Effects of phages on the gut microbiota and their impact on probiotic efficacy in broiler chickens

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Abstract

Background Probiotics are the most recognised alternative to antibiotic growth promoters (AGPs) for poultry production. They adhere to the intestinal epithelial cells to exert their beneficial effects on the host. However, this is often restricted by indigenous gut microbes that compete for adhesion sites and nutrients. Therefore, phage application has been proposed to reduce the competition of probiotics with gut microbiota. The main objective of this study was to investigate the effects of phages on the gut microbiota and their impact on probiotic efficacy in chickens. Results Four best Escherichia coli lytic phages were selected and characterised. The in vivo trial was conducted to investigate the effects of dietary treatments of phage cocktail at 0.1% (1P) and 0.2% (2P), 0.1% PrimaLac ® probiotic (P) and their combinations (1P, 2P) on growth performance and gut microbiota of chickens. The results from the in vivo trial showed that chickens in the 1P group had significantly (P < 0.05) better body weight (BW), body weight gain (BWG), and feed conversion ratio (FCR), lower serum triglycerides and higher ileal villus height than the control. Chickens in the 1P group also had higher (P < 0.05) ileal villus height, and lower crypt depth than those in the probiotic group. The gut microbiota profiles revealed that 1P supplementation significantly (P < 0.05) reduced E. coli population, increased (P < 0.001) short chain fatty acids (SCFAs) producers, and improved (P < 0.001) gene expressions that are related to carbohydrate and amino acid metabolisms, and nutrient uptake. Conclusions The results from this study showed that 1P treatment could modulate the gut microbiota and enhance growth performance in chickens. This finding could be considered as a potential alternative to AGPs for poultry.

Background

Gut microbiome plays a crucial role in the overall process of the host. Several studies have documented that healthy gut boosts growth performance (feed intake to body weight gain ratio) and immune health of the host [17, 18, 19] This is why modulation of gut microbes has been routinely performed in poultry industry through the use of antibiotics growth promoters (AGPs), probiotics or other feed additives [20, 21]. The host improvement through gut modulation is mainly achieved through reduction of gut microbes (commensals or pathogens) which are competing for nutrients, releasing toxic by-products and causing diseases [22].

Probiotics are microorganisms that can confer beneficial effects to the host when consumed in adequate amounts [1, 2]. The use of probiotic in poultry production has shown an improvement on growth performance and health status of chicken [3, 4, 5]. However, some studies on probiotic supplementation have shown no beneficial effects on the host [6, 7]. These variations in probiotic efficacy on the host could be due to several factors, such as indigenous gut microbes that compete with probiotic for adhesion sites and nutrients, and also their production of harmful metabolites, which reduced probiotic efficacy [9, 10]. The reduction of specific gut microbes using phages, might be useful in enhancing beneficial microbes or supplemented probiotic.
Phage is a bacteria-infecting virus that can multiply by utilising bacteria replication systems, and eventually kill them at the end of the phage lytic cycle [11]. They have a very narrow killing range (e.g., strain, species or rarely genera) [12]. This feature can then be exploited to design a targeted gut microbiota modulation that precludes the need of AGPs, and thus reduce the formation of bacterial resistance [12]. The ‘auto-dosage’ system is also one of the intriguing characteristics, where more phages will be produced from the initial infection, increasing their effectiveness on eliminating target bacteria [12]. Recently, phage has been studied for modulation of gut microbiota that has shown improvement on performance and health of chickens and swines [8, 14, 16].

The gut microbiota can be one of the main factors affecting probiotic efficacy in the gut where they can influence the availability of adhesion sites and nutrients. This is because probiotic need to adhere to the intestinal epithelial cells before they can exert their beneficial effects to the host [9, 10, 23]. Little is known about the effects of phages on probiotic efficacy in chickens. Therefore, the aim of this study was to investigate the effects of dietary supplementation of isolated phage cocktail at low 0.1% (1\( \% \)) and high 0.2% (2\( \% \)) dosages, 0.1% PrimaLac® probiotic (P) and combinations of 0.1% PrimaLac® probiotic with 1\( \% \) (1\( \% \)P) and 2\( \% \) (2\( \% \)P) on chicken growth performance and gut microbial dynamics.

**Materials And Methods**

**Isolation of bacterial hosts and phages**

Both *Escherichia coli* (*E. coli*) and phages were originally isolated from ileal and caecal contents of broiler chickens. Four phages (C1, C2, C3 and C4) were selected where they specifically infect unique *E. coli* strains.

**Phage morphological study by transmission electron microscopy (TEM)**

The concentrated and purified phage lysates were dropped onto a carbon-coated copper grid and incubated at room temperature for 6 min. The lysates were then negatively stained with 2% (w/v) uranyl acetate and left for another 10 min before being observed under Philips HMG 400 transmission electron microscope (TEM) (Philips, The Netherlands) at magnification of 300,000 x. The morphology of each phage was analysed, compared and classified based on International Committee of Taxonomy of Viruses (ICTV) that has been described in details by Ackermann [24].

**Chicken trial**

**(i) Chicken management**

A total of 288 one-day-old male Cobb 500 broiler chickens (initial body weight (BW) = 42.95 ± 2.26 g) were obtained from a local commercial hatchery. They were housed in stainless steel three-tiered battery cages with raised wire floors (Dimension: 116 cm width x 89 cm length x 46 cm height) in an open house facility at Animal Research Centre (ARC), Institute of Tropical Agriculture, Universiti Putra Malaysia, Malaysia. The cages were cleaned and disinfected through fumigation beforehand, and strict hygiene
and biosecurity measures were practiced throughout the experiment. The feeders and drinkers were cleaned and filled with fresh feed and water daily. The temperature and relative humidity were recorded twice daily in the morning and the afternoon. For the chicken brooding period that lasts for the first 14 days, lighting from 100 W bulb per cage was provided for each replicates cages. The raised wire floors were covered with newspaper and cleaned daily. Procedures pertaining to chicken management, experimental design, procedures and analyses have been approved by Institutional Animal Care and Use Committee (IACUC) Universiti Putra Malaysia (Ref: UPM/IACUC/AUP-R101/2015).

(ii) Experimental design

One-day-old chicks were randomly allotted to 1 of 6 dietary treatments with six replicates cages containing eight chicks per cage. The dietary treatments consisted of: (i) basal diet (BD)(Control, C), (ii) BD + 0.1% phage cocktail (1), (iii) BD + 0.2% phage cocktail (2), (iv) BD + 0.1% probiotic (P), (v) BD + 0.1% phage cocktail + 0.1% probiotic (1P), (vi) BD + 0.2% phage cocktail + 0.1% probiotic (2P). The phage cocktail consisted of freeze-dried powder of C1, C2, C3 and C4 phages at a titre of $10^{10}$ PFU/g each. The PrimaLac® probiotic (Starlabs, USA) used was consisted of *Lactobacillus acidophilus*, *Lactobacillus casei*, *Bifidobacterium termophilum*, *Enterococcus faecium* and *Aspergillus oryzae* at a concentration of $10^9$ CFU/g each. Both phage cocktail and probiotic for respective treatments were supplemented at the expense of corn to achieve an equal final percentage for each treatment group. The basal diet formulated for starter (1 to 21 days) and finisher (22 to 35 days) periods were antibiotic-free, in mash form, and ensured to meet or exceed the energy and nutrient requirements as recommended by Nutrient Requirements of Poultry (NRC 1994) for each growing phase. Both feeds and water were provided ad libitum. The phage cocktail and commercial probiotic were mixed with basal feed fresh daily before being fed to the chickens. The formulated starter feed, finisher feed, phage cocktail and probiotic were chemically analysed, based on feed proximate analyses (Malaysian Agricultural Research and Development Institute (MARDI)) on crude protein, crude fat, crude fibre, calcium, phosphorus and sodium.

(iii) Data collection and analyses

The chicken body weight (BW) and body weight gain (BWG) were individually recorded on a weekly basis (1 d, 7 d, 14 d, 21 d, 28 d and 35 d), while feed intake (FI) and replicate cages were recorded daily. Mortality rate was checked and recorded daily. The BWG, feed conversion ratio (FCR and mortality rate were calculated as described by Naidoo and McGaw [25] and Wang and Xu [26].

$$\text{Body weight gain (BWG)} = \frac{\text{Final BW} - \text{Initial BW}}{\text{Number of birds}}$$

$$\text{Feed conversion ratio (FCR)} = \frac{\text{FI}}{\text{BWG}}$$

$$\text{Mortality rate} = \frac{\text{Number of dead birds in treatment group}}{\text{Number of initial birds in each treatment group}} \times 100$$
For every sampling period on day 21 and 35, twelve chickens per treatment (2 chickens per replicate cage) were randomly selected, weighed and euthanised by severing the jugular veins. Blood samples were immediately collected from the jugular veins into 10 ml non-coagulated clot activator tubes (BD, USA), and coagulated K2EDTA tubes (BD, USA) for biochemical and serum immunoglobulins analyses and hematological analysis, respectively. Gastrointestinal tract such as ilea and caeca were cut open, and gently scraped by using sterile microscope slide. The samples were used for gut microbiota study based on real-time quantitative PCR (qPCR) and high-throughput next-generation sequencing (HT-NGS) of 16S rRNA gene amplicons. The ilea tissues were also collected for measurement of the villus height and crypt depth. All samples were kept on ice every time before the respective samples were processed, and frozen in -80°C freezer until analysis.

(iv) Serum lipid

The blood samples from non-coagulated clot activator tubes (BD, USA) were processed immediately by centrifugation using JA-20.1 rotor of Beckman Avanti J-25I Centrifuge (Beckman, USA) at 3,000 x g for 10 min. The serum samples were collected and transferred into sterile 2 ml microcentrifuge tubes and used for biochemistry analyses of triglycerides, cholesterol, high-density lipoprotein (HDL) and low-density lipoprotein (LDL) using COBAS reagents (Roche Diagnostics, USA) and analysed using Hitachi 902 Automatic Biochemistry Analyser (Hitachi, Japan). The same serum samples were also stored at -20°C for serum immunoglobulins analysis.

(v) Serum immunoglobulins

Serum samples of three chickens from separate cages were selected for IgG, IgA and IgM analyses of every treatment for day 21 and 35 sampling. Serums were diluted at appropriate dilutions and then quantified in technical duplicates for each sample. Serum immunoglobulins were quantified by using double-antibody sandwich enzyme-linked immunosorbent assay (ELISA) kit of chicken IgG, IgA and IgM (Bethyl Laboratories Inc., USA) by following manufacturer instructions. Absorbance was measured by using BioTek EL800 Absorbance Reader (BioTek, USA) at a wavelength of 450 nm. The standard curve was plotted from the graph of average absorbance measured versus known concentration of chicken IgG, IgA and IgM, to determine and calculate the concentration of unknown samples. The data was then multiplied by the dilution factor used earlier to determine the amount of antibody in the original undiluted sample.

(vi) Ileal villus height and crypt depth

The 1 cm midpoint ileum was excised for measurements of the villi height and crypt depths from 12 chickens per treatment (2 chickens per replicate cage) for day 21 and 35 sampling. The tissue samples were rinsed in phosphate-buffered saline (PBS) buffer (pH 7.4) and fixed in 10% buffered formalin overnight. There were dried in an automatic tissue processor for 16 h using Leica ASP 3000 fully-enclosed tissue processor (Leica, Japan) and embedded in paraffin wax by using Leica EG1160 paraffin embedding station (Leica, Japan). The tissue samples with the thickness of 4 µm were cut using Leica
RM2155 microtome (Leica, Japan), placed on glass slides and then stained with haematoxylin and eosin. The sections were examined under Olympus light microscope QG2-32 (Olympus, Japan) with digital camera attached. The villus height was measured by using Fiji ImageJ [27] from the top end of the villus to the bottom of the villus-crypt junction, while crypt depth from the basement membrane to the villus-crypt junction. An average of 3 villus heights and crypt depth were measured for each sample.

Chicken gut microbial dynamics

(i) DNA extraction

Genomic DNA was extracted by using QIAamp Fast DNA Stool Mini Kit (QIAGEN, Germany) according to the manufacturer instructions with some modifications. The mucosal contents weighing between 180-220 mg were treated with lysozyme lysis buffer [25 mg/ml lysozyme (Vivantis, Malaysia); 20 mM Tris-Cl, pH 8.0; 2 mM EDTA, pH 8.0; 1% Triton X-100] and incubated for 30 min at 37°C to facilitate the lysis of Gram-positive bacteria. The 1 ml InhibitEX Buffer was added to each sample where it was vortexed continuously for 1 min. The mixtures were heated for 5 min at 95°C and vortexed for 10 s to lyse Gram-positive bacteria. Subsequently, the solution was centrifuged at 16,100 x g for 1 min to pellet intestinal debris. The supernatants were treated with 4 µl of 5 µg/ml RNase A (Epicentre, USA) and incubated for 30 min at 37°C to remove RNA. The eluted genomic DNA was then stored in -20°C freezer.

(ii) Real-time quantitative PCR (qPCR)

The population of specific gut microbes which are *Escherichia coli*, *Lactobacillus spp.*, *Bifidobacterium spp.* and *Clostridium perfringens* in ileal and caecal contents at 21 and 35 d were analysed based on absolute quantification in real-time qPCR. The *E. coli* population was quantified as it was the phage-target bacteria of the dietary treatments (1P, 2P, 1PP and 2PP). The *Lactobacillus spp.* and *Bifidobacterium spp.* were part of supplemented Primalac® probiotic dietary treatments (P, 1PP and 2PP). The *Clostridium perfringens* is the common indicator of pathogens.

The genomic DNA from samples of each treatment for ilea and caeca at 21 d and 35 d (three replicate cages (1 chicken per cage) were used as the templates for real-time qPCR study. The procedure was conducted by using SensiFAST SYBR No-ROX Kit (Bioline, UK) in CFX96 Real-Time PCR Detection System (Bio-Rad, USA). The 20 µl reaction mixtures contained 10 µl SensiFAST SYBR No-ROX mix, normalised genomic DNA template at 80 ng/µl, 1 µl of forward and reverse primers (10 pmol/µl) (refer Table 4) and sterile Mili-Q water. Both standard curve samples and no template control (NTC) were prepared in every run. The technical duplicates were prepared for each sample. The PCR cycling reaction included an initial denaturation step at 95°C for 3 min followed by 40 cycles of denaturation at 95°C for 5 s, annealing and extension at 72°C for 20 s. The specificity of amplicons were then verified based on the melting curve analysis where they were incubated by increasing the temperature from 65°C to 95°C (0.5°C increment) and hold for 5 s. The final results were then analysed by using Bio-Rad CFX Manager version 3.1 (Bio-Rad, USA).
(iii) Illumina sequencing of the V3-V4 region of the 16S rRNA gene

V3-V4 hypervariable region of 16S rRNA gene was amplified using forward primer (5’-TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGNNNNCTACGGGNGCWWGACAG-3’) and reverse primer (5’-GTCTCTCAGGCTGTAAGACAGAGACTACHVGGGTATCTAATCC-3’) (Integrated DNA Technologies (IDT), Singapore) as per described by Klindworth, Pruesse et al. (2013) with some modifications. Four degenerate bases (N) were added to maximise the diversity for unique clusters identification. The library preparation condition and procedure is given in Table S2. The purified amplicons were quantified by using Qubit Fluorometer (ThermoFisher Scientific, USA) and normalised to 2 nM and subjected to Illumina Miseq desktop sequencer by using paired 300 bp reads Miseq Reagent Kit v3 (600-cycle) (Illumina, USA) at Monash University Malaysia Genomic Facility.

(iv) 16S sequence analyses

Sequences in FASTQ format were assembled and quality filtered using Mothur software package (v. 1.38.1) [28]. The processed sequences were subsampled to 9998 prior to alpha diversity analyses. Indices including observed OTUs, ACE, Shannon and Inverse Simpson index were calculated and compared with one-way Analysis of Variance (ANOVA) using Statistical Package for Social Science (SPSS) Statistics version 22 (IBM, USA). A follow-up Post Hoc Duncan's multiple range test (Duncan 1955) further conducted for pairwise comparison.

The raw OTUs Table was normalised using 'cumNorm' command. In addition, OTUs with significant difference in abundance were selected based on zero-inflated log-normal model using 'fitFeatureModel' command. Both 'cumNorm' and 'fitFeatureModel' were implemented in metagenomSeq package [29].

The normalised OTUs Table was exported into PRIMER7 with PERMANOVA add on programme package (Plymouth Marine Laboratory, UK) [57] and converted to Bray-Curtis Similarity index for group comparison [i.e. Permutational Multivariate Analysis of Variance (PERMANOVA) and statistical ordinations [i.e. canonical analysis of principal coordinates (CAP), principal coordinate analysis (PCO)]].

Microbial predicted functional metagenomes based on PICRUSt and STAMP. The prediction of metagenome function from 16S rRNA marker gene was performed by using Phylogenetic Investigation of Communities by Reconstruction of Unobserved States (PICRUSt) version 1.1.0 [37]. Briefly, the Greengenes-based biom file was generated using Mothur software and uploaded into online galaxy PICRUSt (http://huttenhower.sph.harvard.edu/galaxy/). The OTUs table provided was first normalised for multiple 16S copy number where the genome was then predicted based on Kyoto Encyclopedia of Genes and Genomes (KEGG) ortholog abundances. The output files were then analysed by using Statistical Analysis of Metagenomic Profiles (STAMP) version 2.1.3 bioinformatics software package [30]. The Storey's FDR of multiple test correction methods was used to calculate the statistical significance and the features were filtered by effect size at 0.05.

(v) Nucleotide sequence accession numbers
The V3-V4 region of 16S rRNA gene sequences from this study have been deposited in the NCBI sequence read archive (https://www.ncbi.nlm.nih.gov/sra) under BioSample Accession numbers of SAMN06027949-SAMN06028092.

Results

Phage morphology

A final four phages were used for the phage cocktail study. They were observed under transmission electron microscopy (TEM) and their morphologies were analysed. All phages exhibit a hexagon nucleocapsid head structure with a short tail for C1 and a long tail for C2, C3 and C4 phages (Fig. 1).

Chicken trial

Growth performance

The effects of phage cocktail at different dosages, probiotic and their combinations on chicken growth performance are shown in Table 1. Chickens supplemented with 1P had significantly (P < 0.05) better BW (35 d) and BWG (22-35 d, 1-35 d) than those of the control, but not chickens of the other supplemented groups (1, 2, P and 2P). There were no significant differences in the FI among all groups throughout the experimental period. Chickens of all supplemented groups at 22-35 d and 1-35 d had significantly (P < 0.05) lower FCR than the control group. However, at 1-21 d, the FCR of the P group (1.40) was not significantly different from that of the control (1.43). The highest mortality rate was recorded in both control and 2 groups at 4.17%, while no mortality was observed in the P group.

Serum Lipid Analysis

Chickens in all supplemented groups, except the 1 group, showed significantly (P < 0.05) lower triglycerides than control chickens at 35 d, but at 21 d, only chickens in the1 and P groups had significantly (P < 0.05) lower triglycerides than the control. There were no significant (P > 0.05) differences in total cholesterol and HDL among all groups of chickens at 21 and 35 d. For LDL, chickens in the 1 and 2 groups showed significantly (P < 0.05) lower levels than the control group at 21 d, but there were no significant differences among all groups at 35 d. The concentrations of IgA and IgM in chickens were not affected (P > 0.05) by dietary treatments at 21 and 35 d (Table 2). This was also similar for IgG at 35 d. However, at 21 d, chickens supplemented with 1, 1P and 2P showed significantly (P < 0.05) lower IgG than the control.
Table 1

Effects of dietary treatments of phage cocktail at different dosages, probiotic and their combinations on growth performances and mortality rate of broiler chickens.

| Item¹ | Age | C   | 1posición | 2posición | P   | 1P | 2P |
|-------|-----|-----|-----------|-----------|-----|----|----|
|       |     |     | BW (g)    | BW (g)    | BW (g) | BW (g) | BW (g) |
|       | 1 d | 43.00 ± 1.10 | 42.78 ± 1.09 | 42.98 ± 0.78 | 42.82 ± 0.96 | 42.78 ± 1.05 | 43.32 ± 0.38 |
|       | 21 d | 859.58 ± 14.20 | 874.07 ± 8.85 | 864.67 ± 11.05 | 871.78 ± 6.09 | 874.02 ± 12.31 | 855.10 ± 13.74 |
|       | 35 d | 1605.30 ± 39.45a | 1734.17 ± 47.55ab | 1741.27 ± 56.58ab | 1690.43 ± 40.05ab | 1785.75 ± 31.93b | 1733.63 ± 56.00ab |
|       |     | 816.63 ± 13.97 | 861.32 ± 9.47 | 821.70 ± 11.09 | 828.98 ± 6.04 | 831.25 ± 11.91 | 811.80 ± 12.95 |
|       | 22–35 d | 745.72 ± 40.37a | 860.10 ± 50.69ab | 876.60 ± 46.20ab | 818.65 ± 41.87ab | 911.73 ± 32.82b | 878.53 ± 47.67ab |
|       | 1–35 d | 1562.35 ± 40.42a | 1691.42 ± 47.67ab | 1698.30 ± 56.55ab | 1647.63 ± 39.82ab | 1742.98 ± 32.76b | 1690.33 ± 55.75ab |
|       |     | 1169.88 ± 18.03 | 1153.10 ± 10.46 | 1111.35 ± 30.52 | 1104.87 ± 37.66 | 1160.58 ± 20.14 | 1131.58 ± 10.33 |
|       | 22–35 d | 1748.38 ± 24.66 | 1761.45 ± 51.10 | 1650.32 ± 104.62 | 1653.68 ± 40.06 | 1740.27 ± 63.75 | 1760.85 ± 44.20 |
|       | 1–35 d | 2918.27 ± 38.40 | 2914.55 ± 55.02 | 2922.88 ± 158.00 | 2758.53 ± 51.98 | 2900.85 ± 70.40 | 2892.42 ± 53.92 |
|       |     | 1.43 ± 0.17c | 1.35 ± 0.17a | 1.37 ± 0.15ab | 1.40 ± 0.10bc | 1.37 ± 0.11ab | 1.34 ± 0.15a |
|       | 22–35 d | 2.53 ± 0.12b | 2.10 ± 0.08a | 2.06 ± 0.06a | 1.97 ± 0.06a | 1.89 ± 0.04a | 2.01 ± 0.07a |
|       | 1–35 d | 1.87 ± 0.04b | 1.68 ± 0.03a | 1.65 ± 0.03a | 1.61 ± 0.03a | 1.60 ± 0.03a | 1.61 ± 0.03a |
| Mortality rate (%) | 1–21 d | 0.35 | 0.35 | 0.35 | NIL | NIL | 0.35 |
|       | 22–35 d | 0.46 | NIL | 0.46 | NIL | 0.46 | NIL |

Each value is mean ± SE of 6 replicate cages with 8 chickens each. a,b,c Means within the same row that have different superscripts differ significantly (P < 0.05). ¹C = control (basal diet); 1posición = BD + 0.1% phage cocktail; 2posición = BD + 0.2% phage cocktail; P = BD + 0.1% probiotic; 1P = BD + 0.1% phage cocktail + 0.1% probiotic; 2P = BD + 0.2% phage cocktail + 0.1% probiotic; BW = body weight; BWG = body weight gain.
Table 2
Effects of dietary treatments of phage cocktail at different dosages, probiotic and their combinations on blood biochemistry of broiler chickens at 21 and 35 d.

| Item ¹ | Age | C         | 1       | 2       | P       | 1P      | 2P      |
|--------|-----|-----------|---------|---------|---------|---------|---------|
| TG (mg/dL) | 21 d | 0.44 ± 0.10 b | 0.28 ± 0.04 a | 0.34 ± 0.03 ab | 0.27 ± 0.01 a | 0.32 ± 0.04 ab | 0.33 ± 0.01 ab |
|         | 35 d | 81.04 ± 9.09 c | 66.43 ± 7.93 bc | 47.83 ± 7.66 ab | 39.12 ± 5.52 a | 43.99 ± 7.15 a | 45.02 ± 3.96 ab |
| CHL (mg/dL) | 21 d | 135.67 ± 10.74 | 114.98 ± 7.68 | 123.23 ± 6.47 | 124.91 ± 4.82 | 121.36 ± 6.67 | 122.78 ± 6.37 |
|         | 35 d | 120.52 ± 4.32 ab | 105.63 ± 3.39 ab | 121.75 ± 7.70 b | 112.40 ± 8.60 ab | 98.61 ± 6.18 a | 120.72 ± 9.53 ab |
| HDL (mg/dL) | 21 d | 85.72 ± 4.73 | 80.88 ± 4.75 | 89.26 ± 3.38 | 88.49 ± 5.96 | 88.30 ± 3.84 | 85.46 ± 3.69 |
|         | 35 d | 66.77 ± 3.32 ab | 60.33 ± 3.44 a | 74.76 ± 4.02 b | 74.18 ± 6.16 ab | 62.07 ± 2.27 ab | 75.92 ± 6.31 b |
| LDL (mg/dL) | 21 d | 21.85 ± 2.52 b | 13.73 ± 2.33 a | 13.92 ± 1.40 a | 17.08 ± 1.35 ab | 17.72 ± 0.85 ab | 18.17 ± 1.82 ab |
|         | 35 d | 27.01 ± 6.39 | 25.33 ± 4.59 | 27.78 ± 3.61 | 23.72 ± 5.14 | 18.95 ± 2.91 | 24.17 ± 3.09 |

Each value is mean ± SE of 6 replicate cages with 1 chicken each. ¹Means within the same row that have different superscripts differ significantly (P < 0.05). ¹C = control (basal diet); 1 = BD + 0.1% phage cocktail; 2 = BD + 0.2% phage cocktail; P = BD + 0.1% probiotic; 1P = BD + 0.1% phage cocktail + 0.1% probiotic; 2P = BD + 0.2% phage cocktail + 0.1% probiotic; GLU = glucose; TP = total protein; ALB = albumin; TG = triglycerides; CHL = cholesterol; HDL = high density lipoprotein; LDL = low density lipoprotein.

Serum Immunoglobulins

The concentrations of IgA and IgM in chickens were not affected (P > 0.05) by dietary treatments at 21 and 35 d (Table 3). This was also similar for IgG at 35 d. However, at 21 d, chickens supplemented with 1, 1P and 2P showed significantly (P < 0.05) lower IgG than the control.
Table 3
Effects of dietary treatments of phage cocktail at different dosages, probiotic and their combinations on serum immunoglobulins of broiler chickens at 21 and 35 d.

| Item  | Age | C    | 1  | 2 | P   | 1P | 2P |
|-------|-----|------|----|---|-----|----|----|
| IgA (g/L) | 21 d | 0.126 ± 0.019 | 0.106 ± 0.007 | 0.133 ± 0.015 | 0.117 ± 0.005 | 0.117 ± 0.003 | 0.105 ± 0.024 |
|       | 35 d | 0.285 ± 0.026 | 0.233 ± 0.036 | 0.240 ± 0.066 | 0.321 ± 0.006 | 0.206 ± 0.020 | 0.253 ± 0.125 |
| IgG (g/L) | 21 d | 3.000 ± 0.308 b | 1.211 ± 0.139 a | 2.478 ± 0.737 ab | 2.074 ± 0.575 ab | 1.252 ± 0.344 a | 1.500 ± 0.273 a |
|       | 35 d | 6.949 ± 2.141 | 5.036 ± 1.770 | 2.505 ± 0.641 | 4.616 ± 2.782 | 4.255 ± 1.412 |
| IgM (g/L) | 21 d | 0.170 ± 0.009 | 0.143 ± 0.010 | 0.178 ± 0.038 | 0.183 ± 0.053 | 0.155 ± 0.025 | 0.147 ± 0.013 |
|       | 35 d | 0.246 ± 0.043 | 0.274 ± 0.010 | 0.323 ± 0.066 | 0.223 ± 0.011 | 0.234 ± 0.053 | 0.280 ± 0.092 |

Each value is mean ± SE of 3 replicate cages with 1 chicken each. a,b Means within the same row that have different superscripts differ significantly (P < 0.05). 1C = control (basal diet); 1 = BD + 0.1% phage cocktail; 2 = BD + 0.2% phage cocktail; P = BD + 0.1% probiotic; 1P = BD + 0.1% phage cocktail + 0.1% probiotic; 2P = BD + 0.2% phage cocktail + 0.1% probiotic.

Ileal Villus Height And Crypt Depth

There were no significant differences between the supplemented treatment groups and the control in villus heights at 21 d, but at 35 d, the 1P and 2P groups had significantly (P < 0.05) higher villus heights than the 1, P and control groups. Chickens fed probiotic (P group) had significantly (P < 0.05) greater crypt depth than those of the control and other supplemented groups at 21 d (Table 4). At 35 d, there were no significant differences between all supplemented groups and control group. The villus to crypt ratio was not significantly (P > 0.05) different among all groups of chickens at 21 d. At 35 d, only chickens of the 2P group had significantly (P < 0.05) higher villus to crypt ratio than control chickens.
Table 4
Effects of dietary treatments of phage cocktail at different dosages, probiotic and their combinations on ileal villi height and crypt depth of broiler chickens at 21 and 35 d.

| Item\(^1\) | Age  | C       | 1\(\) | 2\(\) | P       | 1\(\)P   | 2\(\)P   |
|------------|------|---------|-------|-------|---------|---------|---------|
| Villus height (µm) | 21 d | 640.10 ± 48.95 \(^{abc}\) | 535.34 ± 43.16 \(^{a}\) | 589.17 ± 33.72 \(^{ab}\) | 662.27 ± 46.02 \(^{abc}\) | 737.64 ± 43.37 \(^{c}\) | 682.90 ± 46.54 \(^{bc}\) |
|            | 35 d | 640.94 ± 44.23 \(^{a}\) | 629.07 ± 28.03 \(^{a}\) | 680.60 ± 29.48 \(^{ab}\) | 648.90 ± 37.06 \(^{a}\) | 781.31 ± 44.71 \(^{b}\) | 761.635 ± 34.52 \(^{b}\) |
| Crypt depth (µm) | 21 d | 123.19 ± 9.54 \(^{ab}\) | 101.33 ± 11.03 \(^{a}\) | 123.96 ± 12.21 \(^{ab}\) | 149.23 ± 16.65 \(^{c}\) | 135.31 ± 10.40 \(^{ab}\) | 129.75 ± 12.16 \(^{ab}\) |
|            | 35 d | 179.08 ± 15.05 \(^{ab}\) | 216.17 ± 17.25 \(^{b}\) | 190.88 ± 62.95 \(^{ab}\) | 169.70 ± 10.12 \(^{a}\) | 173.98 ± 14.10 \(^{ab}\) | 158.13 ± 9.21 \(^{a}\) |
| Villus to crypt ratio | 21 d | 5.53 ± 0.65 | 5.71 ± 0.58 | 5.20 ± 0.47 | 4.99 ± 0.60 | 5.73 ± 0.44 | 5.74 ± 0.68 |
|            | 35 d | 3.85 ± 0.40 \(^{ab}\) | 3.04 ± 0.19 \(^{a}\) | 3.82 ± 0.29 \(^{ab}\) | 3.96 ± 0.31 \(^{ab}\) | 4.67 ± 0.30 \(^{bc}\) | 5.00 ± 0.39 \(^{c}\) |

Each value is mean ± SE of 6 replicate cages with 2 chickens each. \(^{a,b,c}\)Means within the same row that have different superscripts differ significantly (P < 0.05). \(^1\)C = control (basal diet); 1\(\) = BD + 0.1% phage cocktail; 2\(\) = BD + 0.2% phage cocktail; P = BD + 0.1% probiotic; 1\(\)P = BD + 0.1% phage cocktail + 0.1% probiotic; 2\(\)P = BD + 0.2% phage cocktail + 0.1% probiotic.

Multivariate Analysis On Gut Microbiome

The principal coordinate analysis (PCO) plot of Bray-Curtis similarity index was employed to investigate the overall similarities of gut microbiota structure. It was indicated that there was a clear separation of gut microbial memberships between age and part of intestine. Moreover, the gut microbial memberships between \(\)P (1\(\)P and 2\(\)P) and non-\(\)P group (C, 1\(\), 2\(\) and P) were tightly clustered, indicating a unique gut
Table 5
Alpha diversity of measured number of observed OTUs, ACE, Shannon and Inverse Simpson from sequences that were normalised to 9998.

| Group   | Sobs          | Coverage | ACE      | Shannon  | Inverse Simpson |
|---------|---------------|----------|----------|----------|-----------------|
| 21I_C   | 562.17 ± 26.81 | 0.973 ± 0.001 | 1018.91 ± 86.10 | 3.47 ± 0.09 | 11.76 ± 1.43 |
|         | 550.50 ± 20.29 | 0.978 ± 0.003 | 811.77 ± 70.43 | 3.43 ± 0.08 | 9.74 ± 1.10 |
| 21I_2   | 496.83 ± 37.30 | 0.982 ± 0.002 | 683.37 ± 63.29 | 3.28 ± 0.22 | 10.10 ± 2.24 |
| 21I_P   | 567.83 ± 20.94 | 0.976 ± 0.002 | 868.52 ± 46.20 | 3.49 ± 0.15 | 11.47 ± 1.75 |
| 21I_1P  | 501.50 ± 33.03 | 0.982 ± 0.003 | 712.75 ± 79.78 | 3.52 ± 0.22 | 13.52 ± 3.11 |
| 21I_2P  | 437.17 ± 18.93 | 0.982 ± 0.002 | 709.14 ± 68.39 | 3.06 ± 0.19 | 8.18 ± 1.65 |
| 35I_C   | 503.67 ± 40.89 | 0.983 ± 0.003 | 734.80 ± 107.38 | 3.33 ± 0.20 | 9.46 ± 1.96 |
| 35I_1   | 620.40 ± 146.55 | 0.979 ± 0.002 | 817.34 ± 80.40 | 3.64 ± 0.19 | 11.28 ± 1.87 |
| 35I_2   | 611.80 ± 33.81 | 0.976 ± 0.003 | 890.51 ± 82.10 | 3.44 ± 0.21 | 9.27 ± 1.91 |
| 35I_P   | 691.33 ± 78.00 | 0.978 ± 0.004 | 896.65 ± 113.08 | 3.88 ± 0.16 | 13.86 ± 2.58 |
| 35I_1P  | 694.60 ± 36.49 | 0.978 ± 0.001 | 905.07 ± 30.98 | 3.87 ± 0.08 | 11.73 ± 1.03 |
| 35I_2P  | 514.83 ± 26.48 | 0.990 ± 0.001 | 586.11 ± 45.91 | 3.91 ± 0.10 | 15.41 ± 1.73 |
| 21C_C   | 306.67 ± 9.13  | 0.992 ± 0.001 | 383.20 ± 19.06 | 3.35 ± 0.09 | 9.24 ± 1.42 |

Each value is mean ± SE of 6 replicate cages with 1 chicken each.

Means within the same column that have different superscripts differ significantly (P < 0.05). For treatment (C = control (basal diet); 1I = BD + 0.1% phage cocktail; 2I = BD + 0.2% phage cocktail; P = BD + 0.1% probiotic; 1I_P = BD + 0.1% phage cocktail + 0.1% probiotic; 2I_P = BD + 0.2% phage cocktail + 0.1% probiotic), age (21 = 21-day-old; 35 = 35-day-old) and part of intestine (I = ilea, C = caeca). Sobs = number of observed OTUs.
### Group Sobs Coverage ACE Shannon Inverse Simpson

| Group          | Sobs          | Coverage       | ACE     | Shannon       | Inverse Simpson |
|----------------|---------------|----------------|---------|---------------|-----------------|
| 21C_1          | 339.50 ± 19.31 | 0.992 ± 0.001  | 413.89 ± 24.88 | 3.44 ± 0.13 abcde | 10.94 ± 3.32 abc |
| 21C_2          | 334.00 ± 24.25 | 0.993 ± 0.001  | 418.19 ± 32.44 | 3.36 ± 0.18 abcde | 10.55 ± 3.46 abc |
| 21C_P          | 344.00 ± 21.64 | 0.992 ± 0.001  | 432.26 ± 18.66 | 3.56 ± 0.20 bcde  | 13.16 ± 2.47 abc |
| 21C_1P         | 334.33 ± 19.54 | 0.992 ± 0.001  | 399.60 ± 27.01 | 3.35 ± 0.18 abcde | 8.80 ± 1.30 abc  |
| 21C_2P         | 307.50 ± 24.22 | 0.993 ± 0.001  | 399.60 ± 27.23 | 3.35 ± 0.18 abc   | 8.80 ± 1.58 ab  |
| 35C_C          | 299.75 ± 31.80 | 0.995 ± 0.003  | 340.26 ± 54.14 | 3.46 ± 0.21 abcde | 13.13 ± 4.83 abc |
| 35C_1          | 323.17 ± 8.29  | 0.992 ± 0.001  | 388.56 ± 13.29 | 3.38 ± 0.10 abcde | 8.15 ± 1.62 abc  |
| 35C_2          | 297.33 ± 7.14  | 0.994 ± 0.000  | 345.39 ± 7.26 | 3.21 ± 0.13 abc   | 7.05 ± 1.44 ab  |
| 35C_P          | 340.67 ± 18.68 | 0.992 ± 0.001  | 405.73 ± 26.97 | 3.31 ± 0.16 abcd  | 9.66 ± 1.91 abc  |
| 35C_1P         | 342.50 ± 19.01 | 0.992 ± 0.002  | 410.87 ± 33.72 | 3.00 ± 0.12 bcde  | 12.62 ± 2.77 abc |
| 35C_2P         | 314.00 ± 14.76 | 0.993 ± 0.000  | 365.88 ± 13.62 | 2.99 ± 0.25 abc   | 5.88 ± 2.02 ab  |

Each value is mean ± SE of 6 replicate cages with 1 chicken each.

Means within the same column that have different superscripts differ significantly (P < 0.05). For treatment (C = control (basal diet); 1 = BD + 0.1% phage cocktail; 2 = BD + 0.2% phage cocktail; P = BD + 0.1% probiotic; 1P = BD + 0.1% phage cocktail + 0.1% probiotic; 2P = BD + 0.2% phage cocktail + 0.1% probiotic), age (21 = 21-day-old; 35 = 35-day-old) and part of intestine (I = ilea, C = caeca). Sobs = number of observed OTUs.

Microbial memberships between those two groups (Fig. 4a). Further analysis based on the canonical analysis of principal coordinates (CAP) plot was performed to study the difference of gut microbiota structure based on dietary treatments. The CAP plot showed that the gut microbial communities between P (1P and 2P) and non-P groups (C, 1, 2 and P) were distinctively different especially in ilea (Fig. 4b) and ilea of 35 d chickens (Fig. 4c).
Further verifications were performed to validate the earlier patterns observed from the PCA plot. The hypothesis test on difference among gut microbial community between age, part of intestine, and dietary treatments was verified based on permutational multivariate analysis of variance (PERMANOVA) marginal test. Based on PERMANOVA marginal test, the gut microbial community was significantly different between age ($p = 0.002$), part of intestine ($p = 0.001$), dietary treatments in ilea ($p = 0.005$) (Table 6) and dietary treatments in ilea of 35 d chickens ($p = 0.001$). Interestingly, the PERMANOVA pairwise test calculated based on dietary treatments in ilea (Table 7) and ilea of 35 d chickens further verified the patterns observed in CAP plot, where a significant difference in gut microbial communities was observed between $\bar{P}$ and non-$\bar{P}$ groups.

Table 6: PERMANOVA (a) marginal and (b) pairwise test based on Bray-Curtis similarities for gut microbiota structure differences from ilea of chickens. The test includes degrees of freedom (Df), sum of squares (SS), mean square (MS) and P value under Monte-Carlo correction ($P_{MC}$).

a. Marginal test

| Source    | Df | SS          | MS          | Pseudo-F | $P_{MC}$ |
|-----------|----|-------------|-------------|----------|----------|
| Treatment | 5  | 23528       | 4705.6      | 1.855    | 0.005    |
| Residual  | 65 | 1.6488E + 05| 2536.7      |          |          |
| Total     | 70 | 1.8841E + 05|             |          |          |

b. Pairwise test
### Table 7: PERMANOVA (a) marginal and (b) pairwise test on Bray-Curtis similarities for gut microbiota diversity based on ilea of 35 d chickens. The test includes degrees of freedom (Df), sum of squares (SS), mean square (MS) and P value under Monte-Carlo correction ($P_{MC}$).

#### a. Marginal test

| Groups | Df | SS   | MS   | Pseudo-F | $P_{MC}$ |
|--------|----|------|------|----------|----------|
| 1, 1P  | 5  | 28910| 5782.1| 4.0189   | 0.001    |
| Residual| 29 | 41723| 1438.7|          |          |
| Total  | 34 | 70634|      |          |          |

For treatment (C = control (basal diet); 1P = BD + 0.1% phage cocktail; 2P = BD + 0.2% phage cocktail; 1 = BD + 0.1% probiotic; 2 = BD + 0.2% phage cocktail + 0.1% probiotic).

#### b. Pairwise test

| Groups | t     | Unique perms | $P_{MC}$ |
|--------|-------|--------------|----------|
| 1, 1P  | 1.0853| 998          | 0.299    |
| 1, 2P  | 0.73964| 998         | 0.81     |
| 1, 2P | 1.5589| 998          | 0.043    |
| 1, C   | 0.96796| 999         | 0.457    |
| 1, P   | 0.66535| 997         | 0.867    |
| 1P, 2P | 1.2802| 998          | 0.137    |
| 1P, 2P | 1.1864| 999          | 0.179    |
| 1P, C  | 1.6169| 996          | 0.026    |
| 1P, P  | 1.4116| 998          | 0.051    |
| 2P, 2P | 1.9504| 998          | 0.004    |
| 2P, C  | 1.0898| 999          | 0.325    |
| 2P, P  | 0.86084| 998       | 0.595    |
| 2P, C  | 2.2436| 999          | 0.001    |
| 2P, P  | 2.0671| 999          | 0.002    |
| C, P   | 0.73884| 999       | 0.804    |
| Groups   | t       | Unique perms | $P_{MC}$ |
|---------|---------|--------------|----------|
| $1^\circ, 1^\circ P$ | 2.2179  | 403          | 0.011    |
| $1^\circ, 2^\circ$ | 0.99346 | 405          | 0.419    |
| $1^\circ, 2^\circ P$ | 2.7819  | 405          | 0.002    |
| $1^\circ, C$ | 0.97549 | 408          | 0.437    |
| $1^\circ, P$ | 0.78783 | 413          | 0.672    |
| $1^\circ P, 2O$ | 2.6116  | 401          | 0.002    |
| $1^\circ P, 2OP$ | 1.179   | 418          | 0.234    |
| $1^\circ P, C$ | 2.4102  | 403          | 0.006    |
| $1^\circ P, P$ | 2.2141  | 410          | 0.005    |
| $2^\circ, 2OP$ | 3.2675  | 401          | 0.002    |
| $2^\circ, C$ | 0.80525 | 408          | 0.651    |
| $2^\circ, P$ | 0.98786 | 397          | 0.393    |
| $2^\circ P, C$ | 3.167   | 399          | 0.001    |
| $2^\circ P, P$ | 2.7201  | 400          | 0.002    |
| $C, P$ | 1.0209  | 394          | .3850    |

For treatment (C = control (basal diet); $1^\circ = BD + 0.1\%$ phage cocktail; $2^\circ = BD + 0.2\%$ phage cocktail; $P = BD + 0.1\%$ probiotics; $1^\circ P = BD + 0.1\%$ phage cocktail + $0.1\%$ probiotics; $2^\circ P = BD + 0.2\%$ phage cocktail + $0.1\%$ probiotics).

**Significant OTUs present in the $\circ P$ compared to the non-$\circ P$ groups**

The OTUs that were significantly expressed in phage cocktail and probiotic combinations treatment groups ($\circ P$) were identified based on ‘fitFeatureModel’ in the metagenomeSeq package (Table 8). The value of logFC is directly related to their abundance in the $\circ P$ groups. Out of the top 50 OTUs selected, the most common bacterial genera or species that were significantly elevated in $\circ P$ groups, compared to non-$\circ P$ groups were *Bacteroides, Odoribacter, Alistipes, Anaerotruncus, Ruminococcaceae, Lachnospiraceae, Ruminococcus, Desulfovibrio, Anaerostipes, Clostridium, Coprobacillus, Butyrivibrio, Faecalibacterium prausnitzii* and *Oscillopira.*
Table 8
List of OTUs that significantly higher in $\text{P}$ than the non-$\text{P}$ groups.

| OTUs     | Taxonomy                  | LogFC    | Standard error (SE) | P-values  | Adjusted P-values |
|----------|---------------------------|----------|---------------------|-----------|-------------------|
| Otu000006 | Bacteroides uniformis     | 3.23846  | 0.513486            | 2.85E-10  | 1.64E-08          |
| Otu000001 | Bacteroides               | 3.08242  | 0.511617            | 1.69E-09  | 6.49E-08          |
| Otu000585 | Odoribacter               | 2.88466  | 1.034084            | 0.005278  | 0.02529           |
| Otu000009 | Alistipes                 | 2.76496  | 0.432556            | 1.64E-10  | 1.64E-08          |
| Otu000139 | Alistipes finegoldii      | 2.72394  | 0.680663            | 6.28E-05  | 0.000723          |
| Otu000269 | Ruminococaceae_unclassified | 2.72069 | 1.02679             | 0.008056  | 0.034313          |
| Otu000070 | Alistipes                 | 2.64718  | 0.526343            | 4.92E-07  | 1.41E-05          |
| Otu000624 | Ruminococaceae UCG-014    | 2.61844  | 1.056871            | 0.013229  | 0.04612           |
| Otu000111 | Anaerotruncus             | 2.53915  | 0.862369            | 0.003236  | 0.018607          |
| Otu000014 | Ruminococcus              | 2.49589  | 0.525048            | 2.00E-06  | 4.59E-05          |
| Otu000031 | Lachnospiraceae_unclassified | 2.47050 | 0.558793            | 9.82E-06  | 0.000188          |
| Otu000117 | Ruminococaceae UCG-005    | 2.44562  | 0.891623            | 0.00609   | 0.028015          |
| Otu000048 | Bacillaceae_unclassified  | 2.30877  | 0.862055            | 0.007401  | 0.032737          |
| Otu000170 | Bacteroides               | 2.24517  | 0.57669             | 9.89E-05  | 0.001034          |
| Otu000319 | Rhodospirillaceae         | 2.16229  | 0.88805             | 0.014897  | 0.047586          |
| Otu000005 | Alistipes onderdonkii     | 2.12116  | 0.521993            | 4.83E-05  | 0.000695          |
| Otu000210 | Anaerotruncus             | 2.02594  | 0.945538            | 0.032142  | 0.080355          |

Note: LogFC is directly related to their abundance in $\text{P}$ group, where the highest number showed the highest abundance. For phage cocktail and probiotic combinations groups ($\text{P}$; $\text{1P}$ and $\text{2P}$) and other groups (Non-$\text{P}$; $\text{C}$, $\text{1P}$, $\text{2P}$ and $\text{P}$).
| OTUs   | Taxonomy                          | LogFC   | Standard error (SE) | P-values | Adjusted P-values |
|--------|-----------------------------------|---------|---------------------|----------|------------------|
| Otu000015 | Clostridium X1Vb                   | 2.019893556 | 0.617525             | 0.001072 | 0.007704         |
| Otu000337 | Desulfovibrio                      | 2.014867537 | 0.841755             | 0.016682 | 0.049954         |
| Otu000675 | Anaerostipes                       | 2.013275931 | 1.087486             | 0.064125 | 0.122906         |
| Otu000032 | Clostridium X1Va                    | 1.982675782 | 0.536143             | 0.000217 | 0.002082         |
| Otu000643 | Vampirovibrio                      | 1.967914419 | 1.072608             | 0.06655  | 0.125463         |
| Otu000353 | Alistipes putredinis                | 1.899173634 | 0.770005             | 0.013646 | 0.046157         |
| Otu000204 | Ruminococcaceae UCG-014            | 1.859164848 | 0.750237             | 0.013208 | 0.04612          |
| Otu000157 | Ruminococcaceae_unclassified       | 1.841791976 | 0.706923             | 0.009178 | 0.036394         |
| Otu000866 | Clostridium                        | 1.840566564 | 0.885046             | 0.03756  | 0.086387         |
| Otu000367 | Coprobacillus                      | 1.822128533 | 1.039682             | 0.079674 | 0.143164         |
| Otu000694 | Lachnospiraceae_unclassified       | 1.815009851 | 1.076386             | 0.091756 | 0.158407         |
| Otu000066 | Bacteroides fragilis               | 1.803001647 | 0.592086             | 0.002325 | 0.014857         |
| Otu000025 | Lachnospiraceae_unclassified       | 1.784599464 | 0.409758             | 1.33E-05 | 0.000218         |
| Otu000426 | Ruminococcaceae UCG-014            | 1.782185523 | 0.793533             | 0.024711 | 0.063151         |
| Otu000027 | Eisenbergiella                     | 1.766953157 | 0.495082             | 0.000358 | 0.00317          |
| Otu000067 | Butyricimonas                      | 1.753087241 | 0.611499             | 0.004146 | 0.02165          |
| Otu000075 | Lachnospiraceae_unclassified       | 1.747367694 | 0.612453             | 0.00433  | 0.02165          |
| Otu000247 | Bacteria_unclassified              | 1.741509274 | 0.770742             | 0.023851 | 0.062338         |

Note: LogFC is directly related to their abundance in P group, where the highest number showed the highest abundance. For phage cocktail and probiotic combinations groups (P; 1P and 2P) and other groups (Non-P; C, 1, 2 and P).
| OTUs       | Taxonomy                             | LogFC       | Standard error (SE) | P-values     | Adjusted P-values |
|------------|-------------------------------------|-------------|---------------------|--------------|-------------------|
| Otu000022  | Faecalibacterium prausnitzii        | 1.738699859 | 0.433282            | 6.00E-05     | 0.000723          |
| Otu000104  | Lachnospiraceae_unclassified        | 1.715040783 | 0.493786            | 0.000514     | 0.004224          |
| Otu002794  | Lachnospiraceae_unclassified        | 1.702047151 | 1.080272            | 0.115124     | 0.194695          |
| Otu000095  | Oscillospira                        | 1.686705907 | 0.725797            | 0.020129     | 0.055115          |
| Otu000338  | Ruminococcaceae UCG-014             | 1.677289282 | 0.969175            | 0.083517     | 0.14776           |
| Otu000186  | Anaerotruncus                       | 1.668325958 | 0.655373            | 0.010909     | 0.041816          |
| Otu000148  | Oscillospira                        | 1.665393273 | 0.699204            | 0.017226     | 0.049954          |
| Otu000947  | Clostridiales                        | 1.659154189 | 1.306282            | 0.204037     | 0.312856          |
| Otu000802  | Clostridium IV                      | 1.654944242 | 0.813506            | 0.041918     | 0.090954          |
| Otu000041  | Clostridium X1Vα                    | 1.644739429 | 0.517848            | 0.001493     | 0.010098          |
| Otu000069  | Ruminococcus                        | 1.601694881 | 0.542143            | 0.003133     | 0.018607          |
| Otu000044  | Lachnospiraceae_unclassified        | 1.575177456 | 0.551342            | 0.004277     | 0.02165           |
| Otu000573  | Ruminococcus                        | 1.546199668 | 1.1055              | 0.16192      | 0.258623          |
| Otu000057  | Ruminococcus                        | 1.517227727 | 0.458007            | 0.000924     | 0.007085          |
| Otu000007  | Phascolarctobacterium               | 1.505713845 | 0.607781            | 0.013234     | 0.04612           |

Note: LogFC is directly related to their abundance in P group, where the highest number showed the highest abundance. For phage cocktail and probiotic combinations groups (P; 1P and 2P) and other groups (Non-P; C, 1, 2 and P).

**Microbial predicted functional metagenomes**

The principal component analysis (PCA) of predicted functional metagenomes study corroborated the earlier findings that the P groups had distinctive gut microbial communities compared to those of non-P.
Discussion

This study investigated the effects of dietary treatments of isolated phage cocktail at different dosages and their combinations with probiotic on growth performance, health and gut microbial communities of chickens. The assessment on phages as dietary supplementation at various dosages and their combinations with probiotic in broiler chickens has never been reported. However, similar experiments have been conducted on pigs in which commercial phages and probiotic were used, and phages were found to be more effective [14, 16]. This study showed that chickens fed with 0.1% phage cocktail and probiotic combination (1P) can positively modulate the gut microbiota based on the significant reduction of E. coli.

The selection of phage-target bacteria was based on the previous study where both genera of Escherichia and Shigella were observed at a moderate concentration in ilea (9–32%) and caeca (1–5%) at different ages [34]. E. coli was selected as the target bacteria due to their consistent representations in various ages in ilea and caeca, the ease of handling (e.g., aerobic, fast growing bacteria), and also due to their non-pathogenic nature for some of the strains.

Chicken has a range of body temperature between 40.5 to 42°C [59]. The survivability of phage within this temperature is required to ensure the efficacy of phage application. In the current study, all phages had an optimal lytic activity at 37°C. Although phage titre for all phages remained high at a high temperature, phage lytic activity (host reduction) was reduced (at 42°C compared to at 37°C). This observation suggested that phage activity will still persist at 42 °C, but phage may take a longer time to clear targeted hosts. The use of an additive such as skim milk might be useful to preserve the phage activity at a high temperature, where it has been demonstrated in Lactococcus phage [53]. At a lower temperature, phage lytic activity was slightly reduced but was stable at 25°C, especially for C2, C3 and C4 phages when compared to C1 phage. This showed that they were stable at room temperature. The E. coli host was nullified at 60°C and hence replications of phages were not possible.

The results of the present study showed that chickens fed with a combination of 0.1% phage cocktail and probiotic (1P) had significantly better BW (35 d), BWG (22–35 d, 1–35d), and lower FCR compared to the groups (Fig. 5). The supplementation of phage cocktail and probiotic combination resulted in changes of gut microbial dynamics. The predicted functional metagenomes by STAMP analysis were performed based on the previous results from multivariate analysis of gut microbiota diversity which has shown significant differences between gut microbiota dynamics between 1P and non-1P groups (Fig. 5). The predicted functional metagenomes of filtered Storey’s FDR multiple test correction analyses showed significant differences between 1P and non-1P groups in metabolic pathways. The pairwise comparison of the microbial predicted functional metagenomes showed that 14 out of 21 KEGG features were significantly higher in 1P, while another 6 were significantly higher in the non-1P groups (Fig. 6). The metabolic pathways of 1P groups that were related to carbohydrate (e.g., fructose and mannose metabolism), amino acids (e.g., amino sugar and nucleotide sugar metabolism) and tyrosine metabolisms were significantly elevated compared to those of non-1P groups.
control. However, the BW (35 d) and BWG (22–35 d, 1–35 d) of chickens supplemented with phage cocktail singly (1, 2) or probiotic (P) singly, or a combination of 0.2% phage cocktail and probiotic (2P) were not significantly different from those of control chickens. This showed that the use of 0.1% phage cocktail with a combination of probiotic (1P) has the potential to improve the growth performance of broiler chickens. Although supplementation of phage cocktail alone did not significantly improve growth performance in broiler chickens, studies on laying hens reported that supplementation of phage cocktail significantly increased their growth performance and egg production [70]. Studies in pigs had also shown that supplementation of phage cocktail singly or in combinations with probiotic significantly improved the growth performance [14, 15].

The results of the present study revealed that 0.2% phage cocktail (2P), probiotic (P) and a combination of 0.1% or 0.2% phage cocktail and probiotic (1P, 2P) significantly reduced serum triglycerides in chickens, but the reduction was not significantly different between the treatments. This suggests that the addition of a phage cocktail to the probiotic did not further reduce the serum triglycerides level. The reduction of serum triglycerides by probiotic has been widely reported in chickens [5, 65, 31, 66, 38], but there is very little information on the effects of phages or their combinations with probiotic on serum triglycerides. The exact mechanism(s) on the reduction of triglycerides by probiotic is still not well understood. Taranto [67] suggested that reduction of triglycerides in mice supplemented with *Lactobacillus reuteri* could be due to hypolipemic effect, which involved the reduction of lipid assimilation or increase of lipid catabolism. Santoso [65], on the other hand, suggested that reduction of triglycerides by *Bacillus subtilis* in broiler chickens was due to their ability to decrease the acetyl coenzyme A carboxylase activity, which has a crucial function on restricting the synthesis of fatty acids. As the fatty acids decrease, it will in turn reduce the triglycerides esterification process [38].

None of the dietary treatments in the present study had any significant effects on serum total cholesterol, HDL and LDL of chickens at 35 d, though LDL was significantly reduced by 0.1% and 0.2% phage cocktails at 21 d and the serum total cholesterol was numerically the lowest in the 1P group. This is rather surprising as many studies have shown the ability of probiotic to reduce serum cholesterol in broiler chickens [5, 38, 44, 35].

Immunoglobulins are antibodies that are released by plasma cells in response to foreign materials with antigens such as bacteria or viruses. Chickens immunoglobulins can be classified into three main classes: IgA, IgY (IgG) and IgM [47]. IgA is found mainly in bile and intestines of chickens [47]. IgG is the most dominant immunoglobulins found in chickens and it carries out an important function of protecting the host from infection [47]. IgM, on the other hand, is present in the first week post-infection and also appears during immunisation [64].

In the current study, the IgA and IgM concentrations of chickens were not significantly different among all the dietary treatment groups. However, IgG was significantly lower in the 1, 1P and 2P groups when compared with the control at 21 d. The current study also showed that there were no significant differences in IgG between the probiotic (P) group and the rest of the supplemented groups. This showed
that supplementation of probiotic singly or in combination with a phage cocktail did not improve antibody titres. These findings are in contrast to those of other studies, in which probiotic either enhance [63, 39, 40] or have no effects [36] on the chicken humoral immune response. Beneficial effects of probiotic in modulating immune response at the cellular and humoral levels have also been reported in chickens [56]. Immunomodulatory activities of probiotics can be influenced by various factors such as type of probiotic strains, host genetics and types of antigens present [39, 62]. There were also no significant effects on immunoglobulin levels when phage cocktail was fed to pigs [15]. Probiotics are known to enhance immunomodulatory activities to the host by improving antibody response, but this was not observed in this study. It is however important to note that Mountzouris [36] had pointed out that studies which highlighted on enhancement of antibody response with probiotic supplementation [63, 39, 40] were performed against specific model antigens. The immunoglobulin levels in this study showed a general overview of systemic antibody response with supplementation of different diets, but not against specific antigens.

Phage supplementation has been known to initiate an immune response in chickens. This had been demonstrated by Huff [13] who reported that chickens supplemented with phages had significantly higher IgG due to activation of immune responses. Repetitive supplementations of phages would lead to an increase in mortality of birds challenged with pathogenic *E. coli* [13]. This shows that repetitive doses of phages would limit phage action due to the immune response that was initiated. In the current study, significantly lower IgG was observed in birds supplemented with phage cocktails or their combinations with probiotic (1P, 1P1 and 2P), as compared to the control at 21 d. This indicated that IgG was not increased even with ad libitum supplementation of phages, suggesting that supplementation of very high titre of phage cocktail at 10^{10} PFU/g might have overwhelm the host antibody response on the phages. However, this can only be confirmed by measuring the antibodies at short intervals after supplementation of phages.

A study of intestinal villus height and crypt depth would provide valuable information on the changes of chicken intestinal epithelial cells in response to supplementation of phage cocktail, probiotic and their combinations. An increase in villus height is associated with greater nutrient absorption due to the larger surface area for efficient nutrient uptake [55]. Villus crypt is recognised as an important site for generation of intestinal villus [69]. A shorter villus is correlated with accumulation of toxins and a high rate of tissue turnover of villus production due to pathogens, while deeper crypt is associated with toxins that cause inflammation or sloughing [69, 52, 33].

In the current study, supplementation of phage cocktail and probiotic combination to chickens significantly improved in villus height (35 d) and crypt depth (21 d) in the 1P and 2P groups, and villus to crypt ratio (35 d) in the 2P group, when compared to the probiotic (P) group. However, chickens supplemented with probiotic alone or in combination with phage cocktail did not show any significant improvements in villus height, crypt depth or villus to crypt ratio when compared to the control. These results are in contrast with previous reports that chickens with probiotic supplementation showed an increase in intestinal villus height and villus to crypt ratio in broilers [44, 52, 56, 58, 61, 41, 8]. Recently,
Ledaș [46] have reported that villus height was significantly increased in the jejunum and ileum, but not duodenum in probiotic-supplemented chicken. Aliakbarpour [51] also found that *Lactobacillus* supplementation increased the intestinal villus height, but this was not demonstrated in chickens that were supplemented with *Bacillus subtilis*. To date, there has been no previous study on the effect of phage supplementation on intestinal villus height and crypt depth study. These findings show that the effects of probiotic on intestinal villi and crypts are influenced by the types of probiotic used and the part of the intestinal studied.

*E. coli* population was significantly lower in chickens fed with phage cocktail and probiotic combination (1P) than the control in ilea of 21 d chickens and also lower in phage cocktail singly groups (1 and 2), although this was not significant. This showed that *E. coli* phage cocktail employed was potentially effective in reducing *E. coli*, especially in combination with probiotic. Based on the current study, it was difficult to deduce the phage cocktail alone which was responsible for the gut modulation as the concentration of phage titer was not quantified. The caveat is due to the challenge in quantifying C2, C3 and C4 phages as they can cross-infect between each target bacterial hosts. Nevertheless, the qPCR results showed that the *E. coli* population in the phage singly group was significantly reduced, compared to the control. This result potentially suggests that phage cocktail and probiotic worked synergistically in ablating *E. coli* in the gut. The milder reduction of *E. coli* population was also detected in the 1P group at 21 d caeca and 35 d caeca than the control, although this was not significant. The lack of difference may be explained by the fact that gut microbiota became more complexed as chicken aged, thus was more resistant to modulation. Indeed, several studies have shown that gut modulation was effectively performed in chickens of young age between 3 to 25-day-old, where these chickens still harboured transient gut microbiota than the adult chickens that had complex gut microbial communities [45, 50, 68]. Furthermore, *E. coli* population was abundant in ilea compared to caeca, where it was expected that greater reduction of the bacterium was mainly detected in ilea [34].

The PrimaLac® probiotic used in this study was comprised of *Lactobacillus acidophilus*, *Lactobacillus casei*, *Bifidobacterium termophilum*, *Enterococcus faecium* and *Aspergillus oryzae*. In the current study, gut population study based on real-time qPCR showed that there was no significant different for *Lactobacillus* spp. population between dietary treatments for all sampling (age and part of intestine). The populations of *Lactobacillus* spp. and *Bifidobacterium* spp. were expected to be higher in probiotic supplemented groups (P, 1P and 2P), but Bifidobacterium population was found to be higher in chickens fed with phage cocktail singly instead (1 and 2). This suggests that the indigenous gut commensals replaced the niche created by the clearance of *E. coli* in phage cocktail singly groups. However, this was not observed in probiotic supplemented groups. It could be due to the inefficacy of PrimaLac® probiotic to colonise in the intestines. This result was in contrast with the previous studies, where probiotic supplementation was found to increase the level of probiotic bacteria in chicken intestines [36, 49, 54]. It also need to be highlighted that in the current study, PrimaLac® probiotic microbes such as *Enterococcus faecium* and *Aspergillus oryzae* were not quantified, where their populations might be elevated in probiotic supplemented groups.
In the current study, chickens from probiotic supplemented groups (P, 1P and 2P) had significantly lower population of *C. perfringens* compared to the control at 21 d caeca. This result was consistent with previous studies, in which *C. perfringens* population was significantly lower in chickens supplemented with probiotic [49]. This was due to the protective roles of probiotic in reducing pathogens based on the reported mode of actions of competitive exclusion [48, 42] and antagonistic activity [44, 43, 32, 60].

**Conclusions**

Chicken gut microbiota was effectively modulated with supplementation of both *E. coli* phage cocktail and probiotic. Specifically, an increased in beneficial bacteria such as SCFAs producers was detected. Such positive modulation might facilitate carbohydrate and amino acid metabolism, and nutrient uptake, which provides energy for the chicken growth. This is the first study that demonstrated a significant gut modulation using a combination of *E. coli* phage cocktail and probiotic. A further study is warranted to understand the mechanism involved in the gut modulation based on phage cocktail and probiotic combination.

**Abbreviations**

AGPs Antibiotic growth promoters  
BW Body weight  
BWG Body weight gain  
FCR Feed conversion ratio  
SCFAs Short chain fatty acids  
d day-old  
P Probiotic  
HDL High density lipoprotein  
LDL Low density lipoprotein  
IgG Immunoglobulin G  
IgA Immunoglobulin A  
IgM Immunoglobulin M  
FI Feed intake  
OTUs Operational taxonomic units
TG Triglycerides
CHL Cholesterol
Phage
PCO Principal coordinate analysis
CAP Canonical analysis of principal coordinates
PERMANOVA Permutational multivariate analysis of variance
KEGG Kyoto Encyclopedia of Genes and Genomes

Declarations

Ethics approval and consent to participate
All animals were handled in strict accordance with guidelines for animal care and use provided by the Institutional Animal Care and Use Committee (IACUC) Universiti Putra Malaysia, and all animal work was approved by Institutional Animal Care and Use Committee (IACUC) Universiti Putra Malaysia (Ref: UPM/IACUC/AUP-R101/2015).

Consent of publication
All authors critically revised the manuscript for important intellectual contents and approved the final manuscript.

Availability of data and material
Not applicable

Competing interests
The authors declare that they have no competing interests.

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71. Declarations.

Figures
Figure 1

Morphology of isolated phages. Transmission electron micrograph (TEM) of (a) C1, (b) C2, (c) C3 and (d) C4 phages that were negatively stained with 2% uranyl acetate under 300 k magnification. Bar = 100 nm.
Figure 2

Effects of dietary treatments of phage cocktail at different dosages, probiotics and their combinations by real-time qPCR in caeca of 21 d broiler chickens. (a) E. coli were significantly lower in all treatments compared to Control (C) group, especially in 1\(\Phi\) group. There were no significant differences observed for Lactobacillus, Bifidobacterium and C. perfringens between treatments. (b) There were no significant differences observed for E. coli and Lactobacillus between treatments. Probiotics supplemented group (P, 1\(\Phi\)P and 2\(\Phi\)P) had significantly lower Bifidobacterium and C. perfringens compared to non-probiotics group (C, 1\(\Phi\) and 2\(\Phi\)). Each bar is mean of 3 replicate cages with 1 chicken each. Error bar represents standard error (SE). a,bBars that have different superscripts differ significantly (\(P < 0.05\)). C = control.
(basal diet); 1 = BD + 0.1% phage cocktail; 2 = BD + 0.2% phage cocktail; P = BD + 0.1% probiotics; 1 P = BD + 0.1% phage cocktail + 0.1% probiotics; 2 P = BD + 0.2% phage cocktail + 0.1% probiotics.
Figure 3

Effects of dietary treatments of phage cocktail at different dosages, probiotics and their combinations by real-time qPCR in ilea of 35 d broiler chickens. (a) There were no significant differences observed for E. coli and Lactobacillus between treatments. Bifidobacterium were the most abundant in probiotics (P) group. C. perfringens were significantly lower in C, 2P and 1P group. (b) There were no significant differences observed for E. coli, Lactobacillus, Bifidobacterium and C. perfringens between treatments. Each bar is mean of 3 replicate cages with 1 chicken each. Error bar represents standard error (SE). a,bBars that have different superscripts differ significantly (P < 0.05). C = control (basal diet); 1P = BD +
0.1% phage cocktail; 2 = BD + 0.2% phage cocktail; P = BD + 0.1% probiotics; 1 = BD + 0.1% phage cocktail + 0.1% probiotics; 2 = BD + 0.2% phage cocktail + 0.1% probiotics.
Figure 4

Gut microbiome analysis. (a) Principal coordinates analysis (PCO) of gut microbial communities based on Bray-Curtis similarity index. (b) Canonical analysis of principal coordinates (CAP) of ileal gut microbial communities based on Bray-Curtis similarity index. (c) Canonical analysis of principal coordinates (CAP) of 35 d ileal gut microbial communities based on Bray-Curtis similarity index. For treatment (C = control (basal diet); 1P = BD + 0.1% phage cocktail; 2P = BD + 0.2% phage cocktail; P = BD + 0.1% probiotic; 1P = BD + 0.1% phage cocktail + 0.1% probiotic; 2P = BD + 0.2% phage cocktail + 0.1% probiotic), age (21 = 21-day-old; 35 = 35-day-old) and part of intestine (I = ilea, C = caeca).

Figure 5

Principal component analysis (PCA) of the predicted functional metagenomes based on P versus non-P groups. For phage cocktail and probiotic combinations groups (1P; 1P and 2P) and other groups (Non-P; C, 1, 2 and P).
**Figure 6**

Pairwise comparison of the predicted functional metagenomes using Storey’s FDR multiple test correction methods based on $\uparrow$P versus non-$\uparrow$P groups. For phage cocktail and probiotic combinations groups ($\uparrow$P, 1$\uparrow$P and 2$\uparrow$P) and other groups (Non-$\uparrow$P; C, 1, 2 and P).