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Carbon Quantum Dots Essential Oils Complex as A Substitute for Antibiotic Growth Promoter in Broiler Chicken Diet

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ABSTRACT

Essential oils (EO) are volatile in nature. To maintain their efficacy as antimicrobial compounds, seven EO were complexed with carbon quantum dots (CQEC). The *in vitro* antimicrobial efficacy in terms of minimum inhibitory concentration and minimum bactericidal concentration of the CQEC was either similar or higher to that of antibiotic growth promoter (AGP i.e. BMD) against common pathogens (*Salmonella, E. coli*, and *Clostridia*). To synthesize a complex of CQ-EOs, and assess the antimicrobial activity, and their potential benefits on performance, carcass traits, immune responses, antioxidant variables, and caecal microbiota composition in broiler chicken-fed diets without AGP. Two feeding trials were conducted to study the *in vivo* efficacy of CQEC as an alternative to BMD. In experiment 1, CQEC was tested at 200 and 250g/ton, while in experiment 2, CQEC was included at 100, 250, 500, and 1000g/ton feed. A positive control (PC) with BMD and a negative control (NC) without BMD or CQEC were fed in both experiments. The diets were in mash form, and each diet was fed to 10 replicates having 25 birds in each floor pen. The results indicated that feed efficiency (FE) reduced significantly in the NC group compared to the PC. The regression analysis indicated nonlinear improvement in FE with supplementation of different concentrations of CQEC to the NC diet. Similarly, nonlinear improvement in immune responses (CMI response and HI titres), and activity of superoxide dismutase, reduced lipid peroxidation and caecal colony count of pathogenic bacteria (*E. coli* and *Salmonella*) were observed when CQEC was supplemented to the NC diet. The gut microbiome analysis indicated upregulation of the Bacteroides population in CQEC-supplemented groups compared to the NC group. Based on the results, it is concluded that the broiler performance can be maintained with supplementation of CQEC to the AGP-free NC diet. The improved performance with CQEC supplementation could be due to the reduction in gut pathogens (*E coli* and *Salmonella*), improvement in the Bacteroides population in the gut, and improvement in immune and antioxidant variables in broilers fed the alternative compound (CQEC) to AGP.

KEYWORDS: Antioxidation, Broilers, Carbon quantum, Essential oils, Gut microbiome, Immune responses.,performance.

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INTRODUCTION

The improved chicken strains are reared in large numbers in a closed environment in the commercial farming system, which has forced to use of antimicrobial compounds in diet to realize maximum performance. Over a half-century, antibiotics at sub-therapeutic concentration () were considered as the essential feed additives in broiler diet to attain higher production and for a balanced gut ecosystem (Huyghebaert et al., 2011). One of the major reasons to use antibiotics as growth promoters (AGP) in poultry diets was their cost effectiveness (Fernandez-Rubio et al., 2009). In recent times, there has been increased resistance

from consumers on the use of AGPs in poultry diets. But, the withdrawal of AGPs in poultry diets resulted in an increased incidence of necrotic enteritis () and other gut-related disorders. Therefore, a need was felt to use alternative and safe compounds that mimic the activity of AGP by improving the function of the gastrointestinal tract and sustaining the broiler performance without leaving any residues in poultry meat or causing antimicrobial resistance in the chicken gut microbiota.

Several alternatives have been tested as non-antibiotic growth promoters in broiler meat production like probiotics, prebiotics, microbial enzymes (), herbal products (Diaz-Sanchez et al., 2015), organic acids (Rama Rao et al., 2023) and essential oils (). Among the several alternatives, essential oils (EO) were reported to have positive effects on broiler performance by improving weight gain (Kim et al, 2016), feed efficiency (Pirgozliev et al., 2019), enzyme secretion in the gut (Zeng et al., 2015), and nutrients digestibility (Amad et al., 2011). Therefore, supplementation of EO was reported to replace AGP in the broiler diet (Basmacioğlu-Malayoğlu et al, 2016, Attia, et al., 2019). The blend of essential oils in diets was reported to improve the intestinal microbial balance by a reduction of coliform bacteria and an increase in *Lactobacillus* spp. counts resulted in an improvement in the weight gain of broiler chickens (Cetin et al, 2016).

Essential herbs oils (EO) like thyme, cinnamon, tea tree, eucalyptus, oregano*, citral*, and mentha are known to have several beneficial roles in human health and livestock farming (Gheorghita et al., 2022). However, the beneficial role of EO in poultry production is quite inconsistent (Agung Irawan et al., 2021**).** As these EO are volatile in nature, coating, tagging/binding these volatile substances with safe organic bases like carbon quantum dots makes this complex (carbon quantum essential oil complex – **CQEC**) stable and retains the activity of EOs (Vishal et al., 2021). EO tagged with carbon quantum dots has been explored for their effectiveness as antibacterial and antifungal properties (Vishal et al., 2021). Carbon quantum dots (CQ) consists of oxygenous carbon-based nanomaterials in the size range of 2–10 nm with multiple surface functional groups. The CQ are non-toxicity, biocompatible, high water-solubility, photostability, and tuneable surface capacity. The CQ is doped with EOs to potentiate their antimicrobial properties. Recent literature demonstrated the antimicrobial properties when the CQ complex with curcumin (Chin-Jung Lin et al., 2019*) or* orange juice (Nguyen et al., 2021). The potential benefits of CQ-EO complex (CQEC) in diets devoid of AGP on the performance of broilers are scanty. Therefore, a study was conducted to synthesize a complex of CQ-EOs, assess the antimicrobial activity, and their potential benefits on performance, carcass traits, immune responses, antioxidant variables, and caecal microbiota composition in broiler chicken-fed diets without AGP.

MATERIALS AND METHODS

Synthesis of CQEC involved two stages as reported by Wang et al. (2019B). The first one is a synthesis of carbon quantum (carbon dots) and the second stage involves conjugation of carbon quantum with essential oils.

Synthesis of carbon quantum dots

The method described briefly. Citric acid (2.1 g) and ethylenediamine (670 uL) were dissolved in 20 mL Milli-Q water. Then the mixture was transferred into a Teflon-lined autoclave (125 mL acid digestion vessel no. 4748, Parr, France) and heated at 250 °C for 5 h. The resulting product was cooled to room temperature and dialyzed against Milli-Q water using a cellulose ester dialysis membrane for 3 days (Biotech CE N°131093, pore size 500-1000 Da) to remove unreacted small molecules and dried at 100C for overnight. Then, the dry mass of 200 mg solution was weighed by microbalance (Sartorius, TG 209 F3 Tarsus, Netzsch) and further dissolved with water for evaluation of the biological activity. The yield of carbon quantum dots was transformed from citric acid which was about 60 % as a carbon source. The final solution was stored at room temperature till further use.

Preparation of CQ–essential oil conjugates

Seven essential oils (thyme (*Thymus vulgaris*), cinnamon (*Cinnamomum verum*), eucalyptus (*Eucalyptus* globulus), oregano (*Origanum vulgare),* and mentha (*Mentha piperita*)) were used in preparations of CQEC. The CQEC was prepared by doping the EO blends with CQ (Nanobiotics TM,

M/s. Imkuraq Animal Health Private Limited, Hyderabad, India) which was in powder form. The conjugate was prepared by using the CQ as a nanocarrier for essential oils. Briefly, the procedure includes CQ–EO conjugation was performed by a single-step method suggested by Wang et al. (2019B). The typical procedure includes 0.5 ml of EOs and 0.1 ml of synthesized CQ (5 : 1 ratio) dispersed in 100 mL of 0.05 M H_2SO_4. The resulting homogeneous mixture was refluxed at 110 °C for 3 hours. Subsequently, the reaction mixture was cooled to room temperature and 20 mL of cold water (4°C) was added to the mixture. The formed conjugated material was separated using diethyl ether. Two distinct aqueous and organic layers were observed and the aqueous layer containing the CDs–essential oil conjugate was separated and dried at 100°C in a vacuum oven (HLT-VO, Hi-Tech lab Solutions, India). The solid product obtained was stored at room temperature for further use as an alternative to AGP. The active components in CQEC were analysed with Adams (2005) method. The brief procedure includes isolation of essential oils with steam distillation. The extract was diluted in methanol (25µL in 10 mL), and the diluted samples (10 µL) were analyzed by GC (Agilent 7890B, Santa Clara, California, USA), and the results were compared to chromatograms of standard essential oil extracts.

In vitro efficacy of CQEC

The anti-microbial activity of CQEC was evaluated by studying minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) against three microorganisms. The MIC and MBC values of CQEC against the *Salmonella typhimurium* strain (ATCC 14028), *Escherichia coli* (ATCC 25922), and *Clostridium perfringens* (ATCC 13124), as a measure of antimicrobial efficacy of the product, were determined by the micro broth dilution method (CLSI Performance Standards for Antimicrobial Susceptibility Testing, 32nd Edition). The MIC was determined by incubating 50 µL of the individual test cultures of *E coli* (1 X10^7 CFU/mL) in nutrient broth medium (50 µL) containing (g/l) glucose 1.0, yeast extract 2.5, and tryptone 5.0. with decreasing concentrations of extracted CQEC as well as Bacitracin Methylene Disalicylate (BMD) as a negative control (2000-2 µg/mL) in 96-well flat-bottom microtiter plates for 24 h, where each plate included a positive control as nutrient broth. All the wells were added with (1 X 10^7 CFU/mL test bacterial culture). The lowest concentration of compound without visible growth was designated as the MIC. For MBC determination, approximately 10 uL of the seeded inoculum was drawn from each well, having no visible growth, and placed on a Nutrient agar plate. The lowest concentration that produced 99.90% killing of the test culture was considered the MBC value of the compound.

In Vivo efficacy of CQEC

Two feeding trials were conducted to study the efficacy of CQEC on broiler performance in an open-sided poultry house with litter floor pens having 26 sft floor area (per pen/replicate) at Sri Ramdhoota Poultry Research Farm Pvt Ltd, Kothur, Hyderabad, India. The conditions and standards of rearing animals used in this experiment were approved by the Institute Animal Ethics Committee (ICAR-Directorate of Poultry Research, Hyderabad, India, IAEC/DPR/17/1; 21/10/2017).

Diets and treatments

Maize-soybean-meat and bone meal-based basal diets (BD) were prepared in the form of mash for three different phases i.e., pre-starter (1-14 d), starter (15-28 d), and finisher (29-42 d) phases (Table 1). The CQEC is a complex of CQ and EO as described earlier (Nanobiotics, Imkuraq Animal Health Pvt Ltd, Hyderabad, India) and tested as an alternative to AGP in boiler chicken diet. In experiment 1, the BD was fed without supplementing AGP (BMD) i.e. negative control (NC). Another three diets were prepared by supplementing BMD (500g/ton) i.e. positive control (PC), two doses of CQEC (200 and 250g/ton). The second experiment was conducted to further explore the benefits of lower and higher concentrations of CQEC and also to test the repeatability of the results of experiment 1. In experiment 2, both the PC and NC diets were fed, and also CQEC was supplemented to the NC at 4 different concentrations (100, 250, 500, and 1000g/ton). The lower level of CQEC (100g/ton) was tested in experiment 2 to study the possibility of reducing the dose of the product in the broiler diet, which was not tested in experiment 1. Each diet was randomly allotted and fed *ad libitum* to birds in 10 pens (replicates) at the rate of 25 broiler male chickens per pen from d 1 to 42 d of age.

Table 1. Ingredient and nutrient composition (g/kg) of control diet

Ingredient	Pre starter (1-14d)	Starter (15-28d)	Finisher (29-42d)
Maize	565.5	610.1	661.6
oil-veg	26.1	33	31.360
Soya DOC 45%	336.1	289.4	243.9
Meat cum bone meal	40	40	40
Salt	3.666	3.667	3.665
Sodium bi-carbonate	1.000	1.000	1.000
Dicalcium phosphate	10.440	8.51	5.94
LSP-powder	7.351	5.338	4.349
DL-Methionine	3.259	2.671	2.199
L-Lysine Hcl	2.187	2.034	2.033
L-Threonine	0.659	0.386	0.216
Premix[1]	4.20	4.20	4.20
Nutrient, g/kg			
M.E (MJ/kg)	12.55	12.97	13.18
Protein	225.9	207.7	190.9
Dig. Lysine	12.5	11.2	10.1
Dig.Methionine	6.4	5.6	4.9
Dig TSAA	9.29	8.32	7.50
Calcium	8.80	7.60	6.60
Available Phosphorus	4.20	3.80	3.30
Sodium	1.80	1.80	1.80
dig. Threnine	8.29	7.42	6.69
dig. Leucine	17.34	16.30	156
dig. Iso-leucine	8.42	7.64	6.90
dig. Valine	9.67	8.65	7.87

[1] Supplied per kg of diet: retinol acetate 2.75 mg, cholecalciferol 0.03 mg, α tocopherol 10 mg, thiamin 1 mg, pyridoxine 2 mg, cyanocobalamine 0.01 mg, niacin 15 mg, pantothenic acid 10 mg, riboflavin 10 mg, biotin 0.08 mg, menadione 2 mg, choline 650 mg, copper 8 mg, iron 45 mg, manganese 80 mg, zinc 60 mg, selenium 0.18 mg monensin sodium 50 mg and hydrated sodium calcium aluminosilicate 800 mg.

[2]calculated concentrations; [3]calculated based on analysed ingredient composition.

Birds and management

In experiments 1 and 2, 1000 and 1500 one-day-old broiler male chicks (Cobb 430, Venkateswara Hatcheries Pvt. Ltd., Hyderabad, India), respectively were evenly distributed into 40 and 60 pens, respectively at the rate of 25 birds in each pen (198 x 122 cm). The floor of each pen was covered with built-up litter at about 8 cm thickness. Built-up litter was used as the bedding material in the house and also mean and bone meal was included (40g/ton) in the BD to create a passive pathogen challenge in experimental animals. The litter and feed samples were analysed for *Clostridium perfringens* and total bacterial count (Smith et al., 1998). Briefly, litter and feed samples were collected aseptically in 0.5 ml sterile test tube to quantify *Clostridia perfringens* and total bacterial count by following the serial dilution method, which was expressed as log 10 values. A ten-fold serial dilution of each sample was made in a sterile normal saline solution. *Clostridia perfringens* counts were determined using Clostridium Agar Base supplemented with *Clostridium perfringens* supplement (Himedia laboratories Pvt. Ltd., Mumbai), whereas the total bacterial count was determined using nutrient agar. The Clostridia plates were incubated anaerobically, while the total bacteria count plates were incubated aerobically for 48 hrs. The colonies were counted and expressed as Log_{10} cfu/g of sample content. *Clostridium perfringens* and total bacterial counts in the litter material were 4.62 and 5.745 log10/g, respectively and the respective counts in a gram of feed were 0.123 and 0.182 log10. The litter was covered with old newspaper to prevent intake of litter material by chicks during the initial 4 days of age after which the

paper was removed. Brooding was done with incandescent bulbs (100 watt/pen) and coal to provide the required temperature (about 35°C in week 1, 32°C in week 2, and 27°C in week 3) in the experimental shed after which the birds were exposed to ambient temperature. The ambient temperature ranged from 24.1+1.66 to 30.5+4.65°C during experiment 1 and 24.1+1.81 to 36.3+2.99°C during experiment 2. Fluorescent bulbs were used to provide light during night-time from 4 to 6 weeks of age.

Performance and slaughter variables

Body weight and feed intake (FI) were recorded at two-week intervals, and body weight gain (BWG) and feed efficiency (FE) were calculated. Feed left in the feeder was placed back in the respective feed drum to calculate the amount of feed consumed by the birds in each pen. All the birds present in each pen were weighed to calculate the BWG. The FE was calculated as FI per unit BWG. At the end of experiment 2 (day 42), one bird representing the mean body weight of the respective pen (replicate) was selected to study carcass traits including ready-to-cook yield (RTC), breast weight, liver weight, and abdominal fat content. The carcass yields were expressed as g/kg live weight of the respective bird.

Caecal Bacterial count

At 42nd d of age, caecal digesta was collected aseptically in 0.5 ml sterile test tubes to quantify *E. coli, Salmonella,* and *Clostridia perfringens* count by following serial dilution method, which was expressed as log 10 values. The caecal digesta samples were collected aseptically and diluted in normal saline (0.1gin 1mL) using a vertex mixer. A ten-fold serial dilution of each sample was made in a sterile normal saline solution. *E. coli* counts were assayed using Eosin Methyline Blue agar (Himedia laboratories Pvt. Ltd. Mumbai) and inoculated plates were incubated for 24 hours. Clostridia were determined using Clostridium Agar Base supplemented with *Clostridium perfringens* supplement (Himedia laboratories Pvt. Ltd., Mumbai) and inoculated plates were incubated anaerobically for 48 hrs. The colonies were counted and expressed as Log10 cfu/g of caecal content (Smith and Macfarlane, 1998).

Immune responses

Immune responses were measured in experiment 2 in terms of HI titer to Newcastle disease vaccine and cell-mediated immune response to phytohemaglutinin-P (PHA-P) inoculation.

HI titre against ND vaccine

The chickens were vaccinated against Newcastle disease (ND) by an ocular route at 5 and 21 d of age with the Lasota strain (ND Lasota Vac-500, Indovax Pvt., Ltd., Hyderabad, India). The humoral immune response was measured as antibody titre against ND vaccine by collecting blood from the brachial vein on the 35th day of age, which was 14 d post inoculation of the vaccine. For this, 2 mL of blood was collected from one bird per replicate and the antibody titres in sera against ND virus were measured (Reynolds and Maraqa, 2000) by haemagglutination test. The antibody titre against the disease was expressed as $\log_2$ values. The reciprocal of the highest dilution where there was complete agglutination was taken as the titre.

Cell mediated immune (CMI) response

The CMI response was assayed by cutaneous basophilic hypersensitivity test *in vivo* by using phytohemaglutinin-P (PHA-P) (TC 226, HiMedia Laboratories Pvt Ltd, Mumbai, India) employing the method as described by Corrier and Deloach (1990). On 35th d of age, one bird from each replicate was selected and the thickness of both right and left wattles was measured by micrometer (p no 7301, Mitutoyo, Japan). 100 µg of PHA-P suspended in 0.10 mL of phosphate buffer saline (PBS) and 0.1 mL of the PBS without PHA-P were injected intradermally into right and left (acted as a control) wattles, respectively. The thickness of both the wattles was measured at 24 h post-injection. The CMI response was calculated as the difference in thickness between right and left wattles due to PHA-P inoculation, which was expressed in relation to the increased thickness due to PBS alone.

Serum anti-oxidant variables

A blood sample (about 2.5 to 3 mL) was collected from the brachial vein of one layer in each replicate at 74 weeks of age. The oxidative parameters like lipid peroxidation (LP) and the activities of antioxidant enzymes like superoxide dismutase (SOD) and glutathione peroxidase (GSHPx) in serum were measured. About 2.0mL of blood sample from each bird were placed into a centrifuge tube containing citrate buffer (1.5 mL/10 mL blood) for erythrocyte separation and antioxidant enzyme estimation. The blood samples were centrifuged at 500×g for 15 min at 4°C to separate buffy coat (WBC) and form erythrocyte

pellet. The erythrocytes were washed thrice with PBS (pH 7.4). The packed RBC obtained was mixed with an equal volume of PBS and then diluted as per the requirement with distilled water.

The LP was estimated in serum by quantifying malonyl dialdehyde (MDA). The MDA reacts with 2-thiobarbituric acid to form a trimethine-colored substance (pink chromogen), which was extracted into butanol. The color intensity was measured at 548 nm. The LP activity in the erythrocytes was expressed in nmol MDA/mg protein (Placer et al., 1966). The activity of SOD and GSHPx were estimated following the method of Paglia and Valantine (1967).

Caecal microbiome analysis

Sample collection and DNA extraction

On day forty-two, ten chickens each group were chosen at random and executed via jugular vein exsanguination. The DNA/RNA Shield TM Fecal Storage Tube (Zymo Research, CA, USA) was used to aseptically collect the caecal contents, which were then kept at room temperature until DNA extraction. DNA was extracted and library was prepared with LSK-SQK114.96 kit of Oxford Nanopore. Sequencing was performed through Mk1C device (Name, number of the equipment, address of the company) of Oxford Nanopore.

Microbiome analysis

FastQC was used to verify the quality of the Illumina paired-end V3-V4 reads (2 bp × 300 bp) after they had been demultiplexed using the bcl2fastq1 tool. The biome data was extracted in advance for more sophisticated analysis and visual aids. Twelve5,000 sequences were chosen per sample (rarefaction) to equalize sequence counts among samples and serve as a foundation for comparing OTU abundances. With the help of the Quantitative Insights into Microbial Ecology (QIIME) pipeline (Caporaso et al., 2010), the microbial composition and diversity in the stitched reads were examined. UCLUST was used to pick Open-reference Operational Taxonomic Units (OTUs) (Edgar 2010). After generating raw files, the abundance was identified and estimated by aligning them against microbial genomes. The raw data was examined and normalized. Different alpha diversity metrics (the diversity within each treatment groups) were estimated based on rarified data to assess different aspects of the groups. The Shannon, Simpson, Chao, and observed OTUs were used to compute alpha diversity. This technique, known as pair-wise analysis, is a beta diversity measure that looks at variations in the overall microbial community structure between samples by taking into account the evolutionary divergence among OTUs. Differental_Abundance_EdgeR was used to find significant group changes over treatment. It can show whether type of bacteria is affected more or less by a treatment.

Statistical analysis

The GraphPad Prism Software, CA, USA was used to examine the relative abundances of bacterial communities. $P < 0.05$ was considered statistically significant. One-way analysis of variance (ANOVA) was used to compare relative abundances at each level of classification (phylum, class, order, family, and genus). Using the Krona tools (Ondov et al., 2011), which provide quantitative phylogenetic data for every sample, Krona charts were created. The QIIME pipeline is used to create PCoA (Caporaso et al., 2010). The performance data were analyzed by considering the pen as an experimental unit, and other (carcass, immune responses, antioxidant variables, caecal bacterial count). The individual bird data were considered as a unit for statistical analysis. The effect of graded inclusion levels CQEC on dependent variables was assessed by ANOVA (experiment 1) regression analysis (experiment 2), i.e., linear ($y = a + bx$), and nonlinear ($y = a+bx+cx^2$, x = the inclusion level of CQEC, y = response in dependant variable). The response of supplementing CQEC to NC diet vis a vis the PC was compared with simple contrast analysis (ANOVA) (SAS Institute, 1994).

RESULTS AND DISCUSSION

MIC and MBC of CQEC

As an antibacterial indicator, the MIC and MBC values of CQEC were determined against *Escherichia coli* (ATCC 25922), *Salmonella typhimurium* strain (ATCC 14028) and *Clostridium perfringens* (ATCC 13124). The MIC values of the CQEC tested ranged from 31.25 to 62.5ug/mL while MBC values were in the range of 62.5 to 125ug/ml for these bacteria while BMD showed similar activity which ranged from 31.25 to 62.5ug/mL, while MBC values ranged between 62.5 to 250ug/ml (Table 2). The analyzed concentrations of thyme, cinnamon, eucalyptus, oregano, and mentha in CQEC (5.21, 4.89, 5.01, 4.18 and 5.41%, respectively) were close to the estimated values (5% each).

Table 2. Determination of MIC and MBC values of carbon quantum dots essential oil complex (CQEC) against *Escherichia coli* (ATCC 25922), *Salmonella typhimurium* strain (ATCC 14028) and *Clostridium perfringens* (ATCC 13124)

Treatment	*Escherichia coli* (ATCC 25922)		*Salmonella typhimurium* strain (ATCC 14028)		*Clostridium perfringens* (ATCC 13124)	
	MIC (ug/ml)	MBC (ug/ml)	MIC (ug/ml)	MBC (ug/ml)	MIC (ug/ml)	MBC (ug/ml)
CQEC	62.5	125	31.25	62.5	62.5	125
BMD	125	250	31.25	32.5	62.5	125

CQEC carbon quantum dots essential oil complex; BMD bacitracin methylene di salicylate; MIC minimum inhibitory concentration; MBC minimum bactericidal concentration

The antimicrobial parameters of CQEC and BMD indicated that the bactericidal (MBC) dose of both compounds was almost double the dose of MIC concentrations. Further, it is also evident that the dose of both CQEC and BMD for antimicrobial effect was almost similar, which means the *in vitro* efficacy of CQEC is similar to that of AGP tested (BMD) in the current study. Since the bacteria tested in the current study are the most common pathogens that prevail in commercial poultry operations, it is expected that CQEC will have a similar response to BMD on these pathogens. The antibacterial concentration of both CQEC and BMD for *Salmonella typhimurium* was lower, while the concentrations of CQEC were similar for both *Escherichia coli* and *Clostridium perfringens*. Higher doses of BMD were required against *E coli* compared to *Clostridium perfringens*. This variation in MIC and MBC values could be attributed to the differences in bacterial strain, variations in virulence factors, or structural differences in the bacterial cell membrane (Abishad et al., 2021). In this study, CQEC exhibited similar antimicrobial properties against *Salmonella typhimurium* and *Clostridium perfringens* and a higher response against *E coli* as compared to the BMD.

Experiment 1

Body weight gain during all the periods (1-2, 1-4, and 1-6 weeks of age) and feed efficiency during 1-4 weeks of age were not affected (P>0.05) by supplementation of either AGP or CQEC compared to those fed the NC diet (Table 3). The FE during the initial 2 weeks (1-2 weeks) was significantly reduced in broilers fed the NC compared to those fed the AGP (PC). Supplementation of CQEC improved the FE compared to those fed NC, and at 250g CQEC the FE was similar to those fed the PC diet. Similarly, at the end of the experiment (1-6 weeks) the FE in NC diet-fed groups was lower than those fed the PC diet. Supplementation of CQEC at 250g/ton significantly improved the FE compared to the NC diet-fed broilers. However, the FE in the latter group was significantly lower than those fed the AGP-supplemented diet.

Table 3. Performance of broiler chicken fed diet containing carbon quantum dots essential oil complex (CQEC) in place of antibiotic growth promoter

Treatment	1-2 weeks		1-4 weeks		1-6 weeks	
	BWG	FE	BWG	FE	BWG	FE
PC	448.0	0.857^{A}	1510	0.690	2574	0.603^{A}
NC	439.4	0.842^{C}	1487	0.681	2526	0.592^{C}
CQEC-200	440.7	0.848^{BC}	1494	0.686	2543	0.595^{BC}
CQEC-250	443.4	0.855^{AB}	1504	0.688	2560	0.598^{B}
P	0.564	0.005	0.654	0.195	0.603	0.001
N	10	10	10	10	10	10
SEM	2.269	0.0018	6.735	0.0016	13.04	0.0010

BWG body weight gain; FI feed intake; AGP antibiotic growth promoter; PC positive control with AGP; NC negative control without AGP/alternatives; CQEC carbon quantum dots essential oil complex 200 and 250g/ton; P probability; N number of replicates; SEM standard error mean

^{ABCD} means having no common superscripts in a column varies significantly (P<0.05)

Maximum and minimum temperature and humidity = 30.9+4.65 & 24.2+1.66°C and 81.4+8.85 & 56.7+18.11%, respectively

Experiment 2

Performance

In general, the BWG and FE in experiment 2 were marginally lower than the experiment 1. The lower performance could be due to higher ambient temperature during Experiment 2 (Maximum and minimum temperature 35.3+2.99 & 24.1+1.81°C, respectively) compared to Experiment 1 (Maximum and minimum temperature 30.9+4.65 & 24.2+1.66°C, respectively). The BWG was not affected (P>0.05) by supplementation of CQEC to the NC diet during the pre-starter phase. However, the BWG during 1-4 and 1-6 weeks and FE during all the phases were improved non-linearly (<0.05) with the level of CQEC in the broiler diet (Table 4). The contrast analysis indicated that the FE in the NC group was significantly (P<0.05) lower than those fed the PC diet. However, supplementation of CQEC at different concentrations improved the FE similar to the PC group during pre-starter phase. The FE in during 1-4 and 1-6 weeks of age in 100 g CQEC groups was similar to the PC group. Supplementation of higher concentration, i.e. 250g CQEC, significantly improved the FE compared to those fed the PC diet. Higher concentrations of CQEC (500 g/kg) also improved the FE during 1-4 weeks of age and further higher concentrations did not improve the FE compared to the PC diet during 1-4 or 1-6 weeks of age.

Table 4. Performance of broiler chicken-fed diets containing graded concentrations of carbon quantum dots essential oil complex (CQEC) in place of antibiotic growth promoter

Treatment	1-2 weeks		1-4 weeks		1-6 weeks	
	BWG	FE	BWG	FE	BWG	FE
PC	345.2	0.821	1329	0.718	2265	0.593^{BC}
NC	332.8	0.814	1320	0.713	2226	0.588^{D}
CQEC-100	342.5	0.819	1322	0.715	2250	0.592^{CD}
CQEC-250	349.1	0.821	1360	0.721	2277	0.599^{A}
CQEC-500	351.3	0.823	1362	0.724	2296	0.595^{ABC}
CQEC-1000	339.7	0.816	1357	0.720	2288	0.597^{AB}
SEM	3.137	0.0009	6.09	0.0006	13.29	0.0062
P values						
Regression						
Linear	0.420	0.319	0.015	0.001	0.140	0.001
Quadratic	0.182	0.002	0.034	0.001	0.250	0.001
Contrast						
PC vs NC	0.263	0.016	0.673	0.020	0.408	0.020
PC vs C-100	0.805	0.593	0.739	0.249	0.745	0.418
PC vs C-250	0.582	0.337	0.107	0.001	0.519	0.018
PC vs C-500	0.721	0.915	0.131	0.017	0.810	0.495
PC vs C-1000	0.617	0.129	0.166	0.091	0.628	0.150

BWG body weight gain; FI feed intake; AGP antibiotic growth promoter; PC positive control with AGP; NC negative control without AGP/alternatives; CQEC carbon quantum dots essential oil complex 100, 250, 500, and 1000g/ton; P probability; N number of replicates; SEM standard error mean

^{ABCD} means having no common superscripts in a column varies significantly (P<0.05)

Maximum and minimum temperature and humidity = 35.3+2.99 & 24.1+1.81°C and 69.1+17.6 & 25.2+15.94%, respectively

The literature on the specific EOs tested in broiler diets was limited, therefore, the current results are compared with related herbal compounds reported in the literature. The improved broiler performance observed in the current study is consistent with the findings of Parade et al. (2019), who found that feeding 1.5% lemongrass leaf powder increased growth and reduced the market age of the broilers to attain the desired slaughter weight. Comparably, feeding 2% lemongrass leaf powder-supplemented diets was reported to enhance weight gain in comparison to the control group (Shaheed, 2021). The authors attributed the antioxidant and antibacterial properties of lemongrass to the higher performance observed in broilers fed lemongrass.

The improved performance observed in the

current study could be attributed to the non-linear reduction in oxidative stress variables (reduced LP and improved SOD activity) and pathogen count (*E coli* and *Salmonella*) in caecum compared with CQEC supplementation to the NC diet.

Slaughter variables

The regression analysis indicated that the slaughter variables (breast meat weight, abdominal fat, and liver weight) were not affected (P>0.05) by supplementation of CQEC to the NC diet (Table 5). Similarly, these carcass variables were not affected by supplementation of AGP (PC) compared to those fed the NC diet. The contrast analysis also indicated that supplementation of CQEC @ 250g/ton significantly improved the RTC yields compared to the PC diet-fed broilers.

Caecal bacterial count

The Clostridia count in the caecum was not affected (P>0.05) by the supplementation of CQEC or AGP to the NC diet (Table 5). The colony counts of salmonella and *E. coli* were reduced non-linearly with the concentration of CQEC in the NC diet. The contrast analysis indicated that Salmonella count was not affected, but *E coli* count reduced significantly with AGP supplementation compared to the NC group. Similarly, the Salmonella count reduced significantly with CQEC supplementation at all levels compared to the PC group.

Table 5. Slaughter variables (g/kg live weight) and caecal bacterial count (log 10/g) in boiler chicken fed graded levels of carbon quantum dots essential oil complex (CQEC) in place of antibiotic growth promoter

Treat	RTC	Breast	Abdfat	Liver	Caecal bacterial count, log 10		
					Salmonella	E coli	Clostridia
PC	761.0	254.7	14.03	22.38	5.317	5.266	5.406
NC	768.4	253.0	14.06	21.44	5.184	5.865	5.312
CQEC-100	761.2	249.3	14.06	21.02	4.856	5.560	5.201
CQEC-250	776.3	257.5	13.44	21.07	4.599	5.208	5.183
CQEC-500	763.5	246.9	14.23	21.61	4.668	5.238	5.331
CQEC-1000	761.7	256.2	13.36	20.40	4.519	5.177	5.262
SEM	2.026	1.892	0.445	0.359	0.0621	0.0608	0.0442
P values							
Regression							
Linear	0.133	0.796	0.725	0.562	0.001	0.001	0.932
Quadratic	0.318	0.893	0.938	0.794	0.001	0.001	0.836
Contrast							
PC vs NC	0.248	0.801	0.982	0.460	0.452	0.002	0.546
PC vs C-100	0.975	0.419	0.482	0.288	0.011	0.121	0.193
PC vs C-250	0.018	0.678	0.715	0.304	0.001	0.753	0.158
PC vs C-500	0.103	0.245	0.897	0.546	0.001	0.878	0.629
PC vs C-1000	0.914	0.818	0.678	0.123	0.001	0.635	0.358

RTC ready to cook yield, Abdfat abdominal fat, AGP antibiotic growth promoter; PC positive control with AGP; NC negative control without AGP/alternatives, CQEC carbon quantum essential oil complex 100, 250, 500, and 1000g/ton; P probability; N number of replicates; SEM standard error mean

^{ABCD} means having no common superscripts in a column varies significantly (P<0.05)

Immune responses

The regression analysis indicates that both CMI response to PHA-P and HI titers against ND vaccination improved non-linearly (P<0.05) in broilers fed graded concentrations of CQEC in the NC diet (Table 6). The contrast analysis indicated significantly lower HI titers in broilers fed the NC diet with or without CQEC supplementation compared to those fed the PC diet. Though the CMI response was not affected by AGP supplementation (PC) compared to those fed the NC diet, the immune response in groups fed 250 or 1000g/ton was significantly higher than those fed the PC, and the immune response at other concentrations (100 and 500 g/ton) was similar to the PC group. The ND titres in broilers fed the highest concentrations of CQEC (1000g/ton) was similar to the PC group.

Serum anti-oxidant variables

The regression analysis indicated that the LP reduced, and SOD activity increased non-linearly with the concentration of CQEC in the NC diet, such affect was not noticed in the activity of GSHPx (Table 6). Similarly, the contrast analysis indicated no significant affect in LP and GSHPx activity with AGP supplementation compared to the NC group, while the activity of SOD was significantly lower in NC compared to the PC group. The LP in groups fed CQEC at 100, 500 or 1000g/ton was significantly lower than those fed the PC diet. The SOD activity in broilers fed CQEC at all concentrations except 1000g/ton (100, 250 and 500g/ton) was similar to those fed the PC diet.

Table 6. Immune responses and serum antioxidant variables in broiler shed fed graded levels of carbon quantum dots essential oil complex (CQEC) in place of antibiotic growth promoter boiler chicken diet

Treat	Immune responses		Serum antioxidant variables		
	CMI, %	ND titre (Log2)	LP, Nano moles MDA	SOD, u/mg of protein	GSHPx, unit/ml
AGP	51.20	8.500	1.884	5.010	303.0
NC	49.00	5.800	1.852	3.340	257.1
CQEC-100	52.00	7.500	1.667	4.548	288.2
CQEC-250	66.80	7.300	1.887	4.233	367.8
CQEC-500	61.20	6.900	1.399	5.213	287.4
CQEC-1000	71.40	8.100	1.558	3.233	331.2
SEM	2.286	0.277	0.040	0.213	11.84
P values					
Regression					
Linear	0.002	0.001	0.010	0.545	0.205
Quadratic	0.010	0.001	0.011	0.012	0.297
Contrast					
PC vs NC	0.763	0.001	0.790	0.018	0.250
PC vs C-100	0.913	0.003	0.073	0.502	0.709
PC vs C-250	0.036	0.001	0.980	0.261	0.107
PC vs C-500	0.174	0.001	0.001	0.768	0.694
PC vs C-1000	0.007	0.224	0.008	0.012	0.478

CMI cell mediated immune response; ND Newcastle disease; LP li[id peroxidation; SOD superoxide dismutase; GSHPx glutathione peroxidase; PC positive control with AGP; NC negative control without AGP/alternatives, CQEC carbon quantum dots essential oil complex 100, 250, 500, and 1000g/ton; P probability; N number of replicates; SEM standard error mean

[ABCD] means having no common superscripts in a column varies significantly (P<0.05)

Both the immune responses improved non-linearly with supplementation of CQEC to NC diet and the response of HI titre was significantly higher at 250g and CMI at 1000g/ton was similar compared to the PC diet fed broilers. Literature demonstrated a strong positive correlation between gut microbiota and immune responses (Wang et al., 2019B). Bacteroides is essential to the intestinal IgA response. Bacteroides-derived IVA induces M2 polarization of macrophages and the expression of IL-10, IL-4, TGF-β, and BAFF to promote IgA response by activating the mTOR/PPAR-γ/STAT3 signaling pathway in the small intestine, independently of the bacterial TLR ligands, which induce IgA responses through the binding of TLRs. The increased population of Bacteroides might have modulated the immunity and has a promising role in gut immunity (Xinkai et al., 2023). Therefore, improving the population of Bacteroides might have improved gut immunity.

Caecal microbiome study

Sequence data analysis

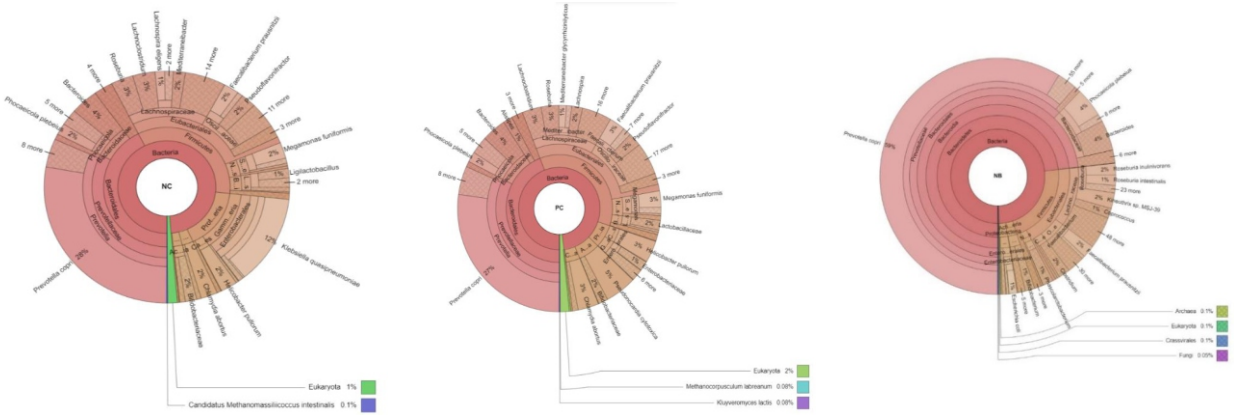
A total of 9.6 million paired-end reads were obtained from 10 pooled cercal samples from each group of birds. After selecting the right reads and removal of chimeric reads, the average clean sequence tags after aligning against microbial genomes were obtained for the NC, PC, and NB250 groups.

At the phylum level, *Firmicutes, Bacteroidetes,* and *Proteobacteria* accounted for more than 96% of the caecal microbiota as normalized % abundance,

in which *Firmicutes* (21%–38%) and *Bacteroidetes* (41%–72%) were the predominant microbes followed by *Proteobacteria* (2%–7%) and *Actinobacteria* (2%–6%) in all the sequences.

Most predominant *Prevotella copri* accounts for 20%–56% of the sequences in the phylum *Bacteroidetes* under class *Bacteroidia*. While *Faecalibacterium prausnitzii* accounts for 1-2% was the most prominent under phylum *Firmicutes*. Furthermore, the *Krona chart* is an interactive plot used to explore the relative abundances and confidences within the complex hierarchies of metagenome classifications. In the present study, NB250 treatment produces an impact on *Bacteroidetes* population which was presented visually in one representative Krona chart (Figure 1).

Figure 1. Krona chart of one representative sample from each group. Bacteroides population has significantly ($p > 0.001$) increased in a carbon quantum dots essential oil complex (CQEC) group a compare to Negative (NC) as well as the positive (PC) control group.



(a) Normal feed group (NC) – without addition- as Negative control

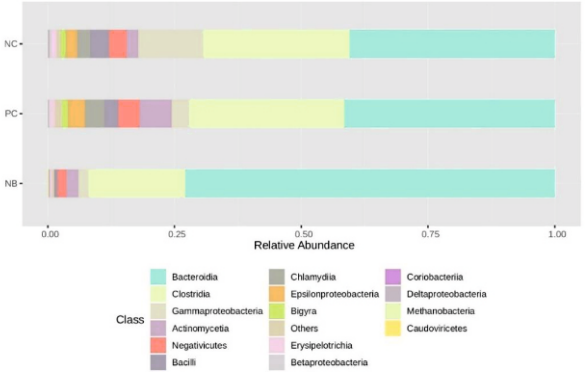
(b) BMD feed group (PC) 500g/TON of feed - as positive control

(c) carbon quantum essential oil complex (CQEC) fed group 250g/TON of feed (NB-250)

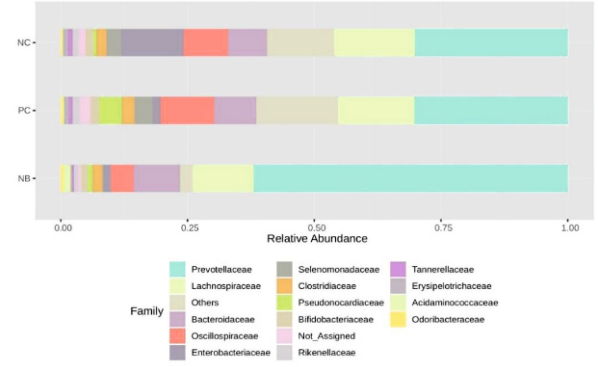
In addition, stacked column plots were generated for the top 16 unique classified organisms identified at phylum, family, genus, and species taxonomic level using relative abundance values for all the samples (Figure 2). Moreover, the *Bacteroides* population was elevated upon supplementation with NB250 in line and with improved body weight and FCR of broiler birds (Table -2 &3).

Figure 2. Stacked Bar plot showing the relative abundance of Phyla and family distribution. carbon quantum dots essential oil complex (CQEC) group a compare to Negative (NC) as well as positive (PC) control group.

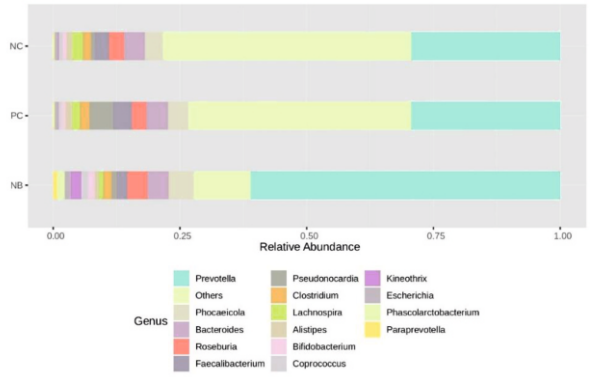
Class level relative abundance plot



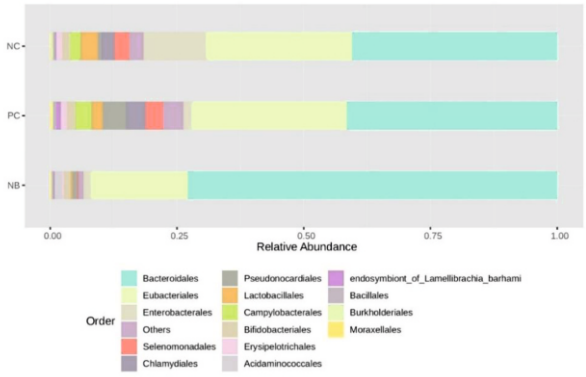
Family-level relative abundance plot



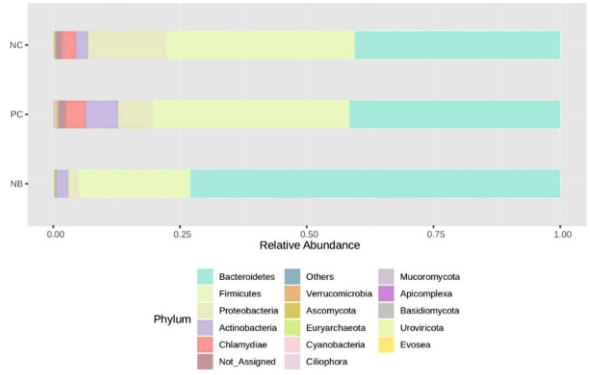
Genus level relative abundance plot



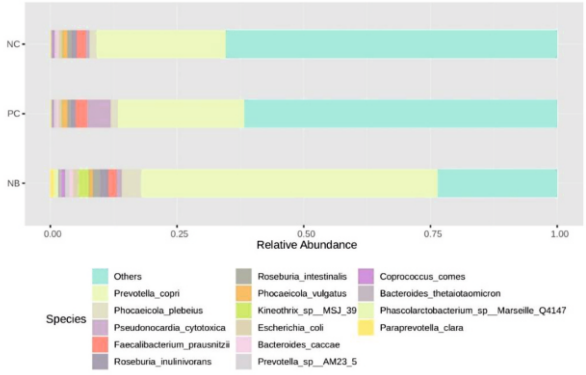
Order level relative abundance plot



Phylum level relative abundance plot



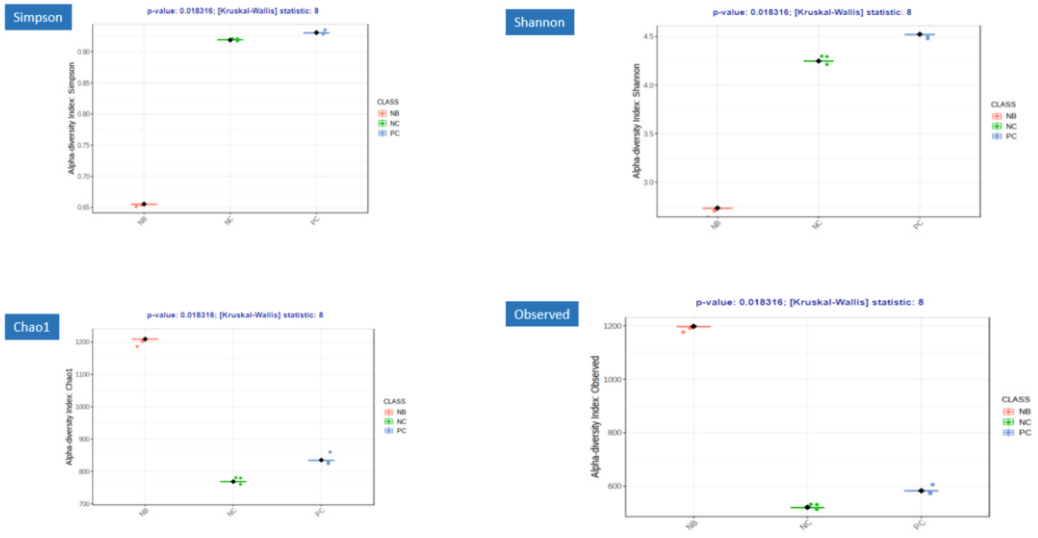
Species level relative abundance plot



Alpha Diversity

Internal sample alpha diversity was estimated through the number of observed species, Simpson, Chao, and Shannon indices (Alpha diversity) Figure 3. NB250 group showed lower 0.66 and 2.5 Alpha diversity index in Simson plot as well as Shannon plot as compared to NC and PC groups values 0.94 - 0.96 and 4.25 - 4.66, respectively (p-value: 0.0183) (Figure 3). While Alpha diversity index was observed with higher values (1200 for NB250) as compared to PC and NC (780-840) for Chao1 and (560 and 590) for Observed, respectively (p-value: 0.0183).

Figure3. Effect of carbon quantum dots essential oil complex (CQEC) supplementation on alpha diversity measures for comparing different statistical parameters (a) Shannon Index, (b) Simpson Index, (c) Chao Index, (d) number of observed species (mean ± standard error of the mean)



The Krona chart (Figure 1) from the current study revealed a favourable relation between the feed efficiency and the *Bacteroides* genus (Tables 3, and 4). It has a similar finding with both cellular immunities as measured by the PHAP test and humoral immunity parameters for the ND vaccination response (Table 6).

The present study's NGS analysis identified a wide range of bacteria in the cecum of broiler chicken and examined the effects of supplementing the essential oil-based carbon quantum dots complex (CQEC). In the current investigation, the impact of CQEC on the caecal microbiome was assessed. The NGS analysis of the broiler chicken cecum revealed the presence of three important phyla in the ceca, namely, *Firmicutes, Bacteroidetes,* and *Proteobacteria* which are in accordance with the literature (Corrigan et al., 2015). The FE and Firmicutes-Bacteroides Ratio (FBR) were higher with supplementation of CQEC at 250 g/ton and are in line with the trend of FE. Essential oils supplementation was reported to improve FBR and performance of chicken (Saravana et al. 2019 and Tesfaye et al., 2023). Similar to our study, the inclusion of certain growth promoters i.e antibiotic growth promoters (Costa et al., 2017), plant extracts (Salaheen et al., 2017), and prebiotics (Choi et al., 2015) were reported to improve the FBR. The effect of polyphenolic compounds from blueberry and blackberry has been shown to improve the performance and FBR in the ceca of broilers (Salaheen et al., 2017).

When compared to the AGP (BMD) group and the NC group, the beta-diversity study showed that the CQEC supplementation resulted in a noticeable shift in the microbiota (Figure 1 and Figure 2). Nonetheless, the beta diversity among the CQEC treatments was statistically significant, suggesting that the microflora is shifted in a way that is similar to the increase in the Bacteroides population. The predominant phylum increased upon CQEC supplementation was *Bacteroides.* These organisms is known to play a vital role in the fermentation of dietary carbohydrates to convert into short-chain fatty acids (SCFA). The increased prevalence of Bacteroides in ceca could indicate a subsequent development in the microbial succession, involving the shift from facultative anaerobes, like Lactobacilli, to stringent anaerobes, like Bacteroides, Ruminococcaceae, and Lachnospiraceae. According to van Der Wielen et al. (2000), an anaerobic gut environment and undigested carbohydrates entering the ceca are necessary for the synthesis of SCFAs, which help for proper function of the gut epithelium and improve nutrient absorption across cellular membranes. The results of this study also showed that the CQEC supplementation changed the caecal microbiota, which is beneficial for gut health. The improvement in the microbiota also leads to an improvement in the FBR, which in turn might have helped to improve the FE. Pie chart showed that the Bacteroidetes population increased with CQEC (250g/ton) supplementation compared to the NC or PC (BMD) group.

To evaluate various facets of the microbial community structure, several alpha diversity metrics (the diversity between groups) were computed using rarified data (Figure 4). Two indices are used to quantify diversity i.e. the Shannon-Wiener and the Simpson. The higher numbers denote less diversity and lower ones suggest more diversity (Harini, 2002). According to Simpson and Shannon analysis, the current study revealed that CQEC had larger diversity of the bacterial community (>1200 species). Alpha diversity, or species diversity within a single community or habitat, is also measured by Chao1. It is employed to calculate the overall number of species present in a community for both known species and uncommon or undiscovered species. The total number of species in a sample, or species richness, is indicated by Chao1. In comparison to both the PC and NC groups, the species diversity of the CQEC group was significantly higher which might provide a better preposition to provide improvement in growth.

The gut microbiota data also indicated a higher abundance of beneficial bacteria in broilers fed CQEC compared to the CD. The caecal microbiota plays a critical role in maintaining gut health and utilization of nutrients left undigested in the small intestine. Several studies have shown the importance of microbes in the digestion of nutrients entering the caeca. It is estimated that 5–10% of the energy from the poultry diet is extracted by caecal fermentation. Improvement in the caecal microflora to extract the undigested nutrients that reach the hindgut provides a great opportunity to improve the productivity of chickens. From our study, it is evident that the prevalence of Bacteroides, which are essential for the fermentation of food substrates improved with the supplementation of CQEC. Bacteroides are crucial for the breakdown of

complex carbohydrates and produce short-chain fatty acids (SCFAs) that are well-suited for the health of the hindgut. Corrigan et al. (2015) demonstrated the improved host's ability to utilize dietary energy with Bacteroides dense ceca. The gut microbiota helps in the fermentation of partially- or non-digestible polysaccharides and produces SCFAs (acetate, propionate, butyrate, and valerate). Liao et al. (2020) found a favorable correlation between the relative abundance of Bacteroides and the caecal SCFA profiles. Though the digestibility of nutrients was not estimated in the current study, the improved feed efficiency observed in our study could also be due to a probable increase in enzyme secretion with CQEC supplementation. The literature reported improved enzyme secretion (Zeng et al., 2015), and nutrient digestibility (Amad et al., 2011) with supplementation of EO in broiler and other monogastric animal diets.

CONCLUSIONS

Based on the results, it is concluded that supplementation of carbon quantum-essential oils (thyme, cinnamon, tea tree, eucalyptus, oregano, citral, and mentha) complex (CQEC) showed potent antibacterial properties comparable to antibiotic growth promoter (BMD) while testing with MIC and MBC methods. Supplementation of CQEC positively affected the bacterial communities in the cecum for improved gut health and enhanced feed efficiency. CQEC improved the population of the beneficial bacteria (Bacteroides), which is known to maintain a balanced microflora in the gut. The increase in the population of the beneficial bacteria is associated with the feed efficiency and meat yields of broilers fed CQEC. The results indicated the possibility of utilizing CQEC as a potent growth promoter in broiler chicken diet in the era of post-AGP ban in poultry and livestock production.

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