Original Research Article

Phylogenetic Biodiversity of Bacteria Community in the gut of Diarrhoaeric Patients in Rivers State, Nigeria.

ABSTRACT

Background: The microbial ecosystem in the human intestine is complex and it plays a great role in health and nutrition. Cultural techniques have been used over years to study the gut microbiota but studies suggest that a greater percentage of these bacteria found in the gut cannot be cultivated using the conventional methods of bacteria isolation.

Aim: To increase understanding in this area, we characterized the bacterial diversity (both cultivated and non cultivated bacteria) in the gut of diarrhoic individuals using 16S rRNA gene (rDNA) sequences.

Methodology: PCR amplification, sequencing and phylogenetic analysis of the 16S ribosomal DNA (rDNA) sequences was done on 10 diarrhoiec stool samples.

Results: After quality filtering and chimeric sequence removal, 72313 sequences from all 10 diarrhoeic stool samples subjected to clustering generated 2767 OTUs of which 2073 were new and unassigned. Representative sequences of the bacteria Operational Taxonomic Unit (OTU) cluster were used to construct a bacteria phylogenetic tree which revealed a wide variety of bacteria *Firmicutes, Bacteroidetes*, *Proteobaacteria, Actinobacteria, Tenericutes* and *Cyanobacteria* and others that could not be detected using the cultural techniques. The evolutionary relationship of the most abundant organisms and their contributions from each sample revealed the phylum *Firmicutes* to be most abundant and therefore have contributed most in the samples followed by *Bacteroidetes*. Fewer contributions were made by the other phyla *Proteobaacteria, Actinobacteria, Tenericutes* and *Cyanobacteria.*

Conclusion: This study was able to identify culturable and unculturable bacteria in the gut of diarrhoeic people in Rivers state and also show the biodiversity and inter relatedness of these microorganisms using molecular methods. Therefore we can say that 16S rRNA techniques for detection and identification of predominant bacteria create new opportunities for noncultivation studies of the human intestinal microflora which will also help in proper diagnosis of infectious diseases and new methods of treatments of diseases

Key words: Phylogenetic tree, Evolutionary relatedness, sequences, gut, operational taxonomic units.

1. INTRODUCTION

The gut is amongst the most important organs of the body and is key in maintaining health and causing diseases [1]. It has been estimated that about 70% of the total microorganisms in the colon are bacteria with about 200 prevalent species and 1000 uncommon species [1, 2]. There are up to 1014 total bacteria in the human intestinal

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tract, which is 10 to 20 times the total number of tissue cells in the entire body [3]. The composition and activity of this flora have a profound influence on health and disease through their involvement in the nutrition, pathogenesis, and immune function of the host [4, 5]. Gut microbiota varies amongst individuals but is greatly controlled by the birth environment and whether an infant is breast or bottle fed. During gestation, humans are naturally sterile but at delivery and birth, their body surfaces become inhabited by different forms of microorganisms. These micoorganisms consists of members of mainly two phyla *Firmicutes* and *Bacteriodetes* with *Actinobacterium* as the next abundant phylum which is mainly comprised of the genus *Bifidobacterium* [6]. Studies by Moles *et al*., [7] revealed that meconium the earliest stool of a mammalian infant contains mainly *Firmucutes* while *Proteobacteria* were abundant in faeces. Other microorganisms that were identified are *Staphylococcus, Enterococcus, Escherichia coli, klebsiella, Serratia and Lactobacilli.* After birth, the intestine becomes colonized by different microorganisms. The mode of delivery of the baby, diet, hygiene, antibiotic treatment and gestational age are major factors that influence the colonisation of these micro organisms [8]. The first colonizers make the environment conducive for new colonizers such as *Bacteroides, Clostridium, and Bifidobacterium species.* The composition of microorganisms in the Gut of neonates is known to be low in diversity and relative abundance of the phyla *Proteobacteria* and *Actinobacteria* and increases in diversity with the emergence of *Firmicutes* and *Bacteriodetes* [6]. Mountzouris *et al*., [9] says that intestinal flora stabilises at 4 weeks after birth until the introduction of solid food. Its composition is relatively simple in infants and increases in complexity as the age increases till adulthood. By the end of the 2 – 3 years of life, infants already have a microbial profile that resembles that of an adult in terms of the type of microorganisms present and how evenly distributed they are [10]. Diarrhoea occurs when the intestine absorbs or secrets fluid more fluid than normal [11]. Most diarrhoea are self limiting mild infections that can be resolved on its own but some can be acute, severe and life threatening. Though some diarrhoea are caused by chemical irritations, metabolism and organic disturbances, a vast majority is by infectious pathogens like virus, fungi, parasite or bacteria [12] with bacteria diarrhoea being more common in developing countries. The commonly associated enteric pathogens include bacteria made up of *Escherichia coli, Salmonella species, Shigella species, Camphylobacter jejuni, Vibrio species, Yersinia species, Aeromonas species, Clostridium difficile., parasites like Cyclospora, Gardia lamblia, Entamoeba histolitica, Cryptosporidium spp., viruses like Rotavirus, Calici virus* and other enteric viruses with Rotavirus as leading cause in young children [13, 14]. All over the world, there are about 1.7 billion diarrhoea cases every year and major reason for malnutrition in children under 5 years [15].

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The most used method for assessing microbial diversity is the culture based method. It has been based on selective and differential plating of samples on culture media and identifying the pure culture of the bacteria to the species level. A major challenge in studying the gut microbiota is the inability of culturing most of the gut micro organisms [16]. Even with these intensive investigations, however, there is much concern that culture based methods does not provide a complete picture of the diversity of the predominant organisms of the gut flora. In fact , molecular genetic tools have indicated that 60 to 80% of the organisms in the total human microflora have not been cultivated [17]. The use of 16S rRNA gene sequences has been by far the most common housekeeping genetic marker used to study bacterial phylogeny and taxonomy [18]. The phylogenetic analysis of bacterial 16S rRNA genes (rDNAs), amplified directly from complex communities, have provided an efficient strategy for exploring the biodiversity of a particular biota. In order to derive a detailed phylogenetic biodiversity of bacteria community in the gut of diarrhoeic patients, we analyzed bacterial 16S rRNAs extracted from 10 diarrrhoeic fecal samples. This method has facilitated access to both cultivated and non cultivated microorganisms. Sequences generated were clustered into OTU's which were used to construct a phylogenetic tree to reveal the wide variety of bacteria and their contribution in the individual samples.

2. MATERIALS AND METHODS

Conventional method of cultivation of microorganisms

An aliquot of each of the 10 faecal samples were inoculated into different media plates (Mac Conkey agar, Salmonella Shigella agar, Deoxycholate Citrate agar, Nutrient agar and Thioglycholate Citrate Bile Salt agar) incubated at 37ºC for 24 hours for the growth of pure single colonies. Suspicious colonies were collated for identification using standard microbiological methods.

DNA Extraction

Total DNA was extracted and purified using a ZR fungal/bacterial DNA mini prep extraction kit supplied by Inqaba, South Africa. 16S rRNA gene of 10 pure cultures of isolated bacteria were mixed with 750 µl of lysis solution and 200 µl of isotonic buffer in a ZR Bashing Bead lyses tubes. A bead beater built in a 2 ml tube holder was used to hold the tubes and spun at maximum speed for 5 minutes. The ZR bashing bead lyses tubes were spun at 10,000*g* for 1 minute. Four hundred (400) µl of the liquid lying above the sediments after centrifugation was put in a collection tube containing the Zymo-Spin IV spin Filter (orange top) and spun at 7000 x*g* for 1 minute. One thousand two hundred (1200) µl of fungal/bacterial DNA binding buffer was put into the collection tube containing the filtrate making the final volume to 1600 µl, 800 µl was now moved to another

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collection tube containing the Zymo-Spin IIC column and spun for 1 minute at 10,000x*g*, the flow through was thrown away from the collection tube. The remaining volume was moved to the same Zymo-spin and spun. Two hundred (200) µl of the DNA Pre-Wash buffer and 500 µl of fungal/bacterial DNA Wash Buffer were added to a new collection tube containing the Zymo-spin IIC and spun for 2 minute at 10,000x*g*. The Zymo-spin IIC column was moved to a clean 1.5µl of fungal/bacterial DNA Wash Buffer centrifuge tube, 100 µl of DNA elution buffer was put into the column matrix and spun 30 seconds at 10,000x*g* to elude the DNA. The ultra pure DNA was then stored at -20 degree for further reactions. The concentration of DNA and size was estimated by agarose gel electrophoresis using DNA of known molecular weight.

16S rRNA Amplification and sequencing

The 16s rRNA region of the rRNA genes of the isolates were amplified using the 27F (AGAGTTTGATCMTGGCTCAG) forward primer and 1492R (CGGTTACCTTGTTACGACTT) reverse primers in an ABI 9700 Applied Biosystems thermal cycler at a final volume of 50 micro litres for 35 cycles. The PCR mix included: the X2 Dream taq Master mix supplied by Inqaba, South Africa (taq polymerase, DNTPs, MgCl), the primers at a concentration of 0.4M and the extracted DNA as template. The PCR conditions were as follows: Initial denaturation, 95ºC for 5 minutes; denaturation, 95ºC for 30 seconds; annealing, 52ºC for 30 seconds; extension, 72ºC for 30 seconds for 35 cycles and final extension, 72ºC for 5 minutes after which the machine keeps the amplicons cool at $4^{o}C$. PCR products were purified and concentrated with a QIA quick spin PCR purification kit (Qiagen, S.A., Courtaboeuf, France) and amplicons detected on an agarose gel by agarose gel electrophoresis. After successful amplification and detection of 16s rRNA gene fragments, reads of all samples were filtered using Illumina sequencing. These reads were generated when trying to determine the relatedness of organisms by subjecting the sequences to BLAST with already known sequenced gene in the gene bank. Taxonomic classification of all sample reads was done. The data retrieved from sequencing using Illumina Miseq (in fastq – format) was demultiplexed and quality screened with MOTHUR software (v.1.39.0) [19]. Only the sequences with minimum length of 250 bp and average quality score 25 were retained. Sequences were aligned to the Silva reference alignment (release 123) [20]. Preclustering was performed in order to remove sequences with possible sequencing errors. Chimeric sequences were identified and removed with UCHIME2 using Silva gold alignment as a reference dataset. The unique sequences were classified using GREENGENES (May, 2013 release) reference taxonomy and assigned to operational taxonomic units (OTUs) with phylotype command in MOTHUR. UPGMA-dendrogram was visualized using FIGTREE. Newick phylogenetic tree of data was done with the R program using Vegan, Phyloseq and BiodiversityR packages [21, 22, 23]. Neighbour

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joining phylogenetic tree was also constructed using MEGA7 [24]. The evolutionary distances were computed using the Jukes-Cantor method with the trees bootstrapped 1000 times.

3. RESULTS

Conventional Cultural method of bacteria isolation identified *Escherichia coli*, *Staphylococcus aureus, Klebsiella pneumoniae, Pseudomonas aeruginosa, Vibrio species, Bacillus species, Salmonella species* and *Clostridium species*. *Escherichia coli* was found to be highest in prevalence amongst the enteric bacteria followed by *Staphylococcus aureus* then *Pseudomonas aeruginosa* while *Vibrio species* was the organism that was least present in the samples. After successful 16SrRNA amplification and sequencing, 72313 sequences were generated. The sequences from all 10 diarrhoeic stool samples subjected to clustering generated 2767 OTUs of which 2073 were new and unassigned. The reads generated where used to try to determine the relatedness of organisms by subjecting the sequences to BLAST with already known sequenced gene in the gene bank.

Similarities between Samples Expressed On A Dendogram

Analysis of the distances between the different samples and sampling parameters using the Jaccard coefficient was used to generate a dendogram as shown below. The dendogram generated is a representation of the similarities between all the samples using the OTUs generated from each sample. The result showed that the dendogram was in 2 groups. Sample V5 on one group and the others Samples V1,V2,V3,V4,V6,V7,V8,V9 and V10 on the other side of the group. Samples V2 and V3, V6 and V8 and V9 and V10 more closely related to each other than the others in the group because they are on the same clade. Sample V5 formed a different phyletic line because its microbial community is not so related with the other samples. The observed clades formed within the tree was tested for statistical significance using tree Pasimony. No significant differences were observed.

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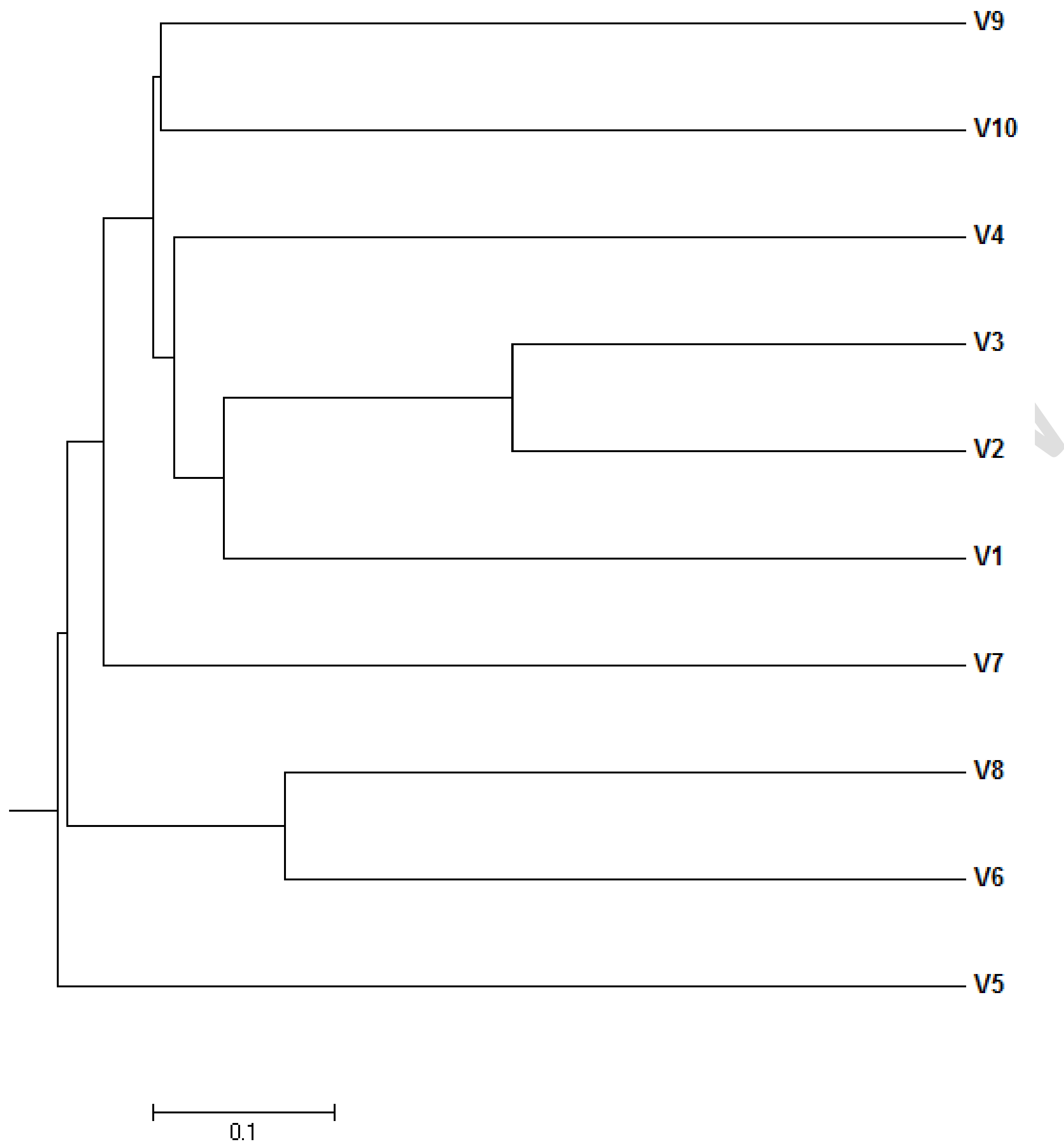

Figure 1: Similarities between samples expressed on a dendogram

Tree parsimony was used to determine if the distances observed within the tree was statistically significant. The Pars significance of 1.0000 indicated that there was no significant difference between the various samples.

Phylogenetic biodiversity of bacteria community in the gut.

Initial phylogenetic identification was made using BLAST. The BLAST search program was used to check for close relatives and phylogenetic affiliation. The search results were used as a guide for phylogenetic tree construction using MEGA7. The evolutionary relationship of the 80 most abundant organisms and their

contribution from each sample is represented in figure 2. The evolutionary relationship of the 50 most abundant organisms per sample is represented in figure 3a-3j.

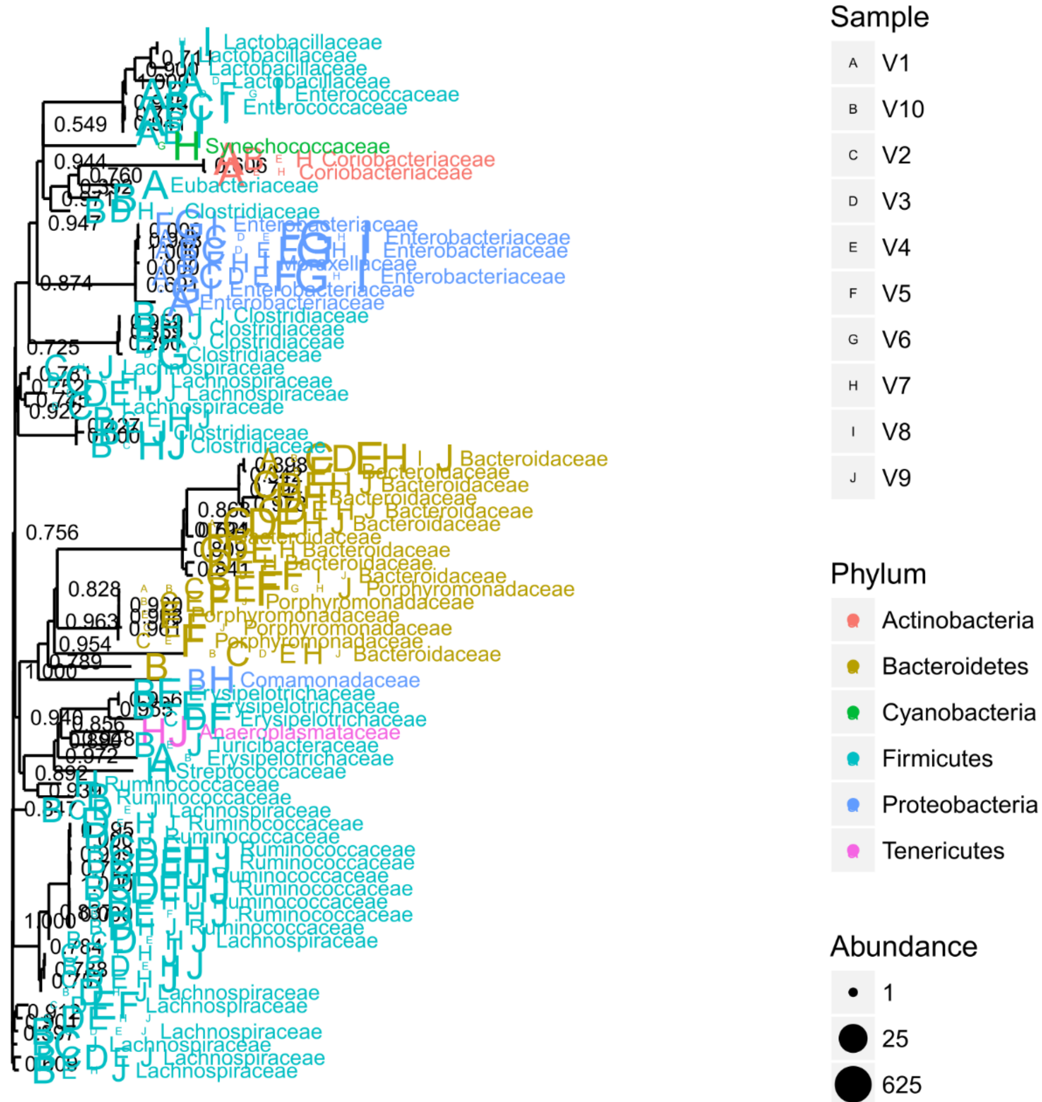

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Figure 2: Evolutionary relationship of the 80 most abundant organisms and the contribution from each sample

Evolutionary Relationship of the 50 Most Abundant Organisms Per Sample

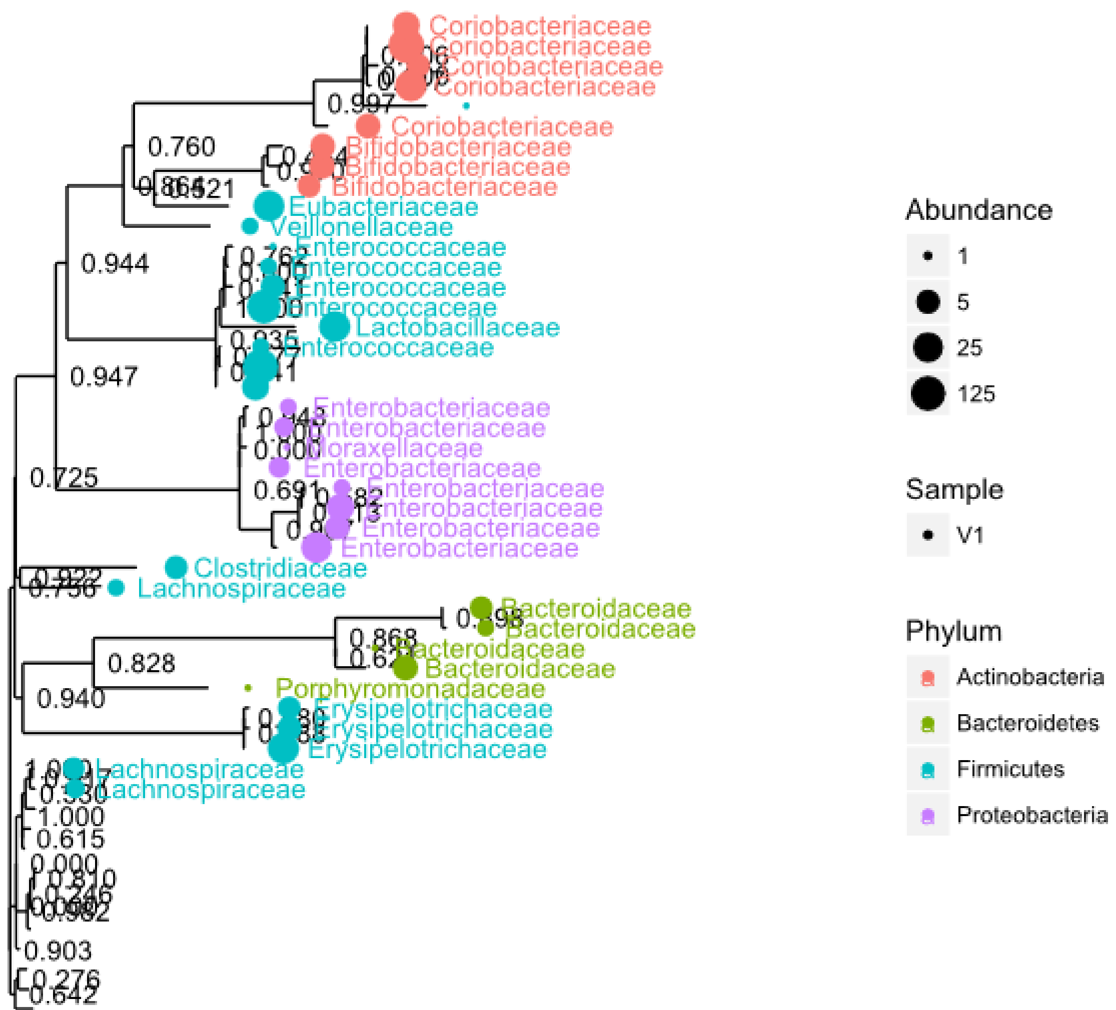

Figure 3a: Evolutionary Relationship of the 50 Most Abundant Organisms in Sample 1

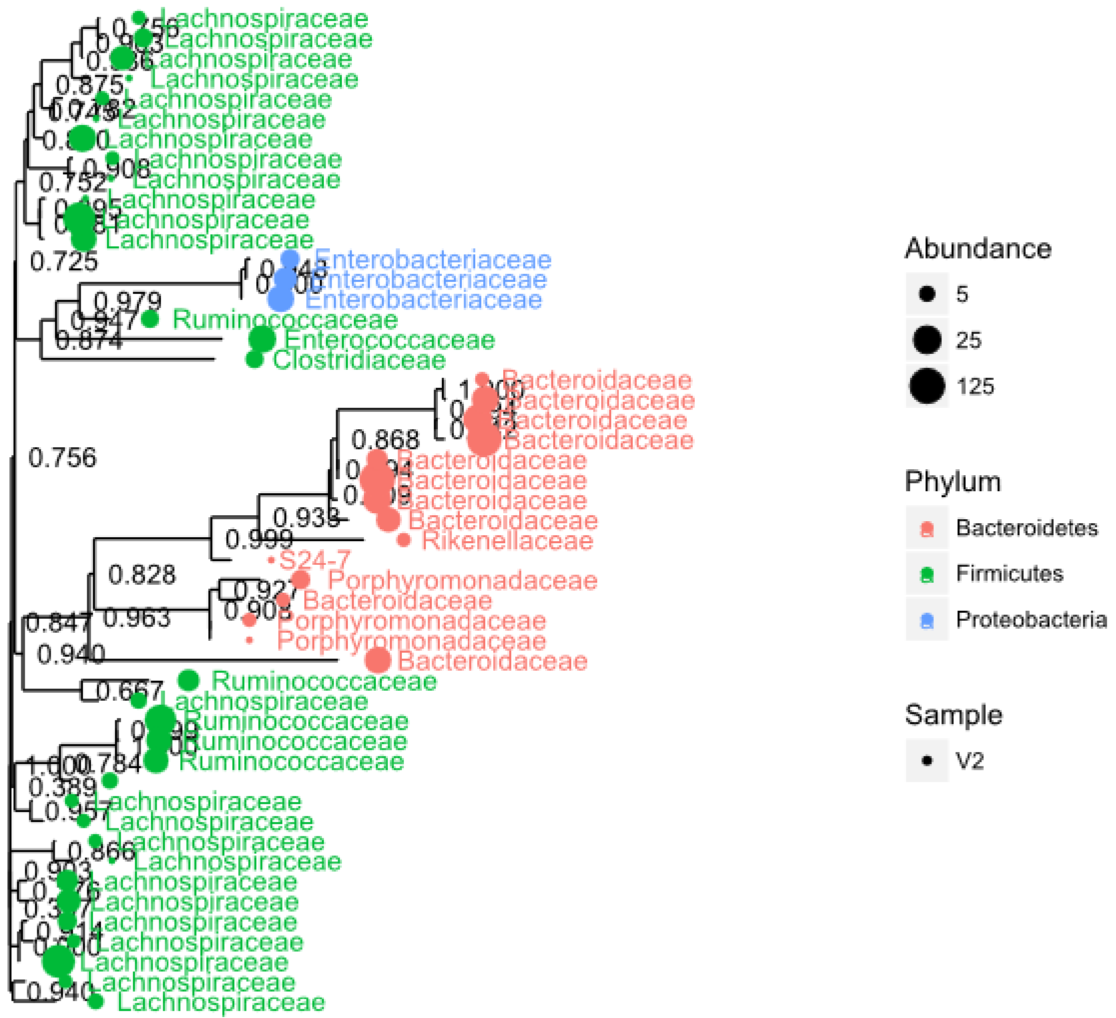

Figure 3b: Evolutionary Relationship of the 50 Most Abundant Organisms in Sample 2

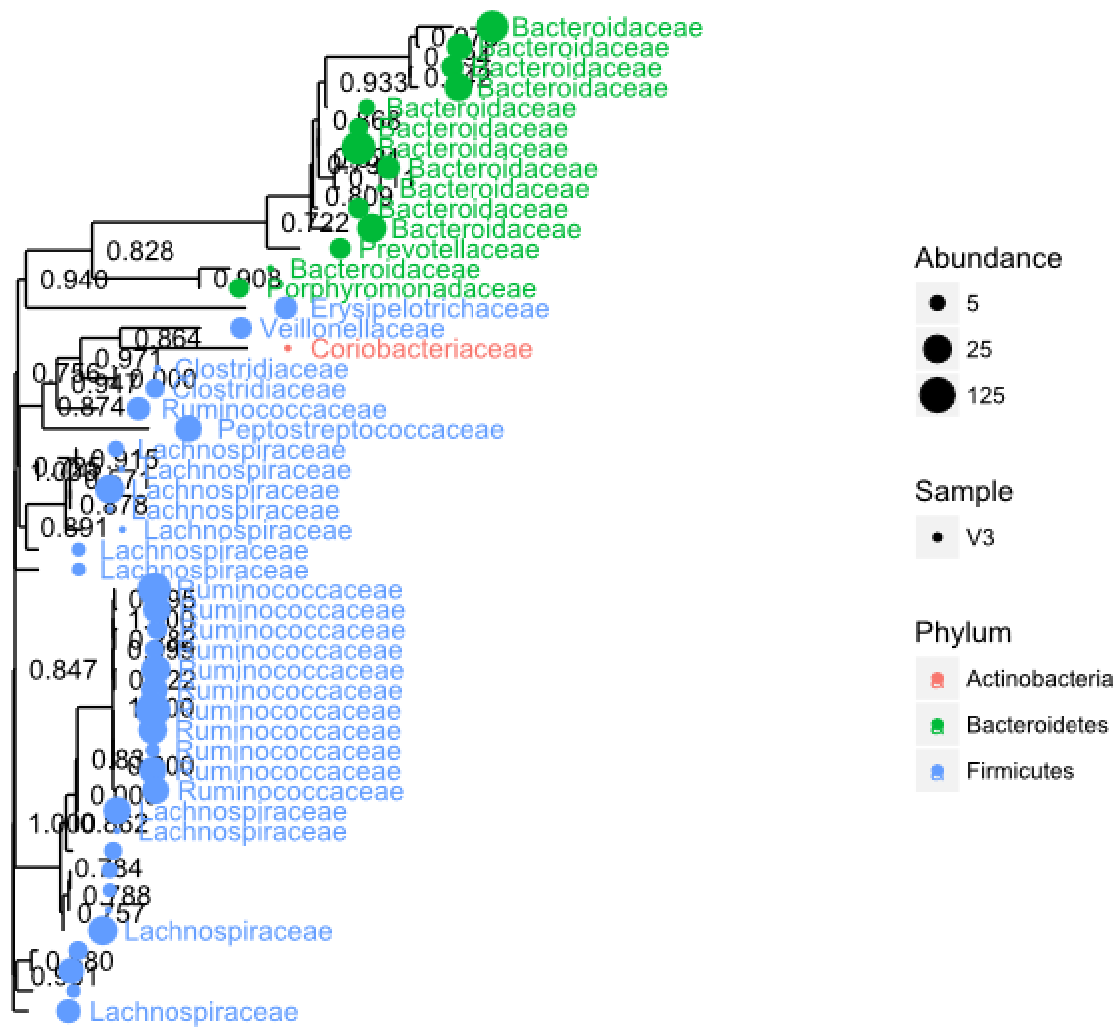

Figure 3c: Evolutionary Relationship of the 50 Most Abundant Organisms in Sample 3

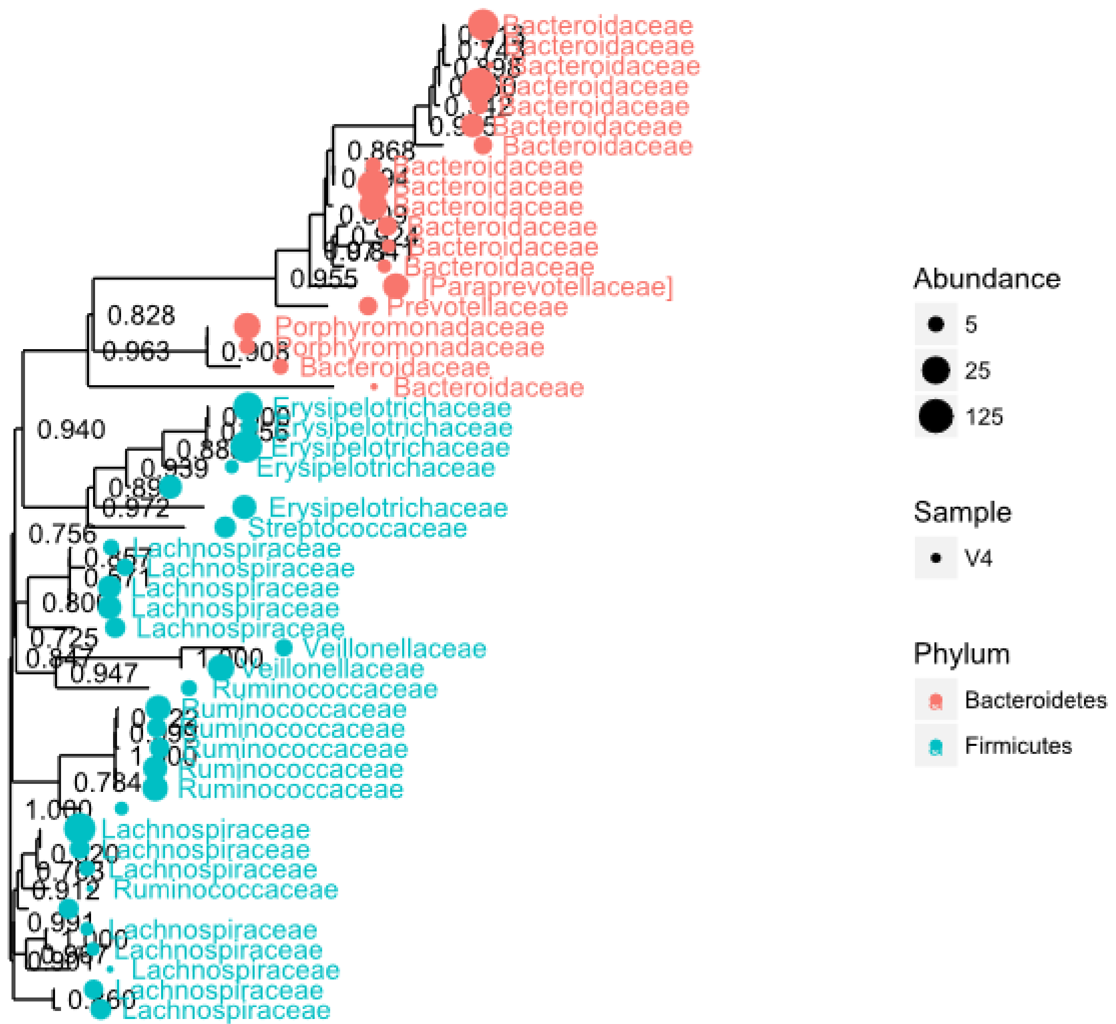

Figure 3d: Evolutionary Relationship of the 50 Most Abundant Organisms in Sample 4

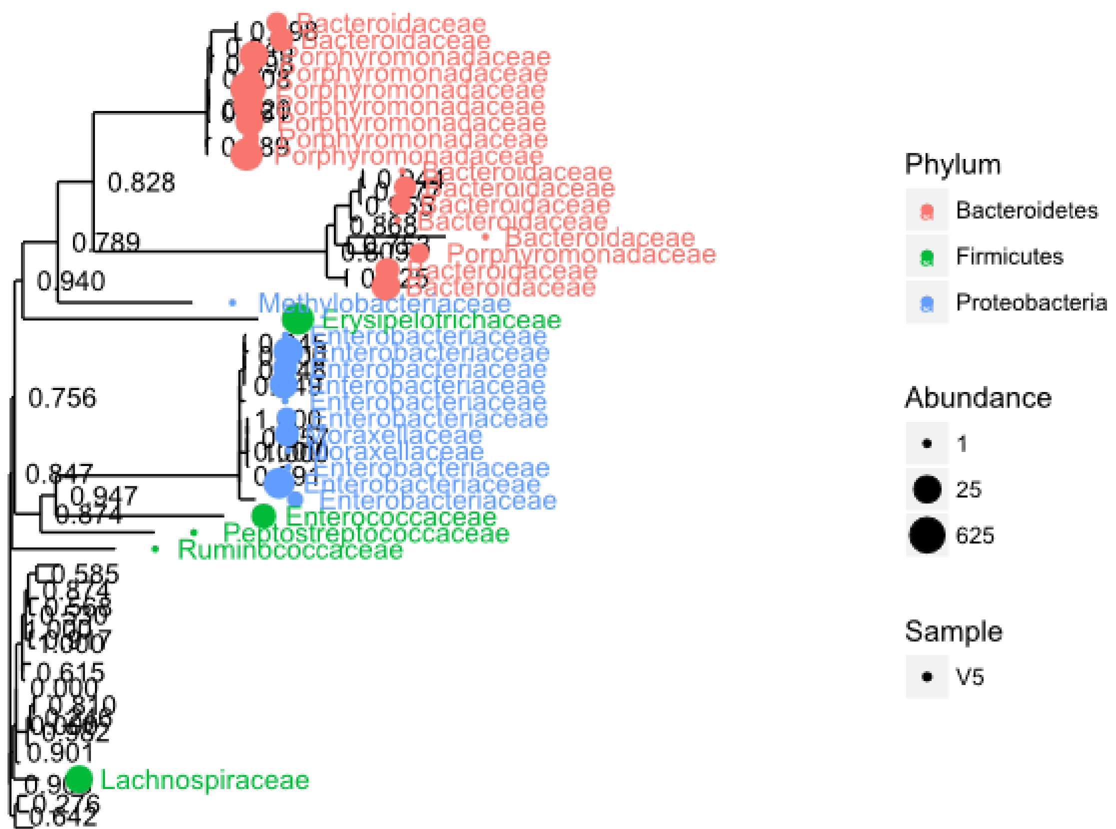

Figure 3e: Evolutionary Relationship of the 50 Most Abundant Organisms in Sample 5

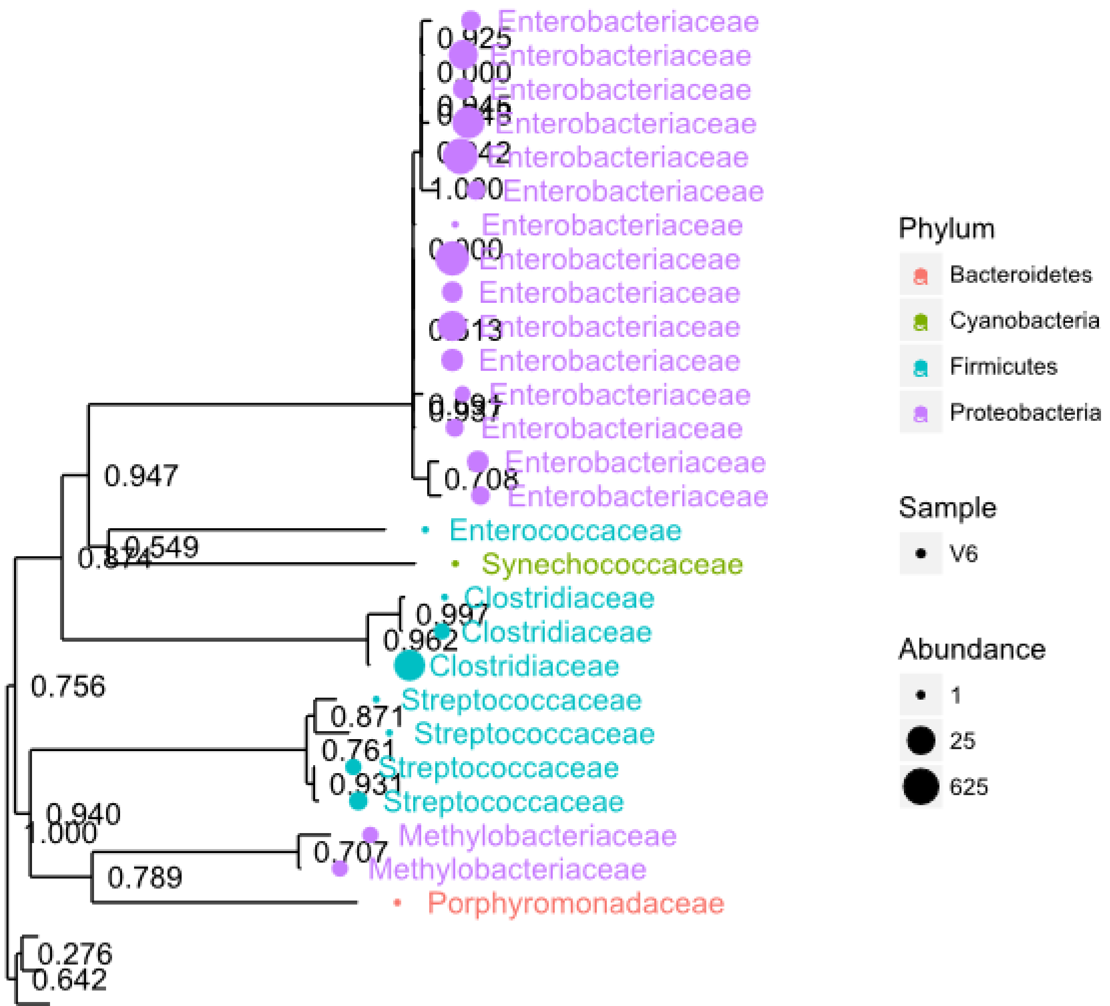

Figure 3f: Evolutionary Relationship of the 50 Most Abundant Organisms in Sample 6

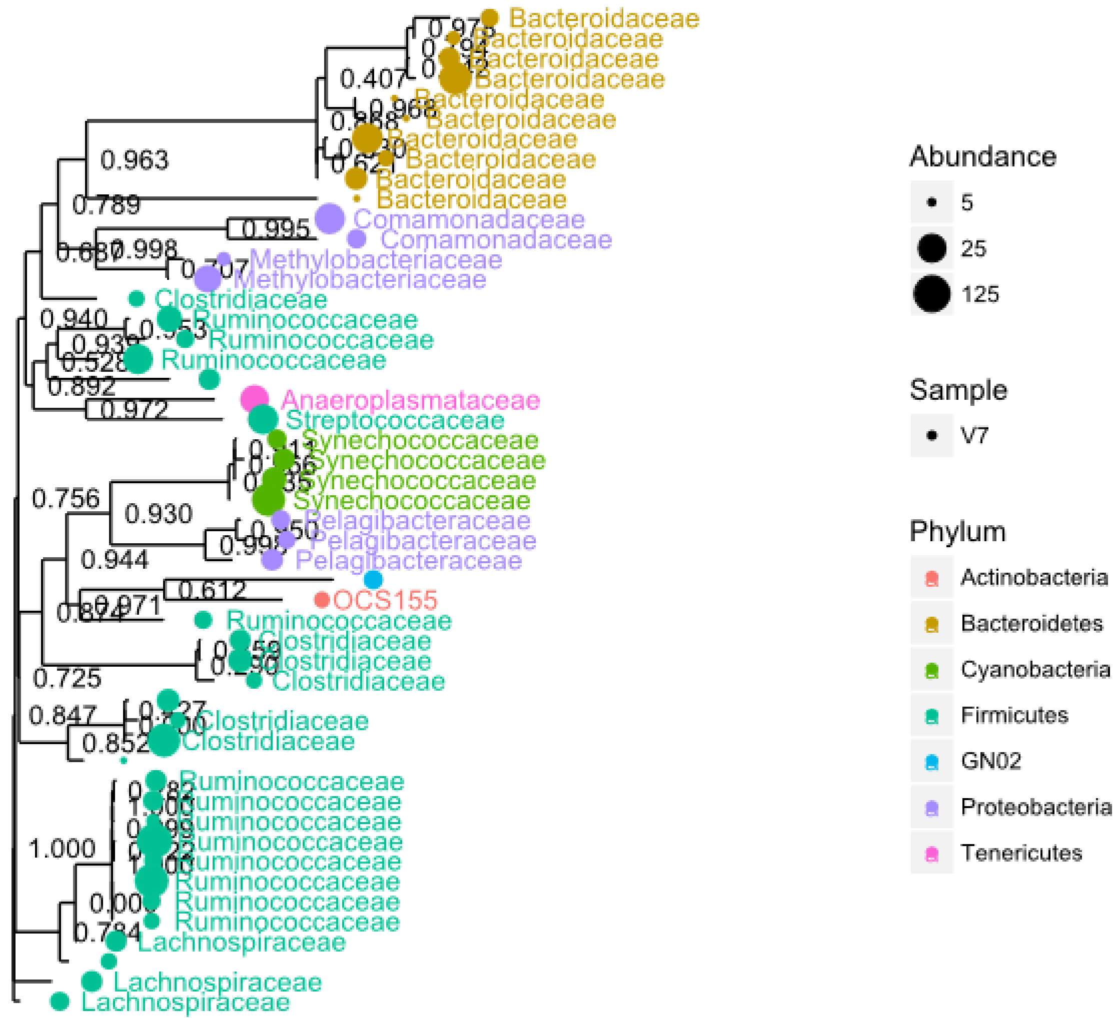

Figure 3g: Evolutionary Relationship of the 50 Most Abundant Organisms in Sample 7

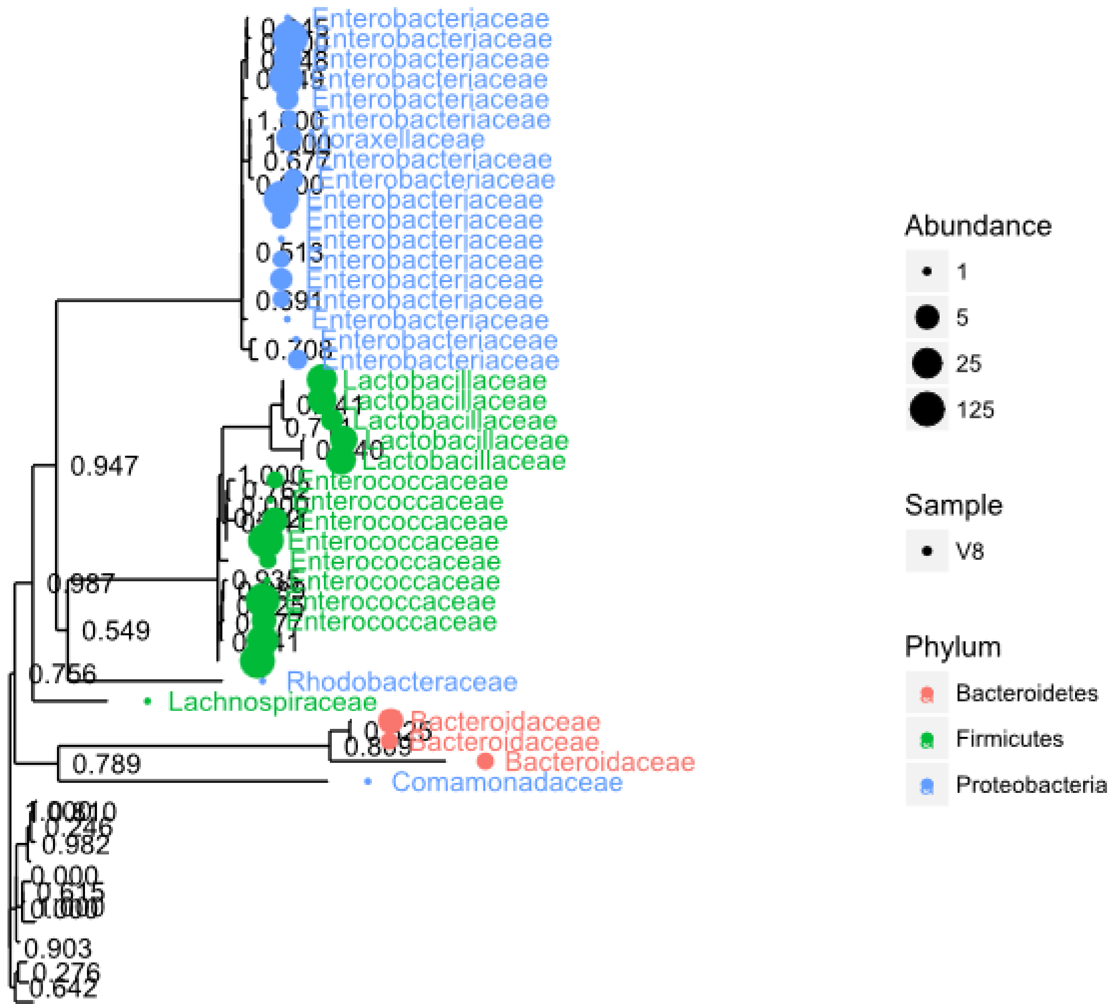

Figure 3h: Evolutionary Relationship of the 50 Most Abundant Organisms in Sample 8

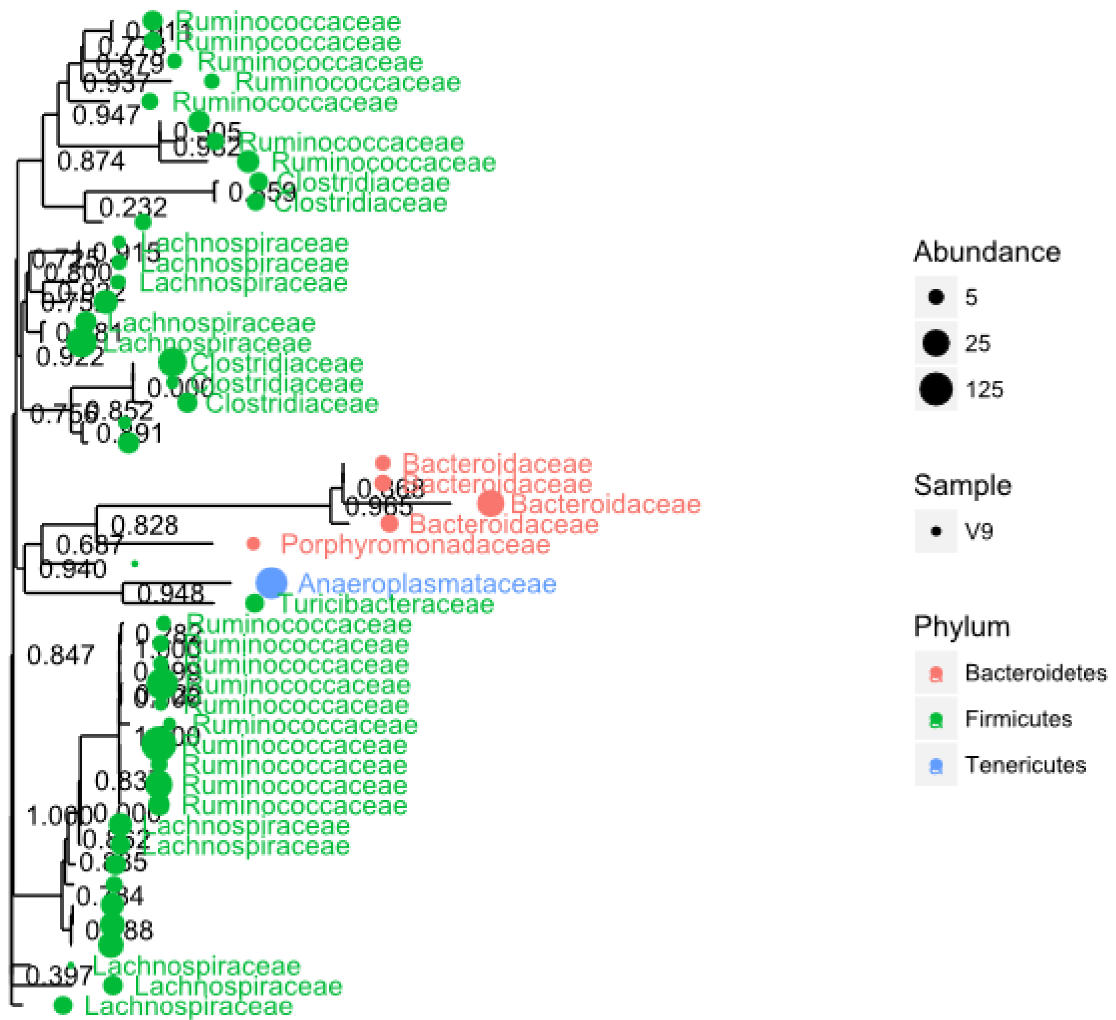

Figure 3i: Evolutionary Relationship of the 50 Most Abundant Organisms in Sample 9

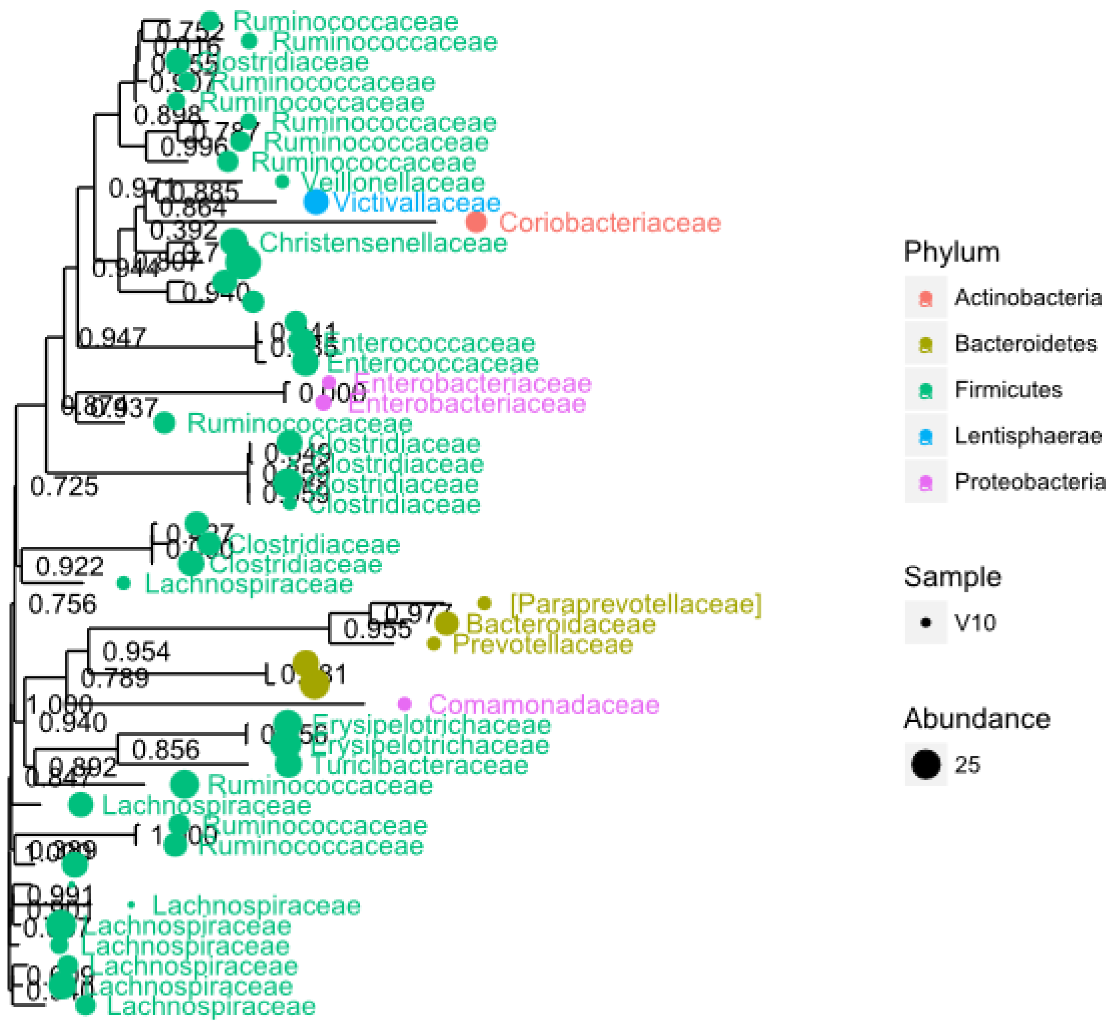

Figure 3j: Evolutionary Relationship of the 50 Most Abundant Organisms in Sample 10

4. DISCUSSION

Human gut microbiota is analyzed mainly by culture-based methods [25]. In this study, examining 10 faecal samples from diarrhoic patients by culture-based method yielded the growth of *Escherichia coli*, *Staphylococcus aureus, Klebsiella pneumoniae, Pseudomonas aeruginosa, Staphylococcus aureus, Vibrio species, Bacillus species, Salmonella species* and *Clostridium species*. Studies have reported cases of *Clostridium species, Bacillus species, Salmonellae species, Pseudomonas aeruginosa* and *Vibrio species* in human faeces [26, 27, 28, 29]. Other studies have also reported the presence of *Escherichia coli, Klebsiella pneumonia and Staphylococcus aureus* in human faeces [30, 31, 32]. The result of this work also agrees with a study in Nigeria in 2010, which reveals *Escherichia coli, Klebsiella pneumonia, Staphylococcus aureus and*

Pseudomonas species as highly prevalent bacteria isolated in diarrhoeic stool samples [33]. Other studies on the other hand have shown that 60 to 80% of the microorganisms in the total human gut microbiota have not been cultivated [9] making the whole human gut microbiota still not yet established. Therefore studying the array of microorganisms in the gut is very important because there are some microorganisms that cannot be detectable using the culture-based methods of bacteria isolation but are responsible for a lot of infections and also known to resist antibiotics used for treatment by these micro organisms. The study of evolutionary relatedness among various groups of organisms in a community is known as microbial phylogeny [34]. The use of 16S rRNA gene sequences has been by far the most common housekeeping genetic marker used to study bacterial phylogeny and taxonomy [35]. In this study, 72313 sequences from all 10 diarrhoeic stool samples subjected to clustering generated 2767 OTUs of which 2073 were new and unassigned. In order to derive a detailed phylogenetic biodiversity of bacteria community in the gut of diarrhoeic patients, we analyzed bacterial 16S rDNAs extracted from the 10 diarrrhoeic fecal samples used for the culture method and it yielded a large percentage of both unculturable and unknown microorganisms available in the gut. A total of 9 Kingdoms, 22 Phyla, 30 Classes, 50 Orders, 74 Families and 670 Blast output results were detected in 16S rRNA metagenomic sequencing of all sample reads. Sequences generated were clustered into OTU's which were used to construct a phylogenetic tree to reveal the wide variety of bacteria and their contribution in the individual samples. The result suggests that several unknown species inhabit the human intestinal tract of which cannot be cultivated. This is in agreement with studies by Stackebrandt and Rainey, [36] which shows that there are difficult to culture bacteria in the human gut which cannot be identified using culture methods of bacteria identification. Suau *et al*., [37] used molecular-biological techniques to overcome the limit of cultivation and reported that 284 clones were classified into 82 species or phylotypes. Of them, 20 (24.4%) were known species. Using PhyloseqR, bacteria phylogenetic tree was created with representative sequences of the bacteria Operational Taxonomic Unit (OTU) and evolutionary distance was computed using the Jukes-cantor method. The results which revealed that the OTUs formed different clades when compared to their closest relatives in the genBank. The evolutionary relationship of the 50 most abundant organisms per sample revealed the phylum *Firmicutes* to be most abundant in samples V1, V2, V3, V4, V7, V9 and V10. The phylum *Protebacteria* is most abundant in samples V6 and V8 while sample V5 had *Bacteroidetes* as the most abundant phylum. Making *Firmicutes* the major phyla in all the samples followed by *Proteobacteria, Bacteroides* and *Actinobacteria*. The evolutionary relationship of the 80 most abundant organisms and their contribution from each sample shows the phylum *Firmicutes* to have contributed most in the samples followed by *Bacteroidetes* and this corroborates the work by Eckburg *et al*.,

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[38] and Sester *et al.,* [39]. Fewer contributions were made by the phylum *Proteobaacteria, Actinobacteria, Tenericutes* and *Cyanobacteria.* Comparison of the top phyla showed that the major phyla in all the samples were *Firmicutes* followed by *Proteobacteria, Bacteroides* and *Actinobacteria*. *Firmicutes* phylum has been identified as the major phyla in the intestine of humans [38]. *Proteobacteria* constituted about 44.39% of the most abundant phyla compared across the samples and this agrees with study by Sester *et al.,* [39] who examined the global pattern of bacterial communities from various habitats and found out that the average level occupied by *Proteobacteria* in the bacterial population was as high as 40%. The abundance of *Clostridia* (43.85%) and *Bacilli* (89.59%) in the comparison of the class classification confirms the Phylum *Firmicutes* as the most abundant throughout the samples. This corroborates with the studies by Eckburg *et al.,* [38] which identifies *Firmicutes* phylum and *Clostridia* class as the most abundant in the human Intestine. In comparison of top order classification of all samples, *Lactobacillales* is the most dominanat order. The *Lactobacillales* also known as lactic acid bacteria play a great role in maintaining a healthy microflora of human mucosal surfaces and is said to preserve the immune function during human Immunodeficiency virus infections [40]. Top blast output results of all samples yielded high relative abundance of uncultured bacteria which includes *uncultured gamma* , *uncultured lachnospiraceae, uncultured organism, uncultured klebsiella, uncultured bacterium, uncultured Escherichia ,uncultured bacilli, Uncultured streptococcus, uncultured marine* , *uncultured acetivibrio*, *Uncultured organism*, *Uncultured romboutsia*, *Uncultured bacteroidetes* and *uncultured ruminococcaceae* . Others bacteria were *Collinsella aerofaciens, Enterococcus faecalis* , *Bifidobacterium longum*, *Bifidobacterium breve*, *Lactobacillus fermentum*, No hits, *[rominococcus]torques, Enterococcus durans, Bacteroides vulgatus, Escherichia coli, Bacteriodes species , Bacteroides vulgatus, Veillonella parvula,* Clostridium species , *Faecalibacterium prausnitzii* , *Bacteroides dorei* , *Parabacteroides distansonis* , *Streptococcus salivarius,* , *Enterobacteriaceae bacterium* , *Clostridium innocuum ,Parabacteroides species , Clostridium saccharobutylicum, Ralstonia solanacearum, Unidentified oral, Roseburia species, Lactococcus species*, *Enterococcus durans, Lactobacilli paracasei, Lactobacilli species, Enterococcus faecalis, , Lachnospiracea bacterium, Bacteroides vulgatus* , *Escherichia albertii*, *Enterococcus durans, Victivallis vadensis* and *Enterococcus faecium* . Most of these bacteria have not yet been characterized. We believe that an improvement of the culture methods would result in the cultivation and identification of new intestinal microorganisms.

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5. CONCLUSION

In conclusion, this research was able to identify culturable and unculturable bacteria in the gut of diarrhoeic people in Rivers state and also show the biodiversity and inter relatedness of these microorganisms using molecular methods. We were able to characterize several diverse microorganisms in the human large bowel by using 16S rRNA libraries and a culture-based method and it has been confirmed that the number of microorganisms identified in the gut of diarrhoeic people in Rivers State using the molecular method of bacteria identification far exceeds that of the conventional cultural method. 16S rRNA metagenomic sequence analysis yielded difficult to culture microorganisms with high level of unknown bacteria of which majority are of public health significance to humans. Therefore, 16S rRNA techniques for detection and identification of predominant bacteria create new opportunities for non cultivation studies of the human intestinal microflora which will also help in proper diagnosis of infectious diseases and new methods of treatments of diseases.

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