Bacteriophage cocktail supplementation improves growth performance, gut microbiome and production traits in broiler chickens

Santi Devi Upadhaya
Dankook University - Cheonan Campus
Je Min Ahn
Dankook University - Cheonan Campus
Jae Hyoung Cho
Dankook University - Cheonan Campus
Hyeun Bum Kim
Dankook University - Cheonan Campus
Jin Young Kim
Dankook University - Cheonan Campus
Dae Kyung Kang
Dankook University - Cheonan Campus
Sung Woo Kim
North Carolina State University College of Agriculture and Life Sciences
Inho Kim (inhokim@dankook.ac.kr)
Dankook University https://orcid.org/0000-0001-6652-2504

Research

Keywords: Bacteriophage, Broiler chickens, Gut microbiome, Performance

Posted Date: October 8th, 2020

DOI: https://doi.org/10.21203/rs.3.rs-86723/v1

Version of Record: A version of this preprint was published at Journal of Animal Science and Biotechnology on April 16th, 2021. See the published version at https://doi.org/10.1186/s40104-021-00570-6.

Abstract

Background: Effective antibiotic alternatives are the urgent need of poultry industry to control disease outbreaks. Phage therapy mainly utilizes lytic phages to kill their respective bacterial hosts and can be attractive solution to combating the emergence of antibiotic resistance in livestock.

Methods: Five hundred and four one-day–old broilers (Ross 308) were allotted into 1 of 4 treatment groups according to a completely randomized design. Dietary treatments consisted of CON (basal diet), PC (CON + 0.025% Avilamax®(antibiotics), TR1 (CON + 0.05% bacteriophage), and TR2 (CON + 0.10 % bacteriophage) groups.

Results: A significant linear effect on body weight gain (BWG) was observed during day 1-7, day 22-35, and overall experiment in bacteriophage (BP) supplemented groups. The BWG tended to be higher (P = 0.08) and the feed intake (FI) was increased (P = 0.017) in birds fed PC than CON diets. A greater (P = 0.016) BWG and trends in increased FI (P = 0.06) were observed during the overall experiment period in birds fed PC than CON diet. A trend in linear (P = 0.0833) increment in excreta *Lactobacillus* counts was observed in birds fed graded level of BP supplemented diets. At the genus level, the relative abundance of *Lactobacillus* was decreased in PC (65.28%), while it was similar in TR1, 2, (90.65%, 86.72%, 81.44%) compared to CON (90.19%). At the species level, relative abundance of *Lactobacillus salivarus* was higher in TR1 (40.15%) and TR2 (38.58%) compared with CON (20.04%) and PC (18.05%). A linear reduction in the weight of Bursa of Fabricus (P = 0.022) and spleen (P = 0.052) was seen in birds fed increasing level of BP diets and a trend in increment (P = 0.059) in the weight of gizzard was observed in birds fed PC than BP diets. Linear and quadratic responses were observed in redness of breast muscle color in birds fed graded level of BP.

Conclusions: The increase in dietary BP supplementation linearly increased BWG, *Lactobacillus* counts and enhanced beneficial microbiota in the gut, and 0.05% BP addition was sufficient for supporting immune organs, bursa and spleen.

Background

In response to the increase in the demand of livestock products such as meat, milk and egg by a global growing population, livestock producers are compelled to significantly increase these products. Thus, large scale intensive farming system is continuing to rise. Unfortunately, such production systems can promote disease transmission very easily due to their low genetic diversity and high stocking density leading to concomitant production and economic losses [1, 2]. Zoonotic pathogens associated with poultry and pigs such as *Salmonella spp*., *E. coli*, *Campylobacter spp*., *Clostridium spp*. and *Listeria spp* have been reported by European Food Safety Authority (EFSA) to be often resistant to several antibiotics [3, 4]. In this context, alternative approaches have become urgent. One option would be the application of lytic bacteriophage to combat the bacterial diseases in livestock [5].

Bacteriophages are viruses that infect and use bacterial resources for their own reproduction. They are very common in all environments and have a high specificity to bacteria at infection [6]. In a review, Domingo et al. [7] suggested that bacteriophages have narrow spectrum activity against bacteria, in opposite to broad spectrum activity of antibiotics against bacteria. Bacteriophages being specific for particular bacteria, the phage therapy is considered to be safe and effective in comparison to antibiotics which is partially manifested as their ability to infect only one species, serotype or strain. This mechanism of action does not inhibit the proliferation of commensal intestinal flora [8, 9]. In a study, Fiorentin et al. [10] noted that the application of single oral cocktail of phages at a dosage of 10^{11} pfu decreased the occurrence of *Salmonella* Enteritidis strains by 3.5 log units.

In addition, other studies have also reported a successful reduction in the *Salmonella spp* counts in chicken internal organs and excreta [11] as well as in poultry products [12, 13] with bacteriophage application. Furthermore, it has been

reported that bacteriophage supplementation improved feed efficiency, liver weight and reduced pathogens in broiler chickens [14] and improved egg production and egg quality in laying hens [15].

The inclusion of phage as a feed additive may potentially provide an integrated solution to modulate the gut microbiome in chicken by reducing specific pathogenic microbial population thereby promoting the proliferation of beneficial microbiota resulting in improved gut health [16].

Under bacterial challenge, bacteriophage has shown to be effective in several studies [17–19], however reports on the usage of bacteriophage cocktail through dietary application in birds without bacterial challenge is scarce. Thus, the objective of the current study was to assess the effect of two different concentrations of cocktail bacteriophage on the performance and production characteristics as well as gut microbiome of broiler chickens raised under normal physiological condition (without inducing infection via bacterial challenge).

Material And Methods

Experimental design, animals and diets

Bacteriophages used in the present study was a commercial product from CJ Cheiljedang Corp. Seoul, South Korea, consisting of *Salmonella gallinarum*, *Salmonella typhimurium*, *S. Enteritidis, Escherichia coli* at the concentrations of 1.0×10^8 pfu/g each and *Clostridium perfringens* (1.0×10^6 pfu/g). A total of 504 1-d-old broilers (ROSS 308) with the initial BW 42.9 ± 1.0 g were used in a 35-d experiment. Chicks were randomly divided into the four experimental groups, and each group had 7 replicate cages, with 18 broilers per cage. The bacteriophage cocktail was administrated by replacing the same amount of corn. The treatment groups were as follows: i) CON group (Control/ basal diet without BP supplementation), ii) PC group (CON + 0.25 g antibiotics; AVILAMIX®/kg feed), iii) TR1 group (CON + 0.5 g bacteriophage/kg feed), and iv) TR2 group (CON + 1.0 g bacteriophage/kg feed). Broiler chickens were raised in a temperature-controlled room with stainless steel pens of identical size (1.75 × 1.55 m2).

Room temperature was maintained at 33 ± 1 °C for the first 3 d, and then gradually reduced by 3 °C a week until reaching 24 °C and maintained for the remainder of the experiment and the relative humidity was around 60%. The basal diet was formulated to meet or exceed all the nutrient requirements of broilers as recommended by National Research Council [20], and supplied in mash form. There were two nutritional phases, including starter (1 to 21 d), and finisher phase (22 to 35 d), and the ingredients and analyzed nutrient composition of the basal diet are shown in Table 1. Artificial light was provided 24 h/d by the use of fluorescent lights. All diets were fed in mash form with feed and water being provided ad libitum throughout the experimental period.

Sampling And Measurements

Growth performance

Broilers were weighed by cage and feed consumption was recorded at day 0, 7, 21 and 35. This information was then used to calculate body weight gain (BWG) average feed intake (FI), and feed conversion ratio (FCR).

Nutrient Digestibility

The apparent total tract digestibility of DM, N and energy was comparable between CON and PC treatments. In addition, inclusion of graded level of bacteriophage to the CON diet did not affect the digestibility of nutrients in birds as shown in Table 3.

Excreta Microbial Counts

For excreta microbial counts, excreta samples were collected from all 7 cages each treatment at day 35. The excreta samples were kept frozen at − 20 °C until microbiota analysis for the enumeration of *Salmonella*, *Escherichia coli* (*E. coli*), *Clostridium spp* and *Lactobacillus*. After thawing, viable counts of bacteria in the excreta were then determined by plating serial 10-fold dilutions (in 10 g/L peptone solution) in respective media. The selective medium used for isolation of *Salmonella* was Salmonella Shigella (Difco, USA), for *E. coli*, Mac Conkey (Difco, USA), for *Clostridia spp*. Cooked Meat Medium (Oxoid, UK) and for *Lactobacillus*, Lactobacilli medium III (Medium 638, DSMZ, Braunschweig, Germany). The Lactobacilli MRS agar plates were incubated for 48 h at 39 °C, and the MacConkey agar and Salmonella Shigella agar plates were incubated for 24 h at 37 °C whereas Cooked Meat Medium agar plates were incubated at 30 °C for 24 h under anaerobic conditions. The colony counts were then enumerated and results are presented as log10-transformed data.

Ileal Mucosa Microbiome

For gut microbiome analysis, six ileal mucosal samples from each group (CON, PC, TR1 and TR2) were collected at day 35 from randomly selected 24 broilers. Briefly, birds were sacrificed by cervical dislocation and exsanguination. After autopsy, the intestinal tract was excised and the intestinal content was removed followed by washing the intestinal segment with distilled water. Then ileal segment (distal ileum) was cut about 10–15 cm proximally to caeca and separated from the intestine and then rinsed in PBS and the mucosal layer was scraped with a glass slide. Mucosal scrapings were collected into a 50 ml conical tube and stored in an ice box and then transferred to Macrogen Inc., (Seoul, Republic of Korea) for gene sequencing. Genomic DNA extraction from the mucosal samples and the preparation of library of amplicons consisting of 16S rRNA gene and sequencing was done by Illumina MiSeq platform at Macrogen Inc. (Seoul, Republic of Korea) using MiSeq sequencing including barcoded 16S rRNA amplicons.

The 16S rRNA gene sequences were processed using the Mothur software to remove low-quality sequences [23]. Briefly, sequences that did not match the PCR primers were eliminated from de-multiplexed sequence reads. The sequences containing ambiguous base calls and sequences with a length less than 100 bp to were trimmed minimize the effects of random sequencing errors. Chimeric sequences were further deleted using the UCHIME algorithm implemented in Mothur. QIIME (Quantitative Insights into Microbial Ecology) software package (version 1.9.1) was used for de novo operational taxonomic unit (OTU) clustering with an OTU definition at an identity cutoff 97% [24]. Taxonomic assignment was performed using the naïve Bayesian RDP classifier and the Greengenes reference database. Beta-diversity was measured using unweighted UniFrac distance metrics using QIIME. The unweighted UniFrace considers the community membership (presence or absence of OTUs) [25]. Principal coordinate analysis (PCoA) plots were generated based on the unweighted UniFrac distance metrics.

Meat Quality

For physicochemical properties of the breast meat, at least one bird per pen (n = 10) from each treatment were selected randomly at day 35 and were individually weighed and killed by cervical dislocation and exsanguinated. The breast muscle (pectoralis major), Bursa of Fabricius, liver, spleen, and abdominal fat were then removed and weighed. Organ weights were expressed as a relative percentage to the whole body weight. The breast muscle Hunter lightness (L*), redness (a*), and yellowness (b*) values were determined using a Minolta CR410 chromameter (Konica Minolta Sensing Inc., Osaka, Japan). The pH of the breast muscle sample was measured by a calibrated, glass-electrode pH meter (Testo 205, Testo, Germany). The water-holding capacity (WHC) was analyzed according to the methods described by Kauffman et al. [26]. Drip loss was measured using approximately 2 g of meat sample according to the plastic bag method described by Honikel [27].

Statistical Analysis

Data were analyzed using the GLM procedure of SAS (version 9.4; SAS Inst., Inc., Cary, NC) in a completely randomized design. Pen served as the experimental unit. Pre-planned contrast was used to test the following: 1) the individual effect of CON versus PC diets 2) the overall effect of Bacteriophage supplementation versus PC diet (PC vs TR1, TR2). Furthermore, linear and quadratic polynomial contrasts were used to examine responses to supplemental graded levels of Bacteriophage at 0%, 0.05% and 0.1%. Variability in the data was expressed as the standard error of means (SEM) and $P \leq 0.05$ was considered to be statistically significant and $P < 0.1$ as trends.

For gut microbiome, analysis of similarities (ANOSIM) to determine whether the microbial compositions between the treatment and control groups were significantly different was done using QIIME software package (version 1.9.1) and was based on the unweighted UniFrac distance metrics.

Results

Growth performance

As shown in Table 2, the BWG tended to be higher (P = 0.089) in birds fed TR1 and TR2 diet during day 1–7 and overall experiment period compared with birds fed CON diet. A significant linear effect on BWG was observed during days 1–7, 22–35, and overall experiment in birds fed the diet supplemented with graded level of BP. During day 1–7, there were no significant differences between PC and CON diet on the growth performance parameters. However, the BWG tended to be higher (P = 0.08) during day 8–22 in birds fed PC diet than CON diet. During day 8–22, the FI was significantly increased (P = 0.017) in birds fed PC than CON diets and FI tended to be higher (P = 0.0796) in birds fed PC than the diet supplemented with BP. A significantly greater (P = 0.016) BWG and trends in increased FI (P = 0.06) were observed during the overall experiment period in birds fed PC diet than CON diet.

Excreta Microbial Enumeration

The effect of dietary bacteriophage supplementation on excreta microbiota counts in broiler chicken is presented in Table 4. The *Lactobacillus* counts tended to be higher (P = 0.058) in birds fed BP supplemented diet than the birds fed PC diet. However, the concentrations of *E. coli*, *Clostiridium perfringens*, and *Salmonella* were comparable between CON and PC diets or PC and TR1 and TR2 diets. A trend in linear (P = 0.0833) increment in *Lactobacillus* counts was observed in birds fed graded level of BP supplemented basal diet.

Gastrointestinal Microbiome

To evaluate the effect of BP on the gut microbiota of broiler chicken, the mucosa-attached microbiome in the ileum were analyzed by deep sequencing. Sequencing of the 16S rRNA genes in the mucosal samples produced a total of 1,121,448 reads after quality-filtering, with a mean sequence number of 36,473 ± 37,381 reads per sample. Analysis of similarities (ANOSIM) of unweighted UniFrace distances indicated that each group was clustered significantly different excluding control group ($P < 0.05$) suggesting that microbiota of the PC and TR1, 2 groups were significantly different. The unweighted UniFrac PCoA plot visually confirmed the distinct separation of microbial communities between groups (Fig. 1).

Comparisons of the relative abundances of the gut microbiota compositions between 4 groups at the phylum and genus levels are shown in Fig. 2. At the phylum level, the bacterial sequences from the CON samples were composed predominatly of the phyla Firmicutes (94.56%), Bacteroidetes (3.89%), Proteobacteria (1.38%) and 4 other phyla that collectively comprised 0.17% of the total sequences analyzed (Fig. 2a). PC group consisted largely of phyla Firmicutes (80.86%), Bacteroidetes (15.09%), Proteobacteria (2.78%), Deferribacteres (1.03%) and 4 other phyla which collectively comprised of 0.24% of the total sequences analyzed (Fig. 2a). In TR1 group, Firmicutes (94.57%) and Bacteroidetes (3.76%) were composed as predominant, the rest 6 phyla were comprised of 1.67% of the total sequences (Fig. 2a). In

TR2 group, Firmicutes (91.81%), Proteobacteria (5.45%) and Bacteroidetes (2.42%) were predominant, while other 5 phyla were composed of 0.32% of the total sequences analyzed (Fig. 2a).

At the genus level, *Lactobacillus* was the most enriched genera in all mucosal samples (Fig. 2b). And its relative abundance was decreased in PC (65.28%), while it was similar in TR1, 2, (90.65%, 86.72%, 81.44%) compared to CON (90.19%). The relative abundance of *Prevotella* increased from an average of 1.15% in CON to 2.56% in TR1 and 1.37% in TR2 (Fig. 3a) and *Bifidobacteria* also increased from an average of 0.01% in CON to 0.70% in TR1 and 0.14% in TR2 (Fig. 3b).

At the species level, while *Lactobacillus salivarius* and *Lactobacillus aviarius* represented the 2 most abundant species in all groups, its relative abundance increased from an average of 18.86% in CON to 40.13% in TR1 and 37.80% in TR2 in *L. salivarius* (Fig. 3c), 10.04% in CON to 15.60% in TR1 and 15.87% in TR2 in *L. aviarius* (Fig. 3d).

Meat Quality And Organ Weight

The effect of bacteriophage supplementation on organ weight and meat quality in broilers is shown in Table 5. Except for the significant reduction in relative weight of Bursa of Fabricus in birds fed PC than CON diets, none of the other meat quality and organ weight parameters were affected between CON and PC diets. The relative weight of gizzard showed trends in increment in birds fed PC than TRI and TR2 diets. A linear reduction in weight of bursa of fabricus (P = 0.026) and spleen (P = 0.052) relative to body weight were seen in birds fed diets supplemented with increasing level of bacteriophage. Linear and quadratic responses were observed in redness of breast muscle color for birds fed graded level of bacteriophage.

Discussion

The emergence of multidrug-resistant bacterial pathogens and the imposition of ban on the usage of antimicrobials in animal production have led to a resurgence of interest in phage therapy [28]. Research on reducing zoonotic pathogens with the application of BP as a viable option in food animals has also focused on reducing the impact of infections in the animals themselves [29] thereby improving the production and performance of animals.

In the present study, commercially available BP consisting of *Salmonella gallinarum, S. typhimurium, S. Enteritidis, E. coli and Clostridium perfringes* was assessed for its suitability as feed additive for the enhancement of performance and production of broiler chickens under normal physiological state (without bacterial challenge).

In agreement with the findings of Kim et al. [30] who demonstrated that FI and FCR were unaffected by supplementing the broilers diet with anti-SE bacteriophage, the inclusion of BP as feed additive at 0.05% and 0.1% levels in the present study showed no effects on FI and FCR throughout the trail, except for a trend in linear reduction in FCR during day 22–35. However, the present study showed significant linear effect in increasing the BWG with the increase in BP levels during the initial starter and finisher phases and overall experiment period indicating that BP supplementation had no detrimental effect on feed consumption but promoted the BWG. In contrast, Huff et al. [31] suggested that the BWG were not affected by the inclusion of bacteriophage in broiler chickens without bacterial challenge; and Wang et al. [14] noted that the supplementation of BP consisting of mixture of *Salmonella gallinarum, S. typhimurium*, and *S. Enteritidis* at the ratio of 3:3:4 at the dose level of 0.05% improved FCR during day 1–14 in broiler chickens. In broiler production, increase in body weight is an important parameter since lower body weight equates with increased cost for broiler meat production [30]. The increase in BWG when BP was used as a feed additive instead of antibiotics in animal feed might be due to the inhibitive or lytic effect on harmful bacteria replication in the gastrointestinal tract of broiler chickens [32]. Moreover as expected, the inclusion of sub-therapeutic dose of antibiotic as positive control in the diet of broiler chickens

led to higher BWG and FI than the birds fed basal diet without antibiotics which agrees with several other studies [33–35] suggesting that improvement in BWG might be due to increase in FI.

The supplementation of antibiotics or bacteriophage to the basal diet did not have significant effect on nutrient digestibility. In line with the findings of Wang et al. [14], the ATTD of nutrients was not affected by the supplementation of increasing levels of BP. Further experiments are needed to confirm the lack of response of antibiotics or BP on nutrient digestibility.

Salmonella is the major cause of food borne diseases worldwide with chickens as the main reservoir. Other zoonotic pathogens include *Clostridium, Campylobacter, E. coli*. For the control of these pathogens in poultry, bactericidal bacteriophages may provide a natural, nontoxic, feasible and non-expensive component. Previous works indicated that Salmonellae can be controlled by the use of bacteriophages [18, 36, 37, 38]. Early studies with *E. coli* also demonstrated that phage therapy can be as efficient as antibiotics [39, 40]. The reduction in *E. coli* and *Salmonella* counts in the excreta of broiler chickens by the application of bacteriophage has been reported [14]. Conversely, in the present study, dietary supplementation of BP did not have significant effect on the pathogenic bacteria such as *E. coli*, *Salmonella* as well as *Clostridium* counts isolated from the caecal digesta. However, a trend in linear increase in *Lactobacillus* count was observed in birds fed BP diets. The possible reason for non-significant effect of BP on nutrient digestibility and pathogenic food borne bacterial counts among the treatments might be the birds were raised in hygienic environment and were not experimentally challenged with bacteria due to which gastro intestinal tract might not have been colonized by harmful micro-organisms thereby maintaining gut in healthy state.

The gastrointestinal microbiota plays a crucial role in gut associated host immune system. Moreover, the physiological development, health, and productivity is also influenced by gut microbiota. Poultry diets have tremendous impact on the gut microbiome in regard to diversity and composition [41]. The manipulation of the microbial community through the inclusion of feed additives such as phage is feasible in order to enhance chicken growth and control either human or animal pathogens. Several studies have reported the use of bacteriophages as a feed additive in animals in order to control bacteria transmitted by foodstuffs. These models include the use of phages to control *Salmonella* and *Campylobacter* in broiler chickens [8, 42]. Microbiome analysis showed that Firmicutes, Bacteroidetes, and Proteobacteria are the predominant phyla in the avian gut [43], which is also supported by the results from our study. In TR1 group, Firmicutes and Bacteroidetes were composed as predominant, whereas in TR2 group besides, Firmicutes, and Bacteroidetes the members of phylum Proteobacteria was also predominant. The presence of members of phylum Proteobacteria in TR2 may indicate that BP dose of 0.1% may not be favorable as increase in Proteobacteria may be associated to increase in *E. coli*. In PC group, in addition to Firmicutes, Bacteroidetes, Proteobacteria, members of phylum Deferribacteres (1.03%) was also present. Members of phylum Firmicutes was reduced whereas members of Proteobacteria and Bacteroidetes were increased in pigs receiving antibiotic treatment. The composition of microbiota at the genus and species level was modified, decreasing the abundance of *Lactobacillus* at the genus level in PC as compared with CON, TR1 and TR2 as well as an increase in the relative abundance of *Prevotella* and *Bifidobacteria* in phage treated groups compared with CON and PC groups. The genus *Lactobacillus* plays a crucial role in the homeostasis of the gastrointestinal tract of metazoans [44].

At the species level, the *Lactobacillus salivarus* population in ileum mucosa in phage treated groups was twofold as compared with CON and PC suggesting the efficacy of the phage in promoting the beneficial bacteria which eventually contributes in improved performance and gut health.

With regards to meat quality, a significant quadratic response in the redness and lightness values of meat color was observed with the increase in the level of bacteriophage. Although, the meat color is closely associated with the meat pH [45], we found that the pH of breast muscle was not different among treatments indicating that change in color was not due to pH. In partial agreement to our finding, Wang et al. [14] demonstrated that meat pH as well as meat color were not

affected by the addition of bacteriophage in the basal diet of broiler chickens. Besides pH, other reported factors affecting color inside the muscle include myoglobin content, muscle fiber orientation and the space between the muscle fibers [46]. Further studies on these factors with bacteriophage application could help explain the changes in color observed. With regards to organ weight, a tendency to increase in the relative weight of gizzard in PC than NC and BP groups was observed. The possible reason for increase in the relative weight of gizzard in PC compared with NC may be due to increase in FI in PC groups. The weight of spleen and Bursa of Fabricius relative to the percentage of body weight in NC was higher than PC group during day 35. However, the inclusion of the increasing level of bacteriophage to NC diet linearly reduced the weight of spleen and Bursa of Fabricius relative to the percentage of the body weight indicating BP at 0.05% is better among the levels tested. As the spleen and bursa are associated with immune function (as lymphoid organs) this may explain that BP level higher than 0.05% may not be effective in improving the immune functions.

Conclusions

Collectively, the data from the present study indicate that the application of increasing level of bacteriophage cocktail to the diet of commercially raised broiler chickens could promote body weight gain as well as enhance gut microbiota diversity and increase excreta *Lactobacillus* counts without having significant difference in *Salmonella*, *E. coli* and *Clostridium* counts as compared with broilers fed antibiotic supplemented diet. Furthermore, it was observed that lower levels of bacteriophage cocktail addition were sufficient for supporting bursa and spleen which are immune organs. These findings suggest that phage therapy is effective and a safe alternative feed additive for raising broilers under intensive farming systems.

List Of Abbreviations

ADFI, average daily feed intake; ADG, average daily gain; ATTD, apparent total tract digestibility; BP, Bacteriophage; BW, body weight; DM, dry matter; FCR, feed conversion ratio; GE, gross energy; N, nitrogen

Declarations

Acknowledgments

The authors are grateful to the lab members of the Department of Animal Resource and Science of Dankook University for their valuable assistance in conducting the experiments. We acknowledge CJ Cheiljedang Corp. Seoul, South Korea, for providing the financial support to conduct this research.

Author’s contributions

IHK, HBK, DKK, JYK and SWK designed the research; JMA, JHC and other lab members assisted with sampling, laboratory analyses and data generation. JMA, SDU and HBK analysed the data. SDU, IHK and HBK wrote the manuscript. All authors read and approved the final manuscript.

Availability of data and materials

All data generated or analyzed during this study are available from the corresponding author on request.

Ethics approval and consent to participate

The experimental protocol (DK-1-1942) was approved by the Animal Care and Use Committee of Dankook University, South Korea. The experiment was conducted at the poultry experimental unit of Dankook University (Anseodong, Cheonan, Choongnam, Korea).

Consent for publication

Not applicable

Competing interests

The authors declare that no competing interests exist. The manuscript has not been published previously

References

1. Jones BA, Grace D, Kock R, Alonso S, Rushton J, Said MY, et al. Zoonosis emergence linked to agricultural intensification and environmental change. Proc Natl Acad Sci. 2013;110(21):8399-
2. Nhung NT, Chansiripornchai N, Carrique-Mas Antimicrobial resistance in bacterial poultry pathogens: a review. Front Vet Sci. 2017;4:126.
3. European Food Safety Authority (EFSA). The European Union Summary Report on Trends and Sources of Zoonoses, Trends and Sources of Zoonoses, Zoonotic Agents and Food-borne Outbreaks in 2017 [Internet]. Available from: https://efsa.onlinelibrary.wiley.com/doi/epdf/10.2903/j. efsa.2018.5500.
4. European Food Safety Authority (EFSA). The European Union summary report on antimicrobial resistance in zoonotic and indicator bacteria from humans, animals and food in 2017. EFSA J. 17:e05598.
5. Brussow H, Kutter E. Phage ecology. In: Kutter E, Sulakvelidze A, editor Bacteriophages: Biology and applications. Boca Raton, Florida: CRC Press; 2005. p. 129-163.
6. White HE, Orlova EV. Bacteriophages: Their Structural Organisation and Function. 2019. doi:5772/intechopen.85484.
7. Domingo-Calap P, Georgel P, Bahram S. Back to the future: bacteriophages as promising therapeutic tools. HLA. 2016;87(3):133-40.
8. Wernicki A, Nowaczek A, Urban-Chmiel Bacteriophage therapy to combat bacterial infections in poultry. Virol J. 2017;14(1):179.
9. Cieplak T, Soffer N, Sulakvelidze A, Nielsen A bacteriophage cocktail targeting *Escherichia coli* reduces *E. coli* in simulated gut conditions, while preserving a non-targeted representative commensal normal microbiota. Gut Microbes. 2018;9(5):391-9.
10. Fiorentin L, Vieira ND, Barioni Jr W. Oral treatment with bacteriophages reduces the concentration of Salmonella Enteritidis PT4 in caecal contents of broilers. Avian Pathol. 2005;34(3):258-
11. Toro H, Price SB, McKee AS, Hoerr FJ, Krehling J, Perdue M, et al. Use of bacteriophages in combination with competitive exclusion to reduce Salmonella from infected chickens. Avian Dis. 2005;49(1):118-24.
12. 12 Whichard JM, Sriranganathan N, Pierson FW. Suppression of Salmonella growth by wild-type and large plaque variants of bacteriophage Felix O1 in liquid culture and on chicken frankfurters. J Food Prot. 2003;66(2):220-5.
13. Higgins JP, Higgins SE, Guenther KL, Huff W, Donoghue AM, Donoghue DJ, et al. Use of a specific bacteriophage treatment to reduce Salmonella in poultry products. Poult Sci. 2005;84(7):1141-5.
14. Wang JP, Yan L, Lee JH, Kim IH. Evaluation of bacteriophage supplementation on growth performance, blood characteristics, relative organ weight, breast muscle characteristics and excreta microbial shedding in broilers. Asian-Aust J Anim Sci. 2013;26(4):573-8.
15. Zhao PY, Baek HY, Kim IH. Effects of bacteriophage supplementation on egg performance, egg quality, excreta microflora, and moisture content in laying hens. Asian-Aust J Anim Sci. 2012;25(7):1015-20.
16. Clavijo V, Florez MJV. The gastrointestinal microbiome and its association with the control of pathogens in broiler chicken production: A review. Poult Sci. 2018;97(3):1006-

17. Bardina C, Spricigo DA, Cortés P, Llagostera M. Significance of the bacteriophage treatment schedule in reducing Salmonella colonization of poultry. Appl Environ Microbiol. 2012;78(18):6600-
18. Atterbury RJ, Van Bergen MA, Ortiz F, Lovell MA, Harris JA, De Boer A, et al. Bacteriophage therapy to reduce Salmonella colonization of broiler chickens. Appl Environ Microbiol. 2007;73(14):4543-9.
19. Fischer S, Kittler S, Klein G, Glünder G. Impact of a single phage and a phage cocktail application in broilers on reduction of *Campylobacter jejuni* and development of resistance. PLoS One. 2013;8(10):e78543.
20. National Research Council (NRC). Nutrient Requirements of Poultry. 9th rev. ed. National Academy Press, Washington, DC;
21. AOAC International. Official Methods of Analysis of AOAC International. 16th ed. AOAC International, Gaithersburg, MD; 20
22. Williams CH, David DJ, Lismaa O. The determination of chromic oxide in faeces samples by atomic absorption spectrophotometry. J Agric Sci. 1962;59(3):381-
23. Schloss PD, Westcott SL, Ryabin T, Hall JR, Hartmann M, et al. Introducing mothur: Open source, platform-independent, community-supported software for describing and comparing microbial communities. Appl Environ Microbiol. 2009;75(23):7537-41.
24. Caporaso JG, Kuczynski J, Stombaugh J, Bittinger K, Bushman FD, Costello EK, et al. QIIME allows analysis of high-throughput community sequencing data. Nature Methods, 2010;7(5):335-6.
25. Navas-Molina JA, Peralta-Sánchez JM, González A, McMurdie PJ, Vázquez-Baeza Y, Xu Z, et al. Advancing our understanding of the human microbiome using QIIME. Methods Enzymol. 2013;531:371-444.
26. Kauffman RG, Eikelenboom G, Van Der Wal PG, Merkus GSM, Zaar M. The use of filter paper to estimate drip loss of porcine musculature. Meat Sci. 1986;18(3):191-
27. Honikel KO. Reference methods for the assessment of physical characteristics of meat. Meat Sci. 1998. 49(4):447-
28. Deresinski S. Bacteriophage therapy: exploiting smaller fleas. Clin Infect Dis. 2009;48(8):1096-
29. Johnson RP, Gyles CL, Huff WE, Ojha S, Huff GR, Rath NC, et al. Bacteriophages for prophylaxis and therapy in cattle, poultry and pigs. Anim Health Res Rev. 2008;9(2):201-
30. Kim KH, Lee GY, Jang JC, Kim JE, Kim YY. Evaluation of anti-se bacteriophage as feed additives to prevent Salmonella enteritidis (SE) in broiler. Asian-Aust J Anim Sci. 2013;26(3):386-93.
31. Huff WE, Huff GR, Rath NC, Balog JM, Donoghue AM. Prevention of Escherichia coli infection in broiler chickens with a bacteriophage aerosol spray. Poult Sci. 2002;81(10):1486-91.
32. Yongsheng M, Pacan J, Wang Q, Xu Y, Huang X, Korenevsky A, et al. Microencapsulation of bacteriophage felix O1 into chitosan alginate microspheres for oral delivery. Appl Environ Microbiol. 2008;74(15):4799-805.
33. Gustafson RH, Bowen RE. Antibiotic use in animal agriculture. J Appl Microbiol. 1997;83(5):531-
34. Emami NK, Samie A, Rahmani HR, Ruiz-Feria CA. The effect of peppermint essential Oil and fructooligosaccharides, as alternatives to virginiamycin, on growth performance, digestibility, gut morphology and immune response of male broilers. Anim Feed Sci Technol. 2012;175(1-2):57-64.
35. Chattopadhyay MK. Use of antibiotics as feed additives: a burning question. Front Microbiol. 2014;5:
36. Berchieri Jr A, Lovell MA, Barrow PA. The activity in the chicken alimentary tract of bacteriophages lytic for Salmonella typhimurium. Res Microbiol. 1991;142(5):541-9.
37. Sklar IB, Joerger RD. Attempts to utilize bacteriophage to combat Salmonella enterica serovar Enteritidis infection in chickens. J Food Safety. 2001;21(1):15-29.
38. Lim TH, Lee DH, Lee YN, Park JK, Youn HN, Kim MS, et al. Efficacy of bacteriophage therapy on horizontal transmission of Salmonella gallinarum on commercial layer chickens. Avian Dis. 2011;55(3):435-8.

39. Barrow PA, Lovell MA, Berchieri A. Use of lytic bacteriophage for control of experimental Escherichia coli septicaemia and meningitis in chickens and calves. Clin Diagn Lab Immunol. 1998;5(3):294-
40. Huff WE, Huff GR, Rath NC, Balog JM, Donoghue AM. Therapeutic efficacy of bacteriophage and Baytril (enrofloxacin) individually and in combination to treat colibacillosis in broilers. Poult Sci. 2004;83(12):1944-7.
41. Broda-Molina D, Seifert J, Camarinha-Silva A. Current perspectives of chicken gastrointestinal tract and its microbiome. Comput Struct Biotechnol J. 2018;16:131-9.
42. Grant A, Hashem F, Parveen S. Salmonella and Campylobacter: antimicrobial resistance and bacteriophage control in poultry. Food Microbiol. 2016;53: 104-9.
43. Waite DW, Taylor M. Exploring the avian gut microbiota: current trends and future directions. Front Microbiol. 2015;6:673.
44. Jin LZ, Ho YW, Abdullah N, Jalaludin S. Probiotics in poultry: modes of action. Worlds Poult Sci J. 1997;53(4):351-
45. Fletcher DL. Poultry Meat Colour. In: Richardson RI, Mead GC, editors. Poultry Meat Science. New York: CABI Publishing; p. 159-75.
46. Barbut S. 2015. The Science of Poultry and Meat Processing. Accessed May 2020. http://www.poultryandmeatprocessing.com

Tables

Table 1
Ingredients composition and analyzed nutrient content of basal diets (as fed-basis).

	Phase[1]	
	Starter	**Finisher**
Ingredients, %		
Corn	55.84	61.57
Soybean meal	20.50	18.67
Corn gluten meal	14.73	10.35
Wheat bran	2.00	3.00
Soybean oil	3.00	3.00
Tri-calcium phosphate	1.81	1.29
Limestone	0.94	1.13
Salt	0.46	0.41
DL-Methionine (98%)	0.19	0.09
L-Lysine (98%)	0.23	0.19
Mineral mix[2]	0.10	0.10
Vitamin mix[3]	0.10	0.10
Choline	0.10	0.10
Calculated composition		
Metabolizable energy, kcal/kg	3184	3191
Analyzed composition		
Crude protein, %	22.79	19.90
Crude fat, %	5.51	5.63
Ash,%	5.59	5.06
Ca, %	0.92	0.85
Available P, %	0.40	0.29
Lysine, %	1.06	0.98
Methionine, %	0.45	0.36

[1] Starter diet provided during d 1 to 21; Finisher diet provided during d 22 to 35.

[2]Provided per kg of complete diet: 11,025 IU vitamin A; 1103 IU vitamin D3; 44 IU vitamin E; 4.4 mg vitamin K; 8.3 mg riboflavin; 50 mg niacin; 4 mg thiamine; 29 mg d-pantothenic; 166 mg choline; 33 µg vitamin B12.

[3]Provided per kg of complete diet:12 mg Cu(as CuSO4.5 H_2O); 85 mg Zn (as $ZnSO_4$); 8 mg Mn (as MnO 2); 0.28 mg I (as KI); 0.15 mg Se (as Na2SeO3.5H_2O).

Table 2
The effect of bacteriophage cocktail supplementation on growth performance in broilers1

Items	PC	CON	TR1	TR2	SEM[2]	P- value				
		0%	0.05%	1.0%		PC vs CON	CON vs TR1, TR2	PC vs TR1, TR2	Linear	Quadratic
d 1 to 7										
BWG, g	114.33	109.55	111.37	116.92	2.092	0.1235	0.0896	0.9437	0.0383	0.5101
FI, g	138.68	135.74	139.2	141.19	3.049	0.5077	0.2476	0.6833	0.1746	0.8240
FCR	1.213	1.241	1.253	1.21	0.025	0.4302	0.749	0.5538	0.2499	0.2511
d 8 to 21										
BWG, g	706.35	675.18	683.16	685.25	11.94	0.0813	0.5447	0.1471	0.6080	0.8671
FI, g	1017.19	969.53	985.43	990.12	12.93	0.0178	0.2644	0.0796	0.2770	0.7263
FCR	1.441	1.443	1.444	1.4471	0.032	0.9749	0.9422	0.9134	0.9344	0.9874
d 22 to 35										
BWG, g	977.95	927.46	958.56	982.54	20.62	0.1006	0.1052	0.773	0.0379	0.8647
FI, g	1774.22	1741.25	1744.61	1755.91	27.8	0.4127	0.4943	0.4907	0.6542	0.8881
FCR	1.820	1.883	1.826	1.791	0.044	0.3226	0.1821	0.8333	0.0840	0.7902
Overall										
BWG, g	1798.63	1712.19	1753.09	1784.71	23.00	0.016	0.0593	0.3053	0.0288	0.8576
FI, g	2930.06	2846.52	2869.25	2887.22	30.64	0.0698	0.4902	0.1842	0.2470	0.9359
FCR	1.63	1.665	1.638	1.619	0.023	0.2881	0.2052	0.902	0.1370	0.8915

[1] Abbreviation: CON, Basal diet without antibiotics or bacteriophage; PC, CON + 0.025% AVILAMIX®; TR1, CON + 0.05% Bacteriophage; TR2, CON + 0.10% Bacteriophage.

[2] Standard error of means.

Values represent the means of 7 pens with 18 chickens per pen

Table 3
The effect of bacteriophage cocktail supplementation on apparent total tract nutrient digestibility in broilers1

Items,%	PC	CON	TR1	TR2	SEM[2]	P- value				
		0%	0.05%	1.00%		PC vs CON	CON vs TR1, TR2	PC vs TR1, TR2	Linear	Quadratic
Day 35										
Dry matter	71.50	69.85	70.26	70.68	0.899	0.2104	0.5788	0.3625	0.4715	0.9976
Nitrogen	71.03	68.63	69.99	70.24	1.660	0.3209	0.4747	0.6592	0.4716	0.7731
Energy	71.76	69.92	70.55	70.86	0.918	0.1745	0.4923	0.3638	0.4338	0.8755
[1] Abbreviation: CON, Basal diet without antibiotics or bacteriophage; PC, CON + 0.025% AVILAMIX®; TRT1, CON + 0.05% Bacteriophage; TRT2, CON + 0.10% Bacteriophage.										
[2] Standard error of means.										
Values represent the means of 7 pens with 18 chickens per pen.										

Table 4
The effect of bacteriophage cocktail supplementation on excreta microbial counts in broilers1

Items, log 10 cfu/ml	PC	CON	TR1	TR2	SEM[2]	P- value				
		0%	0.05%	1.00%		PC vs CON	CON vs TR1, TR2	PC vs TR1, TR2	Linear	Quadratic
Day 35										
Lactobacillus	8.93	8.99	9.115	9.206	0.096	0.6578	0.1551	0.058	0.0833	0.8632
E.coli	5.524	5.552	5.566	5.706	0.129	0.8789	0.5985	0.4835	0.3863	0.6798
Clostridium perfringens	5.512	5.601	5.543	5.529	0.142	0.6607	0.7111	0.8911	0.6689	0.8789
Salmonella	4.096	4.168	4.128	4.118	0.114	0.6581	0.7493	0.8478	0.733	0.9058
[1] Abbreviation: CON, Basal diet without antibiotics or bacteriophage; PC, CON + 0.025% AVILAMIX®; TR1, CON + 0.05% Bacteriophage; TR2, CON + 0.10% Bacteriophage.										
[2] Standard error of means.										
Values represent the means of 7 pens with 18 chickens per pen.										

Table 5
The effect of bacteriophage cocktail supplementation on meat quality and organ weight in broilers1

Items	PC	CON	TR1	TR2	SEM[2]	P- value				
		0%	0.05%	1.00%		PC vs CON	CON vs TR1, TR2	PC vs TR1, TR2	Linear	Quadratic
pH value	7.49	7.52	7.54	7.54	0.0287	0.3845	0.6189	0.1392	0.6821	0.8963
Breast muscle color										
Lightness (L*)	56.51	57.81	54.97	59.07	1.011	0.3689	0.5285	0.6803	0.3749	0.0096
Redness (a*)	12.81	13.02	14.05	11.85	0.457	0.7455	0.9000	0.8030	0.0598	0.0050
Yellowness (b*)	10.80	10.73	11.71	11.65	0.67	0.9449	0.2592	0.2932	0.3568	0.5366
WHC, %	54.79	55.26	54.69	54.97	2.465	0.8931	0.8886	0.9880	0.9279	0.8783
Drip loss, %										
d 1	3.43	3.96	3.58	3.77	0.304	0.2314	0.4583	0.5141	0.6853	0.4802
d 3	5.29	5.48	5.58	5.34	0.126	0.3022	0.9057	0.2832	0.4853	0.3250
d 5	9.17	9.74	9.65	9.16	0.374	0.2913	0.4701	0.6140	0.2868	0.6650
d 7	13.93	14.66	14.20	14.10	0.414	0.2284	0.3322	0.6660	0.3787	0.7361
Relative organ weight, %										
Breast muscle	24.28	22.97	23.46	24.07	0.592	0.1290	0.2815	0.4844	0.2076	0.9382
Liver	2.74	2.72	2.72	2.62	0.122	0.8969	0.7486	0.6388	0.5869	0.7342
Bursa of Fabricius	0.12	0.13	0.12	0.11	0.006	0.0464	0.0226	0.9944	0.0262	0.6886
Abdominal fat	1.19	1.11	1.21	1.21	0.067	0.4196	0.2395	0.7995	0.3781	0.5717
Spleen	0.18	0.19	0.18	0.17	0.006	0.1444	0.0667	0.8625	0.0521	0.8386
Gizzard	1.13	1.12	1.05	1.05	0.031	0.8796	0.0847	0.0596	0.1135	0.2432

[1] Abbreviation: CON, Basal diet without antibiotics or bacteriophage; PC, CON + 0.025% AVILAMIX®; TR1, CON + 0.05% Bacteriophage; TR2, CON + 0.10% Bacteriophage.

[2] Standard error of means.

Values represent the means of 10 chickens that are randomly selected (including at least 1 from each pen)

Figures

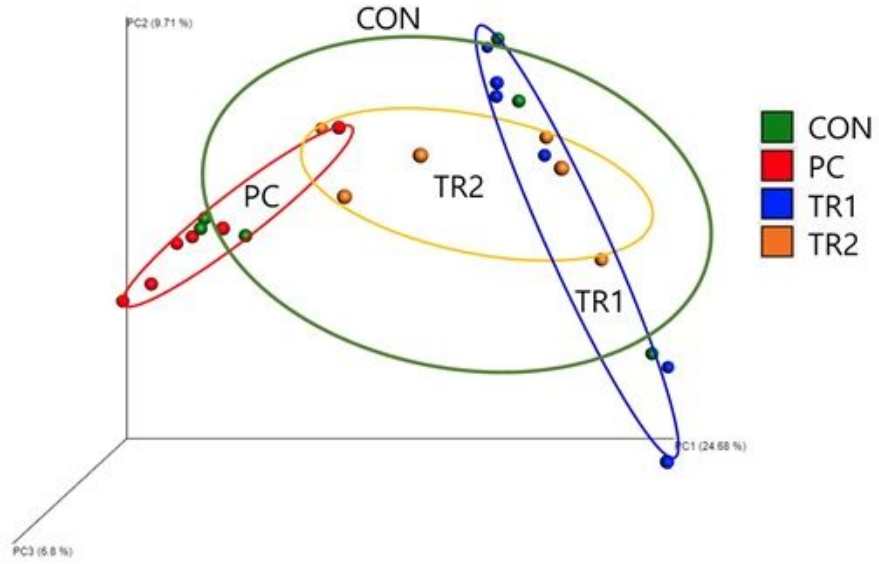

Figure 1

Principal coordinates analysis (PCoA) plots based on unweighted UniFrac distance metrics showing difference in microbial community structure between CON, Basal diet (green), PC, CON + 0.025% Avilamix (red), TR1, CON + 0.05% Bacteriophage (blue), and TR2, CON + 0.10% Bacteriophage (orange) group.

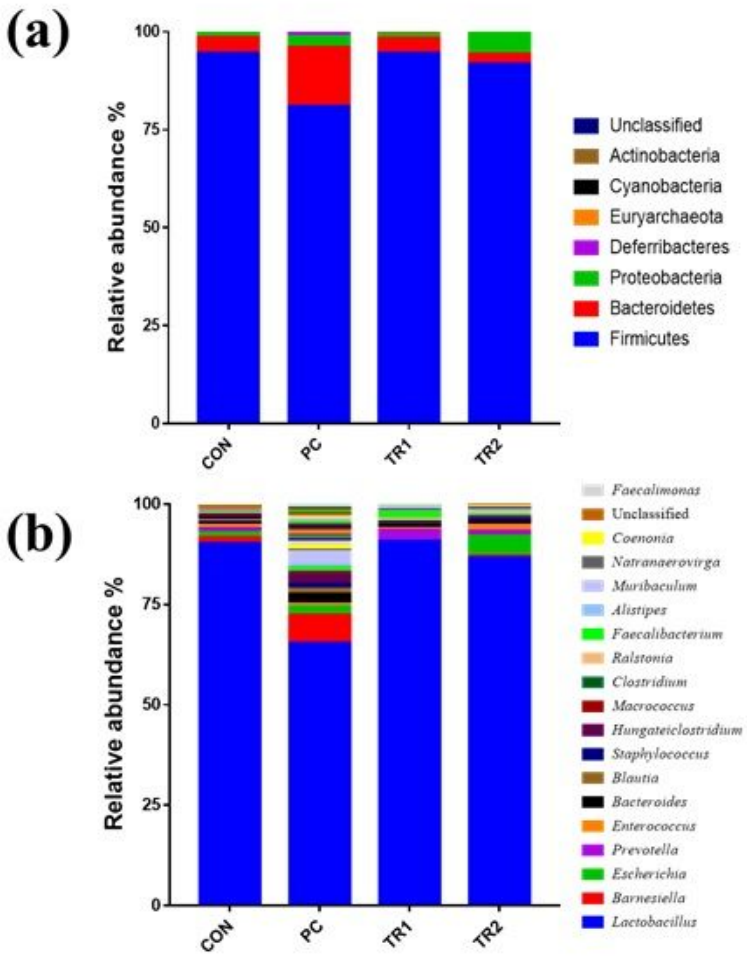

Figure 2

Taxonomic classification of the 16S rRNA gene sequences at the (a) phylum and (b) genus levels in the gut microbiome of broiler fed CON, Basal diet without antibiotics/bacteriophage; PC, CON + 0.025% Avilamix®; TR1, CON + 0.05% Bacteriophage; TR2, CON + 0.10% Bacteriophage.

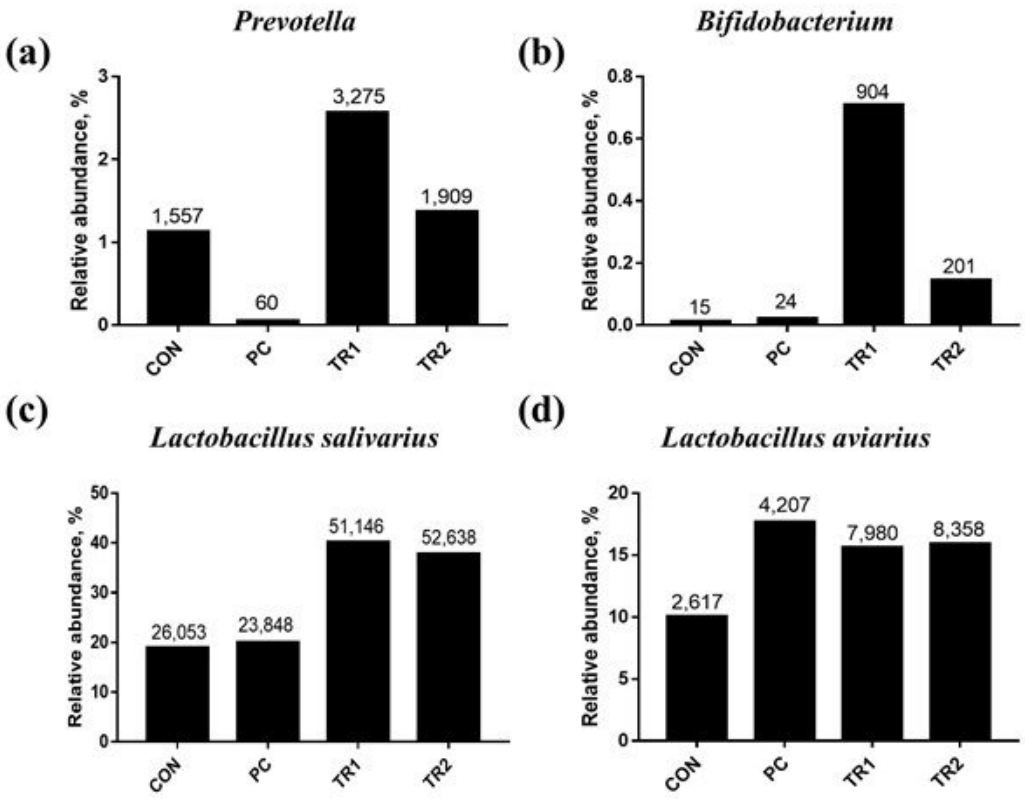

Figure 3

The bar plot identifying the difference in taxa between the gut microbiome of broiler fed CON, Basal diet without antibiotics or bacteriophage; PC, CON + 0.025% Avilamix®; TR1, CON + 0.05% Bacteriophage; TR2, CON + 0.10% Bacteriophage groups at the genus (a, b) and species (c, d) level. The numbers on each bar indicates the normalized abundance of each strains.