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Phylogenetic diversity of caecal microbiota in Krishibro broilers of India

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Abstract

Gut microbiota is attributed to the bird's health, metabolism, immunity and has implications on food safety and public health. Among food producing animals chicken meat is consumed to a large extent across the globe. Improving production performance of food animals could be achieved by understanding and optimizing microbial communities of gastro-intestinal tract. To prevent Dysbacteriosis, a common problem in broiler chicken single or a mixture of antibiotics and coccidiostats are supplemented in broiler chicken diets. A total of 120 krishibro broiler chicken of day old were distributed randomly into three groups (CON, ACFG and CFG) with five replicates of eight birds, in each group which were maintained on similar management conditions. Control group (CON) was administered basal diet alone with no antibiotic and no coccidiostat. ACFG group was given antibiotics chlortetracycline, tylosin phosphate and a coccidiostat, amproilum hydrochloride @ 50 g/100 kg feed each. CFG group was supplemented with coccidiostat alone at the above mentioned dose. At 6 weeks of age, one bird per replicate was slaughtered and the caeca luminal contents were collected and pooled in each group. Changes in caecal microbiota composition were studied using amplicon sequencing of V3-V4 region of 16SrRNA gene on Illumine Miseq platform. The sequencing data were uploaded on MG-RAST pipeline. All the birds appeared healthy throughout the experimental period. Caecal microbial diversity revealed bacteria as the major domain. The dominating bacterial phyla in all the groups were *Firmicutes* and *Bacteroidetes* accounting to >90% of the caecal microbiome. Higher *Firmicutes/Bacteroidetes* ratio was seen in CFG group. The phylum *Deinococcus-thermus, Chlorobi, Acidobacteria* were represented exclusively in ACFG, CFG, CON groups, respectively. ACFG group was dominated by *Rikenellaceae* where as CFG and CON groups showed *Ruminococcaceae* as the major group at the family level of classification (Figure). The predominant genera with above 10% abundance in the caecum were *Alistipes, Bacteroides* and *Clostridium* in ACFG group, *Faecalibacterium* and *Alistipes* in CFG group, *Bacteroides* and *Faecalibacterium* in CON group. Hierarchical clustering showed similarities in ACFG and CON groups at phylum level. The phylogeny at genus level showed CFG clustering with CON. Supplementation of coccidiostats alone and antibiotics with coccidiostats influenced the changes in microbiota composition of Krishibro broiler chicken.

Keywords: Microbiota, Caecum, Krishibro, 16SrRNA gene

Introduction

Poultry are considered as an economical protein source across the globe and the demand for broiler chicken is growing exponentially. Broiler chicken are the most efficient feed converters with 1.5 to 2.0 feed conversion ratio. Intensive poultry production is very prone to infectious disease outbreaks especially in geographical areas where climatic changes are natural. It was mentioned that certain meat type native chicken breeds of China which were slow-growing than the commercial fast-growing broilers do have perfect meat quality (Zhang *et al.,* 2007; Li *et al.,* 2009) and were preferred. Similarly, In Indian conditions the colored broilers fetch a slightly higher price compared to the commercial fast-growing broiler chicken and are associated with quality meat. Krishibro, a colored broiler chicken of India developed by ICAR-DPR is grown for meat purpose. Gut health is considered synonymous to animal/bird health with effective functionality and health of the gastrointestinal tract (GIT) is considered as an important factor in determining animal performance (Brisbin *et al.,* 2008; Stanley *et al.,* 2014). Major components associated with gut health are diet, GIT mucosa and flora (Celi *et al.,* 2017; Biasato *et al.,* 2019) and maintaining balance between these components is essential for the bird's health and performance (Zhao *et al.,* 2013). Gut microbiota is attributed to the bird's health, metabolism, immunity and has

implications on food safety and public health (O'Hara and Shanahan, 2006; DuPont, 2007). Host genotype is also said to influence the gut microbiota composition (Khachatryan *et al.,* 2008; Salzman *et al.,* 2010; Pandit *et al.,* 2018).
Dysbacteriosis is more common in chicken broilers with non-infectious (non-specific stressors) and infectious cause mainly *Clostridium perfringens* associated with or without coccidiosis (De Gussem, 2007, Teirlynck *et al.,* 2009). Antibiotic growth promoters (AGP's) were used in broiler chicken to improve growth and production performance as well as maintain gut health (Pourabedin *et al.,* 2015). Emergence of antimicrobial resistance and its spread to animals, environment, humans are rising which led to subsequent ban/restricted usage of antimicrobials. The broiler chicken production systems started switching from conventional to organic broiler chicken farming/chicken raised without antibiotics. Requirement of anaerobic conditions simulating GIT for gut microbial cultivation is a tedious task. The advent of high-throughput sequencing of 16S rRNA gene amplicons has enabled the study of bacterial communities at increased depth and resolution (Simon and Daniel, 2011). Previously, many studies on poultry microbiota have used the cecum as sampling site due to its relationship with chicken productivity and the highly diverse bacterial communities that inhabit caecum. To date, there has been a dearth of comparative metagenomic analyses pertaining to the role of gut microorganisms and microbial diversity in krishibro (colored) broiler chicken of India. The purpose of this study is to explore caecal microbiota and compare their alterations in Krishibro broiler chicken at 42 days of age supplemented with antibiotics and coccidiostat, coccidiostat alone and without any growth promoters, using 16S amplicon sequencing technology on Illumina Miseq platform.

Material and Methods

Ethical approval: Experiment was conducted as per the guidelines approved by the Institutional Animal Ethics Committee, ICAR-Directorate of Poultry Research, Hyderabad.

Design of Experiment:

A total of 120 krishibro broiler chicks of day old were distributed randomly into three groups with five replicates of eight birds, in each group. The birds were given basal diet (Corn and Soya based diet) prepared at the Institute feed unit. Among groups, Control group (CON) was administered basal diet alone with no antibiotic and no coccidiostat. Another group was antibiotic and coccidiostat fed group (ACFG), which was given antibiotics, chlortetracycline (CTCmix®) and tylosin phosphate @ 50 g/100 kg feed each along with a coccidiostat (Kampro-H®) @ 50 g/100 kg feed. In the third group i.e., coccidiostat alone fed group (CFG) Kampro-H at the above mentioned dose was administered. All the birds appeared healthy throughout the experimental period. On day 42, from each group, five birds *i.e.,* one per replicate was randomly selected and sacrificed by

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CTCmix®: Each gram premix contains 150 mg of chlortetracycline. Manufactured by Venky's (India) Limited.
®Tylomix premix as Tylosin Phosphate 10 % w/w
Kampro-H®: Each kg premix contains 200 g of Amprolium Hydrochloride and 10 g of vitaminK. Manufactured by Venky's (India) Limited. jugular vein exsanguination. The ceca were incised and the lumen contents were collected.

Pooled samples of each group was prepared by mixing 0.1g of caecal contents from each bird of the same group.

DNA isolation: A total of 300 µg of pooled caeca contents from each group was subjected to DNA isolation using the genomic DNA isolation research kit. The steps involved in isolation include suspension of the lyophilized cells with C-TAB, lysis of cell wall using salts, precipitation of DNA. The isolated DNA was subjected to electrophoresis and casted on 1% agarose gel for further confirmation of results.

16S ribosomal RNA gene (16S rRNA) library preparation:

The steps include amplification of the V3-V4 region, using the target gene-specific sequences for V3 and V4 region were Forward Primer = 5'CCTACGGGNGGCWGCAG and Reverse Primer = 5'GACTACHVGGGTATCTAATCC as described by Klindworth *et al.,* 2012. The template size of the PCR enriched fragments were verified on Agilent Technologies 2100 Bioanalyzer using a DNA 1000 chip. The quantification of the DNA library templates was performed using Illumina qPCR quantification protocol. Library sample concentration was calculated using Roche's rapid library standard Quantification solution and calculator and all the libraries passed QC with concentrations of 54.77 ng/µl, 52.22 ng/µl and 56.57 ng/µl of CON, ACFG and CFG amplicons. Libraries were quantified using a fluorometric quantification method and DNA concentration of libraries revealed 136nM, 129nM and 142 nM in CON, ACFG and CFG groups, respectively.

Library denaturation and paired-end sequencing (2bp x 300 bp) was performed on Illumina Miseq sequencing platform using manufacturer recommended protocols.

Bioinformatics Analysis:

All 16S rDNA raw reads were uploaded to MG-RAST V4.0 open source online server for the phylogenetic classification of metagenomics data analysis (Meyer *et al.,* 2008). Annotations were made for the best hits with a minimum *e* value of 1E-5 and minimum identity of 80% and 50 bp length against the RDP (Ribosomal Database Project). The data were further analysed using METAGENassist (Arndt *et al.,* 2012) after filtering unassigned bacteria and considering normalization using pareto scaling (Smilde *et al.,* 2005). Venny 2.1(Oliveros, 2007), PAST v4.02 (Hammer *et al.,* 2001) and Krona tools were utilized for analyzing core microbiome. The metagenomes were deposited in MG-RAST with MG-RAST id: 4859493.3 (ACFG), 4859499.3 (CFG) and 4859489.3 (CON).

Results

Caecal contents collected from 5 chicken at 42 days of age in three different groups were analyzed. The V3-V4 region of 16SrRNA gene was targeted for amplifying and sequencing. The sequence reads were uploaded onto MG-RAST pipeline. Taxonomic comparisions were made from annotations made against the RDP database with a minimum e-value of 1E-5 and minimum identity of 80% and minimum length of 50 bp of MG-RAST.

Taxonomic/Phylogenetic Abundance of Caecal Microbiota:

Bacteria was the major domain with more than 99% abundance in all groups. At phylum level of taxonomic classification, *Bacteroidetes* and *Firmicutes* contributed >90% of caecal microbiome in all groups (Table 1). All groups shared 57.9% abundance in common at phylum level (Figure 1). CON group showed less number of phyla compared to ACFG and CFG. *Unclassified (derived from bacteria)* was third major phylum in all groups followed by *Actinobacteria* and *Proteobacteria* and contributed to >0.1% abundance. *Fusobacteria, Thermotogae* and *Verrucomicrobia* phyla were represented in ACFG and CFG groups only. The phylum *Deinococcus-thermus, Chlorobi, Acidobacteria* were represented exclusively in ACFG, CFG, CON group, respectively.

The phylogenetic analysis at class level revealed *Clostridia* and *Bacteroidia* as major class accounting to 87.36%, 84.36% and 81.91% in ACFG, CFG and CON groups, respectively. In the caecal microbiome *Clostridia, Bacteroidia, Unclassified (derived from bacteria), Bacilli, Erysipelotrichi, Flavobacteria, Negativicutes* and *Actinobacteria (class)* were abundant with more than 1 % reads. The relative abundance of *Clostridia* was high and *Bacteroidia* was low in CFG compared to ACFG and CON groups. In *Firmicutes* phylum, *Clostridia* was most abundant class in all groups followed by *Bacilli, Erysipelotrichi* and *Negativicutes*. Majority of the bacteria in phylum *Bacteroidetes* belong to *Bacteroidia* class followed by *Flavobacteria* in all groups. In *Proteobacteria* phylum *Deltaproteobacteria* was the major class with highest abundance

in all groups, followed by bacteria belonging to *Epsilonproteobacteria* class in ACFG group, *Alphaproteobacteria* class in CFG and *Gammaproteobacteria* class in CON.

At order level of classification, the top most orders (>1%) were *Clostridiales, Bacteroidales, Unclassified (derived from bacteria), Erysipelotrichales, Lactobacillales, Flavobacteriales, Selenomonadales* and *Bacillales*. The order *Flavobacteriales* was <1% in both ACFG and CFG groups. The order *Selenomonadales, Bacillales* showed <1% abundance in ACFG and CON groups, respectively. The per cent abundance of different bacterial families were shown in Figures 2a-2c. ACFG group was dominated by *Rikenellaceae* where as CFG and CON groups showed *Ruminococcaceae* as the major family.

The predominant genera with above 10% abundance in the caecum were *Alistipes*, *Bacteroides* and *Clostridium* in ACFG group, *Faecalibacterium* and *Alistipes* in CFG group and *Bacteroides* and *Faecalibacterium in* CON group. Overall generic types of bacterial domain shared 55.5 per cent in common and the remaining showed variation in genera. The per cent relative abundance of bacterial genera of predominant phyla were depicted in Figures 3a-3c (*Firmicutes* phylum) and Figures 4a-4c (*Bacteroidetes* phylum) of ACFG, CFG and CON groups, respectively. In the *Firmicutes* phylum the predominant genus was *Clostrdium* followed by *Faecalibacterium* in ACFG group and vice versa in CFG and CON groups The predominant bacterial genera of phylum *Bacteroidetes* were *Alistipes* followed by *Bacteroides* in ACFG and CFG groups whereas in CON it was *Bacteroides* followed by *Alistipes*.

Conclusions and Discussion:

In the gastrointestinal tract (GIT), the microbiota plays a central role in enhancing nutrient absorption and strengthening the immune system, thereby affecting both growth and health of chicken (Choi *et al.*, 2015). Hierarchical clustering using Bray-Curtis Similarity Index and Paired group (UPGMA) algorithm for different groups revealed ACFG clustering with CON at phylum level (Figure 5a). In krishibro broiler chicken, *Firmicutes* and *Bacteroidetes* were the major phyla of all groups supplemented with different products. Many authors have mentioned that in chicken production systems, the caecal microbiota was dominated by *Firmicutes* followed by Bacteroidetes (Torok *et al.*, 2011; Zhu *et al.*, 2019). On contrary, *Bacteoridetes* was reported as major phyla in chickens raised under free range conditions (Xu *et al.*, 2016). The productivity indicator, *i.e.*, higher *Firmicutes* to *Bacteroidetes* ratio (F/B), particularly in the late growth phase, was more marked in conventional diet amplicon sequences given sub-lethal antibiotic dosage in chicken (Banerjee *et al.*, 2018). The present study showed F/B of 2.17 in CFG, 1.62 in CON and 1.53 in ACFG. Xu *et al.*, 2016 emphasized that caecal microbiota composition varied with chicken raised on different feeding modes and higher F/B ratio was seen in cage rising chickens than free-range ones. Correlation between gut microbial composition especially F/B ratio and efficiency of energy extraction in humans and animals was mentioned by many authors (Turnbaugh *et al.*, 2006; Ley *et al.*, 2007; Zhao *et al.*, 2015). On contrary, Parnell and Reimer, 2012 mentioned that high dietary fibre increases *Bacteroidetes* proportion and lower the F/B ratio. Pandit *et al.*, 2019 revealed *Bacteroidetes* as the major phylum in indigenous Indian Aseel, and Kadaknath chicken where as *Firmicutes* was the major phylum in Cobb400. They also mentioned that geographic location and chicken line/breed exert a significant impact on caecal microbial composition. Present finding of high F/B might be due to fact that krishibro broiler chicken were raised on Corn and Soya based diet.

At the class level, the caecal microbiome was dominated by *Clostridia* and *Bacteroidia* and similarly *Clostridiales* and *Bacteroidales* were abundant at order level and corroborates with the findings of Pandit *et al.*, 2019. Heat map based on pearson distance measure with ward clustering algorithm for relative abundance of different orders among groups showed similarity between CFG and CON groups. (Figure 6). CFG and CON groups showed family *Ruminococcaceae* with highest abundance. These findings bear resemblance to finding of Carrasco *et al.* 2019, and as mentioned by these authors, the family *Ruminococcaceae* was found to be associated with an improvement of intestinal health, feed efficiency and productive performance of the birds. On contrary, *Rikinellaceae* was highest in ACFG group fed mixture of antibiotics and

coccidiostats followed by *Ruminococcaceae*. Hierarchical clustering using Bray-Curtis Similarity Index and Paired group (UPGMA) algorithm revealed CFG with close proximity to CON group at generic level (Figure 5b). The genus *Alistipes* belonging to *Rikinellaceae* family was predominant genus in ACFG. In CON and CFG groups *Bacteroides* and *Faecalibacterium* were the most abundant genera, respectively. Carrasco *et al.* 2019 studied composition of cecal microbiota in broiler chickens supplemented with either bacitracin and found Bacitracin consistently decreased *Bifidobacterium.* Banerjee *et al.*, 2018 mentioned that conventional diet showed the prevalence of butyrate-producing genera such as *Faecalibacterium, Ruminococcus, Blautia, Coprococcus* and *Bacteroides*, whereas organic diet groups showed members of *Lactobacillales. Alistipes* genus is beneficial to host gut and they are bile-resistant bacteria with saccharolytic and proteoplytic properties, produce acetic acid by producing fibrinolysin, digest gelatin, and ferment carbohydrates (Abe *et al.*, 2012, Rautio *et al.*, 2003). Many factors influence the composition of gut microbiota such as diet and feeding mode (David *et al.*, 2014; Tan *et al.*, 2019), host genetics (Wen *et al.*, 2019; Zhao *et al.*, 2013), medications (Everard *et al.*, 2014), rearing conditions (Xu *et al.*, 2016). The phylogenetic/taxonomic composition of core microbiota of caecum showed variations between groups with CFG having close similarity to CON rather than ACFG which can be attributed to the influence of medications supplemented in diet. It can also be concluded that the krishibro (colored broiler) chicken of India might be suitable choice for organic broiler chicken production systems as the core microbiota showed predominantly beneficial bacteria in all groups depicting better gut health. Future studies on metabolic potential and interactions among various phylogenetic groups will be useful in understanding and modulating the gut environment.

Acknowledgements:

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Table 1: Relative abundance of various phyla among different groups. Data generated using MG-RAST with a minimum *e* value of 1E-5

PHYLUM	ACFG	CFG	CON
Acidobacteria	0	0	0.0008
Actinobacteria	1.2613	1.2325	0.9896
Aquificae	0.0125	0.0079	0.0040
Bacteroidetes	37.0445	29.5674	35.0636
Chlorobi	0	0.0012	0
Chloroflexi	0.002	0.010	0.002
Cyanobacteria	0.015	0.016	0.016
Deferribacteres	0	0.0004	0.0004
Deinococcus-Thermus	0.0005	0	0
Firmicutes	56.9178	64.2330	56.9621
Fusobacteria	0.0005	0.0004	0
Nitrospirae	0.0005	0	0.0004
Proteobacteria	0.9051	0.9142	0.4144
Spirochaetes	0.0040	0.0151	0.0016
Synergistetes	0.0584	0.0749	0.0424
Tenericutes	0.0105	0.0329	0.0164
Thermotogae	0.0015	0.0008	0
Verrucomicrobia	0.1452	0.0004	0
unclassified (derived from Bacteria)	3.6203	3.8923	6.4859

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Figure 1: Number of phyla shared between groups belonging to bacterial domain. Data generated using RDP of MG-RAST with a minimum *e* value of 1E-5

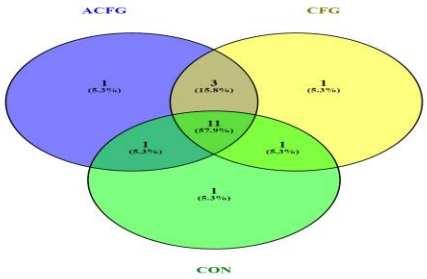

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Figure 2a-2c: **Percent abundance of caecal microbiota >0.1% at family level in ACFG, CFG and CON respectively. Data generated using RDP of MG-RAST with a minimum *e* value of 1E-5.**

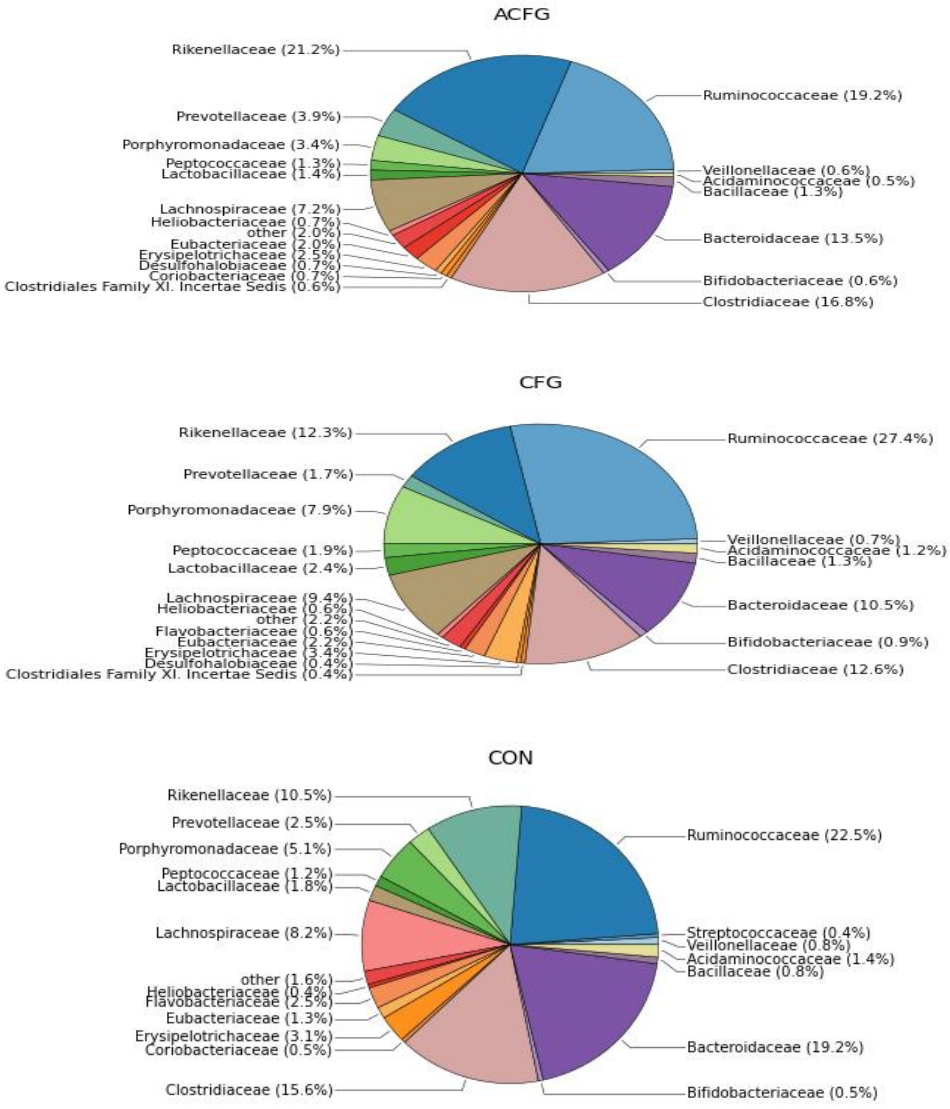

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Figure 3a: **Genera of *Firmicutes* phylum in ACFG group. (Data generated using RDP of MG-RAST with a minimum *e* value of 1E-5. The charts visualize classification levels of Phylum, Class Name, Order, Family and genera)**

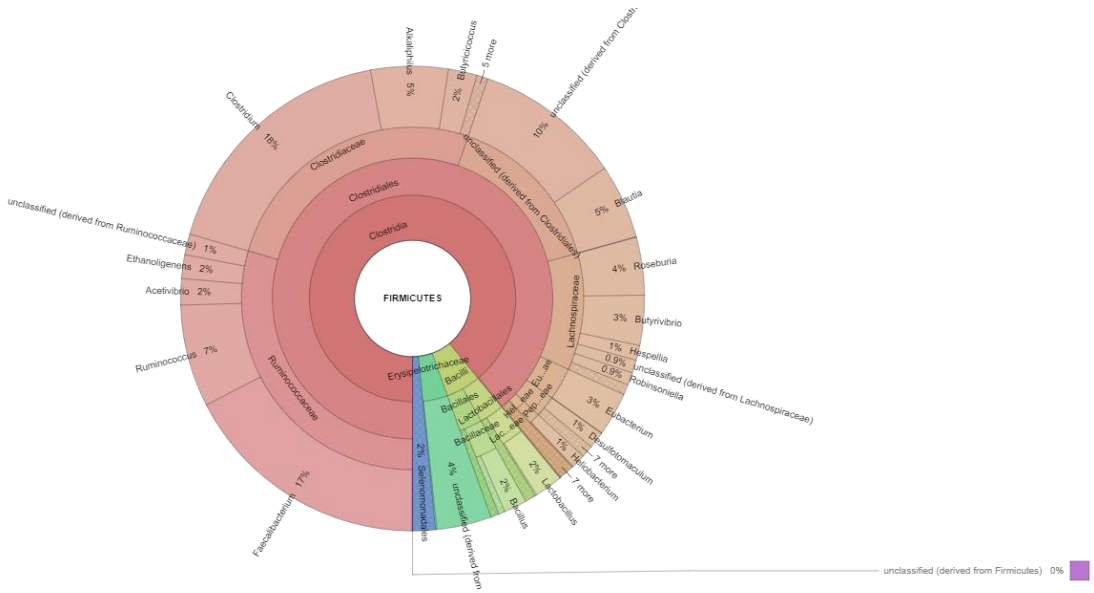

Figure 3b: **Genera of *Firmicutes* phylum in CFG group. (Data generated using RDP of MG-RAST with a minimum *e* value of 1E-5. The charts visualize classification levels of Phylum, Class Name, Order, Family and genera)**

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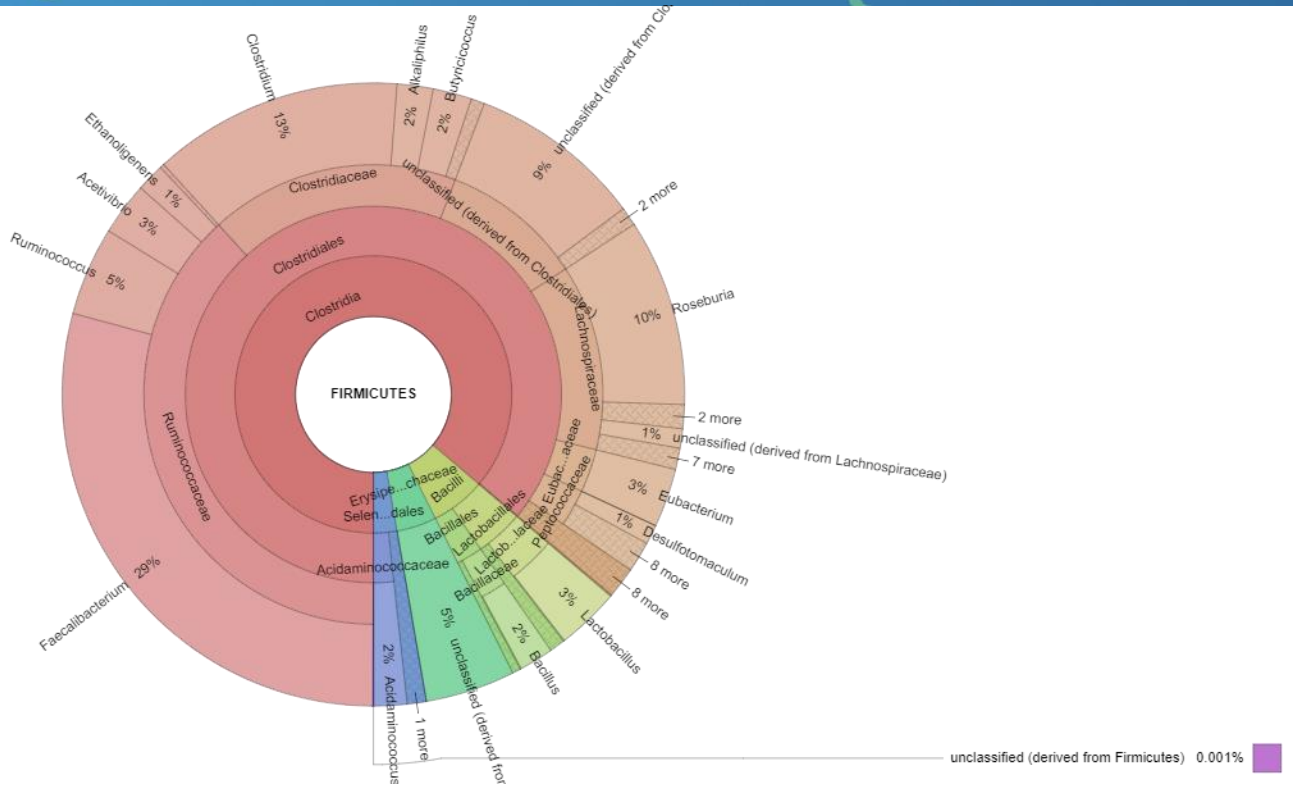

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Figure 3c: **Genera of *Firmicutes* phylum in CON group. (Data generated using RDP of MG-RAST with a minimum *e* value of 1E-5. The charts visualize classification levels of Phylum, Class Name, Order, Family and genera)**

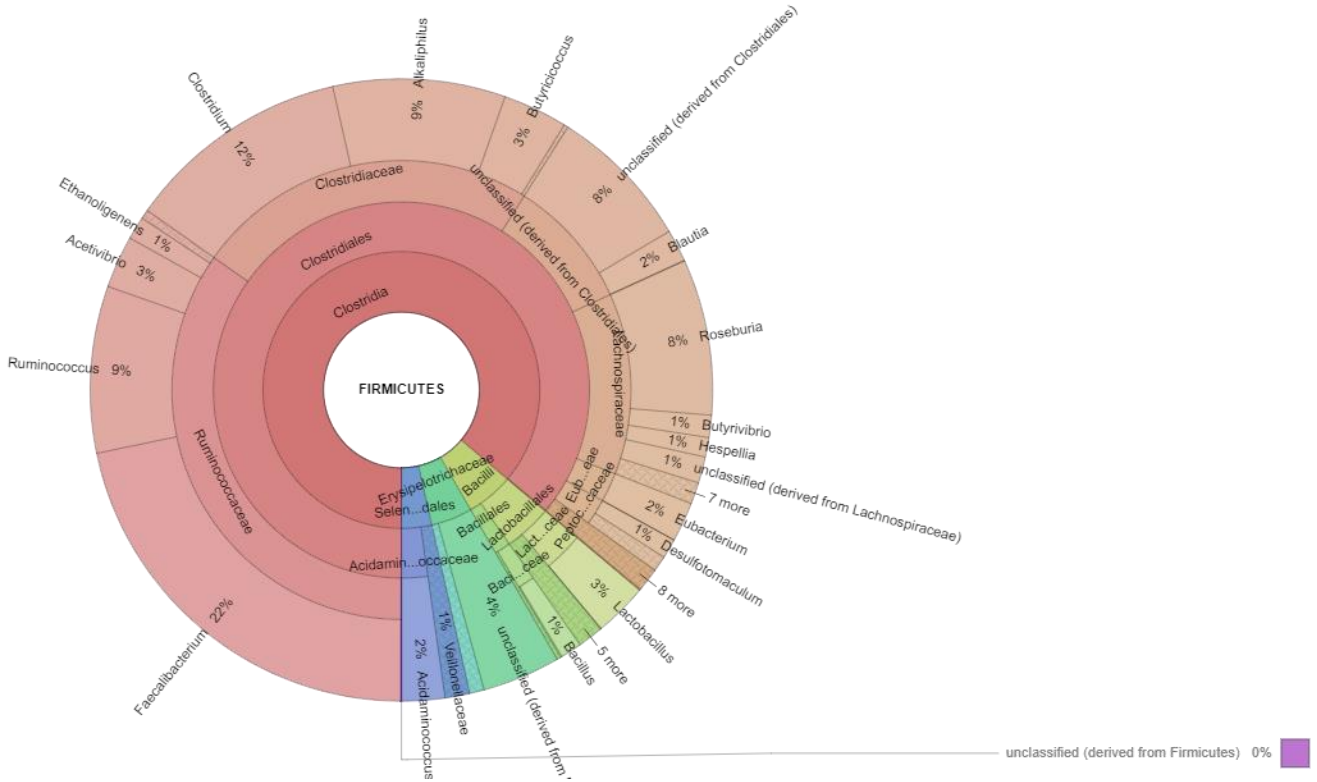

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Figure 4a: **Genera of *Bacteroidetes* phylum in ACFG group. (Data generated using RDP of MG-RAST with a minimum *e* value of 1E-5. The charts visualize classification levels of Phylum, Class Name, Order, Family and genera)**

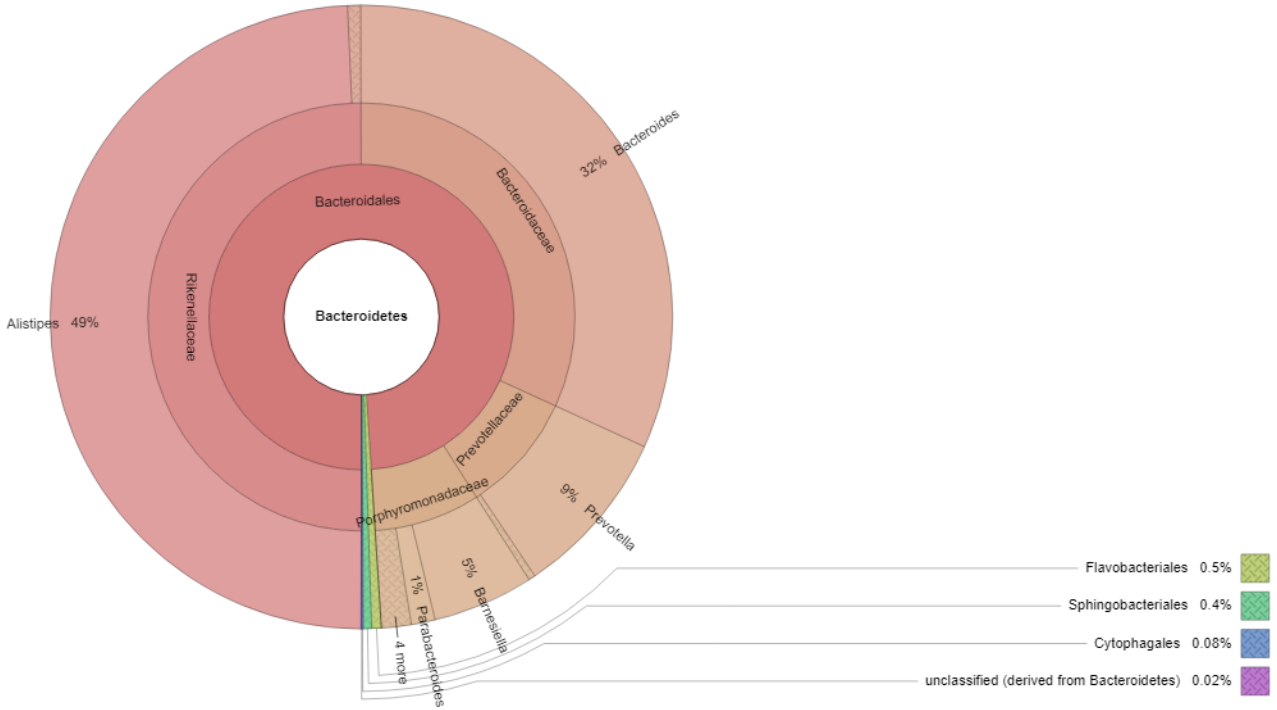

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Figure 4b: **Genera of *Bacteroidetes* phylum in CFG group. (Data generated using RDP of MG-RAST with a minimum *e* value of 1E-5. The charts visualize classification levels of Phylum, Class Name, Order, Family and genera)**

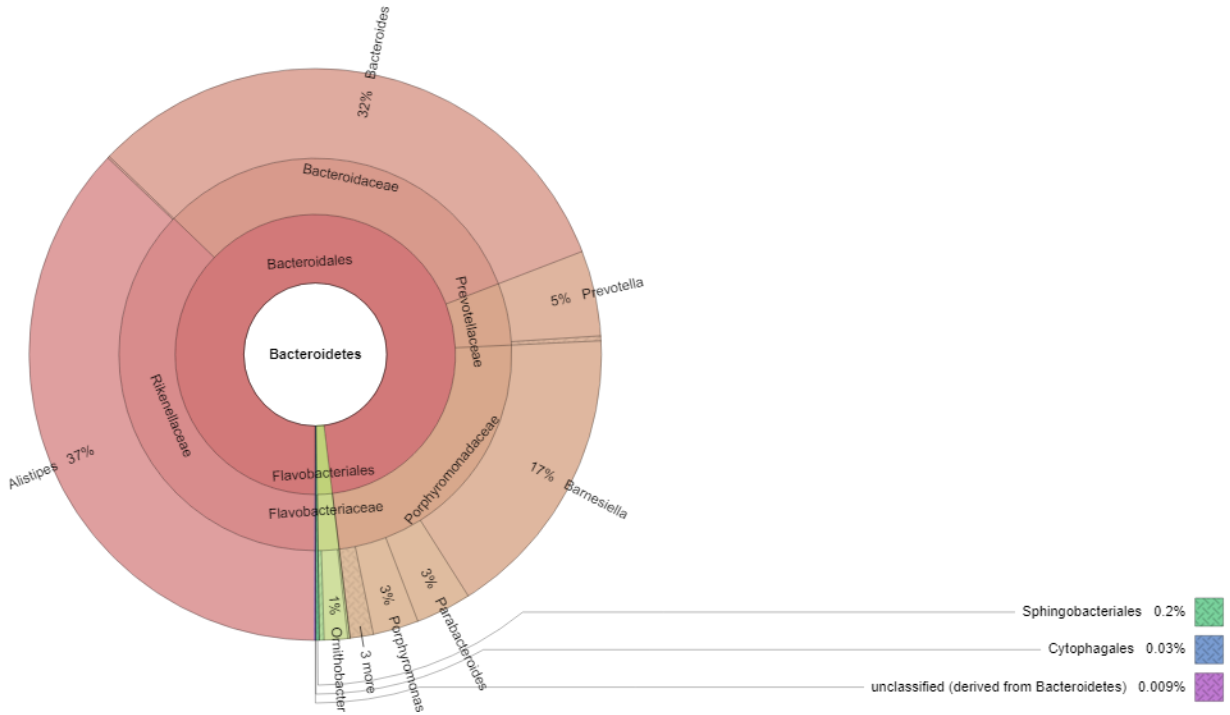

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Figure 4c: **Genera of *Bacteroidetes* phylum in CON group. (Data generated using RDP of MG-RAST with a minimum *e* value of 1E-5. The charts visualize classification levels of Phylum, Class Name, Order, Family and genera)**

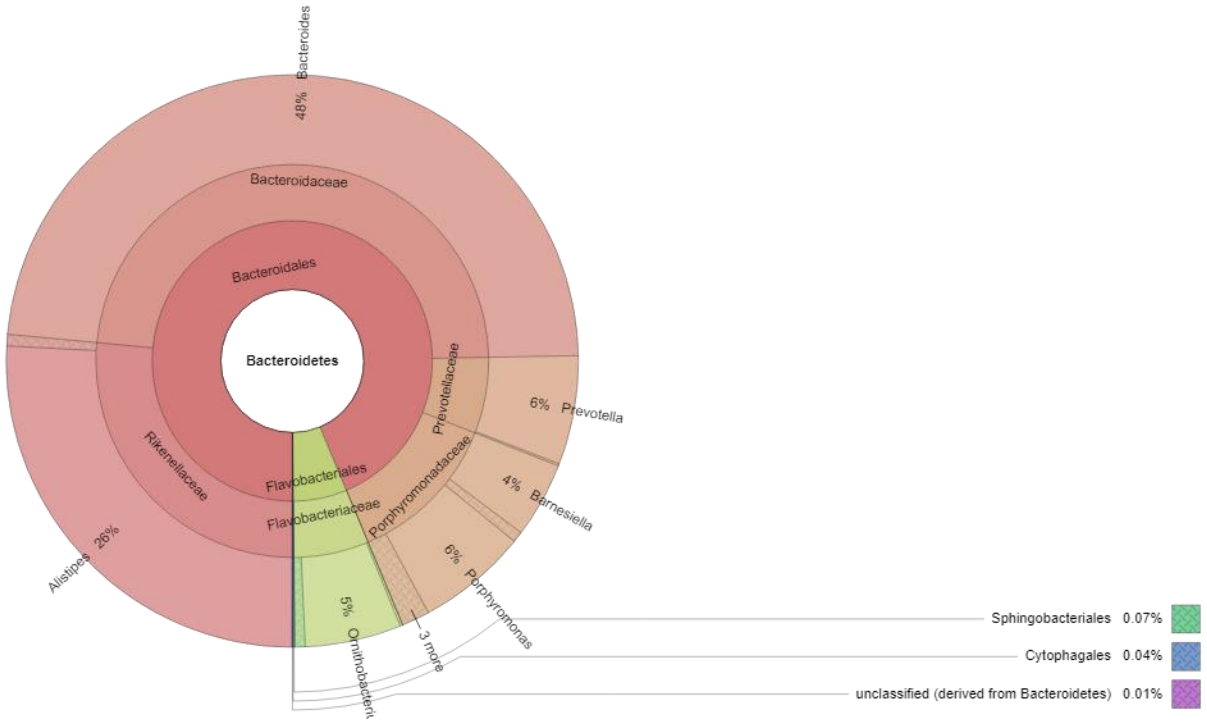

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Figure 5a): Hierrarchial clustering at phylum level among various groups using Bray-Curtis Similarity Index and Paired group (UPGMA) algorithm. Data generated using RDP of MG-RAST with a minimum *e* value of 1E-5.

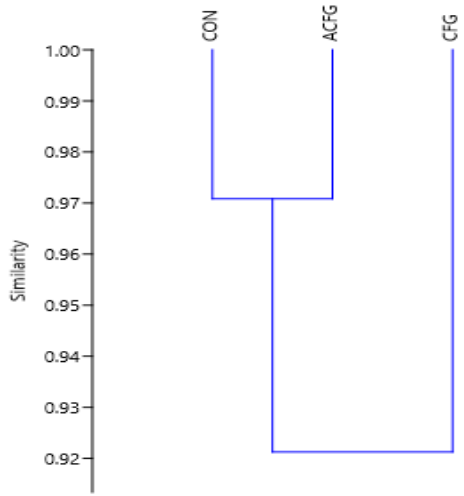

Figure 5b): Hierrarchial clustering at genus level among various groups using Bray-Curtis Similarity Index and Paired group (UPGMA) algorithm. Data generated using RDP of MG-RAST with a minimum *e* value of 1E-5.

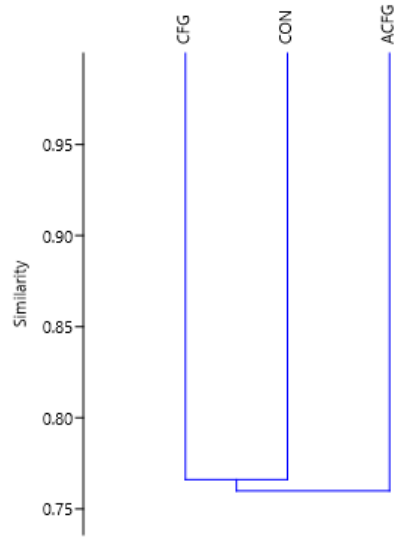

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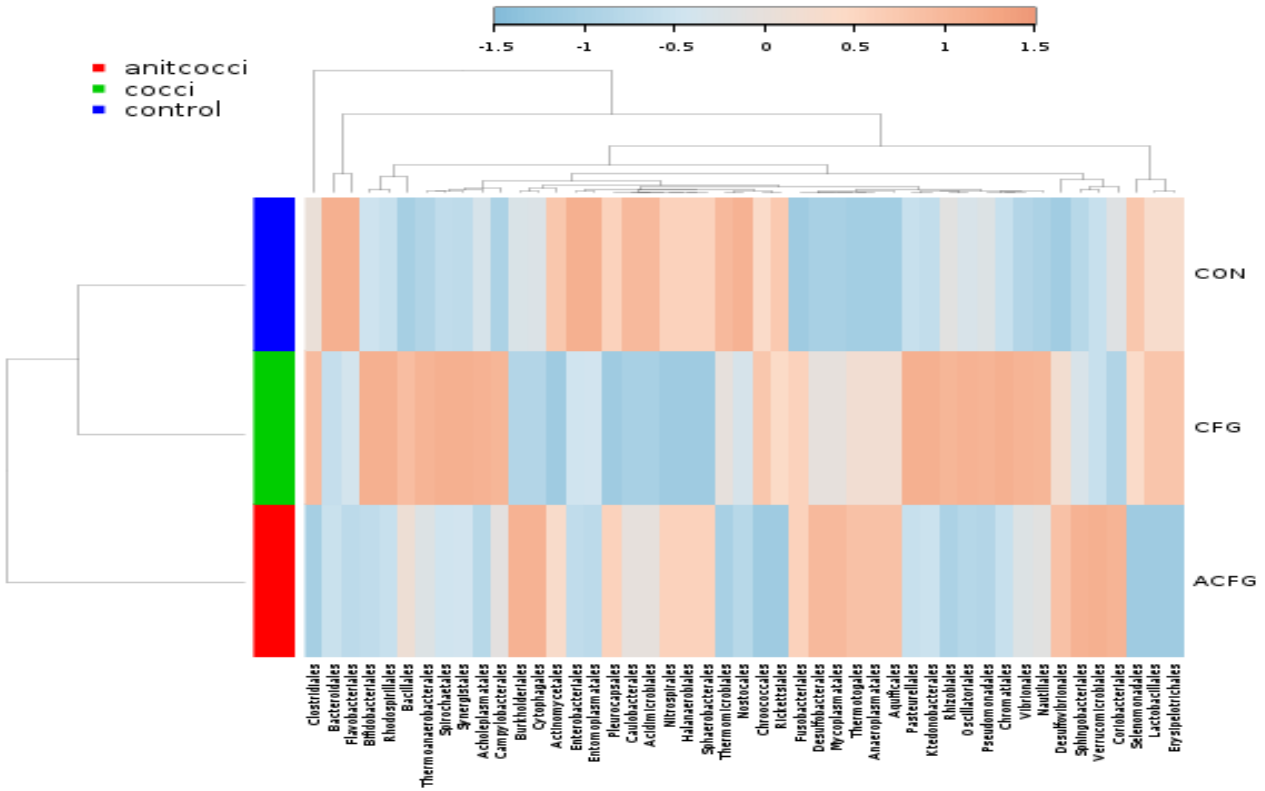
Figure 6: Heat map for relative abundance data of order level among various groups. Data generated using RDP of MG-RAST with a minimum *e* value of 1E-5 using Pearson (anticoc:ACFG group, cocci: CFG group; control: CON group)



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