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Comparative Analysis of Chicken Cecal Microbial Diversity and Taxonomic Composition in Response to Dietary Variation using 16S rRNA Amplicon Sequencing

Zubia Rashid (zubia.rashid2@gmail.com)
Karachi Institute of Biotechnology and Genetic Engineering https://orcid.org/0000-0002-8753-8385
Muhammad Zubair Yousaf
Forman Christian College University
Syed Muddassar Hussain Gilani
University of Karachi
Sitwat Zehra
Karachi Institute of Biotechnology and Genetic Engineering
Ashaq Ali
Wuhan Institute of Virology
Abid Azhar
Karachi Institute of Biotechnology and Genetic Engineering
Saddia Galani
Karachi Institute of Biotechnology and Genetic Engineering

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Abstract

Antibiotic resistance poses a serious threat to human and animal health. As a consequence, their use in conventional poultry feed may be replaced by non-antibiotic additives (alternatives to antibiotics, ATAs). Phytogenic feed additives and organic acids have been gaining considerable attention that could abate the proliferation of pathogenic bacteria and strengthen gut function in broiler chickens. The aim of this study was to evaluate the effects of phytogenic feed additives and organic acids on cecal microbial diversity using 16S rRNA amplicon sequencing of the V3-V4 region. In this study, 240 chicks were divided into five treatments comprising: a controlled basal diet (CON), antibiotic group (AB), phytogenic feed additives (PHY), organic acids (ORG) and a combination of PHY + ORG (COM). A distinctive microbial community structure was observed amongst different treatments with an increased microbial diversity in AB, ORG and COM ($p < 0.05$). The synergistic effects of PHY and ORG increased the population of beneficial bacteria that belonged to the phyla: *Firmicutes*, *Bacteroides* and *Proteobacteria* in the cecum. The presence of the species *Akkermansia muciniphila* (involved in mucin degradation) and *Bacillus safensis* (a probiotic bacterium) were noticed in COM and PHY, respectively. Clustering analysis revealed a higher relative abundance of similar microbial community composition between AB and ORG groups. In conclusion, treatments with PHY and ORG modified the relative abundance and presence/absence of specific microbiota in the chicken cecum. Hence, cecal microbiota modulation through diet is a promising strategy to reduce cross-contamination of zoonotic poultry pathogens.

Introduction

Establishing the provision of safe food to ensure the standard requirements of food safety is a major public health concern globally. Enormous increase in human population, urbanization and income levels contribute to the rise in demand of protein, hence livestock, chicken is the prime source of animal protein [1, 2]. Globally, more than 65 billion chickens are raised annually for meat production which is expected to get 16% higher by 2030 [3]. To address market demand in shortest possible time frame, intense farming practices applied by the producers depends on the incorporation of antibiotics in feed to increase growth performance and prevent infectious diseases. With the emergence of antibiotic resistant organisms and associated genes [4], the European Union has withdrawn approval of antibiotics from poultry [5]. Since, diet has a prominent role in functionality and development of gastrointestinal tract. Dietary nutrients are capable of modifying microbial dynamics in gut. The search for economical, sustainable, safe and less-food competing non-antibiotic feed additives (alternatives to antibiotics, ATAs) in poultry are progressively escalating [6].

Phytogenic feed additives have been obtaining considerable attention lately due to their ability in improving growth performance by sustaining balanced gut ecosystem [7]. The principle role of phytogenic feed additives is the reduction of pathogenic bacteria and effective modulation of favorable intestinal microflora. The antimicrobial effects of phytogenic feed additives are supremely attributed to phenolic compounds and their activity on pathogens along with anti-inflammatory and antioxidative qualities [8]. Investigations conducted on using phytogenic feed additives in broiler chicken demonstrated

their antimicrobial effects on poultry pathogens, such as *Clostridium perfringens* and *Escherichia coli*, reducing the risk of necrotic enteritis and colibacillosis [9–11]. Organic acids, such as acetic, propionic, formic, caprylic, citric, lactic, and benzoic acids are naturally generated in the animal intestinal tract due to microbial fermentation [12, 13]. They are widely used in animal production systems as dietary alternatives to antibiotics for their potential to improve host health, growth performance, amino acid, protein and mineral digestibility, digestive enzymes activities and secretion, maintain gut microecological balance [14] and exert antimicrobial activity against poultry pathogens such as *Salmonella* spp., *Escherichia coli* [15], *Clostridium perfringens* [16], and *Campylobacter jejuni* [17–19].Combination of organic acids and phytogenic feed additives has proven to be efficacious according to the reported synergism between them [20]. In fact, phytogenic feed additives increase permeability of intestinal cell membrane which allows organic acids to easily diffuse into microbial cells. Recently, blend of phytogenic feed additives and organic acids reduced the colonization of pathogenic bacteria (*Salmonella* spp. and *Escherichia coli*) in upper and lower intestine of chicken [21, 22].

The animal's gastrointestinal tract is densely populated with trillions of bacteria outnumbering the host cells [23]. Gut microflora, also known as "forgotten organ", helps the host in numerous ways including absorption of nutrients, angiogenesis, fortification of intestinal barrier, enhancement of immunity, xenobiotic metabolism and combating infections [24, 25]. A diversified microbial composition found in chicken gut which is profound in the cecum and exploration of cecal microbiota diversity could be of great help to unravel their benefits or harmful impacts on host [26]. The promising molecular techniques paved the way to decipher the secret gardens of bacteria. The current era is dedicated to high-throughput sequencing or commonly called as next-generation sequencing (NGS). The NGS technologies (Illumina, 454 pyrosequencing, ABI SOLiD etc.) enable us to unleash the microbial world from any environment [27]. One of the interesting aspects of bacterial genome is the 16S rRNA gene. This gene composed of nine hypervariable regions and the amplicon sequencing of one or more region(s) can detect up to single bacterial genera or species directly from the sample [28, 29]. The choice of hypervariable region is critical. It has been reported that amplicon sequencing of V3-V4 region (469 bp) exhibit the most accurate and reliable bacterial taxonomic identification [30, 31].

On the basis of above reported background, the current study aims to evaluate role of phytogenic feed additives and organic acids as dietary alternatives to antibiotics on cecum microbiota using 16S rRNA amplicon sequencing in broiler chicken.

Methods

Birds, Diets and Experimental Design

Two hundred and forty (240) day old broiler chicks (Hubbard strain) were purchased from a commercial hatchery and reared over 42 days experimental period (IBW = 42.0 g). The chicks were randomly assigned to dietary treatments (3 replicate pens; 16 birds/pen) which are 1) CON: a typical and commercial basal broiler diet without any supplementation 2) AB: basal diet supplemented with sub-therapeutic dose of

enramycin (125 g/tonne diet) 3) PHY: basal diet supplemented with 2 kg phytogenic feed additives/tonne (garlic 10%, cinnamon 10%, peppermint 10%, black cumin 15% & green tea 10%) 4) ORG: basal diet supplemented with 2 kg organic acids /tonne (citric acid 5%, formic acid 26.5%, medium-chain fatty acids (MCFA) 13.1%, lactic acid 16% & mono-di & triglycerides 3.5%) and 5) COM: basal diet supplemented with 2 kg organic acids + phytogenic feed additives/tonne (1 kg each). Chicken diets mainly formulated based on corn and soybean (Table 1) and birds had free access to diet and water *ad libitum*.

Table 1
Ingredients and their compositions

Nutrient composition	0–21 d	21–42 d
Metabolize energy (MJ/kg)	12.1	12.9
Crude protein (%)	21.5	19.1
Calcium (%)	1.0	0.8
Available phosphorous (%)	0.4	0.3
Dig. lysine (%)	1.1	1.0
Dig. methionine (%)	0.5	0.4
Dig. methionine + cysteine (%)	0.8	0.7
Dig. tryptophan (%)	0.2	0.1
Dig. L-threonine (%)	0.8	0.7

Table 2
Operational taxonomic units (> 0.05% coverage) and diversity indices from cecal samples in different study groups

Study Groups	Reads	OTUs	Chao1	ACE	Shannon	Inverse Simpson
CON	72450	280	293.00	294.22	2.15	0.35
AB	104943	292	298.00	299.61	3.27	0.11
ORG	71774	269	288.50	282.72	3.73	0.05
PHY	67241	41	64.75	85.26	0.01	0.99
COM	142660	313	317.23	316.59	3.59	0.07

CON: control, AB: antibiotic group, PHY: phytogenic feed additives, ORG: organic acids, COM: combination.

Chickens were raised under controlled environmental conditions with rice hulls as litter. Lightning was provided up to 32°C on day 1 and then, gradually decreased to 24°C on day 42. All the pens, feeders, and troughs were cleaned and disinfected before the arrival of birds.

Sample Collection and DNA Extraction

Three birds were selected randomly from each replicate group of every treatment and sacrificed on day 42. Cecal contents were collected aseptically to minimize individual variations. Samples were immediately stored in sterile cryogenic vials, snap-shot in liquid nitrogen, transported to the laboratory and frozen at -80°C until DNA extraction. Total bacterial genomic DNA was extracted from 180-220 mg cecal samples using QIAamp DNA stool mini kit (QIAGEN, Germany) following the manufacturer's instructions with some modifications. Initially, cecal samples were treated with lysozyme 25mg/mL (Sigma-Aldrich, USA) in cell lysis buffer (0.5 mM EDTA pH 8.0; 20mM Tris-HCl pH 8.0; 1% Triton X-100) at 37 °C for 30 minutes. Samples were also treated with DNase free RNAse (GeneDireX, USA) for 30 minutes at 37°C. DNA samples were stored at -20 °C until further analysis.

Amplification of V3-V4 region of 16S rRNA Gene and Illumina Sequencing

Microbial diversity in chicken cecum samples was estimated by amplifying V3-V4 hypervariable regions of 16S rRNA gene using forward 5' CCTACGGGAGGCAGCAG 3' and reverse 5' ATTACCGCGGCTGCTGG 3' primers. The strategy of the dual-index paired-end sequencing approach was used in the study. The designed fusion primers included P5 and P7 Illumina adapter sequences, 8 nt-unique index sequence followed by gene-specific primers. PCR amplification was performed in 50µL reaction containing 25µL NEB Phusion high-fidelity PCR Master Mix (New England Biolabs, USA), 4µL PCR primer cocktail (primer 1 100µM, primer 2 100µM, adapter 0.5 µM) and 30ng/ µL DNA template. The reaction conditions include initial denaturation for 3 minutes at 98 °C, then denaturation for 45 seconds at 98 °C, annealing at 55 °C for 45 seconds, extension at 72 °C for 45 seconds for 30 cycles followed by a final extension at 72 °C for 7 minutes. The purification of PCR products was performed by AMPure XP beads (Agencourt, Beckman Coulter, USA). The concentration of individual libraries in nM was determined by estimating the size of amplicons using Agilent 2100 bioanalyzer (Agilent, Canada), and libraries were quantified by real-time quantitative PCR (qPCR) (EvaGreenTM, California, USA). The qualified libraries were sequenced using Illumina MiSeq Sequencer (2 x 300 bp paired-end run) at BGI Genomics, Hong Kong.

Bioinformatics Analysis

The raw data reads were filtered to minimize adapter pollution and low-quality reads subsequently, paired-end reads were merged into tags. The consensus sequence of two overlapped paired-end reads was generated by Fast Length Adjustment of Short reads (FLASH) software (version 1.2.7) [32]. Briefly, sequence reads were trimmed for removal of adapter contamination with maximal 3 bases mismatch permitted. Sequences were truncated for not having an average quality of 20 in a sliding window of 25bp based on *phred* algorithm. The reads with ambiguous bases and low complexity (reads with 10

consecutive same bases) were removed. Paired-end reads after sequencing were analyzed on Quantitative Insights into Microbial Ecology (QIIME) (version 1.8.0) [33].

The paired-end reads were assembled without any quality trimming because error correction was automatically conducted by FLASH before assembly. The assembled reads were denoised using UCHIME algorithm [34] and screened for removal of chimera by mapping to Genomes OnLine Database (GOLD) from UPARSE (version 7.0.1001) [35] for further analyses. The quality-filtered tags were clustered into operational taxonomic units (OTUs, 97% threshold) by using UPARSE and unique OTU representative sequences were obtained. Taxonomic ranks of OTU representative sequences were assigned using the Ribosomal Database Project (version 11) [36] trained against Greengenes datasets with the cut off value of 0.6. For downstream analysis, OTUs were further denoised by discarding rare OTUs representing less than 0.005% of all sequences.

Statistical and Ecological Analysis

Alpha diversity analysis on OTU data showing the microbial community diversity (Shannon and Inverse Simpson) and richness (number of OTUs observed, Chao1 and ACE) were calculated [37]. The rarefaction curve depicting observed OTUs was drawn by software R (version 3.1.1). Beta diversity analysis was conducted to evaluate species complexity differences among treatments. Beta diversity analysis was executed using QIIME and heat map was drawn by software R. Euclidean distance algorithm was performed in software R and the clustering method was 'complete'. Heat map was created in 'gplots' package (software R) with the individual values were transformed to log scale (log 10), prior to heat map generation, and indicated as color key in matrix. The dendrogram evinced phenetic relationship among microbial communities on longitudinal axis while, clustering of groups in different clades were exhibited horizontally. Beta diversity analysis included Bray-Curtis distance (to reflect the difference between two microbial communities) [38], weighted UniFrac (depicting abundance of species) and unweighted UniFrac (comparison of microbial community structure) [39]. Furthermore, Principal component analysis (PCA) plot was drawn illustrating differences among samples according to unweighted UniFrac distance matrix. The closer distance exhibit similar species composition in samples. Based on beta diversity matrix, hierarchical clustering using Unweighted pair group method with arithmetic mean (UPGMA) was performed and a phylogenetic tree was constructed by software R. The analysis method was jackknifing in which 75% of sample sequences were randomly chosen and UPGMA tree from this data was compared to the entire data set in QIIME with repeating the process 100 times randomly. The unique/shared OTUs from aligned sequences were listed and Venn plot was generated using Venn diagram package in R. Hierarchical clustering based on PCA analysis performed for taxonomic assignments of microbial communities to determine differences in levels of classification. A cladogram was constructed in QIIME by aligning representative sequences of abundant genus against SILVA core using Python-based implementation of the Nearest Alignment Space Termination (PyNAST) tool, implemented in the QIIME with a 200-bp minimum length and 75% minimum per cent identity [40]. The relative abundances of each bacterial taxon were compared by conducting Wilcoxon signed rank test. The separation of beta diversity

indices was established by Permutation Multivariate Analysis of Variance (PERMANOVA) (version 4.4.3.4) [41]. The significance was assumed at $p<0.05$.

Ethical Approval

This study was approved by The Karachi Institute of Biotechnology and Genetic Engineering (KIBGE), University of Karachi ethical review board. The animal trial was conducted under the standard operating procedure of guide for the care and use of agricultural animals in agricultural research and teaching [42].

Results

Sequence Data Analysis and Quality Filtering

A total of 1,262,784 paired-end reads were generated from five cecal samples of study groups. After quality filtration, 1,083,070 reads remained with average read number per chicken 96,823 (± 34,814 s.d.) having median read length of 458 (± 9 s.d.) bases in all samples. Altogether, 1,195 distinct OTUs at 97% similarity index threshold were obtained from all samples, out of which, 895 remained for downstream analyses.

Dietary Variation in the Cecal Microbiota

Rarefaction analysis of OTUs from cecal samples indicated good sequencing coverage (~ 90%) in all samples (Figure 1). The cecal microbial diversity among study groups was measured through alpha and beta diversity. Alpha diversity was estimated by using Chao1, ACE, Shannon, Simpson, and number of observed species (Table 2). Alpha rarefaction curves of observed species, chao1, ACE, shannon and simpson against number of sequences in study groups are shown in (Online Resource 1). The ACE and Shannon indexes described higher species richness ($p<0.05$) in COM, AB, and ORG groups. Simpson index illustrated indicated species evenness ($p<0.05$) in phytogenic feed additive (PHY) group. Furthermore, the higher bacterial diversity was achieved in COM and AB groups. Beta diversity analysis were executed through weighted/unweighted UniFrac and Bray-Curtis distances by using PERMANOVA and UPGMA tests to generate heat map and cluster tree. Bray-Curtis and weighted UniFrac distances are quantitative beta diversity traits showing significant differences ($p<0.001$) between CON and feed additive (AB, ORG, PHY and COM) groups. Cluster analysis revealed clear distinction related to abundance of similar species in CON and PHY groups (Figure 2a). Unweighted UniFrac diversity distance illustrated pronounced microbial community presence in PHY group ($p<0.001$) and cluster analysis also placed PHY group in a separate node (Figure 2b). Similar results with Bray-Curtis diversity index was achieved placing PHY in separate branch with CON (Figure 2c). The diversity differences were further exemplified by principal component analysis (PCA) which demonstrated clear demarcation among bacterial assemblages of all groups along principal component axis 1 of PCA plot. The total variance of 83.96% was apparent in cecum samples along the two axes of PCA (Figure 2d). Given that dietary variation contributed to the shifts in microbial communities in chicken cecum, we further evaluated the shared/unique OTUs in cecum samples (Figure 2e). In total, 31 OTUs were shared across all study

groups, accounting for 3.5% of total OTUs, which represents “core” microbiota in chicken cecum. The analysis of shared microbial communities between each study groups were also analyzed as shown in figure (Online Resource 2).

Hierarchical Clustering of Microbiome in Chicken Cecum Across Dietary Treatments

The bacterial community distribution pattern under various taxonomic classification levels encompassing phylum, class, order, family, genus, and species were compared against RDP classifier database using PCA. The total quality filtered reads (1,083,070) from cecal samples were assigned to 1,195 OTUs, which further distributed into ten phyla and one unclassified phylum (Figure 3). The dominant phylum in chicken cecum was *Firmicutes* in all groups with higher abundance in PHY (99%) and CON (64%) groups. The second most abundant phylum was *Bacteroides* followed by *Proteobacteria*, *Deferribacteres*, *Synergistetes* and *Verrucomicrobia* with relative abundance of 24.6%, 9.8%, 4%, 3.8% and 2% respectively. *Bacilli* (70-90%) was the abundant class in PHY and CON groups from phylum *Firmicutes*, with the members of order *Bacillales* being most prominent. Interestingly, a diverse bacterial community distribution at class level was noticed in COM, AB, and ORG groups. *Clostridia* was the most abundant class in all study groups with relative abundance of 56%, 50% and 35% in AB, ORG and COM groups respectively. Class *Bacteroidia* was the third most abundant class in chicken cecum (Figure 4). Order *Bacteroidales* and *Clostridiales* were the abundant orders in COM, AB, and ORG groups. The presence of orders *Acidaminococcales* and *Burkholderiales* were also noticed in ORG (Figure 5). Notably, a large proportion of unclassified OTUs at order level were seen in COM and ORG groups. The taxonomic composition at family level showed highly diversified bacterial community in AB, ORG and COM groups (Online Resource 3). *Campylobacteraceae* and *Bacteroidaceae* (20-30%) were prominent families in AB. At genus level, PHY group was dominated by *Bacillus* (99.2%) from family *Bacillaceae* (Online Resource 4). The abundant bacterial genera in all samples except PHY were *Helicobacter, Blautia, Ruminiclostridium, Campylobacter, Desulfovibrio* and *Bacteroides*. The only species found in PHY group was *Bacillus_safensis*_FO-36b (Figure 6). The dominant species in CON and AB was *Bacteroides_barnesiae* (20-30%). ORG group showed high abundance of unclassified species (58%) along with *Helicobacter_pullorum*_NCTC_12824 (18%), *Ruminococcus_torques*_ATCC_27756 (7%) and *Mucispirillum_schaedleri* (9%).

Abundance Differences of Microbiota Among Study Groups

To further investigate the differences in relative abundance of microbiota and their association with the study groups, heat maps were drawn. The heat map showed presence of all bacteria at phylum level ($p<0.05$) in groups (Figure 7a) whereas species showing low abundance (less than 0.5) were later classified as ‘others’ in subsequent ranks. Phytogenic feed additives showed highest value of 1 for phylum *Firmicutes* and formed distinct branch depicting presence of discreet bacterial phyla composition. Based on the occurrence of microbiota at class level, COM and ORG were placed in a similar branch in clustering analysis showing relative abundance of similar bacteria (Figure 7b). At order level, AB and ORG groups shared phenetic relatedness (Figure 7c). Similarly, presence of order

Verrucomicrobiales was only noticed in control and combination groups. The presence of family *Akkermansiaceae* was noticed in COM group only with high abundance (Figure 7d). The pattern of bacterial families present in AB and ORG group were similar in relative abundance. In COM, presence of genus *Akkermansia* (family *Akkermansiaceae*) was observed (Figure 7e) with the presence of species A*kkermansia_muciniphila_*ATCC_BAA-835. Surprisingly, AB and COM groups shared similar branch in heat map at species level depicting species richness in both groups (Figure 7f) thus, validating the synergistic effect of natural feed additives in maintaining bacterial diversity in chicken cecum.

Phylogenetic Analysis of Dominant Bacterial Genera in Chicken Cecum

Besides species composition and abundance analysis, a cladogram was constructed from the tags of abundant bacterial genera to elucidate the species relatedness present in chicken cecum (Figure 8). Majority of the genera belonged to phylum *Firmicutes* (56%) which was bifurcated into two branches. The first branch having genus *Ruminiclostridium* showed evolutionary relatedness with genera from *Proteobacteria* (15%) whereas the second branch (*Lactobacillus*, *Enterococcus*) formed a distinct clade. Phylum *Bacteroides*, second most abundant phylum (17%), formed separate clade depicting phenetic relatedness with phylum *Lentisphaerae*. Phylum *Deferribacteres* and *Synergistetes* were closely related with each other while, *Elusimicrobia* formed a discreet branch in cladogram.

Discussion

In-depth investigation of gut microbiota is paramount to infer their contribution in host health. The most recent studies on chicken gut bacteria emphasize using culture-independent approaches for taxonomic profiling of co-metabolizing microbial community [43]. Nevertheless, available literature on chicken gut bacteria composition mainly related to culture-dependent procedures [44,45] and fingerprinting techniques [46-48]. Moreover, on the subject of gut microbiota characterization, studies involving variation in environmental factors (climatic region, breed, biosecurity measures, production systems, genetics, diet etc.) which promote ‘beneficial gut microbiota’ are scarce [49,50]. To define ‘beneficial gut microbiota’ is challenging, but comparison of gut microbiome structure and composition that have been linked with improved host performance, health, and immunity enhancement may provide valuable information coupled with genomic analyses for identification of variation(s) in host sequence associated with gut microbiota composition [51]. In addition, modulation of gut microbiota with dietary changes, preferably alternative to antibiotics, can provide another strategy for improvement of poultry production and health. Previously reported role of phytogenic feed additives and organic acid alone on gut bacteria modulation in chicken demonstrated promising results [52,53]. However, characterization of chicken cecal bacteria by adding phytogenic feed additives and organic acids in combination using next-generation sequencing are still unclear. Here, we used high-throughput sequencing of V3-V4 region of 16S rRNA gene to examine cecal microbiota of chickens fed either sub-therapeutic level of enramycin (antibiotic growth promoter), phytogenic feed additives, organic acids, or combination of phytogenic feed additives + organic acids over a 42 days production cycle. Sequence of 16S rRNA gene has extensively analyzed for phylogenetic organization of metagenomes. Given the fact that 16S rRNA gene consists of nine

hypervariable regions, and available sequences data generated employing any of these regions alone or in combination generally reveal differences in microbial diversity profiling. Hence, optimal selection of hypervariable region(s) and combination of primers differ among various ecological communities. Literature integrating V3-V4 region and longer Illumina MiSeq read chemistry present better resolution of microbial diversity, OTU classification and species richness, that may validate or rectify some of the discrepancies between culture-dependent and independent evaluation of chicken gut microbiota [29,54]. Therefore, in the present study, primer set targeting V3-V4 regions of 16S rRNA gene was selected to investigate chicken gut microflora.

Based on our findings, characteristic differences in cecal microbial diversity and richness among different study groups were noticed. This was displayed based on diversity indices (Table 2) and rarefaction curve (Figure 1) where, COM has significantly higher richness, variation, and diversity in microbial community. Rarefaction curve of observed OTUs revealed good sequence depth in samples except ORG, which showed lower coverage of OTUs. Alpha diversity indices (ACE, Chao1, Shannon and Simpson) also indicated higher microbial diversity in COM group. Amongst the few available 16S rRNA amplicon studies using phytogenic feed additives and organic acids in chicken, Chang et al. reported feeding with *Bidens pilosa* (medicinal plant) successfully altered chicken gut dynamics with increased microbial diversity richness which correlated positively with host weight gain [55]. Abdelli et al. investigated effects of microencapsulated blends of organic acids (fumaric, citric and lauric acid) and aromatic compounds (cinnamaldehyde, carvacrol and thymol) on chicken cecal microbiota and found higher species richness in chickens supplemented with microencapsulated blend compared to control [56].

Beta diversity analysis revealed that supplementation with PHY produced a distinct shift in microbiota compared to CON and AB groups (Figure 2a,b,c). Principal component analysis showed no overlapping among samples. The variation in different axes of principal component analysis plot explained that beta diversity between AB and COM was small thus, modulation of cecal microbiota by both groups was analogous. Generally, the digestion process in the fore- and midgut influences the microbiota of hind gut [57]. Moreover, the quantity and type of nutrients reaching the ceca and hind gut might have been affected by ORG and PHY supplementation. The components used in ORG and PHY groups have been reported to possess antibacterial effects. Green tea, being an active constituent of phytogenic feed additives, has reported to exert strong antioxidant and anti-inflammatory properties and improved performance in chickens through regulation of cecal microflora [58]. These effects might have impacted the cecal metagenomic profile of ORG, PHY and COM treated groups.

The present work is mainly focused on cecal microbiota because cecum has higher microbial diversity and thus, involved in fermentation. Also, cecal microbiota has significant roles in breakdown of starch, polysaccharides, cellulose, and uric acid [59,60]. Short chain fatty acids (SCFAs) in the intestine are the end-products of microbial fermentation, which regulates immune responses, blood flow and mucin production [61]. Butyrate, a prominent SCFAs, is the prime energy source for colonocytes and enterocytes. It is also involved in mucin synthesis, intestinal motility, cell differentiation and proliferation while, suppressing inflammatory disorders [62]. Thus, augmenting the growth of butyrate-producing bacteria

would be beneficial for gut health and productivity. It should be noted that findings of this study might differentiate from the previous work because of variation in diet and the differences in sequencing approach. Based on current study, *Firmicutes* was the most dominant phylum in chicken cecum among all groups (Figure 3 &8). These findings agree with the previous reports which described predominant bacterial sequences belonged to phylum *Firmicutes* (50%) in cecum as chicken aged (day 42) [63-65]. However, these findings differ from the cecal sample analyzed by Pandit et al. who highlighted that *Bacteroidetes* was the dominant phylum in chicken cecum followed by *Firmicutes* and Proteobacteria [51]. The class-level analysis depicted abundance of *Clostridia* in AB, COM, and ORG (Figure 4) with genera *Blautia*, *Faecalibacterium*, *Ruminococcus*, *Lachnoclostridium* mainly dominated. *Clostridia* are group of gram-positive rod-shaped bacteria. Among these, *Blautia, Ruminococcus* and *Lachnoclostridium* are butyrate-producing bacteria with beneficial contribution to host [66]. At order level, *Clostridiales* and *Bacteroidales* (Figure 5) are dominant with the higher proportion of *Clostridiales* in a similar manner as previously reported [51]. Order *Clostridiales* (class *Clostridia*) is chiefly responsible for metabolism of SCFAs in chicken cecum [67]. *Bacteroidia*, third abundant order, with the genera *Alistipes, Bacteroides* and *Barnesiella* are mainly involved in the production of propionate in ceca [68]. The presence of genus *Bacteroides*, gram-negative anaerobic bacteria, was found to be consistently higher in chicken cecum as revealed by deeper investigations in similar datasets [60, 69,70]. *Bacteroides* has a role in polysaccharide degradation, mainly glucan and starch. The supplementation of PHY indicated elevated levels of family *Bacillaceae* with the dominant species *Bacillus safensis* (Figure 6). *Bacillus safensis* is a relatively newly identified bacterium [71] and has been used as probiotic in chicken. Atela et al. administered *Bacillus safensis* as alternative to antibiotics in chicken and observed significant differences in feed efficiency [72]. In addition, presence of species *Akkermansia muciniphila* was noticed only in COM group (Figure 7f). Genus *Akkermansia* is a gram-negative anaerobic bacterium involved in degradation of mucin to produce carbon and nitrogen in host [73]. Surprisingly, *Akkermansia* exhibits immunomodulatory and anti-inflammatory responses in host along with strengthening of gut barrier function [74]. The presence of *Akkermansia* in gut protects the host from obesity [75,76], IBDs [77] and diabetes mellitus [78]. The relative abundance analysis of cecal microbiota depicted comparable results of gut microbial community in AB and COM group. The high percentage of unclassified bacterial sequences were noticed in majority of samples, encouraging further investigations to identify or determine their pluses or perils in enteric microbiota.

Conclusion

Taken together, with the high-throughput 16S rRNA amplicon sequencing technique, this study profiled the microbial communities in the chicken cecum using alternative feed additives. The addition of phytogenic feed additives and organic acids affect the cecal microbial diversity in different ways. Besides, the synergistic effects of these two additives in combination reflected comparable results with antibiotic growth promoter in enhancing microbial diversity richness and the proliferation of beneficial bacteria crucial for host digestion and energy metabolism. The description in microbial variation among different groups offers a panel of bacteria that might be amenable in growth promoter selection for use in

poultry production. Therefore, the results provide a promising strategy for cecal microbiota modulation through dietary changes, and further investigations for host-microbe interactions are required for elucidating underlying mechanisms to prevent poultry from zoonotic and pathogenic organisms.

Abbreviations

ACE: Abundance-based coverage estimator, ATCC: American type culture collection, bp: base pair, EDTA: ethylene diamine tetra acetic acid, IBD: inflammatory bowel diseases, nt: nucleotide, OTUs: operational taxonomic units, PCA: principal component analysis, PCR: polymerase chain reaction, RDP: ribosomal database project, s.d.: standard deviation.

Declarations

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No funding was received for this study.

Conflicts of interest/Competing interests

Authors declare no competing interests

Ethics approval

This study was approved by The Karachi Institute of Biotechnology and Genetic Engineering (KIBGE), University of Karachi ethical review board. The animal trial was conducted under the standard operating procedure of guide for the care and use of agricultural animals in agricultural research and teaching

Consent to participate

Not Applicable

Consent for publication

Not Applicable

Availability of data and material

The raw sequence data generated from this study have been submitted to the Sequence Read Archive (SRA) database of the National Center for Biotechnology Information (NCBI) with BioProject accession number PRJNA592164.

Code availability

Not Applicable

Authors' contributions

Zubia Rashid: Conceptualization, Investigation, Methodology, Visualization, Formal analysis, writing-original draft. **Muhammad Zubair Yousaf:** Formal analysis, Writing- Review & Editing. **Syed Muddassar Hussain Gilani**: Investigation, Methodology. **Sitwat Zehra**: Investigation, Methodology. **Ashaq Ali**: Formal analysis, Writing- Review & Editing. **Abid Azhar**: Supervision, Validation. **Saddia Galani**: Conceptualization, Supervision, Writing- Review & Editing.

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