Evaluation of curcumin and copper acetate against Salmonella Typhimurium infection, intestinal permeability, and cecal microbiota composition in broiler chickens

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Research

Keywords: Broiler chickens, Copper acetate, Curcumin, Intestinal permeability, Microbiota composition, Salmonella Typhimurium

DOI: https://doi.org/10.21203/rs.3.rs-42229/v2

Abstract

Background: Interest in the use of natural feed additives as an alternative to antimicrobials in the poultry industry has increased in recent years because of the risk of bacterial resistance. One of the most studied groups are polyphenolic compounds, given their advantages over other types of additives and their easy potentiation of effects when complexes are formed with metal ions. Therefore, the objective of the present study was to evaluate the impact of dietary supplementation of copper acetate (CA), curcumin (CR), and their combination (CA-CR) against *Salmonella* Typhimurium colonization, intestinal permeability, and cecal microbiota composition in broiler chickens through a laboratory *Salmonella* infection model, indirect determination of intestinal integrity by measuring fitc-d in serum and microbiota analysis by DNA extraction.

Results: The obtained results showed that in two independent studies, all experimental treatments were able to significantly reduce the *S*. Typhimurium colonization in cecal tonsils (CT, $P < 0.0001$) compared to the positive control (PC) group. However, CR and CA-CR were the most effective treatments in reducing *S*. Typhimurium counts. Furthermore, the serum fluorescein isothiocyanate dextran (FITC-d) concentration in chickens treated with CR was significantly lower when compared to PC ($P = 0.0084$), which is related to a decrease in intestinal permeability and therefore intestinal integrity. The effect of dietary treatments in reducing *S*. Typhimurium colonization was further supported by the Linear discriminant analysis effect size (LEfSe) analysis of microbiota data, where *Salmonella* was significantly enriched in PC group (LDA score > 2.0 and $P<0.05$). In addition, *Coprobacillus*, *Eubacterium*, and *Clostridium* were significantly higher in the PC group compared to other treatment groups. On the contrary, *Fecalibacterium* and *Enterococcus* in CR, unknown genus of Erysipelotrichaceae at CA-CR, and unknown genus of Lachnospiraceae at CA were significantly abundant respectively.

Conclusions: CR treatment was the most effective treatment to reduce *S*. Typhimurium intestinal colonization and maintain better intestinal homeostasis which might be achieved through modulation of cecal microbiota.

Background

Salmonella, a Gram-negative intracellular bacteria, is a food-borne pathogen that can cause gastroenteritis and severe systemic infections in humans [1–3], as well as significant economic losses in poultry production because it can cause high mortality and affect growth performance parameters in broiler chickens [4,5]. Recently, it has been reported that the global incidence of salmonellosis cases has increased, estimating that of the approximately 94 million reported cases, 155,000 lead to death each year [6]. Furthermore, the estimated costs of medical expenses, sick leaves, and loss of productivity range from US$1.3 to US$4.0 billion a year in the United States of America (USA) [7].

It is known that young chickens are more susceptible to colonization by *Salmonella* [8], being the cecum the target site for establishing chronic infection [9]. Although the genus *Salmonella* consists of more than

2600 serovars, the most common serotypes isolated from chicken-associated outbreaks are *Salmonella enterica* serovar Enteritidis (20%) and *Salmonella enterica* serovar Typhimurium (17%) [10,11].

Nowadays, due to regulations on the use of antibiotics in poultry production derived from the problems of bacterial resistance, and considering that *S*. Typhimurium is a bacterium capable of developing antimicrobial resistance more quickly than other *Salmonella* species [12,13], several strategies have been proposed to treat and control *Salmonella* infections [14]. Among the large number of alternatives that have been tested in recent years, it has been reported that the combination of polyphenolic compounds with metal ions, such as copper, have potentiated antioxidant, anti-inflammatory and antimicrobial effects, having the additional advantage of reducing toxicity of metal ions due to complex formation [15,16].

Copper compounds such as copper acetate (CA) are believed to promote growth by regulating gastrointestinal microbiota through bactericidal and bacteriostatic effects [17]. The mechanisms that explain the antimicrobial effect of the copper ion are related to direct damage to the bacteria's membrane, which generates a loss of membrane potential and cytoplasmic content. Furthermore, reactive oxygen species produced by copper ions induce further damage to cellular structures and even DNA degradation [18].

Another alternative is curcumin (CR), a mixture of polyphenolic compounds obtained from the rhizome or root of the *Curcuma longa* plant, member of the Zingiberaceae or ginger family that is characterized by its excellent antioxidant, anti-inflammatory, and immunomodulatory properties, as well as its antimicrobial and growth-promoting effects [19–21]. However, an essential limitation of CR is its low solubility and permeability. Recent studies performed by our laboratories have shown that the use of solid dispersions with polyvinylpyrrolidone can increase these biopharmaceutical properties [22,23]. Therefore, the objective of the present study was to evaluate the effect of dietary supplementation of CA, CR, and their combination (CA-CR) against *S*. Typhimurium colonization, intestinal permeability, and cecal microbiota composition using a model of *S.* Typhimurium infection in broiler chickens.

Methods

Preparation of experimental treatments and diets

CR treatment consisted of a solid dispersion of curcumin with polyvinylpyrrolidone in a 1:9 ratio previously described [22,23], CA treatment was copper (II) acetate hydrate (98%, catalog no. 341746, Sigma), and CA-CR treatment consisted of a mixture of the previous treatments. Solid dispersion of curcumin was prepared by dissolving 1 part of curcumin in 9 parts of a polyvinylpyrrolidone (PVP) K30 solution, followed by water evaporation at 40 ºC and sieving. Mash corn-soybean-based broiler starter basal diet was formulated to approximate the nutritional requirements of broiler chickens, as recommended by the National Research Council [24] and then adjusted to breeder's recommendations [25]. No antibiotics, coccidiostats or enzymes were added to the feed (Table 1). All animal handling

procedures complied with the Institutional Animal Care and Use Committee (IACUC) at the University of Arkansas, Fayetteville (protocol #18029).

Salmonella strain and culture conditions

The poultry strain of Salmonella Typhimurium was obtained from the USDA National Veterinary Services Laboratory (Ames, IA, United States). This strain was selected for resistance to 25 µg/mL of novobiocin (NO, catalog no. N-1628, Sigma) and 20 µg/mL of nalidixic acid (NA, catalog no. N-4382, Sigma) in our laboratory. In the present study, 100 mL of S. Typhimurium from a frozen aliquot was added to 10 mL of tryptic soy broth (TSB, Catalog No. 22092, Sigma, St. Louis, MO, USA), incubated at 37°C for 8 h, and passed three times every 8 h to ensure that all bacteria were in log phase as previously described [26]. Post-incubation, bacteria were washed three times with sterile 0.9% saline by centrifugation at 1864g for 10 min, reconstituted in saline, quantified by densitometry with a spectrophotometer (Spectronic 20DC, Spectronic Instruments Thermo Scientific, Rochester, NY, USA) and finally diluted to an approximate concentration of 10^4 cfu/mL. Levels of S. Typhimurium were further verified by serial dilutions and plated on brilliant green agar (BGA, Catalog No. 70134, Sigma, St. Louis, MO, USA) with NO and NA for enumeration of actual cfu used in the experiment.

Animal source and experimental design

In the present study, two independent trials with 75 day-of-hatch male Cobb-Vantress broiler chickens (Fayetteville, AR, USA) were conducted. Chicks were individually weighed and randomly assigned to one of five groups (n = 15 chickens/group): 1) Negative control (NC, basal diet); 2) Positive control (PC, basal diet + challenged with 10^4 cfu of S. Typhimurium per bird on hatching day); 3) CA (basal diet supplemented with 250 mg/Kg of Copper (II) acetate hydrate + challenged with 10^4 cfu of S. Typhimurium per bird on hatching day); 4) CR (basal diet supplemented with 0.2% curcumin + challenged with 10^4 cfu of S. Typhimurium per bird on hatching day); and 5) CA-CR (basal diet supplemented with 250 mg/kg of Copper (II) acetate hydrate and 0.2% curcumin + challenged with 10^4 cfu of S. Typhimurium per bird on hatching day). In both trials, chicks were raised in floor pens (300 × 150 cm), provided with their diet, water ad libitum, and maintained at an age-appropriate temperature during all experiments. Body weight (BW), body weight gain (BWG), Feed intake and feed conversion ratio (FCR) were evaluated at 10-days of age. On day ten post-S. Typhimurium challenge, chickens were given an appropriate dose of fluorescein isothiocyanate dextran (FITC-d) by oral gavage one hour before the chickens were euthanized by CO_2 inhalation and only the cecal tonsils (CT) from 12 broilers per group were aseptically collected for S. Typhimurium recovery. Furthermore, blood samples were also collected from the femoral vein for the determination of FITC-d. The concentration of FITC-d administered was calculated based on group body weight at day nine post-S. Typhimurium challenge. For microbiota analysis, the content of the left ceca was collected aseptically and stored at −20°C until analysis.

Salmonella recovery

In both independent trials, the CT samples from 12 chickens per group were individually homogenized and diluted with saline (1:4 w/v), and 10-fold dilutions were plated on Xylose Lysine Tergitol-4 (XLT-4, Catalog No. 223410, BD DifcoTM) with NO and NA for S. Typhimurium recovery. Plates were incubated at 37°C for 24 h to enumerate total S. Typhimurium colony-forming units. Subsequently, the CT samples were enriched in 2× concentrated tetrathionate enrichment broth and further incubated at 37°C for 24 h. Enrichment samples were streaked onto XLT-4 with NO and NA selective media for confirmation of Salmonella presence.

Serum determination of FITC-d leakage

FITC-d (MW 3–5 kDa; Sigma-Aldrich Co., St. Louis, MO, USA) was provided by oral gavage to 12 broiler chickens from each group at a dose of 8.32 mg/kg of body weight one hour before the chicks were euthanized by CO_2 inhalation with the purpose of evaluating the paracellular transport and mucosal barrier dysfunction [27,28]. Three remaining broiler chickens of each group were used as controls. The blood samples were centrifuged (1000×*g* for 15 min) to separate the serum. Then, serum samples obtained were diluted (1:5) and measured fluorometrically at an excitation wavelength of 485 nm and an emission wavelength of 528 nm (Synergy HT, Multi-mode microplate reader, BioTek Instruments, Inc., VT, USA) to determine the serum FITC-d levels [29].

Microbiota analysis

DNA extraction, PCR, and library preparation for sequencing

V4 region of 16S rRNA gene from the genomic DNA of each of the 25 samples of cecal content (5 samples per group x 5 groups) was amplified using the primers 515F [30] and 806R [31]. The library of amplicons for DNA sequencing was prepared according to the 16S Illumina PCR protocol described in the Earth Microbiome Project (http://www.earthmicrobiome.org) with slight modifications [32]. In brief, Q5® High-Fidelity DNA Polymerase user guide protocol (New England Biolabs, Catalog No. M0491S) was used to conduct PCR in a 25 µl final reaction volume via 30 amplification cycles. The length of the amplified product was confirmed with 1% agarose gel electrophoresis, and equal amount (~300 ng) of the amplicons from each sample as measured by Qubit dsDNA BR Assay Kit (ThermoFisher Scientific, Catalog No. Q32850) were pooled together. The pooled amplicons were finally run on 1% agarose gel electrophoresis, purified using Zymoclean Gel DNA Recovery Kit (Zymo Research, Catalog No. D4007), and sequenced with Illumina MiSeq paired-end 300 cycle options at Admera Health, LLC (New Jersey, United States).

Amplicons sequence analysis

Nebula cloud computing platform of the University of Arkansas was used to process raw sequencing reads in QIIME 2 version 2018.8 utilizing the pipelines developed for paired-end data types [33]. In sum, “demux emp-paired” method of q2-demux plugin was used to demultiplex sequencing reads, followed by quality filtering and denoising with “dada2 denoise-paired” method of q2-dada2 [34] plugin available at

QIIME 2. The truncation length of forward and reverse reads were set at 220 and 200 bp, respectively, which was based on the quality score criteria (≥30). Taxonomic assignment was performed using a Naive Bayes classifier pre-trained with Greengenes (Version 13.8) 99% OTUs [35] and q2-feature-classifier plugin, where the sequences have been trimmed to include only the V4 region of the 16S rRNA gene region which is defined by the 515F/806R primer pair. We detected the sequence reads assigned to Chloroplast and Mitochondria, which were subsequencingtly removed using taxonomy-based filtering option in QIIME2. The core-metrics-phylogenetic method at a sampling depth of 69,566 was used to analyze Alpha and Beta diversity. Observed OTUs were used to calculate alpha diversity, while weighted UniFrac distance and unweighted UniFrac distance metrics were used for beta diversity analysis. All figures were created using ggplot2 packages of R [36].

Data and statistical analysis

Data from *S.* Typhimurium counts ($\log_{10}$ cfu/g) and serum determination of FITC-d leakage were subjected to analysis of variance (ANOVA) as a completely randomized design using the General Linear Models procedure of Statistical Analysis System (SAS®) [37]. Significant differences among the means were determined by Duncan's multiple range test at $p < 0.05$. Enrichment data were expressed as positive/total chickens (%), and the percentage of *S.* Typhimurium positive samples were compared by a chi-square test of independence [38], testing all possible combinations to determine the significance ($p < 0.05$).

Statistical differences of bacterial taxa at different levels (family and genus) among treatment groups were determined using Linear discriminant analysis effect size (LEfSe) using all against all comparison mode, where the level of significance was set at LDA score > 2.0 and $P<0.05$ [39]. The significant differences in alpha diversity were calculated using an alpha-group-significance command of QIIME2, which is based on the Kruskal-Wallis test. In the contrary, statistical differences in beta diversity among groups were calculated by PERMANOVA [39] test using a beta-group-significance command of QIIME2 with a pairwise option. For both diversities analysis, the corrected p values for multiple comparisons (q) were used to report a significant difference between the two groups, where the level of significance was set at $q < 0.05$.

Results

The results of the antimicrobial effect of CA, CR, and CA-CR on *S.* Typhimurium colonization in the CT of broiler chickens in trial one and trial two are summarized in Table 2. In both trials, all experimental treatments were able to significantly reduce the *S.* Typhimurium colonization in CT ($P< 0.0001$) when compared to the PC group. However, CR and CA-CR were the most effective treatments, since they reduced the colonization of *S.* Typhimurium more than 2.1 and 2.3 $\log_{10}$ ($P= 0.0019$ and $P= 0.008$), respectively, compared to PC. Although the data are not presented in Table 2, the presence of *Salmonella* was confirmed in all the samples of the experimental groups, with the exception of NC.

Furthermore, Table 2 shows the results of the dietary administration of CA, CR, and CA-CR on serum FITC-d concentration in broiler chickens on day ten post-*S*. Typhimurium challenge. In both trials, there were no significant differences in the serum FITC-d concentration when the CA and CA-CR groups were compared to groups PC and NC. However, the serum FITC-d concentration in chickens treated with CR was significantly lower when compared to PC (P= 0.0084), but there were no significant differences when compared to NC.

The effect of the dietary inclusion of treatment into the feed on growth performance of broiler chickens is summarized in Table 3. At the beginning of the experiment, no significant differences were shown in the weights of the broilers. However, at day 10, only the group treated with CR presented significant differences in BW compared to PC. Furthermore, at the end of the experiments, BWG increased significantly in the group treated with CR when compared to PC. Although the groups treated with CA and CA-CR did not show significant differences in BW on day 10 and BWG, a trend towards improvement in the productive parameters was observed in comparison with PC (P= 0.0853 and P= 0.1192, respectively). Finally, a decrease in feed consumption in PC and a better feed conversion rate in the group treated with CR was observed.

Cecal microbiota was analyzed in samples collected from day ten post-*S*. Typhimurium challenged birds. The relative abundance of different bacterial families recovered across different groups is shown in Fig. 1. In all five groups, either Ruminococcaceae or Lachnospiraceae were the most predominant families. Lachnospiraceae was the most dominant bacterial family in NC (44.10%) and CA (46.37%) followed by Ruminococcaceae (NC, 33.23%; CA, 32.86%). However, Ruminococcaceae was found the highest in PC (36.50%), CR (44.17%), and CA-CR (63.12%) followed by Lachnospiraceae (PC, 31.37%; CR, 31.78%; CA-CR, 17.69%). LEfSe analysis (LDA score > 2.0 and P<0.05) revealed some important differentially abundant taxa at both bacterial family and genus level. As shown in Fig. 2, Enterococcaceae was significantly higher in the PC group while Clostridiaceae was significantly enriched in CR group. Furthermore, at genus level, *Salmonella, Coprobacillus, Eubacterium,* and *Clostridium* were significantly abundant in PC, while the genera *Fecalibacterium* and *Enterococcus* were significantly enriched in CR group and the unknown genera that belong to Erysipelotrichaceae and Lachnospiraceae were significantly enriched in CA-CR and CA groups, respectively (Fig. 3).

Alpha diversity analysis among the groups, as measured by observed OTUs is shown in Fig. 4. Despite there were no significant differences among the groups (Kruskal-Wallis test; $p > 0.05$), it was observed that PC group presented a numerically lower diversity compared to the other groups.

Beta diversities among different groups as measured by weighted and unweighted UniFrac distance metrics are illustrated in PCoA plots (Fig. 5A and 5B, respectively). Results of the Permutational multivariate analysis of variance (PERMANOVA) showed that there were no significant differences in the structure of the microbial community among the groups at $q < 0.05$.

Discussion

Modern animal production has been changing in recent years due to the problems of bacterial resistance derived from the overuse of antimicrobials for prophylactic and growth promotion purposes [40,41]. In this regard, many investigations have focused on probiotics, prebiotics, enzymes, acidifiers, plant extracts, and some metals (copper and zinc) as feed additives, given their antimicrobial properties and effects in promoting growth, mainly [42]. In the present study, selection of CA was based on its advantages over inorganic sources since it has been described that inorganic sources tend to dissociate in the upper part of the gastrointestinal tract, causing a decrease in the availability of copper due to its interaction with other metals (chelation) and therefore a reduction in its activity [18,43]. In contrast, the solubility of organic sources of copper is higher in weak acid enviroments, making their dissolution slower and increasing their availability and activity [43]. Furthermore, lower fecal copper excretion rates have been reported in broilers exposed to an organic source of copper compared to inorganic sources [44]. Although the copper ion is known to be more effective against Gram-positive bacteria [45], dietary supplementation with CA significantly reduced more than 18% the colonization of *S*. Typhimurium in both trials compared to PC group (Table 2). Copper ion has been reported to cause damage at the bacterial membrane level due to its adhesion to membranes and the generation of reactive oxygen species [46]. Additionally, it can be associated with the functional groups of proteins and enzymes, leading to the inactivation or inhibition of some cellular processes, as well as having a direct negative effect on the genetic material of bacteria [45,46]. Furthermore, In addition, this reduction in the colonization of *S.* Typhimurium presented a positive effect on BW and BWG since they tend to improve in both experiments (P= 0.0814 and P= 0.0853, respectively), as well as FI and FCR when compared to PC.

In the case of the group treated with the solid dispersion of curcumin (CR), which was previously described by our research group and is characterized by being more soluble and permeable [22], the colonization of *S*. Typhimurium significantly decreased by more than 35% (more than 2 $\log_{10}$) with respect to the PC group after ten days of treatment (Table 2). These results are due to the antimicrobial action of curcumin, which in general, is associated with damage to the bacterial membrane and inhibition of bacterial cell proliferation [47,48]. Furthermore, it has been published that curcumin can induce some physical and mechanical changes of the *S*. Typhimurium flagellar filament, causing a decrease in motility, adherence, and invasion of the host cells, which results in a reduction or elimination of its virulence [49]. Likewise, curcumin has been reported to decrease bacterial cell division processes since it interacts with the FtsZ protein, a cytoskeleton protein essential for this process [50]. The treatment containing the mixture of CA and CR (CA-CR) reduced 2% and 37% the *S.* Typhimurium colonization compared to the group treated with CR and the PC group, respectively. These results suggest that physical mixtures between CR and CA do not improve or enhance the pharmacological effects of curcumin compared to the complexes that it can form with heavy metals, including copper. These complexes that are chemically synthesized have been shown to have a better effect than curcumin itself and even decrease the toxicity of metals [16,51,52].

After oral infection with *Salmonella*, this pathogen must overcome the conditions of the gastrointestinal tract to interact with the intestinal epithelium [53]. Invasion of epithelial layers by *S*. Typhimurium is

known to increase intestinal permeability in both *in vivo* and *in vitro* models since the expression of some markers such as claudin-1, occludin, and mucin-2, mRNA levels of zonula occludens-1 and E-cadherin was reduced [53,54]. In the present study, FITC-d, a large molecule (3–5 kDa) that, under normal intestinal health conditions, does not leak through the epithelium, was used to assess intestinal permeability. However, when there is damage to the epithelium, the permeability of FITC-d increases so that it can be quantified in serum [55]. In the present study, all treated groups showed lower serum FITC-d concentrations compared to the PC group (Table 2). However, only the group treated with CR had significantly lower concentrations when compared to PC and turned out to have serum FITC-d concentrations comparable to the NC group. Perhaps, this result is due to the ability of CR to restore the intestinal barrier function and the expression of proteins associated with the tight junctions, the proliferation-regeneration of the intestinal epithelium, and its antimicrobial action, resulting in decreased paracellular permeability as has been previously reported [56,57]. Regarding the treatments with CA and CA-CR, although the *S.* Typhimurium counts decreased significantly compared to the PC group, the serum FITC-d concentration only decreased numerically since it has been described that the production of reactive oxygen species by copper affects not only bacteria but also epithelial cells [58].

The chicken gut microbiota are densely populated with complex microbial communities that are involved in digestion and metabolism, regulation of enterocytes, vitamin synthesis, and development and regulation of the host immune system [59]. However, the cecum is by far the most densely colonized microbial habitat in chickens [60]. Despite the absence of any clinical signs of *Salmonella* infection, the composition of the microbiota was affected but, the changes in the cecal microbiota were quite weak [61,62], which supports our results since no significant differences in alpha (measured by the observed OTUs) and beta diversity was observed in the cecal samples at ten days post-*S*. Typhimurium challenge, which means that there were no changes in the relationship of the number of different species per sample (richness) and in the diversity of the microbial community between different samples, respectively [63]. Notwithstanding the above, the taxonomic composition showed some significant differences at the family and genus levels when the groups were compared.

At the family level, Enterococcaceae was significantly higher in the PC group when compared to the other treatment groups. Enterococcaceae, one of the six families of the order Lactobacillales [64], is comprised of the genera *Enterococcus*, *Bavariicoccus*, *Catellicoccus*, *Melissococcus*, *Pilibacter*, *Tetragenococcus*, and *Vagococcus* [65]. So this decrease in Enterococcaceae in other dietary treatment groups as compared to the PC group could be due to copper since it has been described that it alters the intestinal microbiota and decreases the counts of lactic acid bacteria [66]. In contrast, *Salmonella* infection is known to increase the relative abundance of Enterococcaceae, Lactobacillaceae, Clostridiaceae, Lachnospiraceae, Erysipelotrichaceae, Peptostreptococcaceae, and Ruminococcaceae, but decrease that of Enterobacteriaceae [67]. Furthermore, the Clostridiaceae was significantly higher in chickens treated with CR when compared to other groups. Clostridiaceae is one of the responsible families for converting polysaccharides into short-chain fatty acids (SCFAs) [68]. It has been described that SCFAs such as acetate, propionate, and butyrate, are important maintaining intestinal homeostasis due to their immunomodulatory capacity, maintenance of metabolism, proliferation, differentiation and promotion at

low pH, favoring beneficial bacteria, and reducing the growth and viability of pathogenic bacteria [69], thus supporting our findings and relating to the antimicrobial activity of the treatments, as well as to the improvement in the productive parameters since the BW and BWG increased significantly and there were greater efficiency in FI and FCR in the group treated with CR in both experiments when compared to PC.

At the genus level, *Salmonella*, *Coprobacillus*, *Eubacterium*, and *Clostridium* were significantly enriched in the PC group, which is closely related to the severity of the *Salmonella* infection process. *Coprobacillus*, *Clostridium*, and *Eubacterium* have an important role in the production of SCFAs essential amino acids and the digestion of non-starch polysaccharides, which stimulate the production of SCFAs for metabolic balance [68,70]. Likewise, it has been reported that the reduction of *Clostridium* and the maintenance of *Eubacterium* and *Coprobacillus* levels could be related to the effectiveness of the treatments since they represent a positive effect in the maintenance of intestinal homeostasis [70–72]. Finally, high levels of *Salmonella* are related to colonization in cecal tonsils [73] and are closely related to the severity of the infection. Furthermore, the genus *Faecalibacterium* and *Enterococcus* were significantly enriched in the group treated with CR. After infection with *Salmonella*, this pathogenic bacteria alters the intestinal microbiota, causing a decrease in bacteria of the genus *Blautia*, *Enorma*, *Faecalibacterium*, *Shuttleworthia*, *Sellimonas*, *Intestinimonas*, and *Subdoligranulum*, as well as an increase in the abundance of *Butyricicoccus*, *Erysipelatoclostridium*, *Oscillibacter* and *Flavonifractor* [59]. However, in the case of the group treated with CR, the increase in *Faecalibacterium*, a genus of bacteria responsible for the production of butyrate and related to health benefits in poultry, could be mainly due to the prebiotic effect of curcumin, like other substances with the same activity [74]. It has been described that CR could act as a factor of promotion, proliferation, growth, and survival for the beneficial bacteria of the intestinal microbiota from its biotransformation [75]. Finally, the bacterial genera that belong to Erysipelotrichaceae and Lachnospiraceae were significantly enriched in the CA-CR and CA groups, respectively. It has been published that in chickens infected with *Salmonella* this genus of bacteria decreases markedly, which could negatively affect the diversity and development of intestinal bacteria [67]. In the specific case of CA and CA-CR, copper is known to increase the relative abundance of these bacterial genera, which are the most active microbial components in the healthy gut and are responsible for preventing the production of inflammatory cytokines and induce intestinal production of SCFAs by fermenting carbohydrates [76,77].

Conclusion

According to the previous results, it can be concluded that the treatment with CR was the most effective in reducing *S*. Typhimurium counts. Furthermore, it was determined that the antimicrobial activity of CR, when administered at 0.2% into the feed using an *S*. Typhimurium infection laboratory model, is based on a combined mechanism in which direct activity on pathogenic bacteria and the prebiotic effect is mainly involved. Finally, it is clear that the physical mixtures of CR with a metal such as copper (CA) are not comparable with the formation of the corresponding complexes as far as effect is concerned. Studies to confirm and expand these results with a larger number of animals and considering the analysis of inflammatory and antioxidant biomarkers to get a complete description of CR required further investigation.

Abbreviations

BGA: Brilliant green agar; CT: Cecal tonsils; CA: Copper Acetate; CR: Curcumin; CA-CR: Copper acetate and curcumin; FITC-d: Fluorescein isothiocyanate dextran; LEfSe: Linear discriminant analysis effect size; NA: Nalidixic acid; NO: Novobiocin; SCFAs: Short-chain fatty acids; TSB: Tryptic soy broth; NC: Negative control; PC: Positive control; XLT-4: Xylose Lysine Tergitol-4

Declarations

Acknowledgments

The authors thank the CONACyT for the doctoral scholarship number 447447 and the financial support obtained through the program PAPIIT IN218115 of DGAPA-UNAM.

Authors' contributions:

AAL-D, DH-P, BS-C, BA, GT-I Conception, design, and drafting of the manuscript. JDL, XH-V, BF-M, BM-H, GT-I, Drafting the article or revising it critically for valuable intellectual content. AAL-D, DH-P, BS-C, JDL, BA, YMK Acquisition of data. BM-H, RL-A, GT-I, DH-P, BS-C, Analysis, and interpretation of data.

Funding

Research was supported in part by funds provided by USDA-NIFA Sustainable Agriculture Systems, Grant No. 2019-69012-29905. Title of Project: Empowering US Broiler Production for Transformation and Sustainability USDA-NIFA (Sustainable Agriculture Systems): No. 2019-69012-29905.

Data availability statement

The sequencing data of cecal microbiota is available on NCBI Sequence Read Archive (SRA) under BioProject number BioProject ID PRJNA655142.

Ethics approval

All animal handling procedures complied with the Institutional Animal Care and Use Committee (IACUC) at the University of Arkansas, Fayetteville (protocol #18029).

Consent for publication

Not applicable.

Competing interests

The authors declare they have no competing interests.

References

1. Uzzau S, Brown DJ, Wallis T, Rubino S, Leori G, Bernard S, et al. Host adapted serotypes of *Salmonella enterica*. Epidemiol Infect. 2000;125:229–55.
2. Ochman H, Groisman EA. The origin and evolution of species differences in *Escherichia coli* and *Salmonella* Typhimurium. EXS. 1994;69:479–93.
3. Revolledo L, Ferreira CSA, Ferreira AJP. Prevention of *Salmonella* Typhimurium colonization and organ invasion by combination treatment in broiler chicks. Poult Sci. 2009;88:734–43.
4. Marcq C, Cox E, Szalo IM, Thewis A, Beckers Y. *Salmonella* Typhimurium oral challenge model in mature broilers: Bacteriological, immunological, and growth performance aspects. Poult Sci. 2011;90:59–67.
5. Rajagopal R, Mini M. Outbreaks of salmonellosis in three different poultry farms of Kerala, India. Asian Pac J Trop Biomed. 2013;3:496–500.
6. Majowicz SE, Musto J, Scallan E, Angulo FJ, Kirk M, O'brien SJ, et al. The global burden of nontyphoidal *Salmonella* gastroenteritis. Clin Infect Dis. 2010;50:882–9.
7. Jajere SM. A review of *Salmonella enterica* with particular focus on the pathogenicity and virulence factors, host specificity and antimicrobial resistance including multidrug resistance. Vet World. 2019;12:504-21. doi: 10.14202/vetworld.2019.504-521
8. Hinton A, Corrier DE, Spates GE, Norman JO, Ziprin RL, Beier RC, et al. Biological control of *Salmonella* Typhimurium in young chickens. Avian Dis. 1990;34:626.
9. Tellez G, Petrone VM, Escorcia M, Morishita TY, Cobb CW, Villasenõr L. Evaluation of avian-specific probiotic and *Salmonella* Enteritidis, *Salmonella* Typhimurium-, and *Salmonella* Heidelberg-specific antibodies on cecal colonization and organ invasion of *Salmonella* Enteritidis in broilers. J Food Prot. 2001;64:287–91.
10. Babu US, Raybourne RB. Impact of dietary components on chicken immune system and *Salmonella* infection. Expert Rev Anti Infect Ther. 2008;6:121–35.
11. Finstad S, O'Bryan CA, Marcy JA, Crandall PG, Ricke SC. *Salmonella* and broiler processing in the United States: Relationship to foodborne salmonellosis. Food Res Int. 2012;45:789–94.
12. Sharan R, Chhibber S, Reed RH. A murine model to study the antibacterial effect of copper on infectivity of *Salmonella enterica* serovar Typhimurium. Int J Environ Res Public Health. 2011;8:21–36.
13. Wang X, Biswas S, Paudyal N, Pan H, Li X, Fang W, et al. Antibiotic resistance in *Salmonella* Typhimurium isolates recovered from the food chain through national antimicrobial resistance monitoring system between 1996 and 2016. Front Microbiol. 2019;10:985.
14. Cheng G, Hao H, Xie S, Wang X, Dai M, Huang L, et al. Antibiotic alternatives: the substitution of antibiotics in animal husbandry? Front Microbiol. 2014;5:217.
15. Barik A, Mishra B, Shen L, Mohan H, Kadam RM, Dutta S, et al. Evaluation of a new copper (II)–curcumin complex as superoxide dismutase mimic and its free radical reactions. Free Radic Biol Med. 2005;39:811–22.

16. Hieu TQ, Thao DTT. Enhancing the solubility of curcumin metal complexes and investigating some of their biological activities. J Chem. 2019;2019(ID8082195):1-8. https://doi.org/10.1155/2019/8082195
17. Benhalima L, Amri S, Bensouilah M, Ouzrout R. Antibacterial effect of copper sulfate against multi-drug resistant nosocomial pathogens isolated from clinical samples. Pakistan J Med Sci. 2019;35:1322.
18. Parra A, Toro M, Jacob R, Navarrete P, Troncoso M, Figueroa G, et al. Antimicrobial effect of copper surfaces on bacteria isolated from poultry meat. Brazilian J Microbiol. 2018;49:113–8.
19. Menon VP, Sudheer AR. Antioxidant and anti-inflammatory properties of curcumin BT . In: Aggarwal BB, Surh Y-J, Shishodia S, editors. The molecular targets and therapeutic uses of curcumin in health and disease. Boston, MA: Springer US; 2007. p. 105–25. https://doi.org/10.1007/978-0-387-46401-5_3
20. Bose S, Panda AK, Mukherjee S, Sa G. Curcumin and tumor immune-editing: Resurrecting the immune system. Cell Div. 2015;10:6–8.
21. Guil-Guerrero JL, Ramos L, Paredes JCZ, Carlosama-Yépez M, Moreno C, Ruales P. Effects of turmeric rhizome powder and curcumin in poultry production. A review. J Anim Feed Sci. 2017;26:293–302.
22. Hernandez-Patlan D, Solis-Cruz B, Pontin KP, Latorre JD, Baxter MFA, Hernandez-Velasco X, et al. Evaluation of a solid dispersion of curcumin with polyvinylpyrrolidone and boric acid against *Salmonella* Enteritidis infection and intestinal permeability in broiler chickens: A pilot study. Front Microbiol. 2018;9:1289. https://www.frontiersin.org/article/10.3389/fmicb.2018.01289
23. Hernandez-Patlan D, Solis-Cruz B, Pontin KP, Latorre JD, Hernandez-Velasco X, Merino-Guzman R, et al. Evaluation of ascorbic acid or curcumin formulated in a solid dispersion on *Salmonella* Enteritidis infection and intestinal integrity in broiler chickens. Pathogens. 2019;8:229.
24. National Research Council. Nutrient requirements of poultry. 9th ed. Washington, DC, USA: National Academies Press; 1994.
25. Cobb-Vantress I. Cobb 500 broiler performance and nutrition supplement. Cobb-Vantress; 2015.
26. Lin J, Lee IS, Frey J, Slonczewski JL, Foster JW. Comparative analysis of extreme acid survival in *Salmonella* Typhimurium, *Shigella flexneri*, and *Escherichia coli*. J Bacteriol. 1995;177:4097–104.
27. Vicuña EA, Kuttappan VA, Galarza-Seeber R, Latorre JD, Faulkner OB, Hargis BM, et al. Effect of dexamethasone in feed on intestinal permeability, differential white blood cell counts, and immune organs in broiler chicks. Poult Sci. 2015;94:2075–80.
28. Vicuña EA, Kuttappan VA, Tellez G, Hernandez-Velasco X, Seeber-Galarza R, Latorre JD, et al. Dose titration of FITC-D for optimal measurement of enteric inflammation in broiler chicks. Poult Sci. 2015;94:1353–9.
29. Baxter MFA, Merino-Guzman R, Latorre JD, Mahaffey BD, Yang Y, Teague KD, et al. Optimizing fluorescein isothiocyanate dextran measurement as a biomarker in a 24-h feed restriction model to induce gut permeability in broiler chickens. Front Vet Sci. 2017;4:56.

30. Parada AE, Needham DM, Fuhrman JA. Every base matters: assessing small subunit rRNA primers for marine microbiomes with mock communities, time series and global field samples. Environ Microbiol. 2016;18:1403–14.
31. Apprill A, McNally S, Parsons R, Weber L. Minor revision to V4 region SSU rRNA 806R gene primer greatly increases detection of SAR11 bacterioplankton. Aquat Microb Ecol. 2015;75:129–37.
32. Thompson LR, Sanders JG, McDonald D, Amir A, Ladau J, Locey KJ, et al. A communal catalogue reveals Earth's multiscale microbial diversity. Nature. 2017;551:457–63.
33. Bolyen E, Rideout JR, Dillon MR, Bokulich NA, Abnet C, Al-Ghalith GA, et al. QIIME 2: Reproducible, interactive, scalable, and extensible microbiome data science. PeerJ Preprints. 2018;27295. https://www.researchgate.net/publication/328490233
34. Callahan BJ, McMurdie PJ, Rosen MJ, Han AW, Johnson AJA, Holmes SP. DADA2: high-resolution sample inference from Illumina amplicon data. Nat Methods. 2016;13:581.
35. DeSantis TZ, Hugenholtz P, Larsen N, Rojas M, Brodie EL, Keller K, et al. Greengenes, a chimera-checked 16S rRNA gene database and workbench compatible with ARB. Appl Environ Microbiol. 2006;72:5069–72.
36. Wickham H. ggplot2: elegant graphics for data analysis. New York: Springer-Verlag; 2016. https://ggplot2-book.org/index.html
37. SAS Institute Inc. SAS User Guide. No Title. Cary, NC: SAS Institute Inc; 2002.
38. Zar JH. Biostatistical analysis. 2nd. USA: Prentice Hall; 1984.
39. Anderson MJ. Permutation tests for univariate or multivariate analysis of variance and regression. Can J Fish Aquat Sci. 2001;58:626–39.
40. Manyi-Loh C, Mamphweli S, Meyer E, Okoh A. Antibiotic use in agriculture and its consequential resistance in environmental sources: potential public health implications. Molecules. 2018;23:795.
41. Sharma C, Rokana N, Chandra M, Singh BP, Gulhane RD, Gill JPS, et al. Antimicrobial resistance: its surveillance, impact, and alternative management strategies in dairy animals. Front Vet Sci. 2018;4:237.
42. Dhama K, Tiwari R, Khan RU, Chakraborty S, Gopi M, Karthik K, et al. Growth promoters and novel feed additives improving poultry production and health, bioactive principles and beneficial applications: the trends and advances-A Review. Pharmacology. 2014;10:129–59.
43. Olukosi OA, van Kuijk S, Han Y. Copper and zinc sources and levels of zinc inclusion influence growth performance, tissue trace mineral content, and carcass yield of broiler chickens. Poult Sci. 2018;97:3891–8.
44. Attia YA, Abdalah AA, Zeweil HS, Bovera F, El-Din AAT, Araft MA. Effect of inorganic or organic copper additions on reproductive performance, lipid metabolism and morphology of organs of dual-purpose breeding hens. Arch Geflugelk. 2011;3:169–78.
45. Farkhonde Masoule S, Pourhajibagher M, Safari J, Khoobi M. Base-free green synthesis of copper (II) oxide nanoparticles using highly cross-linked poly(curcumin) nanospheres: synergistically improved

antimicrobial activity. Res Chem Intermed. 2019;45:4449–62.

46. Grass G, Rensing C, Solioz M. Metallic copper as an antimicrobial surface. Appl Environ Microbiol. 2011;77:1541–7.
47. Rai D, Singh JK, Roy N, Panda D. Curcumin inhibits FtsZ assembly: an attractive mechanism for its antibacterial activity. Biochem J. 2008;410:147–55.
48. Tyagi P, Singh M, Kumari H, Kumari A, Mukhopadhyay K. Bactericidal activity of curcumin I is associated with damaging of bacterial membrane. PLoS One 2015;10:e0121313. http://www.ncbi.nlm.nih.gov/pmc/articles/PMC4374920/
49. Amol Marathe S, Balakrishnan A, Devi Negi V, Sakorey D, Chandra N, Chakravortty D. Curcumin reduces the motility of *Salmonella enterica* serovar Typhimurium by binding to the flagella, thereby leading to flagellar fragility and shedding. J Bacteriol. 2016;198:1798–811.
50. Silva AC da, Santos PD de F, Silva JT do P, Leimann FV, Bracht L, Gonçalves OH. Impact of curcumin nanoformulation on its antimicrobial activity. Trends Food Sci Technol. 2018;72:74–82.
51. Zorofchian Moghadamtousi S, Abdul Kadir H, Hassandarvish P, Tajik H, Abubakar S, Zandi K. A review on antibacterial, antiviral, and antifungal activity of curcumin. Biomed Res Int. 2014;2014:186864.
52. Călinescu M, Fiastru M, Bala D, Mihailciuc C, Negreanu-Pîrjol T, Jurcă B. Synthesis, characterization, electrochemical behavior and antioxidant activity of new copper (II) coordination compounds with curcumin derivatives. J Saudi Chem Soc. 2019;23:817–27.
53. Tafazoli F, Magnusson K-E, Zheng L. Disruption of epithelial barrier integrity by *Salmonella enterica* serovar Typhimurium requires geranylgeranylated proteins. Infect Immun. 2003;71:872–81.
54. Wang L, Li L, Lv Y, Chen Q, Feng J, Zhao X. *Lactobacillus plantarum* restores intestinal permeability disrupted by *Salmonella* infection in newly-hatched chicks. Sci Rep. 2018;8:1–10.
55. Ruff J, Barros TL, Tellez Jr G, Blankenship J, Lester H, Graham BD, et al. Research Note: Evaluation of a heat stress model to induce gastrointestinal leakage in broiler chickens. Poult Sci. 2020;99:1687–92.
56. Bereswill S, Muñoz M, Fischer A, Plickert R, Haag L-M, Otto B, et al. Anti-inflammatory effects of resveratrol, curcumin and simvastatin in acute small intestinal inflammation. Ahmed N, editor. PLoS One 2010;5:e15099. http://www.ncbi.nlm.nih.gov/pmc/articles/PMC2997083/
57. Ghosh SS, He H, Wang J, Gehr TW, Ghosh S. Curcumin-mediated regulation of intestinal barrier function: the mechanism underlying its beneficial effects. Tissue Barriers. 2018;6(1):e1425085. https://doi.org/10.1080/21688370.2018.1425085
58. Zhao M, Tang S, Xin J, Wei Y, Liu D. Reactive oxygen species induce injury of the intestinal epithelium during hyperoxia. Int J Mol Med. 2018;41:322–30.
59. Khan S, Chousalkar KK. *Salmonella* Typhimurium infection disrupts but continuous feeding of *Bacillus* based probiotic restores gut microbiota in infected hens. J Anim Sci Biotechnol. 2020;11:1–16.

60. Pourabedin M, Zhao X. Prebiotics and gut microbiota in chickens. FEMS Microbiol Lett. 2015;362:fnv122.
61. Videnska P, Sisak F, Havlickova H, Faldynova M, Rychlik I. Influence of *Salmonella enterica* serovar Enteritidis infection on the composition of chicken cecal microbiota. BMC Vet Res. 2013;9:140.
62. Liu L, Lin L, Zheng L, Tang H, Fan X, Xue N, et al. Cecal microbiome profile altered by *Salmonella enterica*, serovar Enteritidis inoculation in chicken. Gut Pathog. 2018;10:34.
63. Prehn-Kristensen A, Zimmermann A, Tittmann L, Lieb W, Schreiber S, Baving L, et al. Reduced microbiome alpha diversity in young patients with ADHD. PLoS One. 2018;13.
64. Monedero V, Revilla-Guarinos A, Zúñiga M. Chapter ome - Physiological role of two-component signal transduction systems in food-associated lactic acid bacteria. In: Sariaslani S, Gadd GM, editors. Advances in Applied Microbiolgy. Academic Press; 2017. p. 1–51. https://doi.org/10.1016/bs.aambs.2016.12.002
65. Sharma N, Angural S, Rana M, Puri N, Kondepudi KK, Gupta N. Phytase producing lactic acid bacteria: Cell factories for enhancing micronutrient bioavailability of phytate rich foods. Trends Food Sci Technol. 2020;96:1-12. https://doi.org/10.1016/j.tifs.2019.12.001
66. Zhang Y, Zhou J, Dong Z, Li G, Wang J, Li Y, et al. Effect of dietary copper on intestinal microbiota and antimicrobial resistance profiles of *Escherichia coli* in weaned piglets. Front Microbiol. 2019;10.
67. Mon KKZ, Saelao P, Halstead MM, Chanthavixay G, Chang H-C, Garas L, et al. *Salmonella enterica* serovars Enteritidis infection alters the indigenous microbiota diversity in young layer chicks. Front Vet Sci. 2015;2:61.
68. Clavijo V, Flórez MJV. The gastrointestinal microbiome and its association with the control of pathogens in broiler chicken production: A review. Poult Sci. 2018;97:1006–21. http://www.sciencedirect.com/science/article/pii/S0032579119310922
69. Purama RK, Raman M, Ambalam P, Pithva S, Kothari C, Doble M. Chapter 30 - Prebiotics and probiotics in altering microbiota: Implications in colorectal cancer. In: Chatterjee S, Jungraithmayr W, Bagchi D, editors. Immun Inflamm Heal Dis 2018. Academic Press; p. 403–13. http://www.sciencedirect.com/science/article/pii/B9780128054178000305
70. Khan S, Chousalkar KK. Short-term feeding of probiotics and synbiotics modulates caecal microbiota during *Salmonella* Typhimurium infection but does not reduce shedding and invasion in chickens. Appl Microbiol Biotechnol. 2020;104:319–34.
71. Li J, Hao H, Cheng G, Liu C, Ahmed S, Shabbir MAB, et al. Microbial shifts in the intestinal microbiota of *Salmonella* infected chickens in response to enrofloxacin. Front Microbiol. 2017;8:1711. https://www.frontiersin.org/article/10.3389/fmicb.2017.01711
72. Bauer BW, Gangadoo S, Bajagai YS, Van TTH, Moore RJ, Stanley D. Oregano powder reduces *Streptococcus* and increases SCFA concentration in a mixed bacterial culture assay. PLoS One. 2019;14.
73. Sivula CP, Bogomolnaya LM, Andrews-Polymenis HL. A comparison of cecal colonization of *Salmonella enterica* serotype Typhimurium in white leghorn chicks and *Salmonella*-resistant mice.

BMC Microbiol. 2008;8:182. https://doi.org/10.1186/1471-2180-8-182

74. Micciche AC, Foley SL, Pavlidis HO, McIntyre DR, Ricke SC. A review of prebiotics against *Salmonella* in poultry: Current and future potential for microbiome research applications. Front Vet Sci. 2018;5:191. https://pubmed.ncbi.nlm.nih.gov/30159318
75. Pluta R, Januszewski S, Ułamek-Kozioł M. Mutual two-way interactions of curcumin and gut microbiota. Int J Mol Sci. 2020;21:1055.
76. Song M, Li X, Zhang X, Shi H, Vos MB, Wei X, et al. Dietary copper-fructose interactions alter gut microbial activity in male rats. Am J Physiol Gastrointest Liver Physiol. 2018;314:G119–30. https://pubmed.ncbi.nlm.nih.gov/29025734
77. Zhang F, Zheng W, Xue Y, Yao W. Suhuai suckling piglet hindgut microbiome-metabolome responses to different dietary copper levels. Appl Microbiol Biotechnol. 2019;103:853–68. https://doi.org/10.1007/s00253-018-9533-0

Tables

Table 1, Ingredient composition and nutrient content of a basal starter diet used in the experiment on as-fed basis

Item	Corn soybean-based diet
Ingredients, (g/kg)	
Corn	574.5
Soybean meal	346.6
Poultry oil	34.5
Dicalcium phosphate	18.6
Calcium carbonate	9.9
Salt	3.8
DL-Methionine	3.3
L-Lysine HCl	3.1
Threonine	1.2
Choline chloride 60 %	2.0
Vitamin premix[1]	1.0
Mineral premix[2]	1.0
Antioxidant[3]	0.5
Calculated analysis	
Metabolizable energy, (MJ/kg)	12.7
Crude protein, (g/kg)	221.5

[1]Vitamin premix supplied per kg of diet: Retinol, 6 mg; cholecalciferol, 150 µg; dl-α-tocopherol, 67.5 mg; menadione, 9 mg; thiamine, 3 mg; riboflavin, 12 mg; pantothenic acid, 18 mg; niacin, 60 mg; pyridoxine, 5 mg; folic acid, 2 mg; biotin, 0.3 mg; cyanocobalamin, 0.4 mg.

. [2]Mineral premix supplied per kg of diet: Mn, 120 mg; Zn, 100 mg; Fe, 120 mg; copper, 10 to 15 mg; iodine, 0.7 mg; selenium, 0.2 mg; and cobalt, 0.2 mg.

[3]Ethoxyquin

Table 2, Evaluation of copper acetate (CA), curcumin (CR) and copper acetate – curcumin (CA-CR) on cecal tonsils (CT) colonization of *Salmonella* Typhimurium[1] and serum concentration of fluorescein isothiocyanate–dextran (FITC-d) in broiler chickens on day ten post-*S*. Typhimurium challenge[2] in trial 1 and trial 2

Treatments	CT Log_{10} cfu/g	FITC-d (ng/mL)
	Trial 1	
CTRL (-)	0.00 ± 0.00 ^{d}	17.03 ± 5.44 ^{b}
CTRL (+)	6.18 ± 0.33 ^{a}	54.99 ± 10.51 ^{a}
CA	4.99 ± 0.32 ^{b}	35.19 ± 8.80 ^{ab}
CR	3.92 ± 0.55 ^{bc}	17.60 ± 7.50 ^{b}
CA-CR	3.76 ± 0.54 ^{c}	32.99 ± 10.34 ^{ab}
	Trial 2	
CTRL (-)	0.00 ± 0.00 ^{d}	19.80 ± 9.26 ^{b}
CTRL (+)	6.09 ± 0.276 ^{a}	59.38 ± 9.81 ^{a}
CA	4.94 ± 0.32 ^{b}	32.99 ± 11.31 ^{ab}
CR	3.91 ± 0.19 ^{c}	15.40 ± 7.60 ^{b}
CA-CR	3.78 ± 0.31 ^{c}	39.59 ± 15.06 ^{ab}

[1] Data expressed in Log_{10} cfu /g of tissue. Mean ± SE from 12 chickens. $^{a–b}$Values within treatments columns for each treatment with different superscripts differ significantly (P<0 .05).

[2] Chickens were orally gavaged with 10^4 cfu of *S*. Typhimurium per chicken at 1-d old, samples were collected at day 10 post-challenge.

Table 3, Evaluation of Evaluation of copper acetate (CA), curcumin (CR) and copper acetate – curcumin (CA-CR) on body weight (BW), body weight gain (BWG), feed intake (FI) and feed conversion ratio (FCR) in broiler chickens on day ten post-*S.* Typhimurium challenge in trial 1 and trial 2[1]

Treatments	BW, g/broiler (D 0)	BW, g/broiler (D 10)	BWG, g/broiler (D 0-10)	FI, g/broiler	FCR
			Trial 1		
CTRL (-)	40.60 ± 0.57	232.33 ± 9.46 ^{ab}	191.73 ± 9.39 ^{ab}	281.33	1.47
CTRL (+)	40.13 ± 0.86	204.97 ± 10.06 ^{b}	164.83 ± 10.05 ^{b}	240.33	1.46
CA	40.67 ± 0.70	227.50 ± 9.32 ^{ab}	186.83 ± 9.40 ^{ab}	270.33	1.45
CR	40.87 ± 1.01	237.93 ± 7.80 ^{a}	197.07 ± 7.60 ^{a}	274.67	1.39
CA-CR	40.47 ± 0.62	226.8 ± 8.75 ^{ab}	186.33 ± 8.83 ^{ab}	267.53	1.44
			Trial 2		
CTRL (-)	40.93 ± 0.64	231.83 ± 7.54 ^{ab}	190.90 ± 7.28 ^{ab}	277.47	1.45
CTRL (+)	40.07 ± 0.95	205.6 ± 7.75 ^{b}	165.53 ± 7.90 ^{b}	239.47	1.45
CA	40.40 ± 0.79	226.83 ± 8.82 ^{ab}	186.43 ± 8.66 ^{ab}	268.40	1.44
CR	41.27 ± 0.71	236.63 ± 8.00 ^{a}	195.37 ± 8.04 ^{a}	270.00	1.38
CA-CR	40.40 ± 0.84	226.6 ± 10.51 ^{ab}	186.2 ± 10.68 ^{ab}	270.67	1.45

[1]Data expressed as mean ± SE from 15 chickens. $^{a-b}$Values within columns with different superscripts differ significantly ($P< 0.05$).

Figures

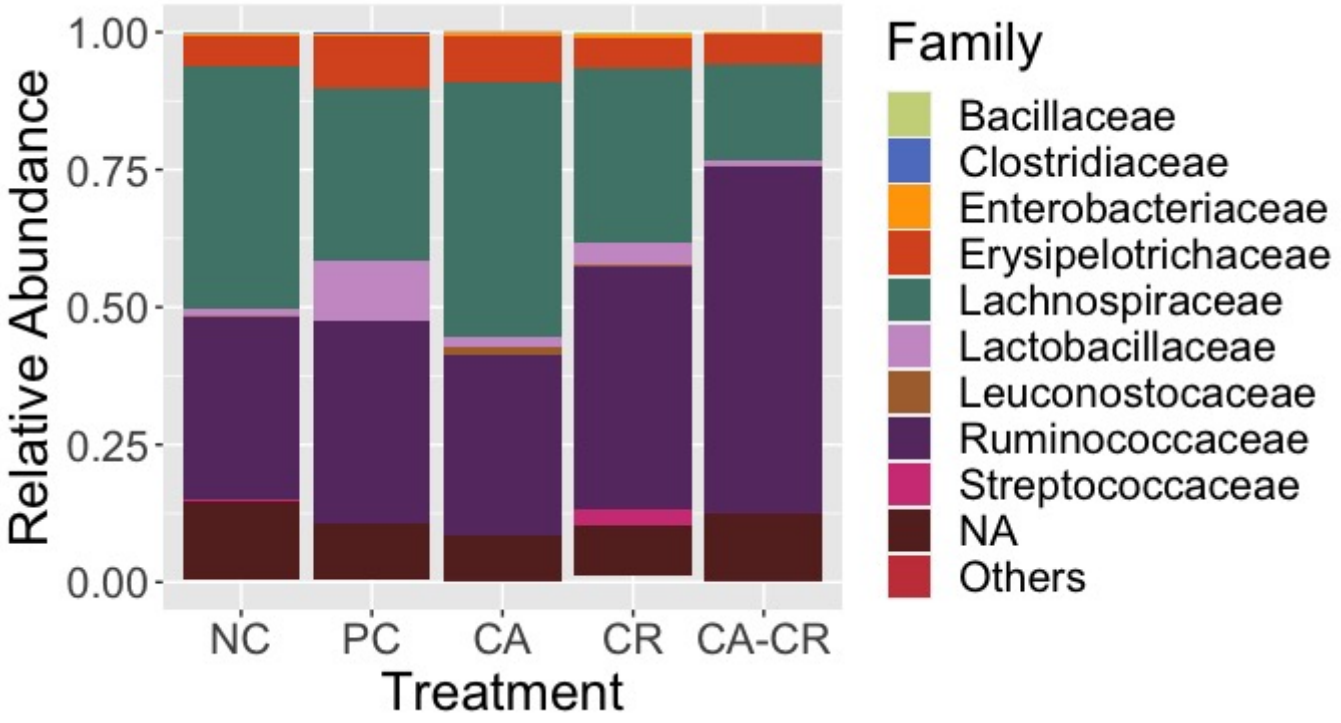

Figure 1

Taxonomic composition of the cecal microbiota in the different treatments at family level. NC: negative control, PC: positive control, CA: cooper acetate, CR: curcumin, and CA-CR: copper acetate – curcumin. "NA" refers to the bacterial taxa that were not assigned to the family level but were assigned at the higher taxonomic level. "Others" represent the minor bacterial families whose relative abundance were <0.1%.

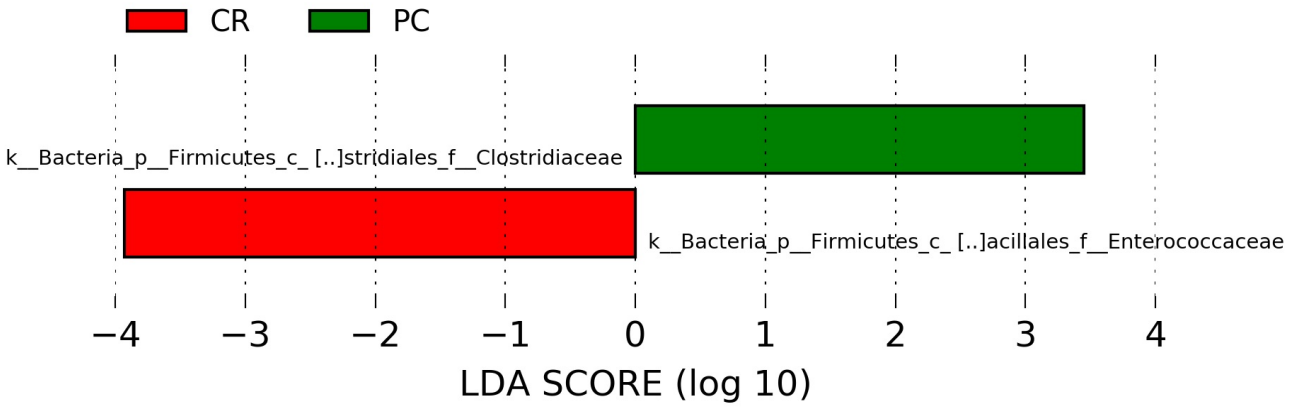

Figure 2

Taxonomic difference of the main families in the microbiota between the curcumin-treated group (CR) and the positive control (PC).

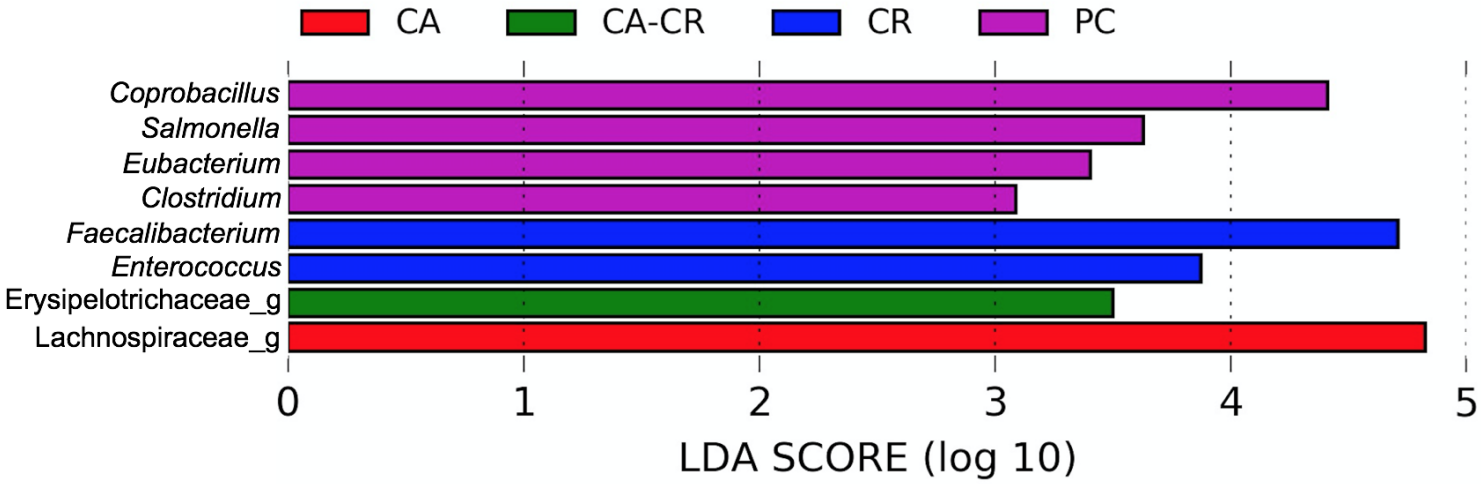

Figure 3

Taxonomic composition of the microbiota in the groups treated at the genus level. PC: positive control, CA: cooper acetate, CR: curcumin, and CA-CR: copper acetate – curcumin.

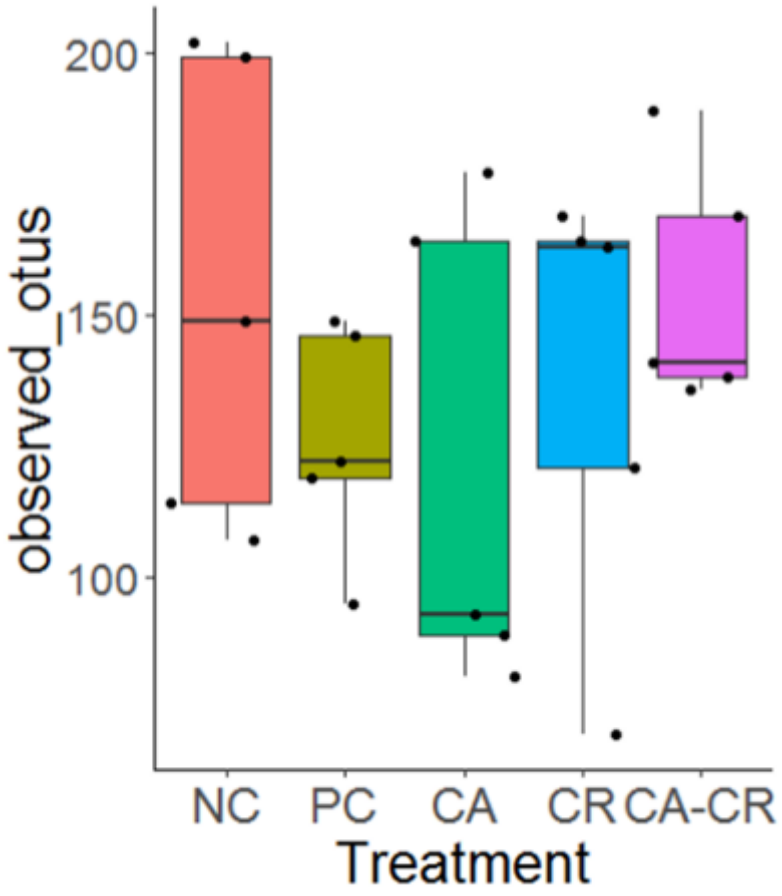

Figure 4

Comparison of the alpha diversities among the groups as measured by observed OTUs.

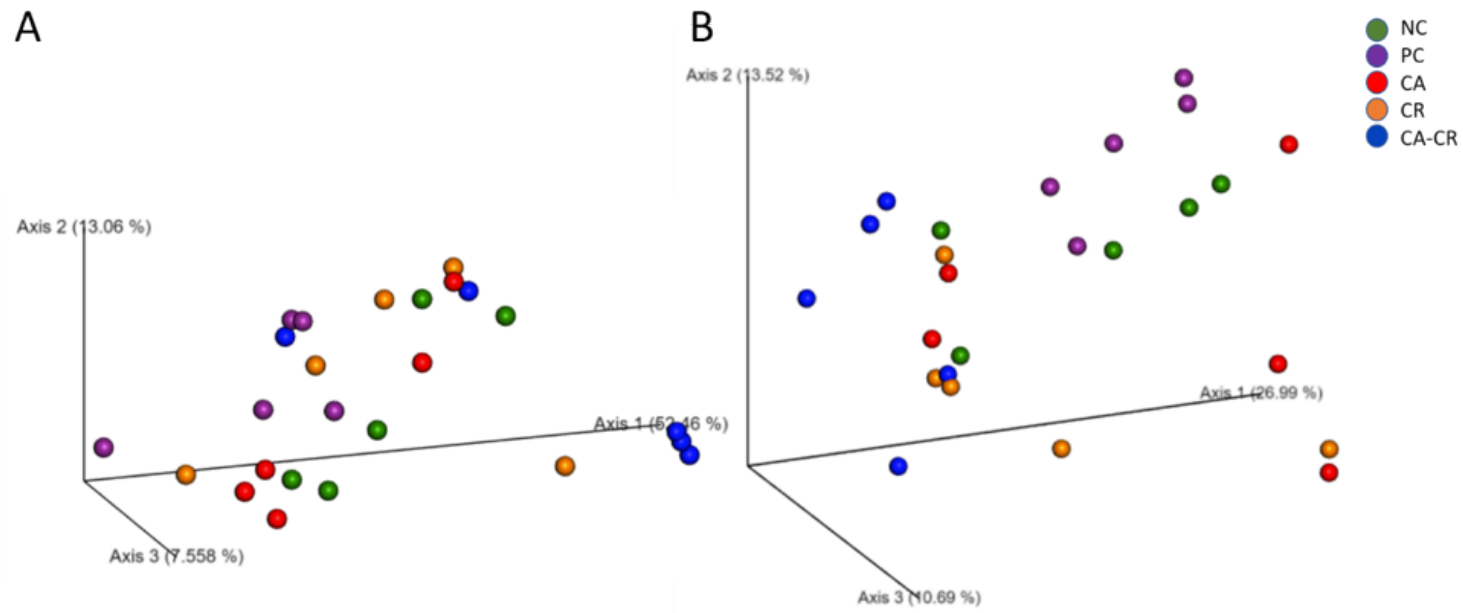

Figure 5

PCoA plots showing beta diversity among the groups according to (A) weighted UniFrac distance metric and (B) unweighted UniFrac distance metric.