
200910	JF-77	LK-892	JF-38	849	BC17
200084	JF-78	LK-65	JF-39	892	BC18
1446	JF-79	LK-1549	JF-40	910	BC19
200909	JF-80	C-43	JF-41	923	BC20
200773	JF-81	C-57	JF-42	73	BC21
200120	JF-82	C-61	JF-43	1377	BC22
200061	JF-83	C-67	JF-44	1476	BC23
200094	JF-84	C-81	JF-45	57	BC24
200779	JF-85	C-84	JF-46	160	BC25
200960	JF-86	C-94	JF-47	1418	BC26
200892	JF-87	C-120	JF-48	832	BC27
1549	JF-88	C-154	JF-49	937	BC28
200833	JF-89	C-773	JF-50	3699	BC29
		C-848	JF-90	1509	BC30
		C-870	JF-91	912	BC31
		C-890	JF-92	948	BC32
		C-909	JF-93	960	BC33
		C-910	JF-94	43	BC34
		C-948	JF-95	1422	BC35
		C-960	JF-96	810	BC36
		C-980	JF-97	3069	BC37
		C-1351	JF-98	154	BC38
		C-1418	JF-99	793	BC39

		C-1438			JF-100	800	BC40
						823	BC41
						830	BC42
						1542	BC43
						1619	BC44
						1477	BC45
						3136	BC46
						2363	BC47
						1446	BC48
						3685	BC49
						1433	BC50
						834	BC51
						835	BC52
						1503	BC53
				2401	BC54		
				1453	BC55		
				3685	BC56		

¹ samples extracted, PCR generated by Jelena Nikolić, 2014 and Miriam Navarro, 2015 and sequenced by Dr Craig Watkins; ² samples extracted, PCR generated and sequenced by Swang Shallangwa 2015-16

Figure 31: Ultraviolet Image of PCR products shown by gel electrophoresis for third collection (Year3).

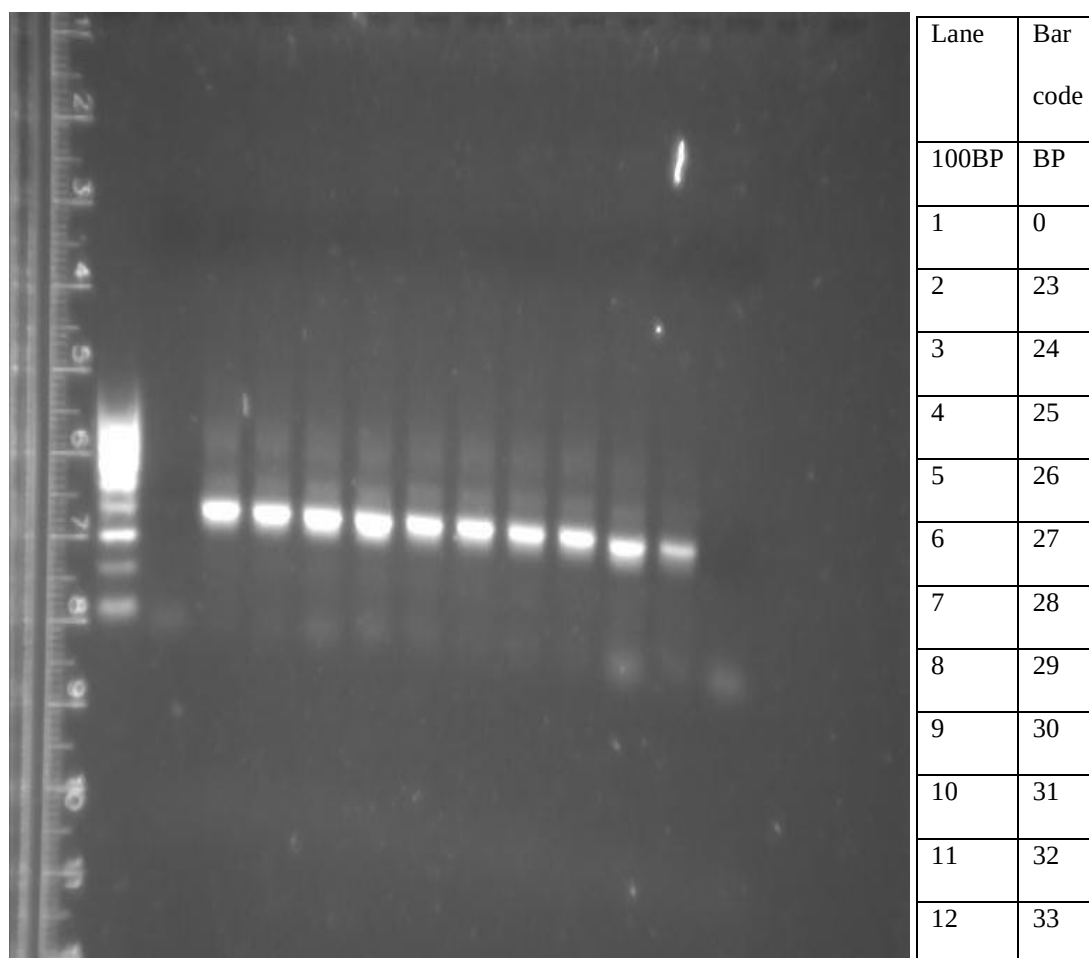


Figure 28 illustrates examples of the ultraviolet images that were obtained after gel electrophoresis of the PCR products. As can be seen from the image, there was no band obtained in well 13 which corresponded to BC33. The PCR was repeated for BC33 which later on revealed a band after imaging.

4.8.1 Analysis of Bacterial and archaeal community.

Following DNA extraction and amplification of the 16SrRNA gene V4 region by PCR , 29 Year 1, 40 Year 2 and 56 Year 3 collections making a total of 125 samples were chosen and forwarded to the Edinburgh Genomics for sequencing through the use of Illumina MiSeq platform.

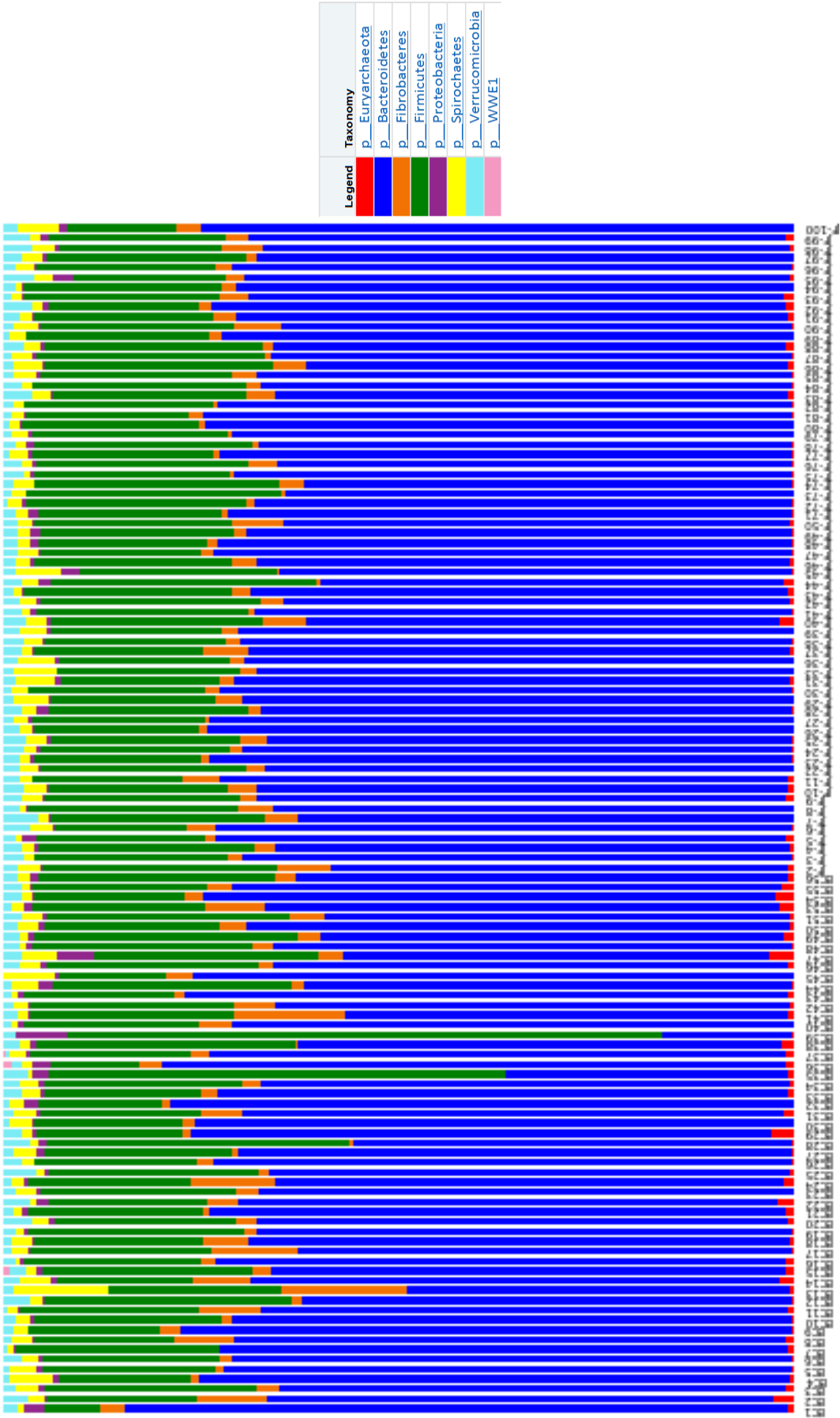
4.8.2 QIIME Taxonomy Results

The data obtained from Edinburgh Genomics was analysed by using the QIIME pipeline. An output file of 10,066,725 sequences was obtained after chimeras were filtered out. The complete QIIME pipeline analysis was performed, including the PyNast alignment with the

Greengenes 13_8 databases. An OTU table excluding the PyNast failures was created. Singletons were also filtered out (164,907 OTUs) from the overall sequence data sets.

4.8.2.1 Taxonomy summary at phylum level.

Figure 32: Bar chart of Taxonomy for Yearly collection at Phylum level.

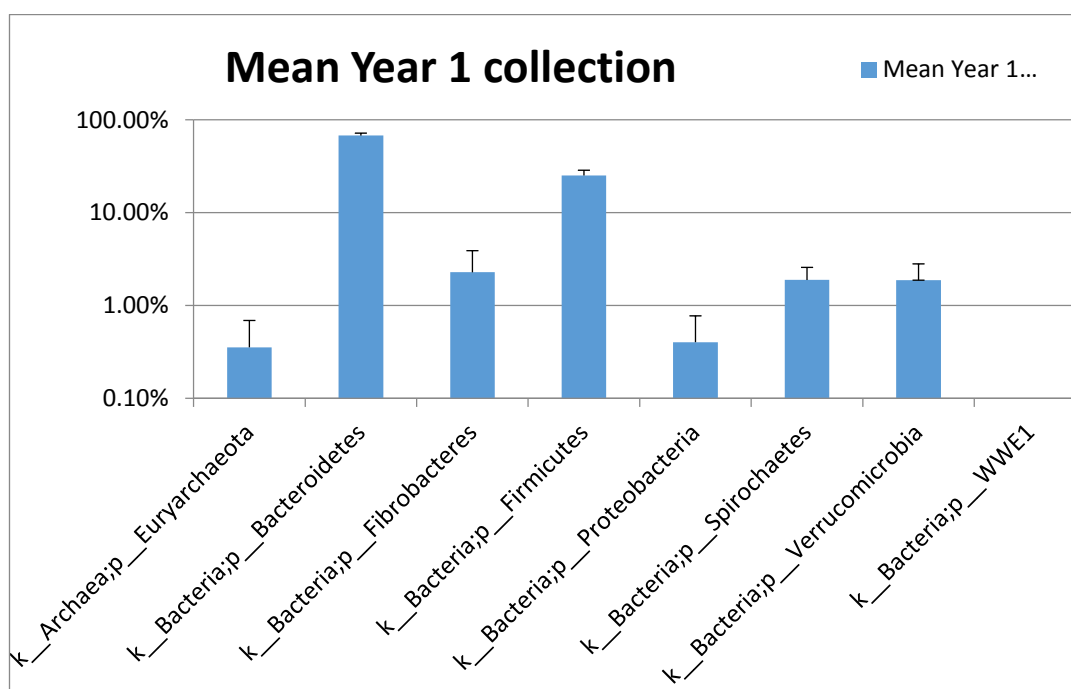


In the Year 1 collection at the Phylum level of taxonomy, a mean of 67.94% and a standard deviation of 4.27% was recorded for *Bacteroidetes*, making it the Phylum with the highest mean relative abundance in the Year 1 collection. *Firmicutes* was a distant second with a mean relative abundance of 25.25% and a standard deviation of 3.52%. *Fibrobacteres* had a mean relative abundance of 2.28% while *Spirochaetes* and *Verrucomicrobia* had a percentage mean relative abundance of 1.89% and 1.88% respectively (Table 32, Figure 33 and Figure 32).

Table 32: Mean percentage of relative abundance at Phylum level, for Year 1 collection.

Taxonomy (Phylum)	Mean Relative Abundance – Year 1 collection	Standard deviation - Year 1 Collection
<i>Euryarchaeota</i>	0.36%	0.34%
<i>Bacteroidetes</i>	67.94%	4.27%
<i>Fibrobacteres</i>	2.28%	1.63%
<i>Firmicutes</i>	25.25%	3.52%
<i>Proteobacteria</i>	0.40%	0.37%
<i>Spirochaetes</i>	1.89%	0.70%
<i>Verrucomicrobia</i>	1.88%	0.94%
<i>Uncultured Phylum WWE1</i>	0.00%	0.00%

Figure 33: Graph showing distribution of mean relative abundance in Year 1 collection (Graph plotted in logarithm scale, based 10).



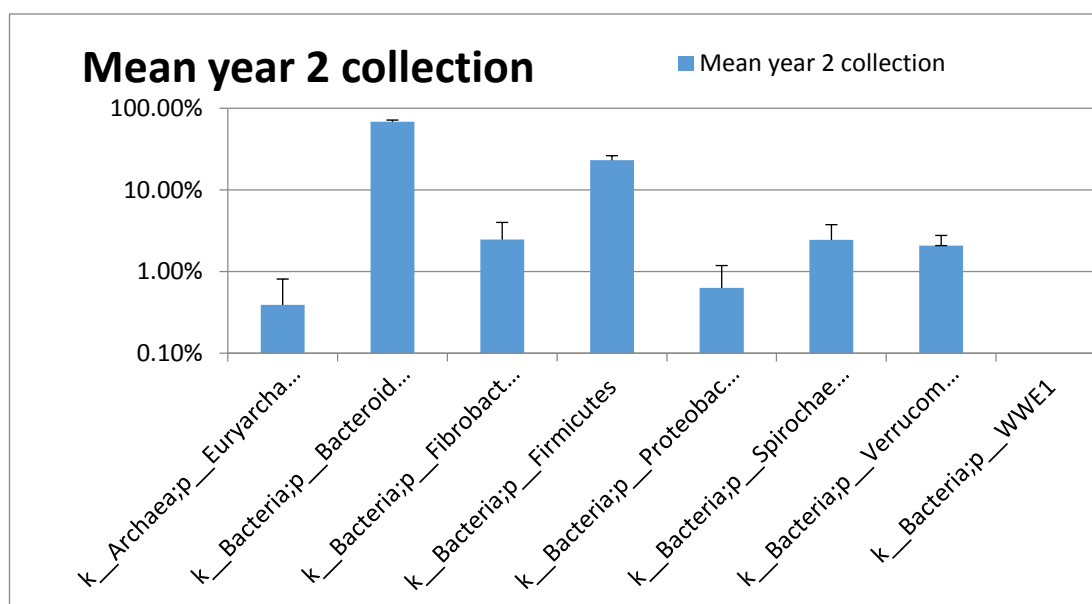
In the Year 2 collection at the Phylum level, *Bacteroidetes* also recorded the highest mean relative abundance of 68.75% with a standard deviation of 3.65%. Just like the Year 1 collection at the Phylum level. *Firmicutes* was a distant second with a mean relative abundance of 23.23% having a standard deviation of 3.23%. *Fibrobacteres* was third with mean relative abundance of 2.48% closely followed by *Spirochaetes* with mean relative abundance of 2.44% (Table 33, Figure 34).

Table 33: Percentage mean relative abundance at Phylum level, for Year 2 collection.

Taxonomy (Phylum)	Mean relative abundance – year 2 collection	Standard deviation year 2 collection
<i>Euryarchaeota</i>	0.39%	0.42%
<i>Bacteroidetes</i>	68.75%	3.65%
<i>Fibrobacteres</i>	2.48%	1.55%
<i>Firmicutes</i>	23.23%	3.23%
<i>Proteobacteria</i>	0.63%	0.56%
<i>Spirochaetes</i>	2.44%	1.34%

<i>Verrucomicrobia</i>	2.09%	0.70%
<i>Uncultured Phylum WWE1</i>	0.00%	0.00%

Figure 34: Graph showing mean percentage relative abundance for year 2 Collection (Graph plotted in logarithm scale base 10).



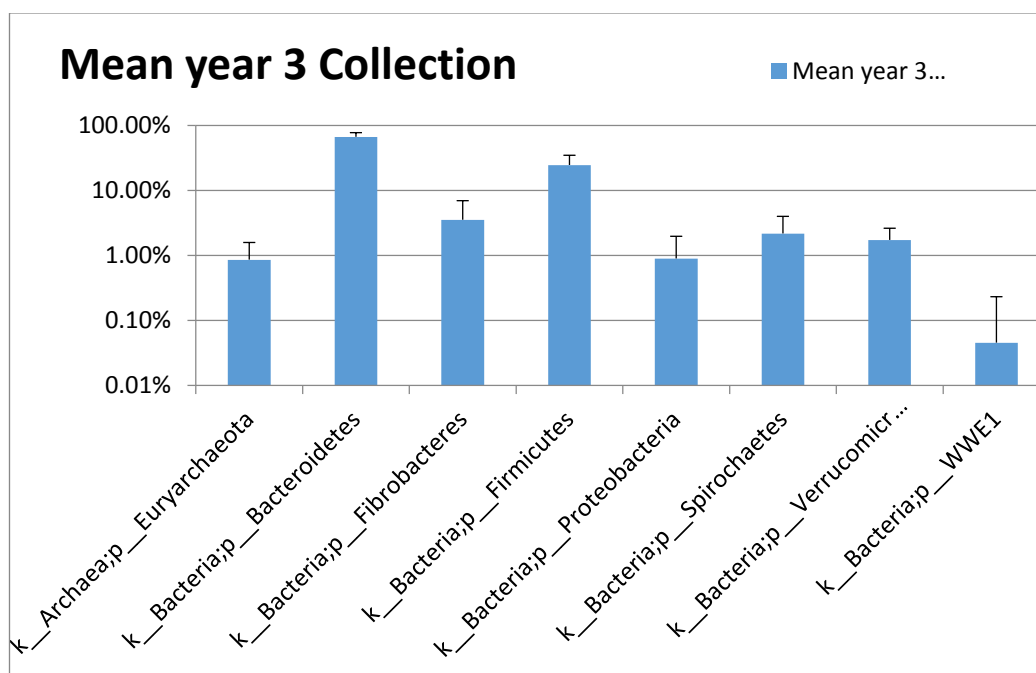
In the Year 3 collection at the Phylum level, we also see *Bacteroidetes* with the highest mean percentage relative abundance of 66.35% having a standard deviation of 10.48%. Like the first and second collection *Firmicutes* was a distant second in the third collection with a mean of 24.45% and a standard deviation of 10.06%. *Fibrobacteres* had a mean percentage relative abundance of 3.52% (Table 34, Figure 35).

Table 34: Percentage mean relative abundance Phylum level Year 3 collection.

Taxonomy (Phylum)	Mean relative abundance - year 3 Collection	Standard Deviation year 3 Collection
<i>Euryarchaeota</i>	0.86%	0.72%
<i>Bacteroidetes</i>	66.35%	10.48%
<i>Fibrobacteres</i>	3.52%	3.40%
<i>Firmicutes</i>	24.45%	10.06%
<i>Proteobacteria</i>	0.89%	1.07%
<i>Spirochaetes</i>	2.17%	1.80%

<i>Verrucomicrobia</i>	1.72%	0.88%
<i>Uncultured Phylum WWE1</i>	0.05%	0.19%

Figure 35: Graph showing mean percentage relative abundance for year 3 collection (Graph plotted in logarithm scale base 10).

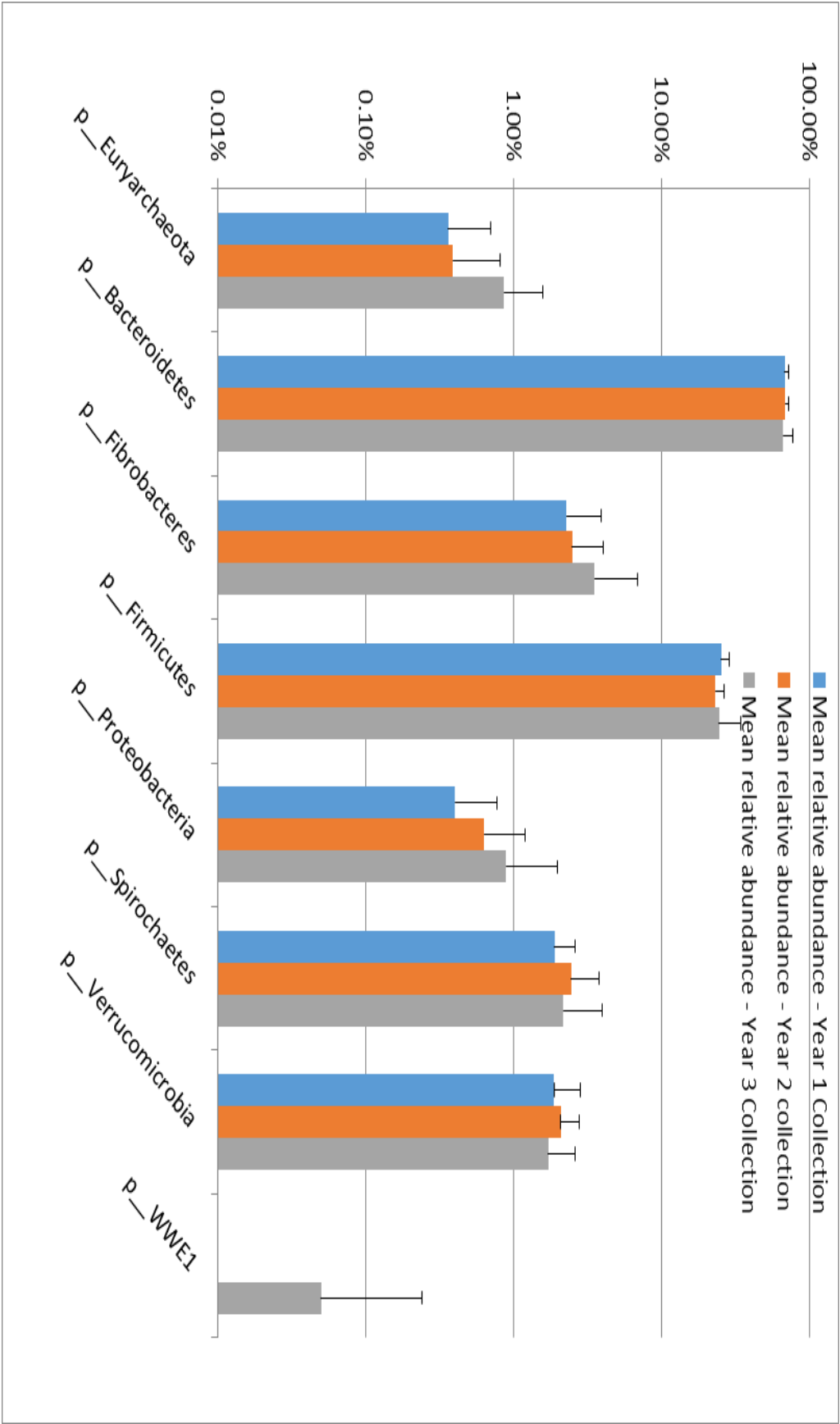


Comparing all 3 collections, it can be seen that the Phylum *Bacteroidetes* recorded the highest percentage mean relative abundance in the Year 2 collection which stood at 68.75% as compared to 67.94% in the Year 1 collection and 66.35% in the Year 3 collection. *Firmicutes* showed the highest percentage mean relative abundance of 25.25% in the Year 1 collection as compared to 23.23% in the Year 2 collection and 24.45% in the Year 3 collection. *Fibrobacteres* showed the highest percentage mean of 3.52% in the Year 3 collection than 2.28% in the Year 1 collection and 2.48% in the Year 2 collection. *Euryarchaeota* recorded 0.86% in the Year 3 collection above 0.36% and 0.39% recorded in the Year 1 and Year 2 collection respectively. *Spirochaetes* was highest in the Year 2 collection with a percentage mean relative abundance of 2.44% (Table 35, Figure 36).

Table 35: Mean percentage of relative abundance in all 3 collections at Phylum level (Year 1, Year 2 and Year 3 collections).

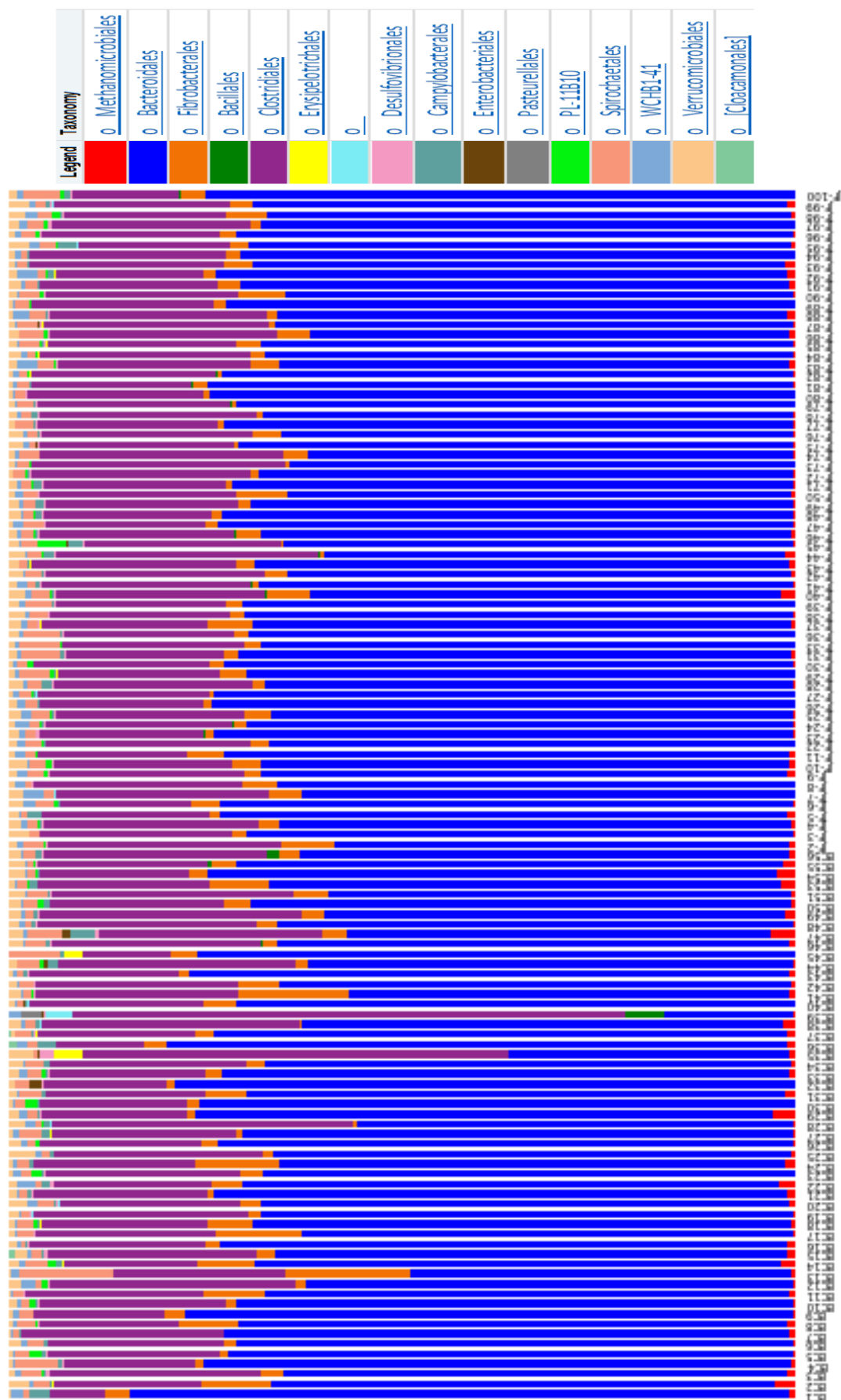
Taxonomy	Mean relative abundance – Year 1 Collection	Mean relative abundance – Year 2 collection	Mean relative abundance – Year 3 Collection
<i>Euryarchaeota</i>	0.36%	0.39%	0.86%
<i>Bacteroidetes</i>	67.94%	68.75%	66.35%
<i>Fibrobacteres</i>	2.28%	2.48%	3.52%
<i>Firmicutes</i>	25.25%	23.23%	24.45%
<i>Proteobacteria</i>	0.40%	0.63%	0.89%
<i>Spirochaetes</i>	1.89%	2.44%	2.17%
<i>Verrucomicrobia</i>	1.88%	2.09%	1.72%
<i>Uncultured, WWE1</i>	0.00%	0.00%	0.05%

Figure 36: Graph showing mean percentage relative abundance in all collections at the Phylum level (year 1, year 2 and year 3).



4.8.2.2 Taxonomy summary at Order level.

Figure 37: Bar chart of Taxonomy at Order level for Yearly Collection.



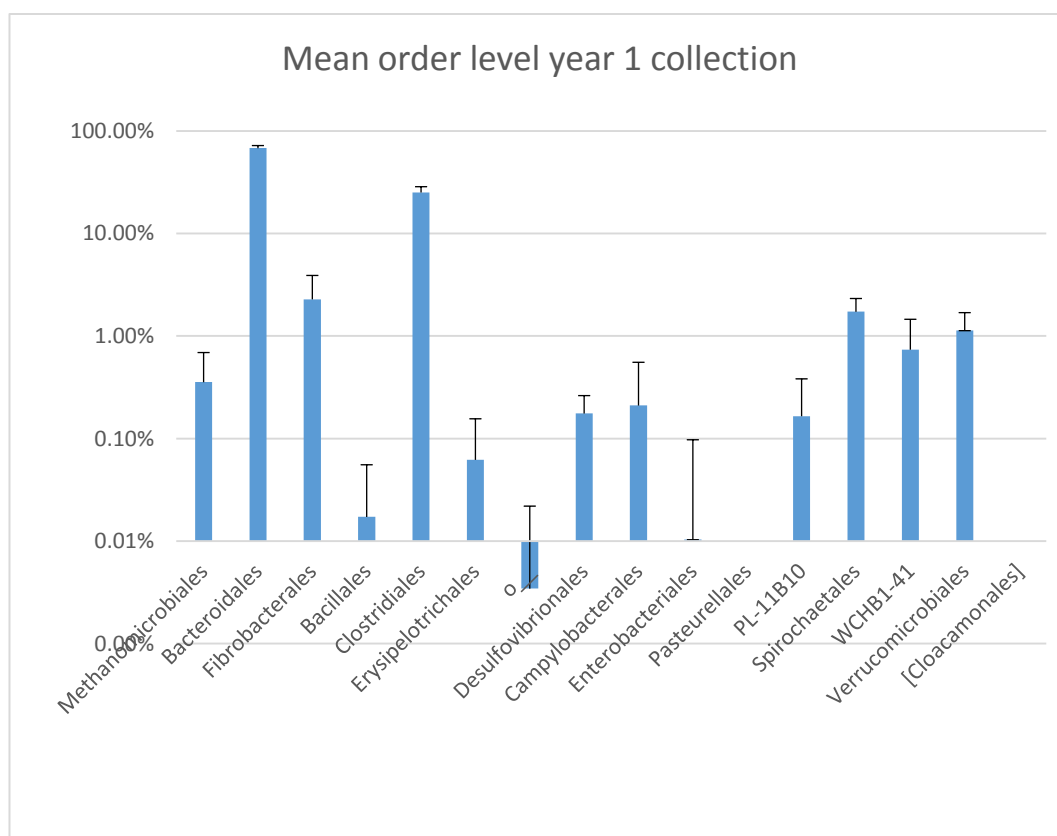
The percentage mean relative abundance of the Order *Methanomicrobiales* in the Year 1 collection was 0.36% with a standard deviation of 0.34%. The Order *Bacteroidales* had the highest mean percentage relative abundance standing at 67.94% with a standard deviation of 4.27 amongst the Year 1 collection. The Order *Clostridiales* was a distant second in the Year 1 collection with a mean percentage relative abundance of 25.17% and a standard deviation of 3.50%. The Order *Fibrobacterales* had a percentage mean relative abundance of 2.28% with a standard deviation of 1.63%. *Campylobacterales* had percentage mean relative abundance of 0.21%. The Order *Bacillales* had a percentage mean relative abundance of 0.02% (Table 36, Figure 38).

Table 36: Percentage mean relative abundance with standard deviation at Order level of year 1 collection.

Taxonomy (Order)	Mean year 1 collection	Standard deviation year 1 collection
<i>Methanomicrobiales</i>	0.36%	0.34%
<i>Bacteroidales</i>	67.94%	4.27%
<i>Fibrobacterales</i>	2.28%	1.63%
<i>Bacillales</i>	0.02%	0.04%
<i>Clostridiales</i>	25.17%	3.50%
<i>Erysipelotrichales</i>	0.06%	0.09%
Uncultured Order Class <i>Alphaproteobacteria</i>	0.00%	0.02%
<i>Desulfovibrionales</i>	0.18%	0.09%
<i>Campylobacterales</i>	0.21%	0.35%
<i>Enterobacteriales</i>	0.01%	0.09%
<i>Pasteurellales</i>	0.00%	0.00%
Uncultured Order PL- 11B10, Phylum <i>Spirochaetes</i>	0.17%	0.22%
<i>Spirochaetales</i>	1.73%	0.60%
<i>WCHB1-41</i>	0.74%	0.72%

<i>Verrucomicrobiales</i>	1.13%	0.56%
[<i>Cloacamonales</i>]	0.00%	0.00%

Figure 38: Graph showing percentage mean relative abundance at Order level in year 1 collection. (Graph plotted in logarithm scale base 10).

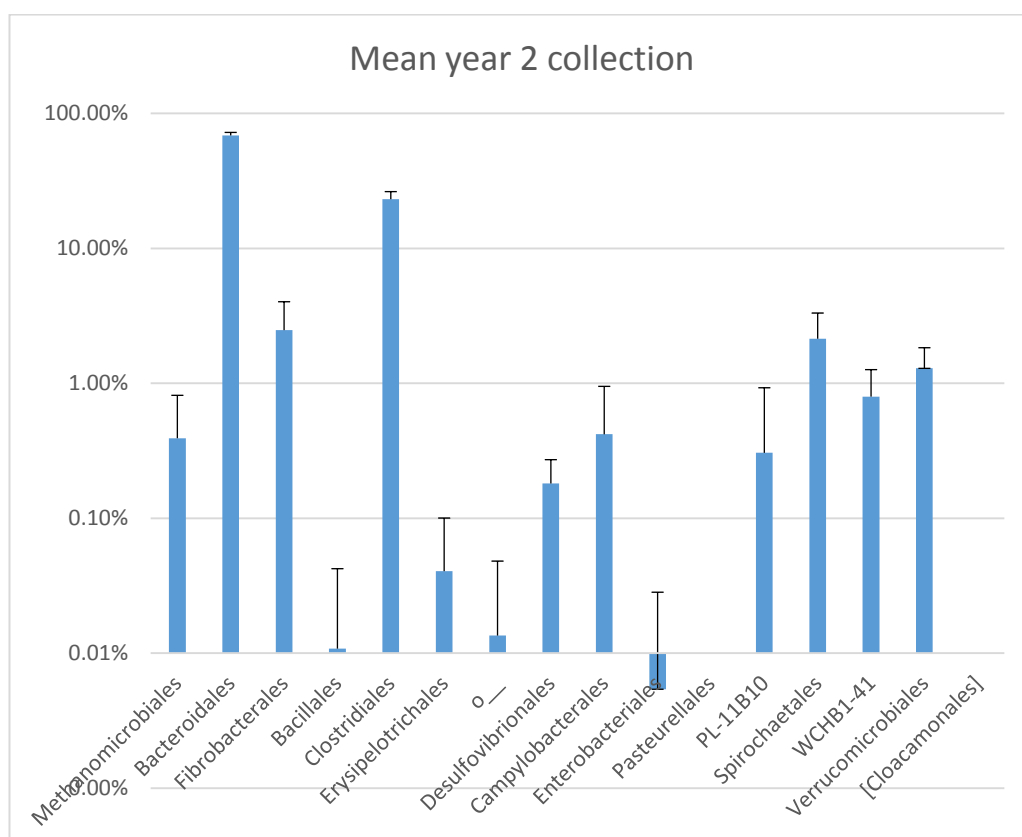


In the Year 2 collection the Order *Methanomicrobiales* had a percentage mean relative abundance of 0.39% with a standard deviation of 0.42%. Just like in the Year 1 collection, the Order *Bacteroidales* also maintained the highest percentage mean relative abundance in the Year 2 with a mean of 68.75% and a standard deviation of 3.65%. *Clostridiales* was second in percentage mean relative abundance at 23.15%. *Fibrobacteriales* stood at a percentage mean relative abundance of 2.48% with a standard deviation of 1.55%. The Order *Erysipelotrichales* had a mean of 0.04% and a standard deviation of 0.06% in percentage relative abundance. *Spirochaetales* had a percentage mean relative abundance of 2.14% while *Campylobacteriales* had a mean of 0.42% respectively. *Verrucomicrobiales* had a percentage mean relative abundance of 1.29% with a standard deviation of 0.54% (Table 37, Figure 29).

Table 37: Mean percentage of relative abundance with standard deviation at Order level of Year 2 Collection.

Taxonomy (Order)	Mean Relative Abundance – year 2 collection	Standard deviation year 2 collection
<i>Methanomicrobiales</i>	0.39%	0.42%
<i>Bacteroidales</i>	68.75%	3.65%
<i>Fibrobacterales</i>	2.48%	1.55%
<i>Bacillales</i>	0.01%	0.03%
<i>Clostridiales</i>	23.15%	3.21%
<i>Erysipelotrichales</i>	0.04%	0.06%
<i>Uncultured Order from the Class Alphaproteobacteria</i>	0.01%	0.03%
<i>Desulfovibrionales</i>	0.18%	0.09%
<i>Campylobacterales</i>	0.42%	0.53%
<i>Enterobacteriales</i>	0.01%	0.02%
<i>Pasteurellales</i>	0.00%	0.00%
<i>Uncultured Order PL-11B10, Phylum Spirochaetes</i>	0.31%	0.62%
<i>Spirochaetales</i>	2.14%	1.18%
<i>Uncultured Order WCHB1-41, Class Verruco-5</i>	0.80%	0.47%
<i>Verrucomicrobiales</i>	1.29%	0.54%
<i>[Cloacamonales]</i>	0.00%	0.00%

Figure 39: Graph showing percentage mean relative abundance at Order level in Second Collection. (Graph plotted in logarithm scale base 10).

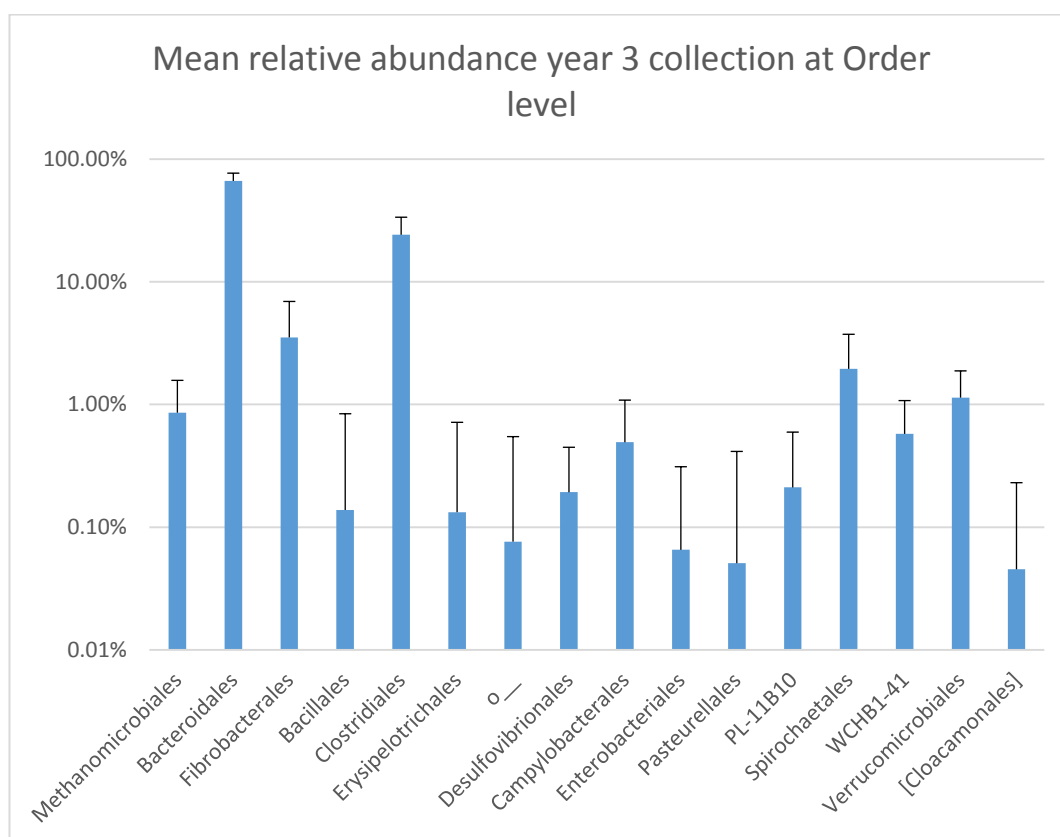


In the Year 3 collection the Order *Methanomicrobiales* had a mean percentage relative abundance of 0.86%. *Bacteroidales* maintain the highest mean percentage relative of 66.35% in the Year 3 collection. The Order *Clostridiales* had a mean percentage relative abundance of 24.16% making it a distant second to *Bacteroidales*. *Fibrobacteriales* had a mean percentage relative abundance of 3.52% with a standard deviation of 3.40%. *Bacillales* recorded a percentage mean relative abundance of 0.14% in the Year 3 collection. *Campylobacteriales* had a mean percentage relative abundance of 0.49% with a standard deviation of 0.59% while the Order *Verrucomicrobiales* had a percentage mean of 1.14% and a standard deviation of 0.74% in the Year 3 collection (Table 38, Figure 40).

Table 38: Percentage mean relative abundance with standard deviation at Order level of Year 3 Collection.

Taxonomy (Order)	Mean relative abundance year 3 collection	Standard deviation year 3 collection.
<i>Methanomicrobiales</i>	0.86%	0.72%
<i>Bacteroidales</i>	66.35%	10.48%
<i>Fibrobacterales</i>	3.52%	3.40%
<i>Bacillales</i>	0.14%	0.70%
<i>Clostridiales</i>	24.16%	9.45%
<i>Erysipelotrichales</i>	0.13%	0.58%
Uncultured Order Class Alphaproteobacteria	0.08%	0.47%
<i>Desulfovibrionales</i>	0.19%	0.26%
<i>Campylobacterales</i>	0.49%	0.59%
<i>Enterobacteriales</i>	0.07%	0.25%
<i>Pasteurellales</i>	0.05%	0.36%
Uncultured Order PL- 11B10, Phylum Spirochaetes	0.21%	0.39%
<i>Spirochaetales</i>	1.95%	1.78%
Uncultured Order WCHB1-41Class Verruco-5	0.58%	0.50%
<i>Verrucomicrobiales</i>	1.14%	0.74%
[<i>Cloacamonales</i>]	0.05%	0.19%

Figure 40: Graph showing percentage mean relative abundance at Order level in Year 3 Collection. (Graph plotted in logarithm scale base 10).



The Order *Methanomicrobiales* had a percentage mean relative abundance of 0.86% in the Year 3, 0.36% in Year 1 collection and 0.39% in the Year 2 collection. The highest percentage mean relative abundance for the Order *Bacteroidales* was seen in the Year 2 collection which stood at 68.75% as compared to 67.94% and 66.35% for Year 1 and Year 3 collections respectively. The Year 1 collection recorded a mean of 25.17% as compared to 23.15% for Year 2 collection and 24.16% in the Year 3 collection for the Order *Clostridiales*. The Year 3 collection recorded 3.52% for the Order *Fibrobacteriales* while in the Year 2 and Year 1 collection it recorded 2.48% and 2.28% respectively (Table 39, Figure 41)

Table 39: Percentage mean relative abundance in all 3 Collections at Order level (Year 1, Year 2 and Year 3 collections).

Taxonomy (Order)	Mean year 1 collection	Mean year 2 collection	Mean year 3 collection
Methanomicrobiales	0.36%	0.39%	0.86%

Bacteroidales	67.94%	68.75%	66.35%
Fibrobacterales	2.28%	2.48%	3.52%
Bacillales	0.02%	0.01%	0.14%
Clostridiales	25.17%	23.15%	24.16%
Erysipelotrichales	0.06%	0.04%	0.13%
Uncultured Order Class Alphaproteobacteria	0.00%	0.01%	0.08%
Desulfovibrionales	0.18%	0.18%	0.19%
Campylobacterales	0.21%	0.42%	0.49%
Enterobacteriales	0.01%	0.01%	0.07%
Pasteurellales	0.00%	0.00%	0.05%
Uncultured Order PL-11B10 Phylum Spirochaetes	0.17%	0.31%	0.21%
Spirochaetales	1.73%	2.14%	1.95%
Uncultured Order WCHB1-41 Class Verruco-5	0.74%	0.80%	0.58%
Verrucomicrobiales	1.13%	1.29%	1.14%
[Cloacamonales]	0.00%	0.00%	0.05%

Figure 41: Graph showing mean percentage relative abundance in all year 1, year 2 and year 3 collections. (Graph plotted in logarithm scale base 10). Data which is ≤ 0.01 is not plotted on this graph due to log10 conversion on the y-axis.

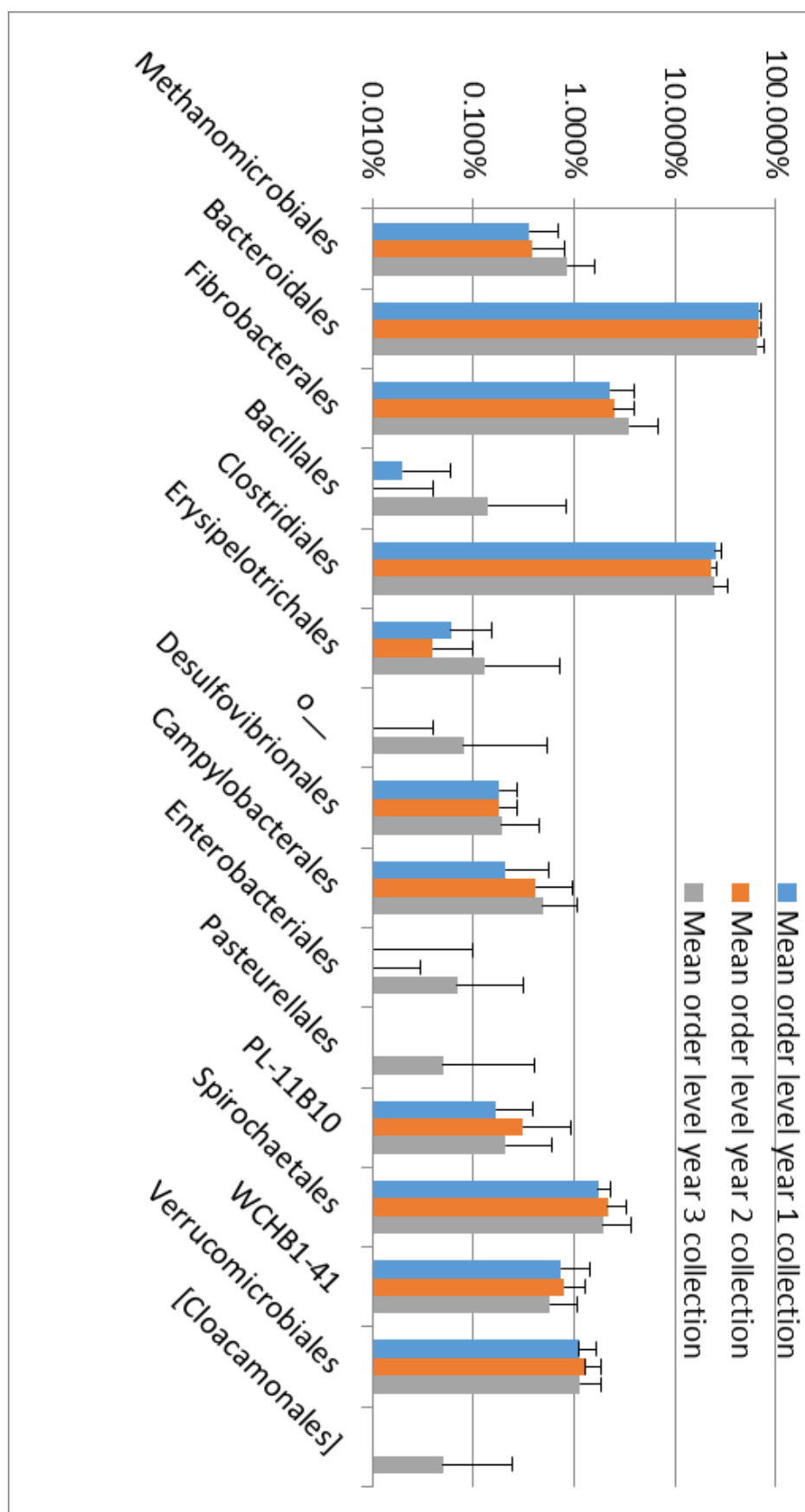


Figure 42: Bar chart of Taxonomy for Yearly collection at Genus level.



In the Year 1 collection an uncultured Genus from the Order *Bacteroidales* was the most dominant with mean of 24.19% and a standard deviation of 4.57%. The second most dominant Genus in the Year 1 collection was another uncultured Genus from the family *Ruminococcaceae* with a mean of 10.02% and a standard deviation of 1.37%. A mean of 0.00% was recorded for 3 Genera identified as *Bibersteinia*, uncultured Genus RFN20 family *Erysipelotrichaceae* and uncultured Genus W5 Family *Cloacamonaceae* (Table 40, Figure 43).

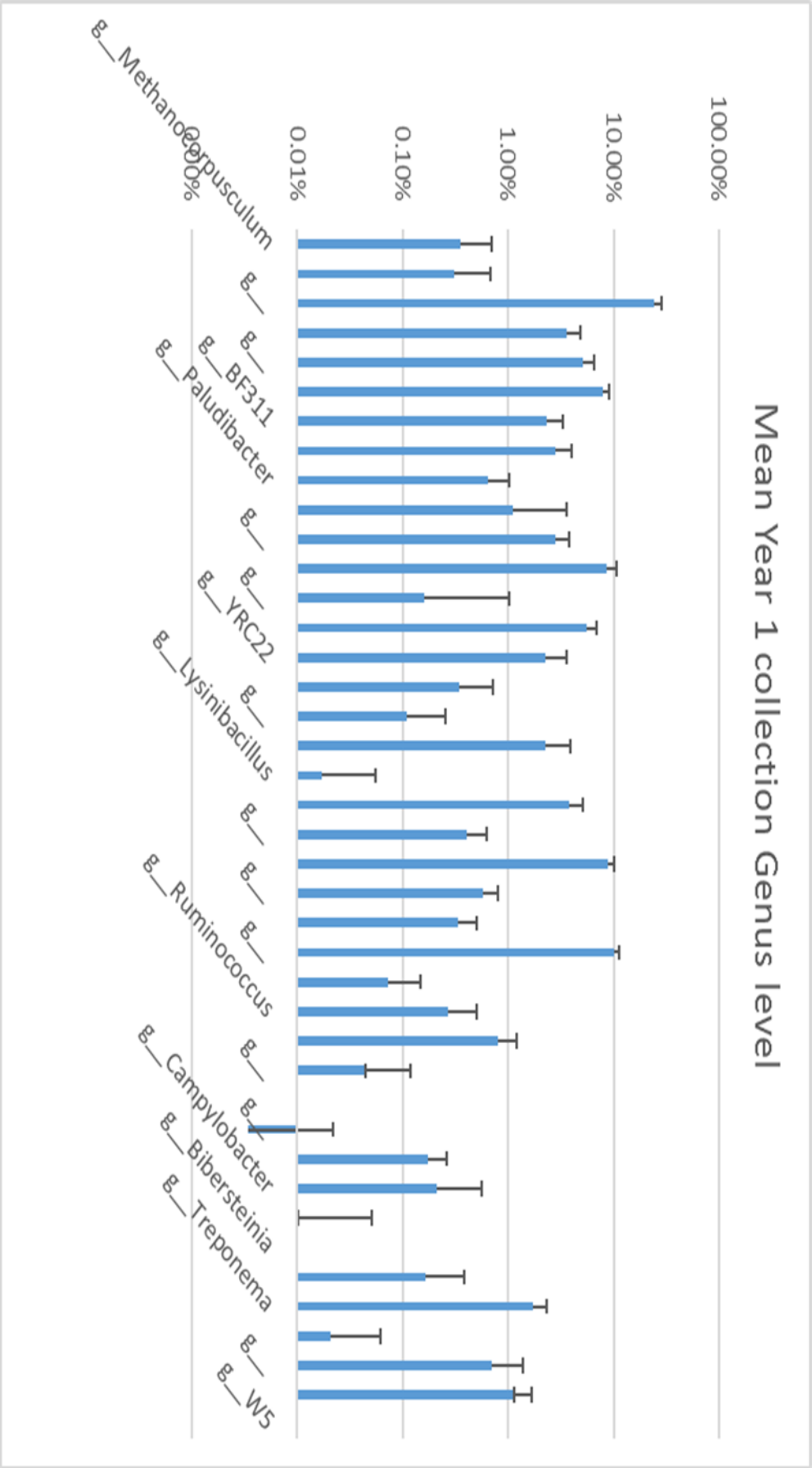
Table 40: Mean percentage relative abundance at Genus level Year 1 collection.

Taxonomy (genus)	Mean Year 1 collection	Standard deviation Year 1 collection
<i>Methanocorpusculum</i>	0.36%	0.34%
<i>Uncultured Genus</i> <i>,Order Bacteroidales</i>	0.31%	0.38%
<i>Uncultured Genus,</i> <i>Order Bacteroidales</i>	24.19%	4.57%
<i>Uncultured Genus,</i> <i>Order Bacteroidales</i>	3.60%	1.26%
<i>Uncultured Genus,</i> <i>Family Bacteroidaceae</i>	5.19%	1.41%
<i>Uncultured Genus 5-</i> <i>7N15, Family</i> <i>Bacteroidaceae</i>	7.96%	1.25%
<i>Uncultured Genus</i> <i>BF311 Family</i> <i>Bacteroidaceae</i>	2.34%	0.94%
<i>Bacteroides</i>	2.80%	1.23%
<i>Paludibacter</i>	0.64%	0.40%
<i>Prevotella</i>	1.13%	2.50%
<i>Uncultured Genus</i> <i>Order Bacteroidales</i>	2.80%	1.00%
<i>Uncultured Genus</i>	8.54%	2.27%

<i>Family Rikenellaceae</i>		
<i>Uncultured Genus</i> <i>Order Bacteroidales</i>	0.16%	0.87%
<i>Uncultured Genus</i> <i>CF231 Family</i> <i>Paraprevotellaceae</i>	5.54%	1.45%
<i>Uncultured Genus</i> <i>YRC22 Family</i> <i>Paraprevotellaceae</i>	2.28%	1.36%
<i>[Prevotella]</i>	0.34%	0.38%
<i>Uncultured Genus</i> <i>Order Bacteroidales</i>	0.11%	0.14%
<i>Fibrobacter</i>	2.28%	1.63%
<i>Lysinibacillus</i>	0.02%	0.04%
<i>Uncultured Genus</i> <i>Order Clostridiales</i>	3.77%	1.37%
<i>Uncultured Genus</i> <i>Family</i> <i>Christendenellaceae</i>	0.41%	0.23%
<i>Clostridium</i>	8.92%	1.19%
<i>Uncultured Genus</i> <i>Family</i> <i>Lachnospiraceae</i>	0.58%	0.22%
<i>Uncultured Genus rc4-4</i> <i>Family</i> <i>Peptococcaceae</i>	0.33%	0.18%
<i>Uncultured Genus</i> <i>Family</i> <i>Ruminococcaceae</i>	10.02%	1.37%
<i>Oscillospira</i>	0.07%	0.08%
<i>g__Ruminococcus</i>	0.27%	0.23%
<i>Phascolarctobacterium</i>	0.79%	0.41%

<i>Uncultured Genus</i> <i>Family</i> <i>Erysipelotrichaceae</i>	0.04%	0.07%
<i>Uncultured Genus</i> <i>RFN20 Family</i> <i>Erysipelotrichaceae</i>	0.00%	0.00%
<i>Uncultured Genus</i> <i>Class</i> <i>Alphaproteobacteria</i>	0.00%	0.02%
<i>Uncultured Genus</i> <i>Family</i> <i>Desulfovibrionaceae</i>	0.18%	0.09%
<i>Campylobacter</i>	0.21%	0.35%
<i>Uncultured Genus</i> <i>Family</i> <i>Enterobacteriaceae</i>	0.01%	0.04%
<i>Bibersteinia</i>	0.00%	0.00%
<i>Uncultured Genus</i> <i>Phylum Spirochaetes.</i>	0.17%	0.22%
<i>Treponema</i>	1.73%	0.60%
<i>Uncultured Genus</i> <i>Class Verruco-5</i>	0.02%	0.04%
<i>Uncultured Genus</i> <i>Class Verruco-5</i>	0.70%	0.70%
<i>Akkermansia</i>	1.13%	0.56%
<i>Unculture Genus W5</i> <i>Family</i> <i>Cloacamonaceae</i>	0.00%	0.00%

Figure 43: Graph representing mean percentage relative abundance in Year 1 collection at Genus level.



The uncultured Genus from the Order *Bacteroidales* was the most dominant in relative abundance with a mean of 28.11% and a standard deviation of 4.41% in the Year 2 collection. The second most dominant in relative abundance in the Year 2 collection was another uncultured Genus from the family *Ruminococcaceae* with a mean of 9.12% and a standard deviation of 1.58%. A mean percentage of 0.00% was recorded for 3 Genera namely *Biberteinia*, an uncultured Genus from the Order *Bacteroidale* and another uncultured Genus W5 from the Family *Cloacamonaceae* (Table 41, Figure 44).

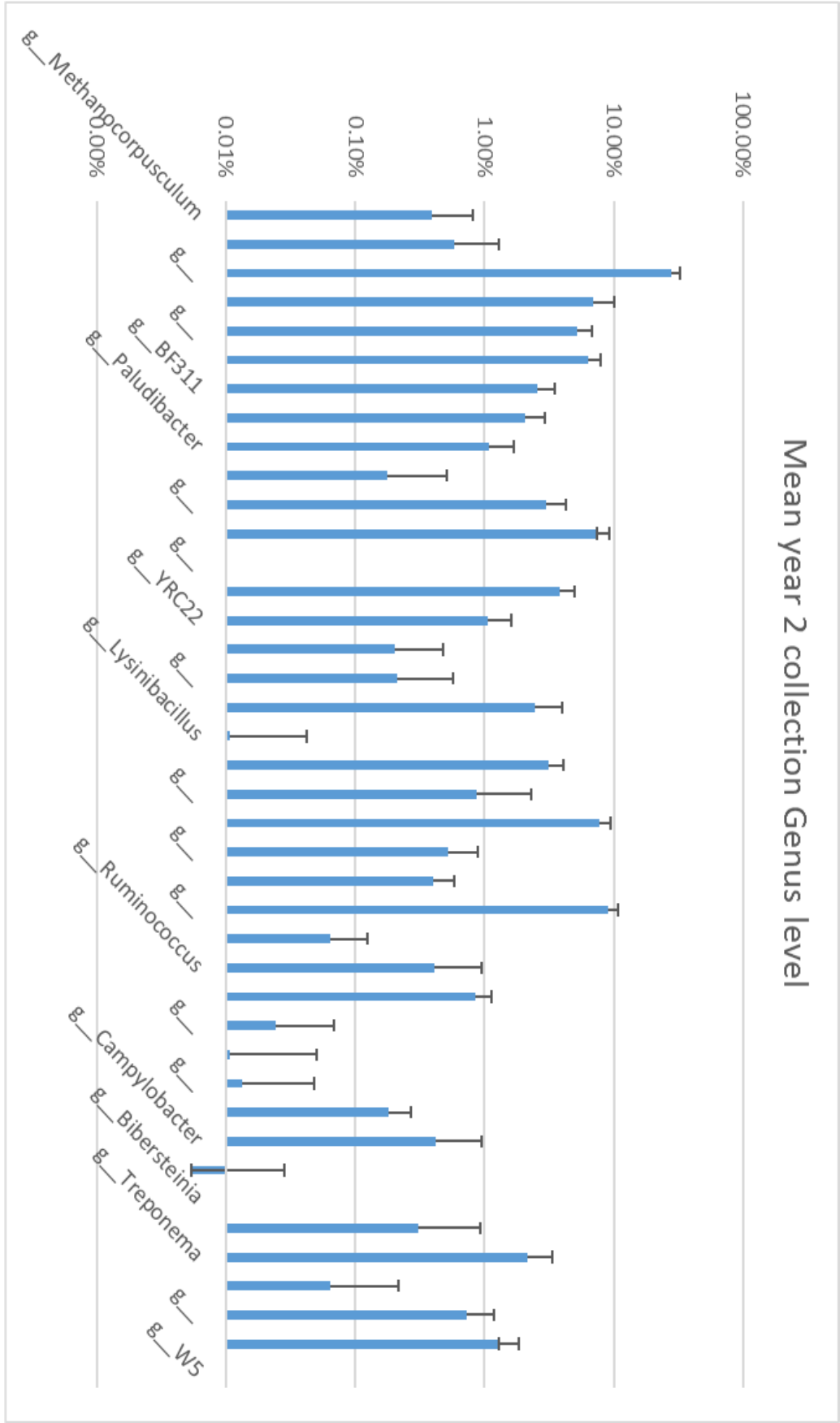
Table 41: Mean percentage relative abundance at Genus level Year 2 collection.

Taxonomy (Genus)	Mean year 2 collection	Standard deviation year 2 collection
<i>Methanocorpusculum</i>	0.39%	0.42%
<i>Uncultured Genus</i> <i>Order Bacteroidales</i>	0.59%	0.71%
<i>Uncultured Genus</i> <i>Order Bacteroidales</i>	28.11%	4.41%
<i>Uncultured Genus</i> <i>Order Bacteroidales</i>	6.91%	3.19%
<i>Uncultured Genus</i> <i>Family Bacteroidaceae</i>	5.19%	1.64%
<i>Uncultured Genus 5-7N15</i> <i>Family Bacteroidaceae</i>	6.31%	1.59%
<i>Uncultured Genus</i> <i>BF311 Family Bacteroidaceae</i>	2.59%	0.92%
<i>Bacteroides</i>	2.07%	0.87%
<i>Paludibacter</i>	1.09%	0.61%
<i>Prevotella</i>	0.18%	0.33%
<i>Uncultured Genus</i> <i>Order Bacteroidales</i>	3.00%	1.32%

<i>Uncultured Genus</i> <i>Family Rikenellaceae</i>	7.43%	1.74%
<i>Uncultured Genus</i> <i>Order Bacteroidales</i>	0.00%	0.00%
<i>Uncultured Genus</i> <i>CF231 Family,</i> <i>Paraprevotellaceae</i>	3.82%	1.13%
<i>Uncultured Genus</i> <i>YRC22 Family,</i> <i>Paraprevotellaceae</i>	1.05%	0.57%
<i>[Prevotella]</i>	0.20%	0.27%
<i>Uncultured Genus,</i> <i>Order Bacteroidales</i>	0.21%	0.36%
<i>Fibrobacter</i>	2.48%	1.55%
<i>Lysinibacillus</i>	0.01%	0.03%
<i>Uncultured, Genus</i> <i>Order Clostridiales</i>	3.12%	1.00%
<i>Uncultured Genus</i> <i>Family</i> <i>Christendenellaceae</i>	0.86%	1.43%
<i>Clostridium</i>	7.78%	1.63%
<i>Uncultured Genus</i> <i>Family</i> <i>Lachnospiraceae</i>	0.52%	0.38%
<i>Uncultured Genus rc4-</i> <i>4 Family</i> <i>Peptococcaceae</i>	0.41%	0.18%
<i>Uncultured Genus</i> <i>Family</i> <i>Ruminococcaceae</i>	9.12%	1.58%
<i>Oscillospira</i>	0.06%	0.06%
<i>g__Ruminococcus</i>	0.41%	0.54%

<i>Phascolarctobacterium</i>	0.85%	0.30%
<i>Uncultured Genus</i> <i>Family</i> <i>Erysipelotrichaceae</i>	0.02%	0.04%
<i>Uncultured Genus</i> <i>RFN20 Family</i> <i>Erysipelotrichaceae</i>	0.01%	0.04%
<i>Uncultured Genus,</i> <i>Class</i> <i>Alphaproteobacteria</i>	0.01%	0.03%
<i>Uncultured Genus,</i> <i>Family</i> <i>Desulfovibrionaceae</i>	0.18%	0.09%
<i>Campylobacter</i>	0.42%	0.53%
<i>Uncultured Genus,</i> <i>Family</i> <i>Enterobacteriaceae</i>	0.01%	0.02%
<i>Bibersteinia</i>	0.00%	0.00%
<i>Uncultured Genus</i> <i>Phylum Spirochaetes.</i>	0.31%	0.62%
<i>Treponema</i>	2.14%	1.18%
<i>Uncultured Genus</i> <i>Class Verruco-5</i>	0.06%	0.15%
<i>Uncultured Genus,</i> <i>Class Verruco-5</i>	0.73%	0.47%
<i>Akkermansia</i>	1.29%	0.54%
<i>Uncultured Genus W5</i> <i>Family</i> <i>Cloacamonaceae</i>	0.00%	0.00%

Figure 44: Graph representing mean percentage relative abundance in Year 2 collection at Genus level.



In the Year 3 collection the uncultured Genus from the Order *Bacteroidales* was the most dominant with mean of 23.23% and a standard deviation of 6.35%. The uncultured Genus from the family *Ruminococcaceae* was a distant second in relative abundance with a mean of 9.30% and a standard deviation of 2.54%. A mean of 0.00% was recorded for an uncultured Genus from the Order *Bacteroidales* in the Year 3 collection (Table 42, Figure 45).

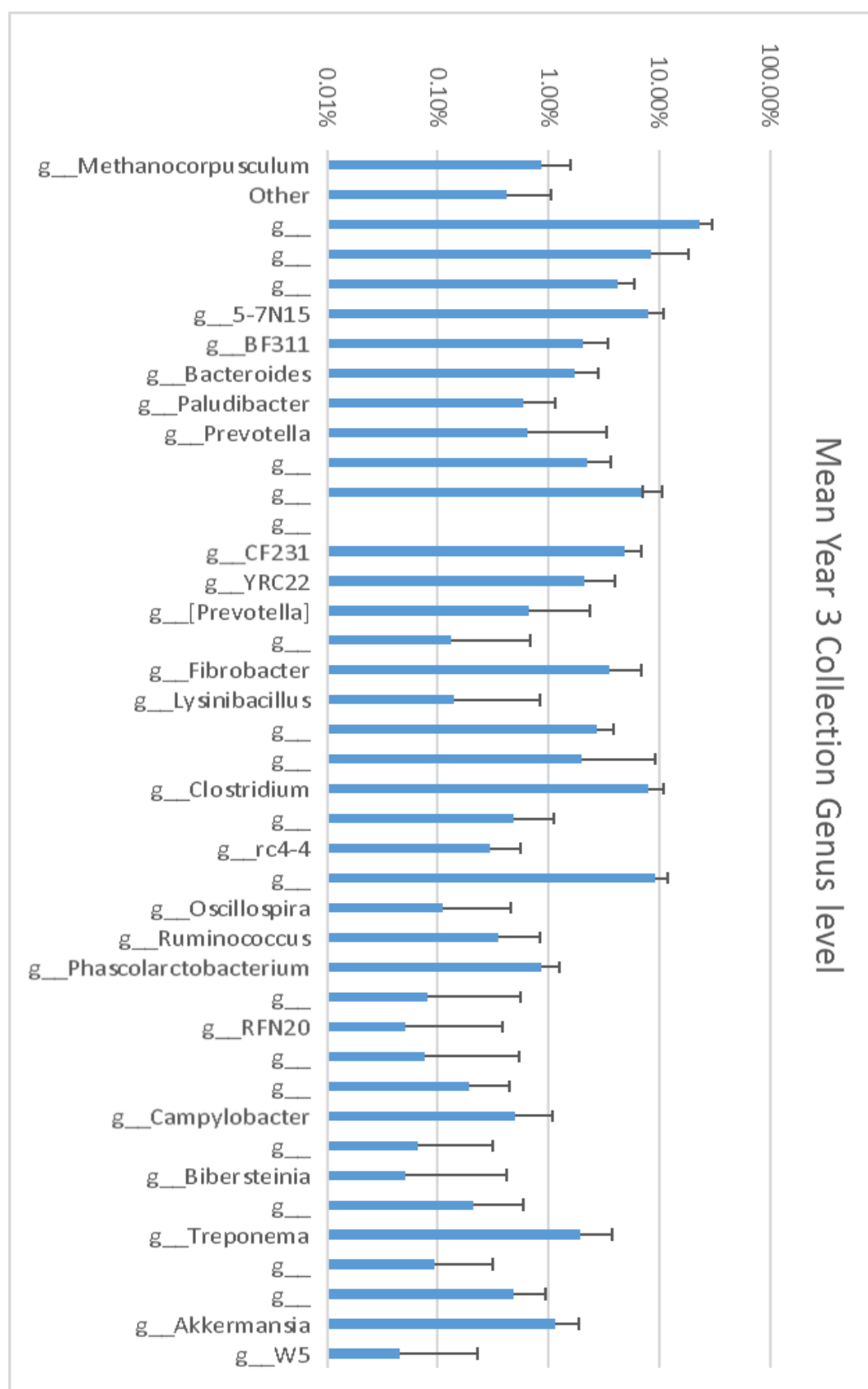
Table 42: Mean percentage relative abundance at Genus level Year 3 collection.

Taxonomy (genus)	Mean Year 3 Collection	Standard deviation Year 3 Collection
<i>Methanocorpusculum</i>	0.86%	0.72%
<i>Uncultured Genus Order Bacteroidales</i>	0.42%	0.63%
<i>Uncultured Genus Order Bacteroidales</i>	23.23%	6.35%
<i>Uncultured Genus Order Bacteroidales</i>	8.41%	10.05%
<i>Uncultured Genus Family Bacteroidaceae</i>	4.23%	1.78%
<i>Uncultured Genus 5-7N15 Family Bacteroidaceae</i>	7.99%	2.90%
<i>Uncultured Genus BF311 Family Bacteroidaceae</i>	2.05%	1.36%
<i>Bacteroides</i>	1.73%	1.07%
<i>Paludibacter</i>	0.59%	0.58%
<i>Prevotella</i>	0.65%	2.65%
<i>Uncultured Genus Order Bacteroidales</i>	2.23%	1.41%
<i>Uncultured Genus Family Rikenellaceae</i>	7.04%	3.50%
<i>Uncultured Genus Order</i>	0.00%	0.00%

<i>Bacteroidales</i>		
<i>Uncultured Genus CF231</i> <i>Family Paraprevotellaceae</i>	4.84%	2.14%
<i>Uncultured Genus YRC22</i> <i>Family Paraprevotellaceae</i>	2.14%	1.86%
<i>[Prevotella]</i>	0.66%	1.68%
<i>Uncultured Genus Order</i> <i>Bacteroidales</i>	0.13%	0.55%
<i>Fibrobacter</i>	3.52%	3.40%
<i>Lysinibacillus</i>	0.14%	0.70%
<i>Uncultured Genus Order</i> <i>Clostridiales</i>	2.76%	1.10%
<i>Uncultured Genus Family</i> <i>Christendenellaceae</i>	2.00%	7.20%
<i>Clostridium</i>	8.01%	2.83%
<i>Uncultured Genus Family</i> <i>Lachnospiraceae</i>	0.48%	0.63%
<i>Uncultured Genus rc4-4</i> <i>Family Peptococcaceae</i>	0.30%	0.26%
<i>Uncultured Genus Family</i> <i>Ruminococcaceae</i>	9.30%	2.54%
<i>Oscillospira</i>	0.11%	0.35%
<i>Ruminococcus</i>	0.35%	0.49%
<i>Phascolarctobacterium</i>	0.85%	0.40%
<i>Uncultured Genus Family</i> <i>Erysipelotrichaceae</i>	0.08%	0.49%
<i>Uncultured Genus RFN20</i> <i>Family Erysipelotrichaceae</i>	0.05%	0.34%
<i>Uncultured Genus Class</i> <i>Alphaproteobacteria</i>	0.08%	0.47%

<i>Uncultured Genus Family Desulfovibrionaceae</i>	0.19%	0.26%
<i>Campylobacter</i>	0.49%	0.59%
<i>Uncultured Genus Family Enterobacteriaceae</i>	0.07%	0.25%
<i>Bibersteinia</i>	0.05%	0.36%
<i>Uncultured Genus Phylum Spirochaetes.</i>	0.21%	0.39%
<i>Treponema</i>	1.95%	1.78%
<i>Uncultured Genus Class Verruco-5</i>	0.09%	0.22%
<i>Uncultured Genus Class Verruco-5</i>	0.48%	0.46%
<i>Akkermansia</i>	1.14%	0.74%
<i>Unculture Genus W5 Family Cloacamonaceae</i>	0.05%	0.19%

Figure 45: Graph representing mean percentage relative abundance in Year 3 collection at Genus level.



The uncultured Genus from the Order *Bacteroidales* had the highest mean percentage relative abundance of 28.11% in the Year 2 collection. Another uncultured Genus from the family *Ruminococcaceae* recorded a mean of 10.02% in the Year 1 collection. The Genus *Bibersteinia* recorded a mean of 0.00% in both Year 1 and Year 2 collection and a mean of 0.05% in the Year 3 collection. Another uncultured Genus W5 from the family *Cloacamonaceae* recorded a mean of 0.00% in both Year 1 and Year 2 collections while recording a mean of 0.05% in the Year 3 collections (Table 43, Figure 46).

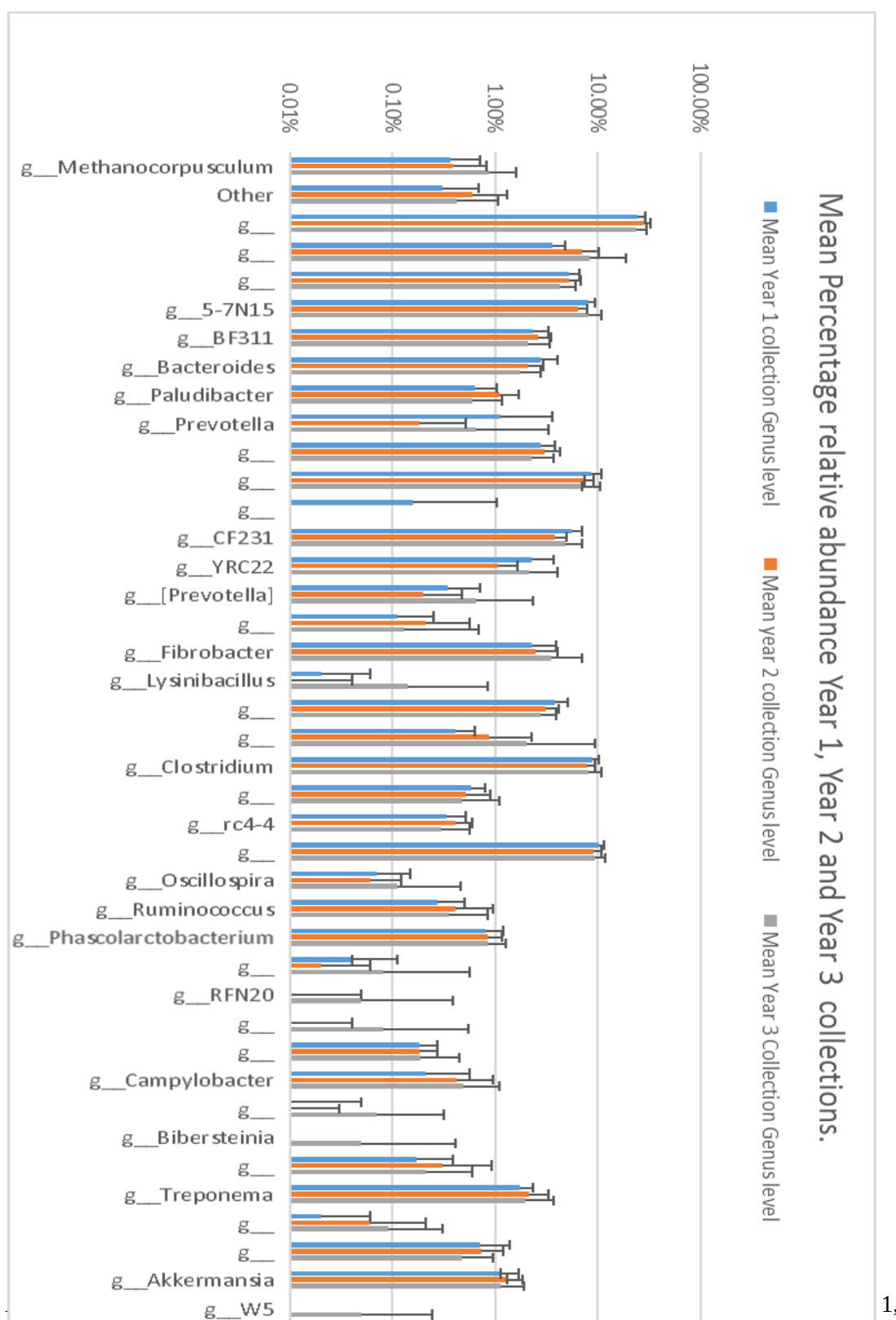
Table 43: Table comparing the mean percentage relative abundance in Year 1, Year 2 and Year 3 collections Genus level of Taxonomy.

Taxonomy (genus)	Mean Year 1	Mean Year 2	Mean Year 3	Standard deviation Year 1	Standard deviation Year 2	Standard deviation Year 3
<i>Methanocorpusculum</i>	0.36%	0.39%	0.86%	0.34%	0.42%	0.72%
<i>Uncultured Genus Order Bacteroidales</i>	0.31%	0.59%	0.42%	0.38%	0.71%	0.63%
<i>Uncultured Genus Order Bacteroidales</i>	24.19%	28.11%	23.23%	4.57%	4.41%	6.35%
<i>Uncultured Genus Order Bacteroidales</i>	3.60%	6.91%	8.41%	1.26%	3.19%	10.05%
<i>Uncultured Genus Family Bacteroidaceae</i>	5.19%	5.19%	4.23%	1.41%	1.64%	1.78%
<i>Uncultured Genus 5-7N15, Family Bacteroidaceae</i>	7.96%	6.31%	7.99%	1.25%	1.59%	2.90%
<i>Uncultured Genus BF311 Family Bacteroidaceae</i>	2.34%	2.59%	2.05%	0.94%	0.92%	1.36%
<i>Bacteroides</i>	2.80%	2.07%	1.73%	1.23%	0.87%	1.07%
<i>Paludibacter</i>	0.64%	1.09%	0.59%	0.40%	0.61%	0.58%
<i>Prevotella</i>	1.13%	0.18%	0.65%	2.50%	0.33%	2.65%
Uncultured Genus, Order Bacteroidales	2.80%	3.00%	2.23%	1.00%	1.32%	1.41%

<i>Uncultured Genus</i> <i>Family Rikenellaceae</i>	8.54%	7.43%	7.04%	2.27%	1.74%	3.50%
<i>Uncultured Genus</i> <i>Order Bacteroidales</i>	0.16%	0.00%	0.00%	0.87%	0.00%	0.00%
<i>Uncultured Genus</i> <i>CF231 Family</i> <i>Paraprevotellaceae</i>	5.54%	3.82%	4.84%	1.45%	1.13%	2.14%
<i>Uncultured Genus</i> <i>YRC22 Family</i> <i>Paraprevotellaceae</i>	2.28%	1.05%	2.14%	1.36%	0.57%	1.86%
<i>[Prevotella]</i>	0.34%	0.20%	0.66%	0.38%	0.27%	1.68%
<i>Uncultured Genus</i> <i>Order Bacteroidales</i>	0.11%	0.21%	0.13%	0.14%	0.36%	0.55%
<i>Fibrobacter</i>	2.28%	2.48%	3.52%	1.63%	1.55%	3.40%
<i>Lysinibacillus</i>	0.02%	0.01%	0.14%	0.04%	0.03%	0.70%
<i>Uncultured Genus</i> <i>Order Clostridiales</i>	3.77%	3.12%	2.76%	1.37%	1.00%	1.10%
<i>Uncultured Genus</i> <i>Family</i> <i>Christendenellaceae</i>	0.41%	0.86%	2.00%	0.23%	1.43%	7.20%
<i>Clostridium</i>	8.92%	7.78%	8.01%	1.19%	1.63%	2.83%
<i>Uncultured Genus</i> <i>Family</i> <i>Lachnospiraceae</i>	0.58%	0.52%	0.48%	0.22%	0.38%	0.63%
<i>Uncultured Genus rc4-4</i> <i>Family</i> <i>Peptococcaceae</i>	0.33%	0.41%	0.30%	0.18%	0.18%	0.26%
<i>Uncultured Genus</i> <i>Family</i> <i>Ruminococcaceae</i>	10.02%	9.12%	9.30%	1.37%	1.58%	2.54%
<i>Oscillospira</i>	0.07%	0.06%	0.11%	0.08%	0.06%	0.35%
<i>Ruminococcus</i>	0.27%	0.41%	0.35%	0.23%	0.54%	0.49%

<i>Phascolarctobacterium</i>	0.79%	0.85%	0.85%	0.41%	0.30%	0.40%
<i>Uncultured Genus</i> <i>Family</i> <i>Erysipelotrichaceae</i>	0.04%	0.02%	0.08%	0.07%	0.04%	0.49%
<i>Uncultured Genus</i> <i>RFN20 Family</i> <i>Erysipelotrichaceae</i>	0.00%	0.01%	0.05%	0.00%	0.04%	0.34%
<i>Uncultured Genus</i> <i>Class</i> <i>Alphaproteobacteria</i>	0.00%	0.01%	0.08%	0.02%	0.03%	0.47%
<i>Uncultured Genus</i> <i>Family</i> <i>Desulfovibrionaceae</i>	0.18%	0.18%	0.19%	0.09%	0.09%	0.26%
<i>Campylobacter</i>	0.21%	0.42%	0.49%	0.35%	0.53%	0.59%
<i>Uncultured Genus</i> <i>Family</i> <i>Enterobacteriaceae</i>	0.01%	0.01%	0.07%	0.04%	0.02%	0.25%
<i>Bibersteinia</i>	0.00%	0.00%	0.05%	0.00%	0.00%	0.36%
<i>Uncultured Genus</i> <i>Phylum Spirochaetes.</i>	0.17%	0.31%	0.21%	0.22%	0.62%	0.39%
<i>Treponema</i>	1.73%	2.14%	1.95%	0.60%	1.18%	1.78%
<i>Uncultured Genus</i> <i>Class Verruco-5</i>	0.02%	0.06%	0.09%	0.04%	0.15%	0.22%
<i>Uncultured Genus</i> <i>Class Verruco-5</i>	0.70%	0.73%	0.48%	0.70%	0.47%	0.46%
<i>Akkermansia</i>	1.13%	1.29%	1.14%	0.56%	0.54%	0.74%
<i>Unculture Genus W5,</i> <i>Family</i> <i>Cloacamonaceae</i>	0.00%	0.00%	0.05%	0.00%	0.00%	0.19%

Figure 46: Graph comparing percentage mean relative abundance at Genus level in Year 1, Year 2 and Year 3 collections.



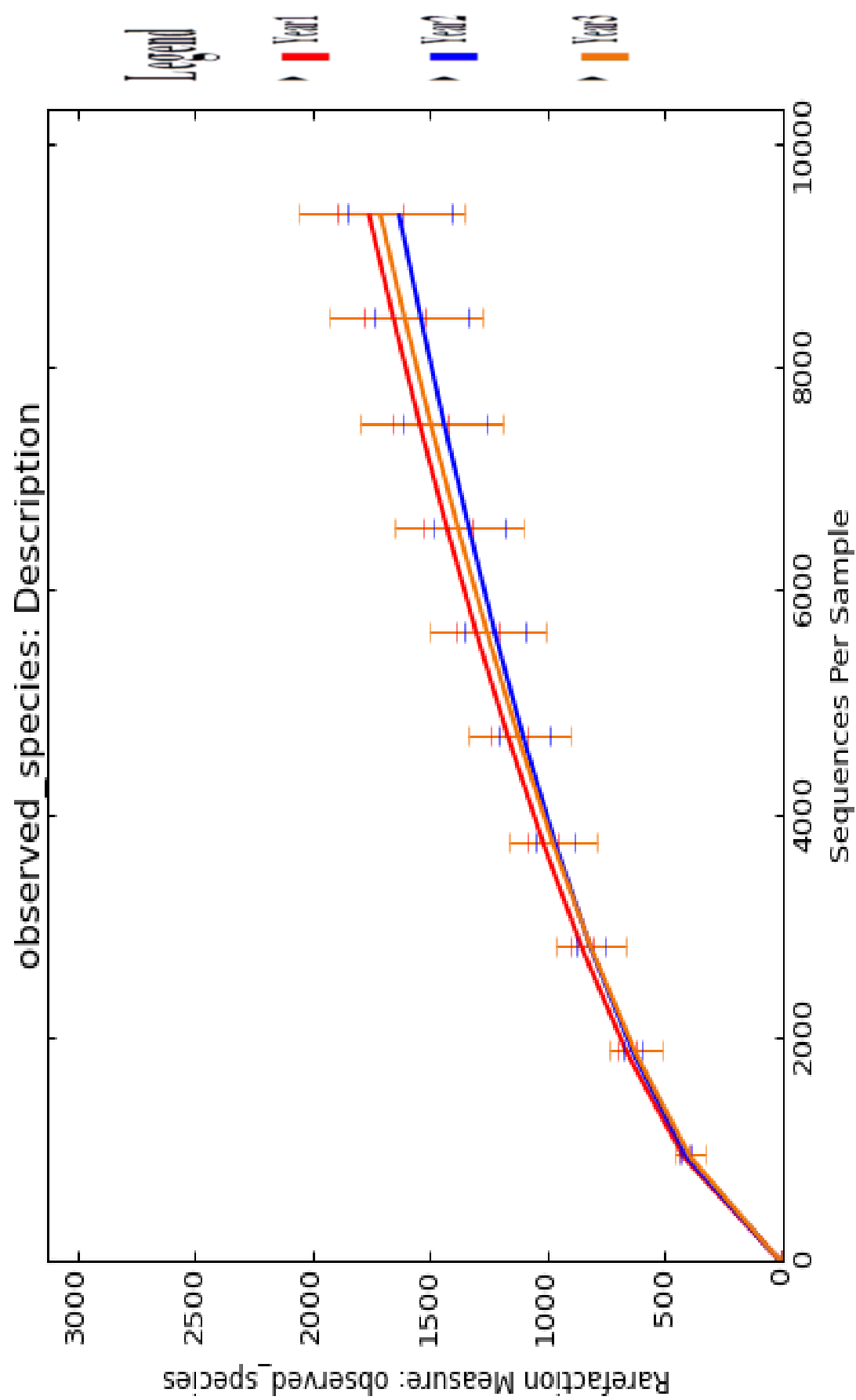
Year 2 and Year 3 collections. Species richness also known as alpha (α) diversity in all the

collection was determined by scaling down the number of sequences in all the samples to a uniform or standardized sequence in all the samples and determining the number of species in standardized samples.

Species richness is determined by the gradient of the rarefaction curve. A steep gradient is indicative of higher species richness or a higher alpha diversity while a lower gradient is indicative of a lower species richness or low alpha diversity. As can be seen in Figure 23, year 1 collection (red curve) had a very slightly higher gradient than both the year 2 collection (blue curve) and year 3 collection (orange curve). What this means is that there are slightly more species in year 1 collection than any of the other 2 collections. Looking closely at the graph it will be observed that though year 1 collection (red curve) is slightly higher in gradient it is very close to year 3 collection (orange curve). This means year 1 collection (red curve) is very slightly higher in alpha diversity but is closely followed by year 3 collection (orange curve).

Year 2 collection (blue curve) has the lowest gradient when compared to Year 1 and Year 3 collections. This means year 2 has slightly lowest alpha diversity. It should also be noted that the gradients of the curves are not widely apart which suggest that the difference in species richness amongst the collections is not markedly different. It can also be noted that the gradient in Year 1 collection (red curve) and year 3 collection (orange curve) is much closer than in Year 1 and Year 2 or in Year 3 and Year 2. This means that alpha diversity in Year 1 is much similar to Year 3 than Year 2 (Figure 47).

Figure 47: Rarefaction curve of year 1, year 2 and year 3 collections.

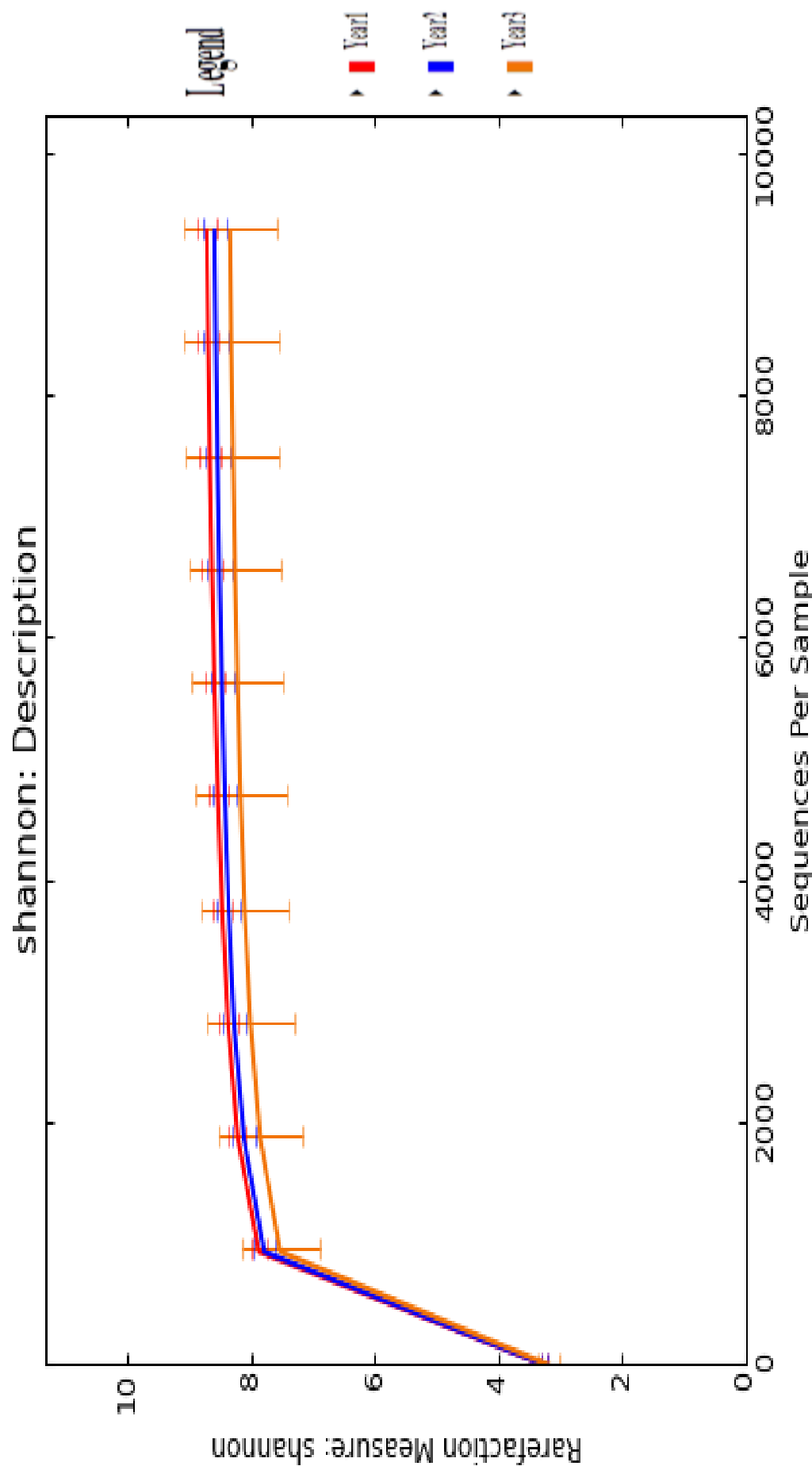


4.8.4 Shannon Diversity – Index

Species richness and the proportion of distribution of each species in the Year 1, Year 2 and Year 3 collection was measured and compared using Shannon diversity index.

Year 1 (red curve) collection has a slightly higher Shannon diversity index than the other 2 collections. This means that Year 1 (red curve) collection has a very slightly higher alpha diversity with a more even distribution of species within the sample than the other 2 collections. The Shannon diversity also shows that year 2 collection has also a narrow higher species richness with species more evenly distributed in the samples than in Year 3 (Figure 48).

Figure 48: Shannon diversity curve for the Year 1, Year 2 and Year 3 Collections.

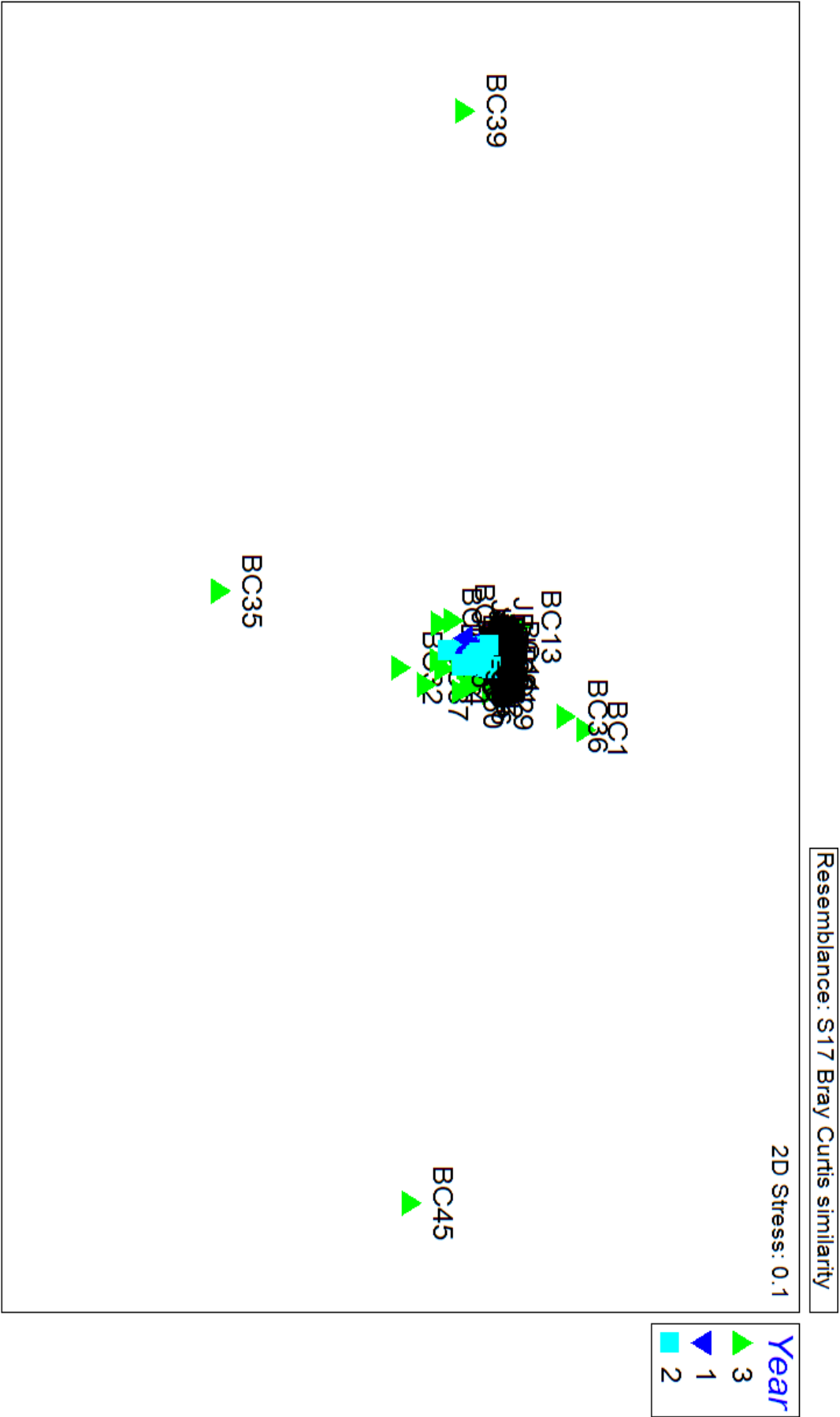


4.8.5 Non – Metric Multidimensional Scaling.

Similarity or resemblance between the samples in all the collection was measured and compared using the Bray – Curtis statistical tool.

Samples were divided into Year 1 samples, Year 2 samples and Year 3 samples. There is a clustering of samples based on resemblance or similarity. Samples that are not similar appear wider apart on the 2 dimensional Bray – Curtis plot. The plot has a stress value of 0.1 revealing the fitness of the values into the 2 dimensional plot (Figure 49a).

Figure 49a: Bray – Curtis MDS plot base on relative abundance of OTUs for Year 1, Year 2 and Year 3 collections.



As can be seen from the MDS plot above (Figure 49a), there is a visual clustering of samples in the Year 1, Year 2 and Year 3 collections. Samples in Year 1 collection (inverted blue triangle), Year 2 collection (light blue square) and Year 3 (green triangle) cluster visually. Samples in the Year 3 collections identified as BC1, BC36, BC35, BC39, BC45 appear as outliers because of their lack of similarity to the other samples in the collections.

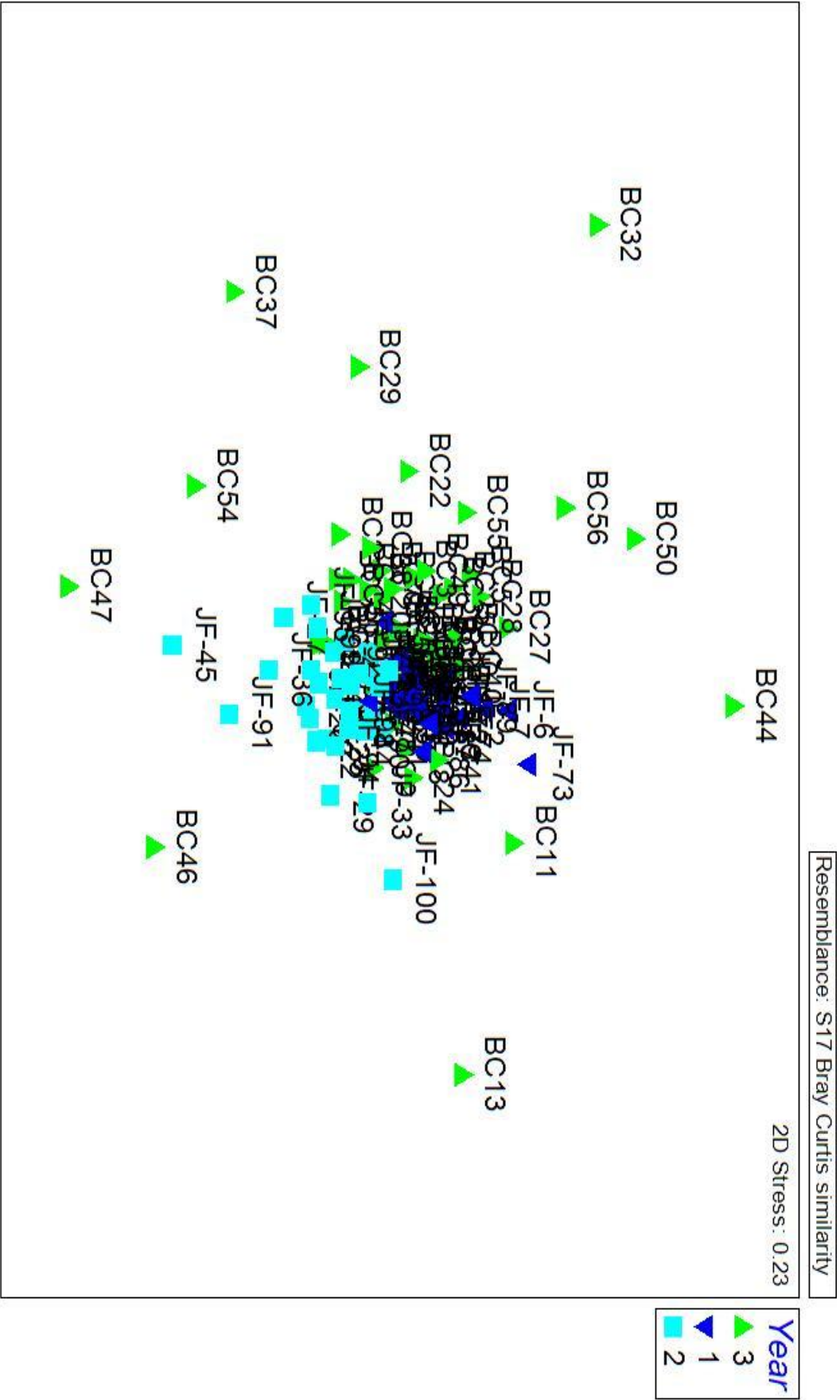
Employing PERMANOVA pair wise test to further analyses the data, a significant difference in the clustering of Year 3 and Year 1 was observed with a P value of 0.001 and a t value of 2.119. PERMANOVA again showed a significant difference between the clustering of Year 3 and Year 2 with a P = 0.001 and t = 2.214. PERMANOVA pair wise test also revealed a significant difference between the clustering of Year 1 and Year 2 collection with a P value of 0.001 and a t value of 2.440.

PERMDISP pair wise test was also performed to further analyse the data. It revealed a significant difference between Year 3 and Year 1 collections with P = 0.001 and t = 4.745. It again showed a significant difference between Year 2 and Year 3 collections with P value of 0.001 and a t value of 4.453. However PERMDISP pair wise test revealed no significant difference between Year 1 and Year 2 collections with P = 0.133 and t = 1.577.

To further analyse the collections another MDS plot was done without the outliers from the Year 3 collections. The MDS plot had a 2D stress of 0.23 (Figure 49b). When the outliers were removed a better appreciation of the visual difference in clustering was seen. With the outliers removed, PERMANOVA pair wise test again revealed a significant difference in clustering between Year 1 and Year 3 collections with a P value of 0.001 and a t value of 2.15. There was a significant difference in the clustering of Year 2 and Year 3 collections with P = 0.001 and t = 2.287. Again PERMANOVA revealed significant difference in clustering of Year 1 and Year 2 collection with P = 0.001 t = 2.440.

PERMDISP pair wise test also revealed significant difference in the clustering of Year 1 and Year 3 collection with P = 0.001 and t = 5.917. There was a significant difference in Year 2 and Year 3 collection with a P value of 0.001 and a t value of 4.743. However PERMDISP also showed no significant difference between Year 1 and Year 2 collections (Figure 49b).

Figure 49b : Bray – Curtis MDS plot base on relative abundance of OTUs for Year 1, Year 2 and Year 3 without outliers.



4.8.6 Statistical View of Outliers at Order level.

From the MDS plot in figure 49a, BC1, BC36, BC35, BC39 and BC45 appear as outliers. All the outliers are located in the Year 3 collection. It should also be noted that BC1 and BC36 are from the same animal identified as 810 and therefore act as biological replicates.

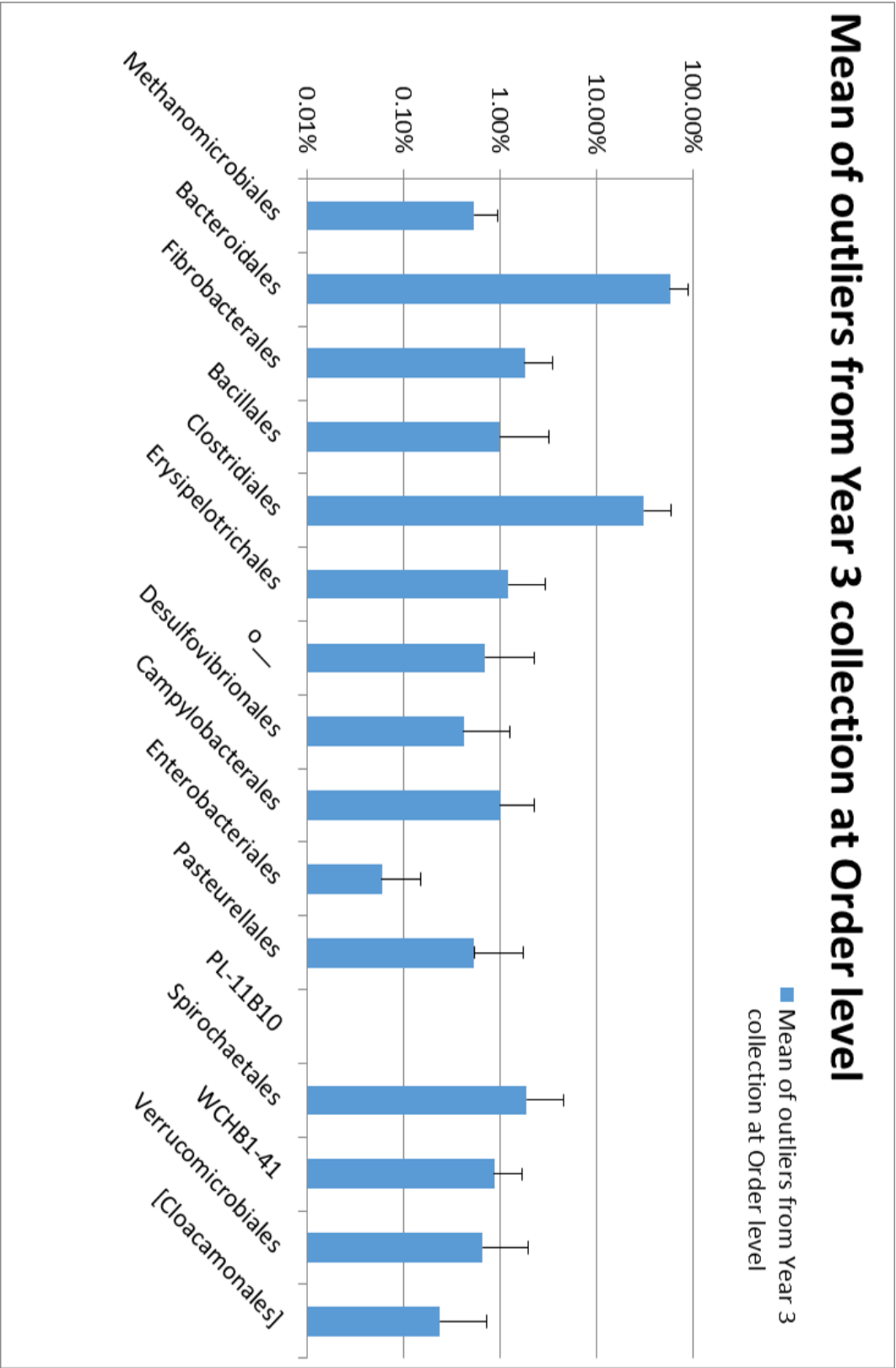
Percentage mean relative abundance for the Order *Methanomicrobiales* in the outliers was 0.54% with a standard deviation of 0.42%. *Bacteroidales* had a mean percentage relative abundance of 58.20% with a standard deviation of 30.26%. *Clostridiales* had a mean of 30.84% and a standard deviation of 29.33%. *Erysipelotrichales* recorded a mean of 1.22% while the Order *Campylobacterales* recorded a percentage mean of 1.02%. *Bacillales* had a mean of 1.00%. *Enterobacteriales* had a mean of 0.06%. *Pasteurellales* recorded a mean of 0.54% while the order *Spirochaetales* had a mean of 1.90% with a standard deviation of 2.62%. *Verrucomicrobiales* had an average of 0.66%. the uncultured Order WCHB1-41 from the Class *Verruco-5* had a mean of 0.88% and a standard deviation of 0.81% (Table 44, Figure 50).

Table 44: Percentage mean relative abundance for the outliers (BC1,BC35,BC36,BC39 and BC45) from the year 3 collection.

Taxonomy	Mean of outliers at Order level	Standard deviation of Outliers
<i>Methanomicrobiales</i>	0.54%	0.42%
<i>Bacteroidales</i>	58.20%	30.26%
<i>Fibrobacterales</i>	1.82%	1.66%
<i>Bacillales</i>	1.00%	2.24%
<i>Clostridiales</i>	30.84%	29.33%
<i>Erysipelotrichales</i>	1.22%	1.72%
Unclassified Order Class <i>Alphaproteobacteria</i>	0.70%	1.57%
<i>Desulfovibrionales</i>	0.42%	0.83%
<i>Campylobacterales</i>	1.02%	1.22%

<i>Enterobacteriales</i>	0.06%	0.09%
<i>Pasteurellales</i>	0.54%	1.21%
<i>Uncultured Order PL-11B10 Phylum Spirochaetes</i>	0.00%	0.00%
<i>Spirochaetales</i>	1.90%	2.62%
<i>Uncultured Order WCHB1-41, Class Verruco-5</i>	0.88%	0.81%
<i>Verrucomicrobiales</i>	0.66%	1.31%
<i>[Cloacamonales]</i>	0.24%	0.48%

Figure 50: Graph representing the average percentage relative abundance in the outliers (BC1, BC35, BC36, BC39, BC45).



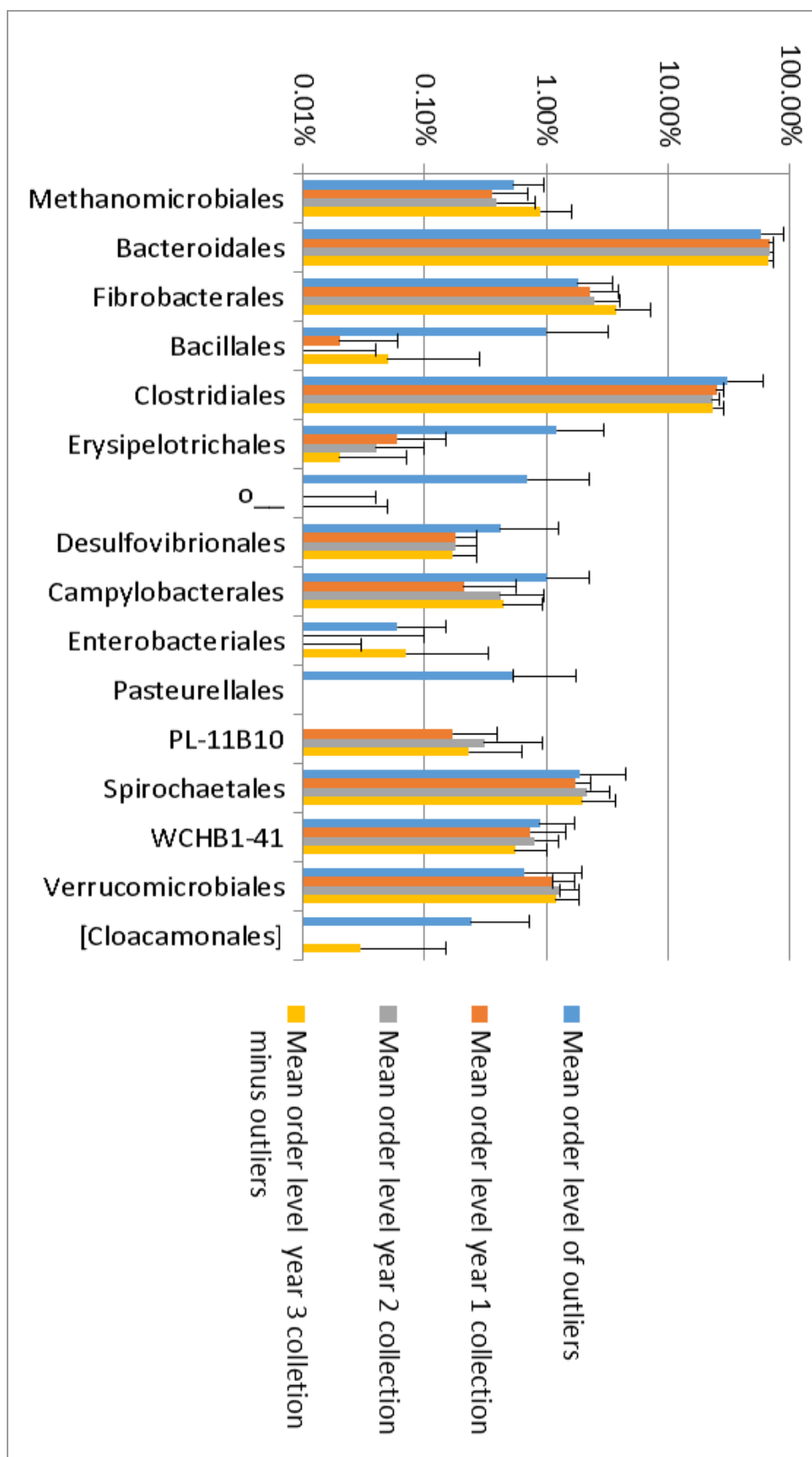
The order *Methanomicrobiales* recorded a mean percentage relative abundance of 0.54% in the outliers and a mean of 0.36% and 0.39% in the Year 1 and Year 2 collections respectively. The Order *Bacillales* recorded a percentage mean relative abundance of 1.00% in the outliers, it however recorded a mean of 0.02% in the Year 1 collection and a mean of 0.01% in the Year 2 collection. *Erysipelotrichales* had a mean of 1.22% in the outliers, 0.06% in the Year 1 collection and 0.04% in the Year 2 collection. The Order *Campylobacterales* recorded a mean of 1.02% relative abundance in the outliers, 0.21% in the Year 1 collection and 0.42% in the Year 2 collection. *Enterobacteriales* had a percentage mean relative abundance of 0.06% in the outliers and a mean of 0.01% in both the Year 1 and Year 2 collection. The Order *Pasteurellales* had a mean percentage relative abundance of 0.54% in the outliers and 0.00% in both the Year 1 and Year 2 collections (Table 45, Figure 51).

Table 45: Percentage mean relative abundance in the outliers, Year 1 collection, Year 2 collection and Year 3 collection minus the outliers at the Order level of Taxonomy.

Taxonomy	Mean of outliers at order level	Mean order year 1 collection	Mean year 2 collection	Mean year 3 collection minus outliers
<i>Methanomicrobiales</i>	0.54%	0.36%	0.39%	0.89%
<i>Bacteroidales</i>	58.20%	67.94%	68.75%	67.17%
<i>Fibrobacterales</i>	1.82%	2.28%	2.48%	3.69%
<i>Bacillales</i>	1.00%	0.02%	0.01%	0.05%
<i>Clostridiales</i>	30.84%	25.17%	23.15%	23.49%
<i>Erysipelotrichales</i>	1.22%	0.06%	0.04%	0.02%
Unclassified Order Class <i>Alphaproteobacteria</i>	0.70%	0.00%	0.01%	0.01%
<i>Desulfovibrionales</i>	0.42%	0.18%	0.18%	0.17%
<i>Campylobacterales</i>	1.02%	0.21%	0.42%	0.44%
<i>Enterobacteriales</i>	0.06%	0.01%	0.01%	0.07%
<i>Pasteurellales</i>	0.54%	0.00%	0.00%	0.00%

<i>Uncultured Order PL-11B10, Phylum Spirochaetes</i>	0.00%	0.17%	0.31%	0.23%
<i>Spirochaetales</i>	1.90%	1.73%	2.14%	1.96%
<i>Uncultured Order WCHB1-41 Class Verruco-5</i>	0.88%	0.74%	0.80%	0.55%
<i>Verrucomicrobiales</i>	0.66%	1.13%	1.29%	1.19%
<i>[Cloacamonales]</i>	0.24%	0.00%	0.00%	0.03%

Figure 51: Graph comparing the percentage mean relative abundance in the outliers (BC1,BC35,BC36,BC39,BC45), year 1 collection , year 2 collection and year 3 collection minus the outliers.



4.8.7 Statistical view of outliers at Genus level.

In the outliers an uncultured Genus from the Order *Bactroidales* was the most dominant with a mean percentage relative abundance of 22.98%. The second most dominant amongst the outliers was another uncultured Genus from the Family *Christensenellaceae* having a mean of 14.72%. A mean percentage relative abundance of 0.00% was recorded for 3 uncultured Genera (Table 46, Figure 52).

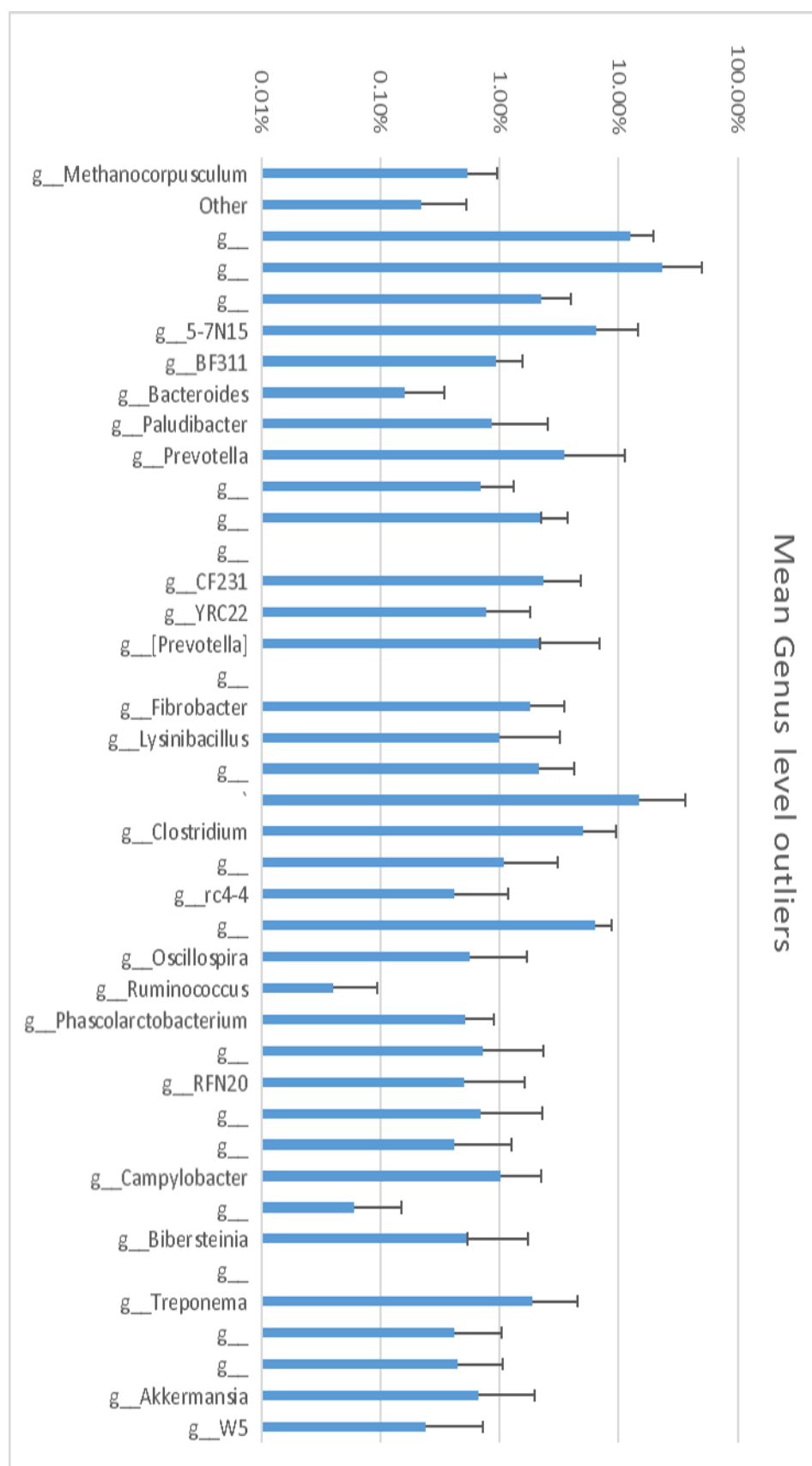
Table 46: Mean percentage relative abundance in outliers.

Taxonomy (genus)	Mean	Standard deviation
<i>Methanocorpusculum</i>	0.54%	0.42%
<i>Uncultured Genus Order Bactroidales</i>	0.22%	0.30%
<i>Uncultured Genus Order Bactroidales</i>	12.64%	6.76%
<i>Uncultured Genus Order Bactroidales</i>	22.98%	26.62%
<i>Uncultured Genus Family Bacteroidaceae</i>	2.26%	1.75%
<i>Uncultured Genus 5-7N15 Family Bacteroidaceae</i>	6.48%	8.01%
<i>Uncultured Genus BF311 Family Bacteroidaceae</i>	0.94%	0.62%
<i>Bacteroides</i>	0.16%	0.18%
<i>Paludibacter</i>	0.86%	1.70%
<i>Prevotella</i>	3.50%	7.83%
<i>Uncultured Genus Order Bactroidales</i>	0.70%	0.62%
<i>Uncultured Genus Family Rikenellaceae</i>	2.24%	1.51%
<i>Uncultured Genus Order Bactroidales</i>	0.00%	0.00%
<i>Uncultured Genus CF231 Family</i>	2.32%	2.45%

<i>Paraprevotellaceae</i>		
<i>Uncultured Genus YRC22</i> <i>Family</i> <i>Paraprevotellaceae</i>	0.78%	1.01%
<i>[Prevotella]</i>	2.18%	4.76%
<i>Uncultured Genus Order</i> <i>Bacteroidales</i>	0.00%	0.00%
<i>Fibrobacter</i>	1.82%	1.66%
<i>Lysinibacillus</i>	1.00%	2.24%
<i>Uncultured Genus Order</i> <i>Clostridiales</i>	2.16%	2.03%
<i>Uncultured Genus Family</i> <i>Christendenellaceae</i>	14.72%	21.73%
<i>Clostridium</i>	4.98%	4.53%
<i>Uncultured Genus Family</i> <i>Lachnospiraceae</i>	1.08%	2.03%
<i>Uncultured Genus rc4-4</i> <i>Family Peptococcaceae</i>	0.42%	0.77%
<i>Uncultured Genus Family</i> <i>Ruminococcaceae</i>	6.30%	2.47%
<i>Oscillospira</i>	0.56%	1.14%
<i>Ruminococcus</i>	0.04%	0.05%
<i>Phascolarctobacterium</i>	0.52%	0.37%
<i>Uncultured Genus Family</i> <i>Erysipelotrichaceae</i>	0.72%	1.61%
<i>Uncultured Genus RFN20</i> <i>Family</i> <i>Erysipelotrichaceae</i>	0.50%	1.12%
<i>Uncultured Genus Class</i> <i>Alphaproteobacteria</i>	0.70%	1.57%
<i>Uncultured Genus Family</i> <i>Desulfovibrionaceae</i>	0.42%	0.83%

<i>Campylobacter</i>	1.02%	1.22%
<i>Uncultured Genus Family Enterobacteriaceae</i>	0.06%	0.09%
<i>Bibersteinia</i>	0.54%	1.21%
<i>Uncultured Genus Phylum Spirochaetes.</i>	0.00%	0.00%
<i>Treponema</i>	1.90%	2.62%
<i>Uncultured Genus Class Verruco-5</i>	0.42%	0.63%
<i>Uncultured Genus Class Verruco-5</i>	0.44%	0.62%
<i>Akkermansia</i>	0.66%	1.31%
<i>Unculture Genus W5 Family Cloacamonaceae</i>	0.24%	0.48%

Figure 52: Graph showing mean percentage relative abundance Genus level in outliers.



The uncultured Genus from the Order *Bacteroidales* recorded its most dominant mean percentage relative abundance of 28.11% in the Year 2 collection. It recorded a mean of 12.64% in the outliers which are originally all members of the year 3 collections. Another uncultured Genus from the Order *Bacteroidales* had a mean percentage relative of 22.98% in the outliers. The Genus *Bibersteinia* had a mean percentage relative abundance of 0.54% in the outliers but recorded a mean of 0.00% in all the other collections. The Genus *Lysinbacillus* had mean of 1.00% in the outliers which exceeds its relative abundance in the other collections. The Genus *Oscillospira* was most dominant in the outliers with mean of 0.56% (Table 47, Figure 53).

Table 47: Table comparing mean percentage relative abundance in Year 1, Year 2, Year 3 minus outliers, and outliers.

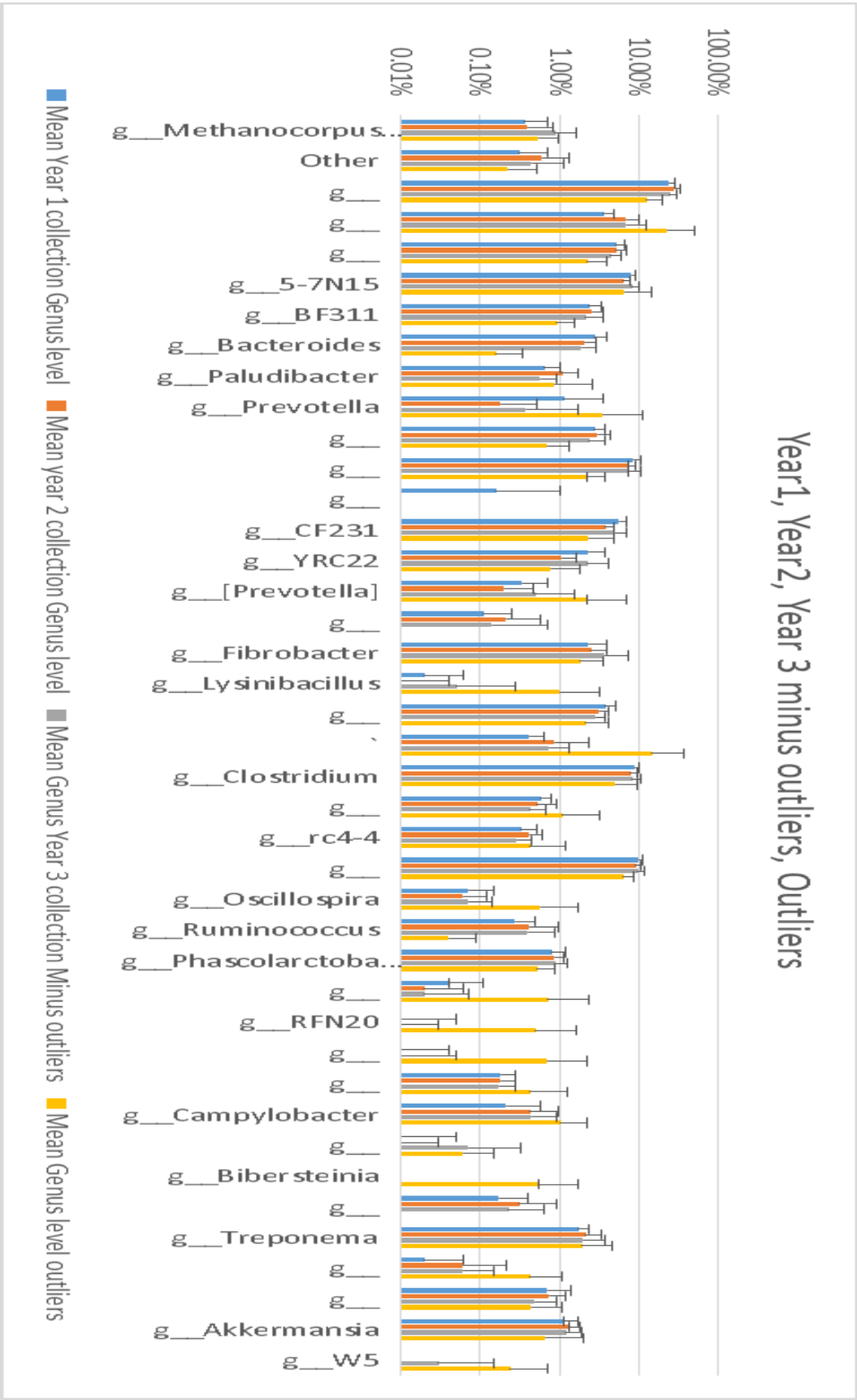
Taxonomy (Genus)	Mean Year 1	Mean year 2	Mean Year 3 without outliers	Mean outliers only	Standard deviation Year 1	Standard deviation year 2	Standard deviation outliers only	Standard deviation year 3 collection without outliers
<i>Methanocorpusculum</i>	0.36%	0.39%	0.89%	0.54%	0.34%	0.42%	0.42%	0.73%
<i>Uncultured Genus Order Bacteroidales</i>	0.31%	0.59%	0.44%	0.22%	0.38%	0.71%	0.30%	0.66%
<i>Uncultured Genus Order Bacteroidales</i>	24.19%	28.11%	24.29%	12.64%	4.57%	4.41%	6.76%	5.30%
<i>Uncultured Genus Order Bacteroidales</i>	3.60%	6.91%	6.95%	22.98%	1.26%	3.19%	26.62%	5.44%
<i>Uncultured Genus Family Bacteroidaceae</i>	5.19%	5.19%	4.43%	2.26%	1.41%	1.64%	1.75%	1.67%

<i>Uncultured Genus 5-7N15</i> Family <i>Bacteroidaceae</i>	7.96 %	6.31 %	8.14 %	6.48 %	1.25%	1.59%	8.01%	1.95%
<i>Uncultured GenusBF311,Bacteroidaeae</i>	2.34 %	2.59 %	2.16 %	0.94 %	0.94%	0.92%	0.62%	1.37%
<i>Bacteroides</i>	2.80 %	2.07 %	1.89 %	0.16 %	1.23%	0.87%	0.18%	0.99%
<i>Paludibacter</i>	0.64 %	1.09 %	0.56 %	0.86 %	0.40%	0.61%	1.70%	0.35%
<i>Prevotella</i>	1.13 %	0.18 %	0.37 %	3.50 %	2.50%	0.33%	7.83%	1.34%
<i>Uncultured Genus</i> Order <i>Bacteroidales</i>	2.80 %	3.00 %	2.39 %	0.70 %	1.00%	1.32%	0.62%	1.37%
<i>Uncultured Genus</i> Family <i>Rikenellaceae</i>	8.54 %	7.43 %	7.52 %	2.24 %	2.27%	1.74%	1.51%	3.28%
<i>Uncultured Genus</i> Order <i>Bacteroidales</i>	0.16 %	0.00 %	0.00 %	0.00 %	0.87%	0.00%	0.00%	0.00%
<i>Uncultured Genus</i> CF231 Family <i>Paraprevotellaceae</i>	5.54 %	3.82 %	5.09 %	2.32 %	1.45%	1.13%	2.45%	1.96%
<i>Uncultured Genus</i> YRC22 Family <i>Paraprevotellaceae</i>	2.28 %	1.05 %	2.27 %	0.78 %	1.36%	0.57%	1.01%	1.88%
<i>[Prevotella]</i>	0.34 %	0.20 %	0.50 %	2.18 %	0.38%	0.27%	4.76%	1.01%
<i>Uncultured Genus</i> Order <i>Bacteroidales</i>	0.11 %	0.21 %	0.14 %	0.00 %	0.14%	0.36%	0.00%	0.58%
<i>Fibrobacter</i>	2.28 %	2.48 %	3.69 %	1.82 %	1.63%	1.55%	1.66%	3.49%
<i>Lysinibacillus</i>	0.02 %	0.01 %	0.05 %	1.00 %	0.04%	0.03%	2.24%	0.23%
<i>Uncultured Genus</i> Order <i>Clostridiales</i>	3.77 %	3.12 %	2.82 %	2.16 %	1.37%	1.00%	2.03%	0.98%

<i>Uncultured Genus Family,Christendene llace</i>	0.41 %	0.86 %	0.73 %	14.72 %	0.23%	1.43%	21.73 %	0.62%
<i>Clostridium</i>	8.92 %	7.78 %	8.31 %	4.98 %	1.19%	1.63%	4.53%	2.48%
<i>Uncultured Genus Family Lachnospiraceae</i>	0.58 %	0.52 %	0.42 %	1.08 %	0.22%	0.38%	2.03%	0.26%
<i>Uncultured Genus rc4-4 Family Peptococcaceae</i>	0.33 %	0.41 %	0.29 %	0.42 %	0.18%	0.18%	0.77%	0.16%
<i>Uncultured Genus Family Ruminococcaceae</i>	10.02 %	9.12 %	9.60 %	6.30 %	1.37%	1.58%	2.47%	2.36%
<i>Oscillospira</i>	0.07 %	0.06 %	0.07 %	0.56 %	0.08%	0.06%	1.14%	0.07%
<i>Ruminococcus</i>	0.27 %	0.41 %	0.39 %	0.04 %	0.23%	0.54%	0.05%	0.50%
<i>Phascolarctobacteu m</i>	0.79 %	0.85 %	0.89 %	0.52 %	0.41%	0.30%	0.37%	0.39%
<i>Uncultured Genus Family Erysipelotrichaceae</i>	0.04 %	0.02 %	0.02 %	0.72 %	0.07%	0.04%	1.61%	0.05%
<i>Uncultured Genus RFN20 Family Erysipelotrichaceae</i>	0.00 %	0.01 %	0.01 %	0.50 %	0.00%	0.04%	1.12%	0.02%
<i>Uncultured Genus Class Alphaproteobacteria</i>	0.00 %	0.01 %	0.01 %	0.70 %	0.02%	0.03%	1.57%	0.04%
<i>Uncultured Genus Family Desulfovibrionaceae</i>	0.18 %	0.18 %	0.17 %	0.42 %	0.09%	0.09%	0.83%	0.10%
<i>Campylobacter</i>	0.21 %	0.42 %	0.44 %	1.02 %	0.35%	0.53%	1.22%	0.49%
<i>Uncultured Genus Family</i>	0.01	0.01	0.07	0.06	0.04%	0.02%	0.09%	0.26%

<i>Enterobacteriaceae</i>	%	%	%	%				
<i>Bibersteinia</i>	0.00 %	0.00 %	0.00 %	0.54 %	0.00%	0.00%	1.21%	0.01%
<i>Uncultured Genus</i> <i>Phylum</i> <i>Spirochaetes.</i>	0.17 %	0.31 %	0.23 %	0.00 %	0.22%	0.62%	0.00%	0.40%
<i>Treponema</i>	1.73	2.14	1.96	1.90	0.60%	1.18%	2.62%	1.72%
<i>Uncultured Genus</i> <i>Class Verruco-5</i>	0.02 %	0.06 %	0.06 %	0.42 %	0.04%	0.15%	0.63%	0.09%
<i>Uncultured Genus</i> <i>Class Verruco-5</i>	0.70 %	0.73 %	0.49 %	0.44 %	0.70%	0.47%	0.62%	0.45%
<i>Akkermansia</i>	1.13 %	1.29 %	1.19 %	0.66 %	0.56%	0.54%	1.31%	0.66%
<i>Unculture Genus W5</i> <i>Family</i> <i>Cloacamonaceae</i>	0.00 %	0.00 %	0.03 %	0.24 %	0.00%	0.00%	0.48%	0.12%

Figure 53: Graph comparing mean percentage relative abundance in Year1, Year2, Year 3 without outliers, and outliers.



4.9 MDS plot for Study 1 and Study 2.

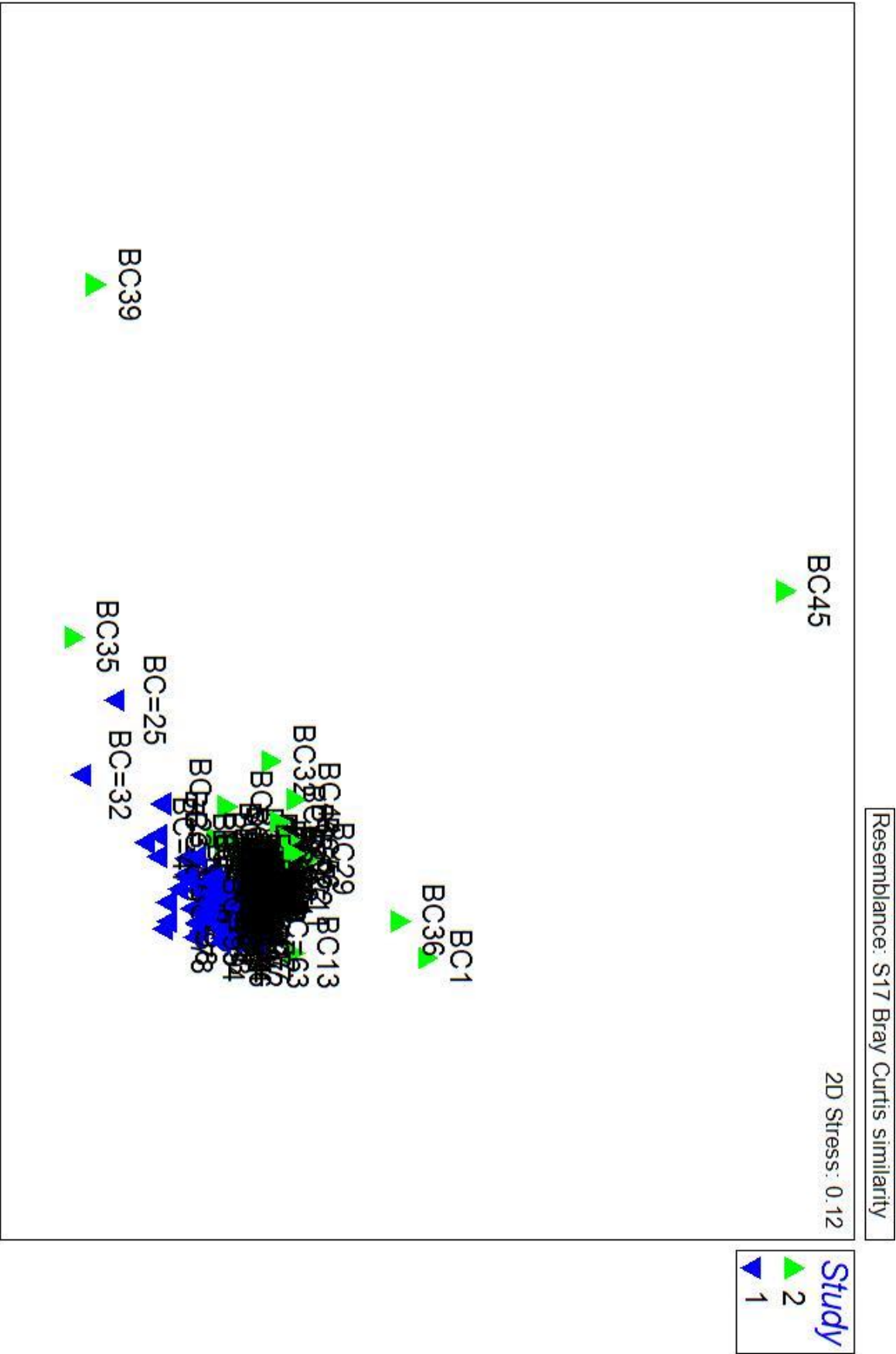
In an attempt to have an overview of the 2 studies namely:

Study 1: Helminth only infected sheep study.

Study 2: MAP and helminth infected sheep study using only the Year 3 collection.

An MDS plot was carried out to give a broader picture on the gastrointestinal microbiome in the 2 studies.

Figure 54: MDS plot of Study 1 (Helminth only infected sheep) and Study 2 (MAP and helminths infected sheep).



PERMANOVA pair – wise test showed a significant difference in the clustering between Study 1 and Study 2 with a P value of 0.001 and a t value of 3.685. PERMANOVA main test also revealed a significant difference in study 1 and study 2 with a P value of 0.001 and a Pseudo F value of 13.581. PERMDISP pair wise comparison did not show significant difference with a P value of 0.758 and t = 0.319.

5. DISCUSSION

In the first part of this study, the gastrointestinal microbiomes of different sheep groups were analysed to understand the effect of helminthiasis on the gut microbiome of sheep that are not infected with *Mycobacterium avium* subspecies *paratuberculosis* (MAP). Illumina MiSeq platform was used to carry out analysis of the V4 region of the 16S rRNA gene. Quantitative Insight Into Microbial Ecology (QIIME) was used to further investigate the raw sequences data obtained from Edinburgh Genomics for Operational Taxonomic Unit (OTU) picking, Taxonomic assignment, alpha diversity analysis and beta diversity analysis. Non-metric multidimensional plots were used to determine relationships between Operational taxonomy units based on similarity. PRIMER and PERMANOVA statistical tools were employed to investigate further the level significant difference between samples.

In the second part of the study, the gastrointestinal microbiome of sheep infected with MAP and helminths were examined and analysed. Illumina MiSeq sequencing was used to examine the V4 region of the prokaryotic 16S rRNA gene. QIIME was used to investigate the raw data obtained from Edinburgh genomics. MDS plots using Bray-Curtis similarity curve were plotted to determine the relationship between OTUs based on resemblance. Statistically analysis were carried out by the PRIMER and PERMANOVA tools.

The two studies were then compared to analyse whether there was a difference in the microbiomes of sheep dually infected with both JD and gastrointestinal roundworms and those only infected with the roundworms. MDS plots were plotted and statistical tools PERMANOVA and PERMDISP were used to analyse significance in difference between the 2 studies.

5.1 Gastrointestinal Microbiome

The main objective of this study is to discover the effect of intestinal infections on the gastrointestinal microbiome of sheep focussing on helminthiasis and Johne's disease of sheep. In Helminth infected group, which is the first part of the study, 38 pre-treatment amplicons and 37 post-treated amplicons were examined by analysing the V4 region of the 16S rRNA gene using Illumina MiSeq platform.

In the MAP infected group which is the second part of the study the V4 region of the 16S rRNA of 29 amplicons from the Year 1 collection, 40 amplicons from Year 2 collection

and 56 amplicons from Year 3 collections were analysed by the use of Illumina MiSeq platform.

5.2 Helminth infected group

Sheep in this group were positive for helminthiasis. The V4 region of the 16SrRNA of 38 pre-treatments and 37 post treated amplicons were analysed by Illumina MiSeq platform. QIIME pipeline was used to analyse the raw data obtained. In all the samples in this group (Pre-treatment plus post-treated) QIIME revealed the Phylum *Bacteroidetes* as the most dominant followed by *Firmicutes* (Henderson et al., 2015). Other Phyla observed amongst the 8 top in the helminth infected group include *Fibrobacteres*, *Proteobacteria*, *Spirochaetes*, *Tenericutes* and *Verrucomicrobia*.

The Phylum *Bacteroidetes* was slightly higher in the post-treated samples (59.26%) than the pre-treatment samples (58.26%), *Fibrobacteres* was also slightly higher in the post-treated (5.25%) than the pre-treatment samples (4.65%). *Firmicutes* was slightly higher in the pre-treatment samples than the post-treated samples while *Tenericutes* was higher in post-treated samples than pre-treatment sample. These marginal variations of change in relative abundance at the Phylum level might suggest that the administration of anthelmintic does not drastically change the microbiome in any particular pattern at the Phylum level.

At the Order level of taxonomy, the Order *Bacteroidales* was slightly higher in the post-treated samples (59.26%) than the pre-treatment samples (58.26%). The same pattern was also seen in the *Fibrobacterales* with post-treated samples recording a mean of 5.25% and pre-treatment samples having a mean of 4.65%. The Order *Clostridiales* was only marginally higher in pre-treatment (29.05%) than in post-treated samples (27.99%). The uncultured *Deltaproteobacterium* GMD14H09 was more dominant in the pre-treatment samples (0.24%) than the post-treated samples (0.08%). The opposite is true for another uncultured Order RF39 which was less dominant in the pre-treatment samples (0.04%) than the post-treated samples (0.12%). Again These variations in percentage relative abundance do not show an obvious pattern in the effect of the anthelmintic on the gut microbiome at the Order level of Taxonomy which might suggest that the anthelmintic drug does not show any effect on the gut microbiome at the Order level of Taxonomy.

At the Genus level the uncultured Genus from the Order *Bacteroidales* was marginally higher in the post treated samples (23.73%) than the pre-treatment (20.85%). In both pre-

treatment and post-treated samples another uncultured Genus from the Family *Ruminococcaceae* was next in relative abundance with its mean slightly higher in post-treated (9.86%) than pre-treatment samples (9.60%). The uncultured Genus from the uncultured Order RF39 from the Class *Mollicutes* was the least dominant in the pre-treatment samples (0.04%) while the least dominant in the post-treated sample was another uncultured Genus from an uncultured Order GMD14H09 from the Class *Mollicutes* (0.08%). Most of the Genera show only slight differences between the pre-treatment and post treated samples in no particular pattern, therefore it can be suggested that even at the Genus level there was no clear distinction between the relative abundance in the pre-treatment and post-treated samples which might suggest that the anthelmintic used is not causing any change in the gut microbiome.

In The post-treated groups (1, 2 and 3) an uncultured Genus from the Order *Bacteroidales* was most dominant followed by another uncultured Genus from the Family *Ruminococcaceae* which was closely followed by the Genus *Clostridium* in all the 3 groups except in group 2 where the Genus *Fibrobacter* (7.21%) was slightly higher than the Genus *Clostridium* (6.21%).

5.2.1 Pre- treatment and Post – treated groups based on anthelmintic.

The similarity between pre-treatment samples and the different groups of the post treated samples were measured by Bray – Curtis similarity plots. PERMDISP pairwise comparison showed no significant difference observed in the clustering of pre – treatment samples and group 2 (Startect®) with $P = 0.265$ and $t = 1.489$. It also showed no significant difference in the pre – treatment and group 3 (Zolvix®), + Startect®) with P value of 0.193 t value of 1.692. PERMDISP revealed a marginal significant difference in the clustering of pre – treatment and group 1 (Zolvix®) with a $P = 0.022$ and $t = 2.621$. There was no significance difference in the clustering of group 2 (Startect®) and group 3 (Zolvix® + Startect®) with $P = 0.689$ and $t = 0.440$. There was also no significance difference in the clustering of group 1 (Zolvix®) and group 2 (Startect®) with a P value of 0.287 and a t value of 1.225. PERMDISP pairwise comparison also showed no significant difference in the clustering of the group 1 (Zolvix®) and group 3 (Zolvix®), + Startec®) with $P = 0.436$ $t = 0.917$. This suggest that the overall PERMDISP clustering of the different groups from the centroid

when the different groups were paired was not significantly different with $P = 0.062$ and an F value of 3.583.

PERMANOVA pair wise test showed there was no significant difference between the clustering of the pre-treatment samples and the clustering of group 2 (Startect®) with $P = 0.166$ and a t value of 1.0841. PERMANOVA revealed a marginal significant difference between the clustering of pre-treatment samples and the group 3 (Zolvix®) + Startect® with $P = 0.052$ and t value of 1.159. It also showed no significant difference when the pre-treatment samples were paired with group 1 (Zolvix®) with $P = 0.06$ and $t = 1.156$. When PERMANOVA paired group 2 (Startect®) and group 3 (Zolvix® + Startect®), it also revealed no significant difference in the clustering with p value of 0.206 and t value of 1.0535. There was also no significant difference in the clustering of group 1 (Zolvix®) and group 2 (Startect®) with $P = 0.161$ and $t = 1.059$. PERMANOVA revealed a significant difference in clustering of group 3 (Zolvix® + Startect®) and group 1 (Zolvix) with a P value of 0.01 and t value of 1.169.

It can be observed from the PERMANOVA pair wise test that a significant difference is seen when the when group 3 (Zolvix® + Startect®) is paired with group 1 (Zolvix®) with P value of 0.01 and also when group 3 (Zolvix® + Startect®) is paired with the pre-treatment with P value of 0.052. this might be as a result of the combined effect of the 2 anthelmintic they related with microbiome. But again this can be mere speculation because the same pattern of difference was not seen when group 3 (Zolvix® + Startect®) is paired with group 2 (Startect®) with P value of 0.206.

Comparing the pre – treatment samples with the different post treatment groups at the Order level revealed that the Order *Bacteroidales* was higher in group 3 (63.13%) than group 1 (57.25%) and group 2 (58.56%) and pre-treatment (58.26%). The Order *Fibrobacterales* was lowest in group 3 (2.28%) and highest in group 2 (7.12%), and recorded a mean of 5.73% and a mean of 4.65% in groups 1 and pre-treatment samples respectively. An uncultured Order GMD14H09 was slightly lower in group 3 (0.06%) than other groups. The same also applies for another uncultured Genus RF39 with a mean of 0.01% in group 3.

Some marginal variations have been observed between the group 3 samples and the other groups, but it is still inconclusive to say that the changes in the microbiome were due to the combination of the 2 anthelmintic drug Zolvix® and Startect®.

At the Genus level, the uncultured Genus from the Order *Bacteroidales* was slightly higher in group 3 (26.07%) than in the pre-treatment (20.85%), group 1 (22.86%), group 2 (22.87%). The Genus *Fibrobacter* had the least dominance in group 3 (2.28%) and most dominance in group 2 (7.12%). The Genus *Ruminococcus* had the least dominance also in group 3 (0.08%) with highest dominance of 0.35% in group 1. An uncultured Genus from the Class *Mollicutes* also recorded the least dominance in group 3 (0.01%) than in pre – treatment (0.04%), group 1 (0.25%) and group 2 (0.01%).

Another uncultured Genus from the Order *Bacteroidales* was also least dominant in group 3 (0.03%) than in all the other groups.

At the Genus level, some degree of variations was observed in all the groups. Some Genera were more abundant in one group and less abundant in another. This does not show any particular pattern so it will be inconclusive to say whether it was the effect of the anthelmintic or not.

A trend that was also observed in the MDS plot is that the outliers appear predominantly in the pre-treatment group which seems to go back into the centre after treatment amongst the post treated group, this is just a trend that might suggest an alteration of the gut microbiome of the outliers after treatment making them more similar to the others.

5.2.2 Pre – treatment outliers compared to group 1, group2 and group 3.

The pre – treatment outliers observed in the MDS plots were analysed on the basis of relative abundance at the Genus level of Taxonomy. The Genus *Methanocorpusculum* was most dominant in the outliers (2.07%) than in group 1 (0.63%), group 2 (1.42%) and group 3 (1.15%). An unknown Genus from the Order *Bacteroidales* was also least dominant in the outliers (15.67%) than the group 1 (22.86%), group 2 (22.87) and group 3 (26.07%). An uncultured Genus from the Order *Bacteroidales* had a mean of 10.43% in the outliers which was the highest as compared to group 1 (4.53%), group 2 (6.16%) and group 3 (6.46%). Another uncultured Genus 5-7N15 from the Family *Bacteroidaceae* recorded the highest mean of 11.13% in the outliers. The Genus *Prevotella* had a mean of 0.00% only in the outliers. Interestingly, the outliers had the highest mean of 5.79% for the Genus

Campylobacter which recorded a mean of 1.17% in group 1, 1.57% in group 2 and 1.22% in group 3.

These variations seen in the relative abundance of the Outliers observed at the Genus level could be attributed to the fact that sheep (gimmers) were sourced from different locations living under different conditions, exposed to different types of diseases and parasites, fed different kinds of diet, administered different kinds of medication (Carding et al., 2015). The period of quarantine might also not have been adequate for the gut microbiome to adjust as to reflect some degree of uniformity with all the other gimmers.

5.3 MAP and round worm dual infected group (Year 1 collection, Year 2 and Year 3 collection).

Sheep in this group had dual infection with MAP and helminths. Rectal faecal samples were collected annually for a period of 3 years identified as Year 1 collection, Year 2 collection and Year 3 collections. Year 1 collection had 29 samples that were extracted, PCR generated by Jelena Nikolić (previous student), in 2014. Year 2 collection had 40 samples that were extracted and PCR generated by Miriam Navarro (previous student). Year 3 collection had 56 samples that were extracted and their PCR generated during this project. All the amplicons in all the collections were sequenced using the Illumina MiSeq platform. QIIME pipeline was used to analyse the raw data obtained from Edinburgh Genomics. QIIME revealed the Phylum *Bacteroidetes* as the most dominant (67.5%) followed by the Phylum *Firmicutes* (24.3%). Other Phyla include *Euryarchaeota*, *Fibrobacteres*, *Proteobacteria*, *Spirochaetes*, *Verrucomicrobia* and an uncultured Phylum WWE1.

PERMANOVA pair-wise test revealed a significant difference in clustering between the Year 3 collection and the Year 1 collection with a $P = 0.001$ and $t = 2.119$. There was also a significant difference in clustering observed by PERMANOVA between the Year 3 collection and the Year 2 collection with a P value of 0.001 t value of 2.214. Again PERMANOVA showed a significant level of difference between the Year 1 collection and the Year 2 collection with $P = 0.001$ and $t = 2.440$. These significant differences observed in the different pair-wise PERMANOVA might be attributed to the changes in the gut microbiome as result of the effects or clinical signs associated with MAP infection and or helminths infection which might alter the gut microbiome. PERMANOVA also revealed that average similarity within Year 1 collection was higher (65.14) than the average similarity within Year 2 (63.276) and within Year 3 (51.488). These might suggest that as the disease condition

progresses there was the likelihood of variations occurring in the gut microbiome. PERMDISP pair wise comparisons revealed a significant difference in the clustering between Year 1 and Year 3 collections with a P value of 0.001 and a t value of 4.745. There was also a significant difference in the clustering between Year 2 and Year 3 collection $P = 0.001$ and $t = 4.453$. PERMDISP however did not show a significant difference between Year 1 and Year 2 collection with P value of 0.133 and t value of 1.577. Again it can be argued here that the progression of the disease might have played a role in detecting the degree of significant difference in the clustering between the collections.

At the Order level of taxonomy, the Order *Methanomicrobiales* had the highest mean percentage relative abundance in Year 3 which stood at 0.86% as compared to Year 1 (0.36%) and Year 2 (0.39%). Also another Order *Bacillales* had the highest mean in Year 3 collection (0.14%) as compared to Year 1 (0.02%) and Year 2 (0.01%). The Order *Erysipelotrichales* again recorded the highest mean in Year 3 at 0.13% when compared to Year 1 (0.06%) and Year 2 (0.04%). *Enterobacteriales* was higher in Year 3 (0.08%) than Years 1 and 2 were both (0.01%). The Order *Pasteurella* was only present in Year 3 with a mean of 0.05%. The Order *Cloacamonales* was only present in Year 3 with a mean of 0.05% as well. At the Order level of taxonomy, it can be seen that Year 3 collection recorded higher mean of relative abundance in quite a number of Orders than what was observed in Year 1 and Year 2. Also Year 3 collection had some Orders (*Pasteurella*, *Cloacamonales* and an uncultured Order from the Class *Alphaproteobacteria*) which were not present in Year 1 and Year 2 collections. Year 3 collection had varied from both Year 1 and Year 2 at the Order level of Taxonomy which might be due to disease conditions or other factors affecting the gut microbiome such as undiagnosed diseases and parasitic infections, diet, toxins (Carding et al., 2015).

At the Genus level of Taxonomy *Methanocorpusculum* had its highest mean in the Year 3 collection at 0.86% when compared to 0.36% in Year 1 and 0.39% in Year 2. Another Genus *Lysinibacillus* also had the highest mean in Year 3 (0.14%) than Year 1 (0.02%) with its lowest recorded in Year 2 (0.01%). The Genus *Bibersteinia* was only seen in the Year 3 collection with a mean of 0.05%. An unknown Genus W5 from the Family *Cloacamonaceae* was also only observed in the Year 3 collection at a mean of 0.05%. A lot of uncultured bacteria at the Genus level were observed making it difficult to say whether variations at this stage can

be clearly seen, but from the Genera observed the Year 3 collection might be showing some degree of variations from Year 1 and Year 2 collections.

5.3.1 Year 3 collection outliers compared to Year 1 and Year 2.

Year 3 outliers observed in the MDS plots were examined based on average relative abundance of the Genera found in them. An uncultured Genus from the Order *Bacteroidales* recorded a mean of 22.98% in the outliers which was about seven times that recorded in the Year 1 collection (3.60%) and three times what was recorded in Year 2 collection (6.91%). The Genus *Bacteroides* had a mean of 0.16% in the outliers, which was lower than what was recorded in the Year 1 (2.80%) and Year 2 (2.07%). The Genus *Prevotella* was highest in the outliers with a mean of 3.50%. An uncultured Genus from the Family *Rikenellaceae* recorded a mean of 2.24% in the outliers which was lower than what was recorded in Year 1 (8.54%), Year 2 (7.43%). The Genus *Lysinibacillus* had a mean of 1.00% in the outliers, 0.02% in Year 1 and 0.01% in the Year 2 collection. Another uncultured Genus from the Family *Christensenellaceae* recorded a mean of 14.72% in the outliers which exceeded what it recorded in the Year 1 (0.41%) and Year 2 (0.86%) collections. The Genus *Campylobacter* had a mean of 1.02% in the outliers, it had a mean of 0.21% and 0.42% in the Year 1 and Year 2 collections respectively. Another variation worthy of note in the outliers is that the Genus *Bibersteinia* and another uncultured Genus W5 from the Family *Cloacamonaceae* were only present in the outliers and not in the Year 1 or Year 2 collections at a mean of 0.54% for *Bibersteinia* and 0.24% for the uncultured Genus W5 respectively.

One of the outliers tested positive by nested real time PCR of IS900 gene (result kindly provided by Dr Celia Leao). Another outlier tested positive for serum antibody. Two of the outliers tested negative to serum antibody test.

It can be suggested that as Johne's disease progresses in sheep, it can cause changes in the gut microbiome with the gut microbiome becoming more different in sick animals when compared to healthy ones. More work is required in order to substantiate that the changes in the gut microbiome are due specifically to Johne's disease and not the consequence of other diseases or conditions within the flock. Although the sheep sampled were from a closed flock, limiting the numbers of sheep being introduced to the farm, it is possible that new diseases could have been introduced by new animals being introduced or co-grazing with other flocks or cattle or wildlife. A vaccination study is currently underway, to

vaccinate sheep against Johne's disease, to compare the vaccinated sheep with those sheep that were not vaccinated within the same flock so as to reduce the spread of the disease and to further determine whether the microbiome of the vaccinated sheep differ from the microbiome of the unvaccinated sheep.

5.4 Overall comparison of study 1 and study 2.

The gastrointestinal microbiome of sheep in study 1 and the gastrointestinal microbiome of sheep in study 2 were compared by plotting an MDS curve. PERMANOVA pair wise test revealed a significant difference between study 1 and study 2 with a P value of 0.001 and t value of 3.685. This difference in the gut microbiome might be as a result of diet, location, drugs, toxins and pathogens that the sheep in the 2 different studies were exposed to (Carding et al., 2015). On the other hand, PERMDISP showed no significant difference between the clustering of Study 1 samples and Study 2 samples with a P value of 0.758 and a t value of 0.319. This might suggest that there is more happening in the sheep which might be as result of exposure to pathogens, diet or other factors in the wider environment that is causing some form of resemblance in the gut microbiome.

6. Conclusion.

In study 1 the effect of helminth infection and anthelmintic treatment on the gastrointestinal microbiome was examined and analysed. The pre-treatment samples on comparison with the 3 groups of post treated samples showed no significant difference in the gastrointestinal microbiome with $P > 0.05$. This study revealed that there are variations in relative abundance at different level of taxonomy observed in the pre-treatment and the different groups of the post-treated samples. It is still immature to say that this differences were as a result of the effect of the anthelmintic used. A more precise experimental design involving animals of the same age and breed, raised from the same environmental conditions and fed the same kind of diet should be organised and carried out so as to obtain more reliable results and outcomes.

In study 2 involving the dually MAP and helminth infected sheep it was discovered that variations in the gastrointestinal microbiome occurred as disease progress in sheep with Year 3 sheep showing significant difference from the Year 1 and Year 2 sheep with $P = 0.001$. This might suggest that as Johne's disease progress in sheep some degree of alterations also occur on the gastrointestinal microbiome. In order to understand the specific interaction of the gastrointestinal microbiome with MAP, more work will be required to discover biomarkers in the microbiome population that supports and potentiate MAP infection.

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Appendix A

Parasite Egg Counts from faeces and Blood Serum Antibody Results

Samples collected 20th Jan 2016

Sheep Tag No.	Results	
	Fecal Egg Count (FEC) Strongyloids/Nematoduris ¹	Serum Antibody Johne's Test ²
00043	42	
00053	42	
00057	15	
00061	30	
00067	57	
00073	18	negative
00081	*	
00084	12	
00094	*	
00130	6	
01377	87	
001418	9	
01422	612	negative
001433	9	
001438	2	
001446	30	
001476	27	
01477	0	negative
001503	57	
001523	18	
00154	2	
001542	27	
00160	57	negative
00176	255	
00219	15	
03136	3	positive
03685	6	
00793	48	positive
0800	27	
00810	9	negative
00818	54	positive
00823	0	

00830	9	
00832	6	positive
00833	0	negative
00834	18	
00835	2	
00848	12	positive
00849	48	
00892	54	
Sheep Tag No.	Results	
	Faecal Egg Count (FEC) Strongyloids /Nematoduris ¹	Serum Antibody Johne's Test ²
00894	0	
00910	45	negative
00912	27	
00923	15	
00934	192	
00937	42	positive
00948	180	
00959	*	
00960	2	
001453	*	
001509	75	
001619	42	positive
02401	39	positive
03069	9 + Strongyloides	positive
03699	27 + Capillaria	
002294	6	negative
02363	279/6	positive
Notes:		

1) Highlighted FEC results are unusually high.

2) Blood samples only taken from selected sheep

Test results provided by Craig Watkins/Dave Bartley at the Moredun Research Institute

19 February 2016