Comparative analysis of chicken cecal microbial diversity and taxonomic composition in response to dietary variation using 16S rRNA amplicon sequencing

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Received: 19 March 2021 / Accepted: 15 September 2021 / Published online: 24 September 2021
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Abstract
Background Antibiotic resistance poses a grave threat to One-Health. By replacing antibiotics with non-antibiotic additives (are alternatives to antibiotics, ATAs) like phytogenic feed additives and organic acids in poultry feed. ATAs are a potential alternative as these decline the proliferation of pathogenic bacteria and strengthen gut function in broiler chickens. In this study, we use 16S rRNA amplicon sequencing of the V3-V4 region to evaluate phytogenic feed additives and organic acids on the cecal microbial diversity of broiler chickens.

Methods and results Two hundred & forty broiler 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 increased microbial diversity in AB, ORG, and COM (p < 0.05). The synergistic effects of PHY and ORG increased bacterial population of phyla: Firmicutes, Bacteroides, and Proteobacteria in the cecum. The presence of 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.

Conclusions 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, led to healthy and economical broiler meat.

Keywords Amplicon sequencing · Broiler · Diet · Gut microbiota · Organic acids · Phytogenic feed additives

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

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Introduction

The requirement of safe and secure food, especially protein of animal origin, to the ever-increasing human population is a global challenge. Broiler chicken is the quickest and economical source of quality protein [1, 2]. Around 65 billion broiler chickens; are raised annually, and a rise of 16% is expected by 2030 [3]. Intense farming practices will result in excessive use of antibiotics in poultry feed to meet this demand. It will add fuel to the already burning issue of antibiotic resistance. The role of ATAs in this scenario will be crucial as an alternate option of feed antibiotics to increase growth performance and prevent infectious diseases in poultry [4]. The European Union has withdrawn approval of antibiotics from poultry and motivating farmers to use ATAs as a substitute. With dietary nutrients modifying microbial dynamics in the gut, the search for economical, sustainable, safe non-antibiotic feed additives in poultry is escalating [5]. Phytogenic feed additives are known to improve growth performance positively by manipulating the gut ecosystem. It is worth exploring the immunomodulation role of ATAs resulting in the decline of pathogenic bacteria and the effective enhancement of favorable intestinal microflora. The antimicrobial effects of phytogenic feed additives are mainly due to the presence of phenolic compounds and their activity on pathogens along with anti-inflammatory and antioxidative qualities. Investigations conducted on using phytogenic feed additives (thymol, carvacrol, and cinnamic aldehyde) 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 [6–8]. 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 [9, 10]. They are widely used in animal production systems as dietary alternatives to antibiotics resulting in improved digestibility of proteins and essential minerals. Additionally, they enhance the effect on digestive enzymes leads to maintain gut micro ecological balance and exert antimicrobial activity against poultry pathogens such as Salmonella spp., Escherichia coli [11], Clostridium perfringens [12], and Campylobacter jejuni [13–15].

Synergistic use of organic acids and phytogenic feed additives has proved to be very effective as they increase the permeability of intestinal cell membrane; moreover, organic acids are responsible for lowering gut pH, which allows organic acids to diffuse readily into microbial cells. Recently, a blend of phytogenic feed additives and organic acids reduced the colonization of pathogenic bacteria (Salmonella spp. and Escherichia coli) in the upper and lower intestine of chicken [16].

The chicken gastrointestinal tract is densely populated, with trillions of bacteria outnumbering the host cells. Gut microflora, known as a “forgotten organ”, helps the host in a variety of ways that include absorption of nutrients, angiogenesis, fortification of the intestinal barrier, enhancement of immunity, xenobiotic metabolism, and combating infections [17, 18]. A diversified microbial composition found in the 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 the host [19]. The promising molecular techniques paved the way to decipher the secret gardens of bacteria. The NGS (Next Generation Technologies) technologies (Illumina, 454 pyrosequencing, ABI SOLiD, etc.) unleash the microbial world from any environment [20]. The bacterial 16S rRNA gene is 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 [21]. The choice of the hypervariable region is critical. It has been reported that amplicon sequencing of the V3-V4 region (469 bp) exhibits the most accurate and reliable bacterial taxonomic identification [22, 23].

Hence, the current study aims to assess the synergistic 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 assigned randomly to dietary treatments (3 replicate pens; 16 birds/pen) that are (1) CON: a typical and commercial basal broiler diet without any supplementation (2) AB: basal diet supplemented with a sub-therapeutic dose of enramycin (125 g/tonne diet) (3) PHY: basal diet supplemented with 2 kg phytogenic feed additives/tonne (garlic 20%, cinnamon 20%, peppermint 20%, black cumin 20% & green tea 20%) (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 35% of active substances, calcium their salts and also lauric acid (C:12)) and (5) COM: basal diet supplemented with 2 kg organic acids + phytogenic feed additives/tonne (1 kg each). The antibiotic dose used in the study was selected to fulfill the requirements for growth-promoting effect based on feed analysis standards
The doses used were organic acids according to Kemira Pro GIT SF3 (Shanghai, China). Chickens were raised under controlled environmental conditions with rice hulls as litter and were fed on the commercial starter and finisher diets ad libitum (Table 1). Lightning was provided at 32 °C on day 1, and then, gradually decreased to 24 °C on day 42. Broiler chicken must be sent to market for sale at 42 days. If not, FCR (Feed Conversion Ratio) will be negatively affected, leading to business loss. The final sample collection was done, at this point, keeping in view this fact. All the pens, feeders, and troughs were cleaned and disinfected before the arrival of the birds.

**Sample collection and DNA extraction**

Three birds were selected randomly from each replicate group of every treatment and sacrificed through throat cutting with the knife on day 42. Cecal contents were collected aseptically to minimize individual variations. Samples were immediately stored in sterile cryogenic vials, snap-frozen in liquid nitrogen, transported to the laboratory, and frozen at − 80 °C until DNA extraction. Total bacterial genomic DNA was extracted from 180 to 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 25 mg/mL (Sigma-Aldrich, USA) in cell lysis buffer (0.5 mM EDTA pH 8.0; 20 mM Tris–HCl pH 8.0; 1% Triton X-100) at 37 °C for 30 min. Samples were also treated with DNase-free RNAse (GeneDireX, USA) for 30 min at 37 °C for 30 min. Samples were also treated with DNase-free RNAse (GeneDireX, USA) for 30 min at 37 °C. Genomic DNA was extracted in triplicates, and later, samples were pooled and 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′CCTACGGGAGGCAGCAG3′ and reverse 5′ATTACCAGGCTGCTGG3′ 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, the fragment of 8 nucleotides-unique index sequence followed by gene-specific primers. PCR amplification was performed in 50 µL reaction containing 25 µL NEB Phusion high-fidelity PCR Master Mix (New England Biolabs, USA), 4 µL PCR primer cocktail (primer 1 100 µM, primer 2 100 µM, adapter 0.5 µM) and 30 ng/µL DNA template. The reaction conditions include initial denaturation for 3 min at 98 °C, then denaturation for 45 s at 98 °C, annealing at 55 °C for 45 s, extension at 72 °C for 45 s for 30 cycles followed by a final extension at 72 °C for 7 min. 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 × 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) [25]. Briefly, sequence reads were trimmed for removal of adapter contamination with maximal three (3) bases mismatch permitted. Sequences were truncated for not having an average quality of 20 in a sliding window of 25 bp based on the 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) [26].

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 the UCHIME algorithm [27] and screened for removal of chimera by mapping to Genomes OnLine Database (GOLD) from UPARSE (version 7.0.1001) [28] 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) [29] 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 using Wilcoxon Rank-Sum test for two groups comparison, while Kruskal–Wallis test is used for multi-groups comparison. 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.' The 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 phylogenetic 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), weighted UniFrac (depicting abundance of species) and unweighted UniFrac (comparison of microbial community structure). Furthermore, a 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. 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). 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 (DG/AA-089). 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 [30].

Data availability

Paired end data of samples from this study have been submitted to the NCBI Sequence Read Archive (SRA) with BioProject accession number PRJNA592164.

Results

Sequence data analysis and quality filtering

A total of 1262784 paired-end reads were generated from five cecal samples of study groups. After quality filtration, 1083070 reads remained with average read number per sample 96823 (± 34814 s.d.) having median read length of 458 (± 9 s.d.) bases in all samples. Altogether, 1195 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 (Fig. 1). The cecal microbial diversity among study groups was measured through alpha and beta diversity. Alpha diversity was estimated 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 the phytogenic feed additive (PHY) group. Furthermore, higher bacterial diversity was achieved in COM and AB groups. Beta diversity analysis were executed through weighted/unweighted UniFrac and Bray–Curtis distances 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 (Fig. 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 (Fig. 2b). Similar results with Bray–Curtis diversity index were achieved placing PHY in separate branch with CON (Fig. 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 (Fig. 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 (Fig. 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 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 (1083070) from cecal samples were assigned to 1,195 OTUs, which further distributed into ten phyla and one unclassified phylum (Fig. 3a). 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 (Fig. 3b). Order Bacteroidales and Clostridiales were the abundant orders in AB, ORG and ORG groups. The presence of orders Acidaminococcales and Burkholderiales were also noticed in ORG (Fig. 3c). 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 analysis statistics of PHY sample revealed increased artificial duplicate reads (65750) in sequence count from the total sequencing reads (67241) post QC which resulted in 1,491 sequences available for downstream analysis and might be attributed for less microbial diversity in PHY sample. 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

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**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
Bacillus_safensis_OF-36b (Fig. 3d). The dominant species in CON and AB was Bacteroides_barnesiae (20–30%). ORG group showed high abundance of unclassified species (58%) along with Helicobacter_pylori_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 (Fig. 4a) whereas species showing low abundance (< 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 (Fig. 4b). At order level, AB and ORG groups shared phenetic relatedness (Fig. 4c). Similarly, presence of order Verrucomicrobiales was only noticed in control and combination groups. The presence of family Akkermansiae was noticed in COM group only with high abundance (Fig. 4d). The pattern of bacterial families present in AB and ORG group were similar in relative abundance. In COM, presence of genus Akkermansia (family Akkermansiae) was observed (Fig. 4e) with the presence...
of species *Akkermansia muciniphila* ATCC_BAA-835. Surprisingly, AB and COM groups shared similar branch in heat map at species level depicting species richness in both groups (Fig. 4f) 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 (Fig. 5). 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**

Defining ‘beneficial gut microbiota’ is challenging. Gut microbiome composition is positively linked to improved host performance, health, and immunity enhancement of birds. It may also provide valuable information in combination with genomic analyses for identification of variation(s) in host associated sequences [31].

Previously we have reported that adding phytogenic feed additives and organic acids had shown promising results on animal performance and health. Growth performance (daily feed intake, body weight gain and feed conversion ratio),
carcass characteristics, and biochemical and quantitative bacterial analyses revealed that variation in diet enhanced performance-related bacteria in the gut. In chicken gut, phytophagenic feed additives and organic acids tend to increase the Lactobacillus spp. count and decrease E. coli and Campylobacter spp. Decreased targeted pathogenic bacterial growth in the NGP (ORG, PHY, and COM) groups together with an increased host weight gain with increased feed efficiency. The data related to animal performance (body weight gain, feed intake and FCR) is not shown in the current study [32, 33]. Here, we used high-throughput sequencing of the V3-V4 region of 16S rRNA gene to examine cecal microbiota of chickens fed either sub-therapeutic level of enramycin (antibiotic growth promoter), phytophagenic feed additives, organic acids, or combination of phytophagenic 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 [34]. 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 various study groups were noticed. It was depicted through diversity indices (Table 2) and rarefaction curve (Fig. 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, that 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 phytophagenic feed additives and organic acids in chicken, Chang et al. reported feeding with Bidens pilosa (medicinal plant) successfully altered chicken gut dynamics with increased richness of microbial diversity which correlated positively with host weight gain [35]. 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 when compared to control [36].

Beta diversity analysis revealed that supplementation with PHY produced a distinct shift in microbiota compared to CON and AB groups (Fig. 2a–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 [37]. 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 phytochemical additives, has reported to exert strong antioxidant and anti-inflammatory properties and improved performance in chickens through regulation of cecal microflora [38]. 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 [39] and thus, involved in fermentation. Also, cecal microbiota has significant roles in breakdown of starch, polysaccharides, cellulose, and uric acid [40, 41]. Short chain fatty acids (SCFAs) in the intestine are the end-products of microbial fermentation, which regulates immune responses, blood flow and mucin production [42]. 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 along with suppressing inflammatory disorders [43]. 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 (Figs. 3, 5). These findings are in accordance with the previous reports which described predominant bacterial sequences belonged to phylum Firmicutes (50%) in cecum as chicken aged (day 42) [44, 45]. 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 [46]. The class-level analysis depicted abundance of *Clostridia* in AB, COM, and ORG (Fig. 3b) with genera *Blautia, Faecalibacterium, Ruminococcus, Lachnocolostridium* mainly dominated. *Clostridia* are group of gram-positive rod-shaped bacteria. Among these, *Blautia, Ruminococcus* and *Lachnocolostridium* are butyrate-producing bacteria with beneficial contribution to host [47]. At order level, *Clostridiales* and *Bacteroidales* (Fig. 3c) are dominant with the higher proportion of *Clostridiales* in a similar manner as previously reported [46]. Order *Clostridiales* (class *Clostridia*) is chiefly responsible for metabolism of SCFAs in chicken cecum [48]. *Bacteroidia*, third abundant order, with the genera *Alistipes, Bacteroides* and *Barnesiella* are mainly involved in the production of propionate in ceca [47]. 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 [41, 49]. *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* (Fig. 3d). *Bacillus safensis* is a relatively newly identified bacterium [50] 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 [51]. In addition, presence of species *Akkermansia muciniphila* was noticed only in COM group (Fig. 4f). Genus *Akkermansia* is a gram-negative anaerobic bacterium involved in degradation of mucin to produce carbon and nitrogen in host [52]. Surprisingly, *Akkermansia* exhibits immunomodulatory and anti-inflammatory responses in host along with strengthening of gut barrier function [53]. The presence of *Akkermansia* in gut protects the host from obesity [54], IBDS [55] and diabetes mellitus [56]. 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 NGS of 16S rRNA ampilon, this study profiled the microbial communities in the chicken cecum using alternative feed additives. The addition of phytogetic 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 the richness of microbial diversity 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 positively responsive 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.

**Supplementary Information** The online version contains supplementary material available at https://doi.org/10.1007/s11033-021-06712-3.

**Acknowledgements** Authors like to acknowledge The Karachi Institute of Biotechnology and Genetic Engineering for their professional support.

**Author contributions** ZR: Conceptualization, Investigation, Methodology, Visualization, Formal analysis, writing-original draft. MZY: Formal analysis, Writing- Review and Editing. SMHG: Investigation, Methodology. SZ: Investigation, Methodology. AA: Formal analysis, Writing- Review and Editing. AA: Supervision, Validation. Saddia Galani: Conceptualization, Supervision, Writing- Review & Editing.

**Funding** No funding was received for this study.

**Data availability** 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. This article has been submitted to a Research Square (Preprint server) with https://doi.org/10.21203/rs.3.rs-201124/v1. This work is licensed under a Creative Commons Attribution 4.0 International License.

**Declarations**

**Conflict of interest** Authors declare no competing interests.

**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.

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