

# Salmonella Phage cocktail, its effects and benefits on the gut of chickens in a commercial farm.

**Viviana Clavijo**

Universidad de Los Andes

**Laura Tatiana Morales**

Universidad de los Andes

**Martha Josefina Vives Flores**

Universidad de los Andes

**Alejandro Reyes** (✉ [a.reyes@uniandes.edu.co](mailto:a.reyes@uniandes.edu.co))

Universidad de los Andes <https://orcid.org/0000-0003-2907-3265>

---

## Research

## Keywords:

**Posted Date:** May 23rd, 2020

**DOI:** <https://doi.org/10.21203/rs.3.rs-29114/v1>

**License:** © ⓘ This work is licensed under a Creative Commons Attribution 4.0 International License.

[Read Full License](#)

---

# Abstract

## Background

The microbiota of broiler chicken gut affects positively or negatively the health, metabolism and immunity of chickens thus it has a significant impact on the animal productivity. Phages, host-specific parasites of bacterial cells, are a promising antimicrobial alternative that selectively target pathogens without disturbing the microbiota. The aim of the study is further characterizing the behaviour of phages and its effect on the commensal microbial community.

## Methods

We used *16S rRNA* gene amplicon sequencing to evaluate the effect of SalmoFree® (a phage cocktail against *Salmonella*) on the microbiota of the cecum of broilers reared in a commercial farming system. Two field trials were carried out including 2 control and 2 experimental houses. Phages were incorporated in the broilers' drinking water using three doses at the grower stage. The core microbiome, differentially abundant taxa between treatments as well as taxa associated with age were identified using Qiime2 and the R-Project for statistics.

## Results

Analyses of similarities among communities over time allowed us to identify two stages of microbiota development at the last stage of the production cycle. The core microbiome identified some key species in the adaptation of the microbiota at the last stage of the cycle. Among these there are important degraders of complex polysaccharides and producers of short chain fatty acids such as *Eisenbergiella* and *Lachnoclostridium*. Additionally, the phage cocktail did not affect the normal development of the microbiota structure while its application contributes to reduce the presence of pathogenic Enterobacteria including *Salmonella*. The addition of the phage cocktail led to a significant reduction in the abundance of *Campylobacter* and *Helicobacter* and increase in *Butyricimonas* and *Rikenellaceae* abundance which are common inhabitants of the chicken gut with known negative and positive effects in the health, respectively.

## Conclusions

Our results suggest that phages can contribute to improve the chicken health and reduce pathogens burden at the end of the production cycle of broilers. Furthermore, it presents for the first time a detailed look of the microbiota composition and changes in broilers in a commercial scenario, information that will be valuable towards the implementation of phage therapy technologies in the field.

## Background

The microbiota is defined as the complete microbial community, including commensal, symbiotic and pathogenic microorganisms that reside on or within a complex multicellular organism, including plants,

animals and humans. This microbiota includes bacteria, archaea, fungi, protists and viruses [1][Belizário, 2018, Microbiome and Gut Dysbiosis;Belizário, 2018, Microbiome and Gut Dysbiosis]. The knowledge about the importance of the microbiota in human and animal health has grown steadily in the past decade. Early studies focused on cataloguing the microbial species that comprises the human microbiota and its correlation with the health or disease of the host [2-4]. Nowadays, studies are going deeper, focusing, for example, on uncovering the associations between the microbiota, the host and pathogenic bacteria [5-7]. From these later studies, it has been clearly established that microbiota and their products are essential not only for gut development, but also for shaping the host innate immune system, thereby performing multifactorial impacts on the host health [2-4].

In poultry, the microbiota of the broiler chicken gastrointestinal tract (GIT) has demonstrated its importance for the host health, as it has a positive impact on the immune system, the physiology of the GIT, and the animal productivity [8]. Likewise, the microbiota of broilers is involved in reducing and preventing colonization by enteric pathogens through the process of competitive exclusion and the production of bacteriostatic and bactericidal substances[9]. Therefore, an unbalanced microbiota can induce inflammation, leaky gut, or other gut-related disorders [10, 11]. In this context, managing gut health is a key aspect to ensure optimal development and health in poultry.

The broiler's microbiota composition is affected by different factors, such as the age of the animal, the diet and especially the use of antimicrobials [12]. The fact that most antibiotics are broad-spectrum implies that antibiotic therapy causes substantial collateral damage in the host microbiota by killing non-targeted and usually beneficial bacteria. This side effect can often lead to dysbiosis, further promoting the emergence of antibiotic resistant bacteria and potentially leading to the horizontal transfer of the corresponding resistance genes [13]. Poultry production systems have used antibiotics extensively, not only for therapeutic purposes, but also as growth promoters. Indiscriminate use of antibiotics leads to a reduction in the stability of the microbiota in broilers as well as to a reduction of the population of *Lactobacillus* in the intestine [14-16]. Furthermore, in a recent study Danzeisen *et al*/has shown that chickens that did not receive antibiotic supplements had a higher diversity of gene families involved in the degradation of starch, cellulose and hemicellulose, potentially leading to a healthier and more adaptive microbial community; supporting the negative effect of the antibiotic use [17]. For these reasons, there is an increasing interest to manage infections caused by antibiotic resistant pathogens by selectively targeting the disease-causing bacteria, without disturbing the commensal microbiota of the GIT.

Among the different bacterial pathogens, one with increasing relevance to poultry producers is *Salmonella*. This bacterium is a gram-negative foodborne pathogen that is one of the most common causes of acute gastroenteritis in humans worldwide and is becoming an important public health concern with high economic impact in society. The main source of human *Salmonella* infections is associated with the consumption of poultry products [18]. Furthermore, although a broad-host-range of *Salmonella* serovars do not produce clinical disease in older birds, they can cause gastroenteritis in young chicks [19]. In consequence, in addition to the public health threat, *Salmonella* also represents an

economic production problem because besides the damage to the intestinal mucosa due to the infection, *Salmonella* can also contribute to a reduction in the broilers' feed intake and their growth rate. This effect on growth rate reduction has been estimated that could be up to 29% [20]. Additionally, two restricted host range serotypes, *Salmonella enterica* serovar Pullorum and *Salmonella enterica* serovar Gallinarum cause septicemic diseases in the chicken causing pullorum disease and fowl typhoid, respectively. These diseases have been largely controlled in Europe and North America, but still cause substantial economic losses in South America and Asia where the poultry is continuing to intensify and where open sided housing is common [21, 22].

Controlling *Salmonella* outbreaks is thus a priority due to its health impacts and large economic losses. The pre-harvest stage in poultry is a potentially relevant control point, since it would aid preventing the introduction of the pathogen into the food chain and consequently reduce food poisoning among consumers [23]. However, to date, the most common practice to control the pathogen is with antibiotics, at the cost of the aforementioned undesirable side effects.

Phages, as host-specific parasites of bacterial cells, are a promising antimicrobial alternative. Particularly, the use of lytic bacteriophages is an alternative that complies selective targeting of particular pathogens without disturbing the microbiota [24]. Phage therapy has been reported to show additional advantages, for example, the modulation of the immune system as well as the modulation of the microbiota of the host with the potential of improving host health [25]. Furthermore, phages have been proposed for animal therapy, prophylaxis and reduction of pathogen loads in food products of animal origin [26], thus, resulting an appropriate alternative for the control of *Salmonella* contamination in poultry. The use and research on *Salmonella* phages in poultry is not new, with reports including isolation and characterization of the phages, safety assessment and effectiveness of selected phages in chicken meat and in chickens in laboratory or controlled environments [27-32]. Although, most of these studies have been successful; however, to date there is only one report on the use of phages targeting *Salmonella* at a productive scale [33].

We have previously isolated, characterized and developed a phage cocktail that selectively targets *Salmonella* strains [33]. The cocktail, called SalmoFree<sup>®</sup>, has the ability to control a broad range of *Salmonella* serotypes. The phages present in the cocktail have been characterized by host range, infection assays, stability in chlorine, transmission electron microscopy, genome sequencing and a safety assessment in broilers kept in cage batteries [33]. We have recently demonstrated the effectiveness of the cocktail to reduce the presence of *Salmonella* in a commercial farm [34], without affecting animals nor the production parameters, thus demonstrating its innocuity at the productive scale.

In order to further characterize, the behaviour of phages and its effect on the commensal microbial community, we studied the effect of SalmoFree<sup>®</sup> in the cecum microbiota of broilers in a commercial farm, when phages were incorporated in the drinking water. This study is based on the same experimental set up recently published [34], and extended the analysis through the use of 16S rRNA gene amplicon sequencing. Altogether, this work expands the knowledge about the effect of phage therapy in the

microbiota in broiler chickens and will help to reveal the performance of phages in the commercial farming conditions. Additionally, this work is the first study to characterize the composition of broilers microbiota at the growth stage of broilers under farming conditions.

## Methods

### Experimental design

This study was approved by the Institutional Committee on Care and Use of Experimental Animals (CICUAL) from Los Andes University, Ref. CICUAL 15 – 008, in the framework of Colombian Law 84/89 and Resolution 8430/93.

Two field trials under commercial rearing conditions were carried out in a commercial broiler farm in Colombia [34]. This farm belongs to an integrated poultry company that typically handles the entire production and processing cycle of a chicken (hatching, feed, production, processing, and marketing).

Four production houses (labeled as houses 1, 2, 3 and 4) were selected due to the existing record of *Salmonella* presence detected during two previous productions cycles (data not shown). Chickens in houses 1 and 2 were treated with a control suspension (see below) whereas houses 3 and 4 were treated with the bacteriophage cocktail SalmoFree®. Treated flocks were separated from the controls by a distance of 300 m approximately. Information about houses' size, breed line, sex, and antimicrobial therapy per house and trial are presented in detail in Clavijo *et al*, 2019 [34]. Houses 1, 2, 3 and 4 correspond to 4, 8, 9 and 10 respectively in Clavijo *et al*, 2019.

The broiler production cycle in Colombia is carried out in two stages. The first stage comprises the period from which one-day old chickens are received at the farm (day 1), until day 13–17. Chickens at this stage are fed with a starter diet. Following this diet, the grower stage spans from days 14 to 18 until day 35 to 42, where chickens receive a grower diet. Variation in the length of each period depends on several factors (market demand, weight of the chickens, schedule at slaughterhouse, among others). At the end of the second stage, chickens are sent to the slaughterhouse. SalmoFree® and the control suspensions were delivered to the animals in the drinking water during the second stage in three doses (one per week): at the beginning (day 18 for both trials), at the middle (day 27 and 26 for the first and second trial, respectively), and one day before slaughter (day 35 and 34 for the first and second trial, respectively). (Fig. 1).

### Phage and control treatments preparation

SalmoFree® was prepared following a standard liquid lysate procedure using *Salmonella* Enteritidis s25pp. This strain was donated by Dr. Pilar Donado from the Colombian Integrated Program for the Antimicrobial Resistance Surveillance (COIPARS - CORPOICA) [35]. Typically, the preparation was carried out individually for each phage in nutritive broth (Sharlau), using an MOI of 0.1. Each lysate was centrifuged at 4 °C at 13,000 x g for 20 min and the supernatant was filtered through a 0.22-µm filter.

Approximately, 5.5L of each phage was produced per trial and stored at 4 °C. Individual phages were mixed in the adequate concentration and volume immediately before administering each treatment.

To evaluate the quality of the cocktail, a quality standard of sterility (zero bacteria) and a concentration of at least  $10^{10}$  PFU/mL were established. All doses were evaluated under these criteria. Sterility was evaluated incubating a 10 mL aliquot of the cocktail in 50 mL tubes at 37 °C for 24 h. Additionally, streaking from this culture was performed onto a nutritive agar plate (Sharlau). After 24 h of incubation, the absence of any type of growth was verified. Cocktail concentration was determined by carrying out serial dilutions from the cocktail suspension and plating the dilutions by the double agar overlay plaque assay [35].

The control suspension was elaborated using a fully-grown *Salmonella* culture that was lysed by the addition of chloroform at a final concentration of 0.1% (v/v). The lysate was centrifuged and filtered as described above in the phage lysate procedure. The final suspension was verified to be free of bacteria and phages. This control allows estimating if the cell residuals found in a normal lysate have any effect on the observed results.

## Delivery of the treatments to the animals

The drinking water supply was removed 30 min before administering the treatments. This is a regular practice in the poultry production that is carried out in order to facilitate the uptake of any treatment due to the temporary shortage of hydration, but does not compromise the health of the chickens in any way.

The water supply tanks found in each farmhouse can store up to 1000 L of water. SalmoFree® and control suspensions were added to these tanks in a 100:1 water to treatment ratio. Thus, the final concentration of the phage suspension was  $10^8$  PFU/mL. Treatments were delivered to the animals for 2.5 h, time in which is estimated that the animals drink the full 1000 L water supply.

## Sampling methods

Cecum samples were taken one day before and after delivery of SalmoFree® treatments, according to Fig. 1. Additionally, samples one day before the start of the growing phase (day 15 and 14 for the first and second trial, respectively) and at the abattoir, after the slaughter, were collected. To get all samples, five female chickens from each of the four houses were randomly selected. These chickens were sacrificed and their cecum was removed in the most aseptic possible way in the farm. Each cecum was placed into sterile plastic bags (Nasco, USA) and transported in liquid nitrogen to the laboratory where they were stored at -80 °C and promptly processed for DNA extraction. A total of 320 samples were collected corresponding to 160 per trial (5 ceca per house per 4 houses per 8 sampling days). Since sampling days occurred at different points of the growth cycle between trial 1 and trial 2 (Fig. 1), we renamed the samples according to each treatment dose. The sampling point corresponds to the number of dose (1–3) followed by a letter indicating whether it was taken one day before (b) or after (a) the corresponding dose.

Besides the ceca samples, individual cloacal swabs samples were taken in order to detect *Salmonella* using a genus-specific PCR as described in Clavijo *et al*, 2019 [34]. Each individual swab corresponded to the same individual chicken sacrificed for cecum extraction.

## DNA Extraction and 16S rRNA gene amplification

From ceca samples, 180–200 mg of the cecal content were aseptically collected in dry ice, avoiding the thawing of the samples. Immediately, the samples were processed through the QIAamp DNA Stool Mini Kit (Qiagen, Valencia, CA) according to manufacturer's instructions. Samples were measured in a Nanodrop ND-1000 spectrophotometer (Thermo Scientific, Wilmington, USA) to assess the DNA quality and were quantified using a Qubit fluorometer (Life Technologies, Paisley, UK). After DNA quantification, samples were diluted with elution buffer (Qiagen, Hilden, GM) to a concentration of 5 ng/μL. From these diluted samples amplification of the V4 hypervariable region of *16S rRNA* gene was achieved using the following primers:

Primer1 5'TACACGACGCTCTTCCGATCT**GTGCCAGCMGCCGCGGTAA** 3' and

Primer2 5'AGACGTGTGCTCTTCCGATCT**GGACTACHVGGGTWTCTAAT** 3'.

The bold region of the primers corresponds to the universal 515F and 806R primers. Each PCR reaction used buffer 1X, dNTP's 10 mM, Primer1 10 μM, Primer2 10 μM, Phusion High-Fidelity DNA Polymerase (0.02U/μL) and 1.5 μL DNA (7,5 ng on average) in a final volume of 20 μL. The configuration of the temperature profile for the reaction was as follows: Initial denaturation 94 °C for 3 min; 30 cycles at 94 °C for 45 s, 56 °C for 30 s y 72 °C for 30 s; and the final extension at 72 °C for 7 min. The PCR procedure was carried out in triplicate with a negative control in which water was added instead of DNA sample. PCR products were visualized using Gelred through 1.5% (w/v) agarose gel electrophoreses. Finally, after the confirmation of the amplification, the PCR triplicates were mixed in one pool and were kept at -20 °C until further processing.

## Libraries preparation and sequencing

After the first amplification, a second PCR was carried out using a pair of primers with the Illumina adapters and indexes to demultiplex the samples. Sequences of the primers are:

Primer3

5' AATGATACGGCGACCACCGAGATCTACACNNNNNNNNNACACTCTTTCCCTACACGA

Primer4

5'CAAGCAGAAGACGGCATAACGAGATNNNNNNNNNGTGACTGGAGTTCAGACGTGTG

The underlined region corresponds to the location of a particular index (the sequences for all primers are presented in Supplementary material Table 1); Each sample is amplified with a pair of primers with a different sequence in this region, in order to allow pooling all samples in the same sequencing run and

demultiplexing afterwards. The second PCR was carried by adding 5  $\mu\text{L}$  of the pooled product from the first PCR to a mixture with 4  $\mu\text{L}$  of water, 10  $\mu\text{L}$  of GoTaq Green Master mix (Promega) and 0.5  $\mu\text{L}$  of each Illumina index primer (0.25  $\mu\text{M}$ ) which was amplified using the following PCR conditions: 3 min at 94°C, and 12 cycles of 45 s at 94°C, 60 s at 55°C and 30 s at 72°C and a final period of 7 min at 72°C and kept at 4°C. Then, a purification process was performed by using 18  $\mu\text{L}$  of AMPure beads (Beckman Coulter) and eluting samples with 15  $\mu\text{L}$  of Tris buffer (10 mM, pH 8.5). Next, the concentration of this mixture of purified amplicons was determined with the Qubit fluorometer (Life Technologies, Paisley, UK) followed by pooling all the libraries into equimolar concentrations. Paired-end sequencing (2  $\times$  250) of this pool was conducted on an Illumina MiSeq platform at Washington University in Saint Louis, Center for Genome Sciences and Systems Biology.

## **Bioinformatics' and statistical analyses**

Sequences were pre-processed, quality filtered and analyzed using QIIME2 version 2018.11[36] (<https://qiime2.org>) and its plugins. The input files used were the demultiplexed paired-end fastq files generated in Casava format (Illumina) and a mapping file. Raw sequencing data was imported and demultiplexed through the Casava 1.8 paired-end demultiplexed fastq protocol. Then, adapters were removed using the cutadapt plugin [37] and subsequently the fastq sequences were merged using FLASH software [38]. DEBLUR software package [39], included in QIIME2, was used for modelling and correcting Illumina sequences. This process integrates chimera removal, truncation of reads and the collapse of reads into amplicon sequence variants (ASVs). All parameters were used by defaults except for truncation of reads: -p-trunc 214. The detection limit of the method ( $1 \times 10^{-4}$ ) was established based the maximum number of rarefied reads per sample (10,000 per sample) implying that any ASV at an abundance less than 1/10,000 will not be detected.

ASVs were filtered using QIIME2 q2-feature-table filter features command, keeping only features with a frequency higher than 10, in general corresponding to a minimum relative abundance of 0.001 and present in at least 2 samples. A second filtration was done retaining samples with more than 3,000 sequences after ASV filtration. Taxonomy assignment to the ASVs was performed using QIIME2 q2-feature-classifier plugin and the Naïve Bayes classifier that was trained on the SILVA database (version SSUParc\_100)[40]. Alpha- and beta- diversity analyses were performed with the q2-diversity plugin at a sampling depth of 10,000. Alpha diversity was calculated using Shannon's diversity index, observed OTUs and Faith's Phylogenetic diversity. Kruskal-Wallis test was used to test for differences in mean alpha-diversity between experimental treatments and trial, farmhouse, genetic line, sampling point and dose variables. Distance matrices for beta-diversity were constructed using Bray Curtis and weighted Unifrac metrics. Permutational multivariate analysis of variance (PERMANOVA,  $P < 0,05$ ) using group significance command was used to analyse spatial variation in beta-diversity and effects of experimental treatments and the other variables. Additionally, Unweighted Pair Group Method with Arithmetic Mean (UPGMA) clustering analysis based on Bray Curtis and weighted UniFrac distance was performed. Significant differences in taxons between the groups were assessed with the ANCOM test in QIIME2. Specific analyses were carried by week collapsing samples between cycle days 15–21 in week 3, days 22–28 in

week 4 and days 29–36 in week 5 (Fig. 1). Additionally, the phyloseq package [41] in R was used to determine the core microbiome and also to analyse the abundance of the predominant phyla over time.

## Bioinformatics' *Salmonella* analyses

Given that none of the identified ASV's were assigned to the genus *Salmonella*, but several of them were annotated as Enterobacteriaceae family, the next step was to determine if some of those ASVs in the feature file were closely related to *Salmonella*. For this purpose, a collection of *Salmonella*, *Escherichia coli* and *Shigella* 16S rRNA genes deposited in the NCBI Refseq collection were retrieved. These sequences (Supplementary material table 2) were used along with the sequences of ASV's assigned to the Enterobacteriaceae family for a multiple alignment using MUSCLE [42]. Following, the alignments were manually inspected and edited using the sequence editor Jalview (version 2) [43], looking for specific positions that constitute specific markers for the *Salmonella* group. Afterwards, a maximum-likelihood phylogenetic tree was constructed using FastTree version 2.1 [44] and visualized in Figtree [45]. From this analysis, it was possible to identify the ASV that most likely corresponds to *Salmonella*.

A similar characterization of the ASVs assigned to *Helicobacter* was performed since some species of the genera *Helicobacter* are considered human or animal pathogens. These ASVs were aligned with the collection of *Helicobacter* 16S rRNA gene sequences deposited in the NCBI Refseq collection (Supplementary material table 3)

## Results

The current study focused on the evaluation of the effect of the *Salmonella* phage cocktail on the cecum microbiota of broilers in a commercial scenario while being incorporated as treatment in the broilers' drinking water. The experimental set-up was the same as previously published, where it was performed the evaluation of the effectiveness of the phage cocktail by comparing the presence of *Salmonella* and the production parameters between the two treatment groups; also, detection of phages was performed [33]. The summary of those published results is presented in Table 1 and Table 2.

## Sequencing information

From a preliminary analysis, we determined that the sequence variants generated for the F2 farmhouse two (Control farmhouse) in trial 2 were significantly different in composition and diversity from the other samples (Supplementary material Fig. 1), likely reflecting a technical rather than a biological effect, thus all samples from that farmhouses were excluded from further analysis. Additionally, sequences generated from most of the samples taken one day before the beginning of the growing phase (day 15 and 14 for the first and second trial, respectively) had a very low extraction yield of DNA leading to a high frequency of failed amplifications and sequencing; likely a consequence of low biomass due to the early age of the chicks. Thus, all the samples from these time points were excluded from further analysis (Supplementary material Fig. 1). Finally, 244 samples were kept for the rest of analysis.

After quality control, 228 samples were retained and we obtained 4,995,664 sequences with a total of 3,993 amplicon sequence variants (ASVs). The frequency of reads per sample varied from 3,527 to 210,082 with an average of 21,911 and a median of 18,130 per sample, which is comparable to previous studies [46, 47].

## Microbial diversity

The evaluation of rarefaction curves based on rarefied Shannon indices indicates that a sufficient sequencing effort was obtained to characterize the most abundant bacteria affecting the diversity index for all samples, as represented by a plateau in the curve (Supplementary material Fig. 2A and B), however, the observed OTUs metric shows that even at a sequencing depth of 11,000 reads per samples, it was still possible to detect new ASVs as a function of sequencing, likely transient or very low abundant species, as they didn't have an effect on the Shannon indices (Supplementary material Fig. 2C and D).

Alpha diversity analyses for both trials using Faith's phylogenetic diversity and number of observed OTUs suggested a slight increase over time. This temporal phenomenon is more prominent in trial 2. However, there was not a high variation trend in diversity between the days of the experiment (17–36) since their Faith indices fall within a comparable range of 20 to 33. This small range might occur because the microbial community is reaching a relatively stable, yet dynamic, state (Fig. 2). This behaviour is in agreement with previous reports where the microbiome stabilizes at approximately day 12 [48]. Regarding the maturation process of the microbiota, the microbial communities have a similar diversity at day 17 between samples (3rd week), at the beginning of the experiment. Also, it is possible to notice that between the 4th and 5th week, the microbiome increases its variability and stabilize again at the last day of the trial, as it remains constant at the slaughterhouse (Fig. 2). Comparing trials, higher alpha-diversity index values (Faith, Shannon and Observed OTUs) were observed in trial 1 compared to trial 2 (Fig. 2).

Alpha diversity indices changed significantly (Kruskall Wallis test,  $P < 0.05$ ) in response to the trial, farmhouse, treatment and markedly by the age of the animal. Conversely, the genetic line did not show significant differences (Kruskall Wallis test,  $P > 0.05$ ) in microbial diversity. Although the treatment showed significant differences, there is no observable pattern to discriminate among treatment groups.

Alpha-diversity results are complemented by the beta-diversity comparison between weeks, treatments and trials (Fig. 3A, B, C, D). Principal Coordinates Analysis (PCoA) plots based on treatment did not reveal any clustering pattern (Fig. 3A) while PCoA plot based colored by age and trial showed that microbial communities were driven mainly by these two variables (Fig. 3B, 3C). Interestingly, regardless of the trial, the microbial community showed significantly higher similarity at the beginning of the experiment (Fig. 3B, 3D), and diverged as a function of time, becoming more distant at the second dose of the treatments. The trial dependency suggests a high contribution of the environment in the development of the microbiota, which is expected due to the uncontrolled variables and their variation in a broiler farm, such as temperature, humidity, feed composition, litter replacement, feeding and antibiotic intervention, among others. The observation of complex but highly similar diversity pattern in older birds agrees with

previous studies where microbial communities exhibit similar patterns as chickens reached their marketing age [48].

## Taxonomic Composition of the Bacterial Community

We performed a general analysis of the taxonomic composition at phylum level of the community in order to characterize their behaviour in a commercial scenario throughout the last stage of the production cycle. In addition, we analysed the minimum community of microbes that is essential for the host (i.e. core microbiome) for both trials, at genus level.

First, the taxonomic composition of trials 1 and 2 indicated that Firmicutes is the most predominant phylum in the gut of broilers (46.4% abundance), followed by Bacteroidetes (37%). Together, both phyla account for more than 80% of the relative abundance within the community. Then, in lower proportion, the phylum Epsilonbacterota (4.47%), Proteobacteria (3.2%), and Tenericutes (1.21%) are found, while unassigned bacteria at phylum level accounted for 6.41% of the abundance.

Regarding phyla dynamics, Firmicutes and Bacteroidetes remain relatively constant throughout the experiment (Fig. 4), only a slight decrease in Firmicutes, with a corresponding increase in Bacteroidetes, was observed, the day after the second dose (Day 28 and 27 for trial 1 and 2, respectively) suggesting a complementation between this two phyla. Proteobacteria behaves similarly in the two trials, maintaining relatively constant numbers over time where the lowest point was found after the second dose as well. The presence of bacteria of the phylum Tenericutes is higher at the beginning of the experiment with a slight reduction over time (Fig. 4). Epsilonbacteria phylum was present at a very low abundance at the beginning of the experiment but increased rapidly starting at the 4th week of the cycle (before the second dose) and remain constant until the end of the experiment (Fig. 4).

To better understand the shared taxa occurring over time, we analysed the core microbiome discriminated by trial and week (Table 3). Core microbiomes were defined as those taxa that were present in more than 50% of samples of each week sampling point. When comparing the core microbiome for both trials over time, it was observed that the genera conforming the broilers' microbiome is highly conserved since most genera (78%) were present in both trials (Table 3). This result is interesting because despite being different in terms of a) alpha and beta diversity; b) breeds; and c) antimicrobial regime, both trials share the core genera.

Likewise, the analysis of the core microbiome over time showed that the microbiota, during the period examined, was also conserved: 65% of the genera were found in most of the samples during the three weeks (Table 3).

The core microbiome at the slaughterhouse contained all the genera found during the experiment. Consequently, the main members of the community were maintained during transportation of the broilers to the slaughterhouse (Table 3).

# Taxonomic dynamics at the grower stage of the production cycle

The differential analysis discriminated by week allowed the identification of some key species in the adaptation of the microbiota during the grower phase of broilers. For instance, in both trials several genera revealed an increasing abundance trend over time, including *Alistipes*, *Rikenellaceae*, *Phascolarctobacterium*, *Desulfovibrionaceae* and *Megamonas*; while *Bacillales*, *Coprobacter*, *Barnesiellaceae* and *Ruminococcaceae* decreased. Another taxon with an intriguing abundance was *Odoribacter*, which increases between the 3rd and 4th week and then remains relatively constant until the end of the cycle. In contrast, *Hydrogenoanaerobacterium* is only detected at the beginning of the cycle (3rd week) and then disappears (Fig. 5).

## Abundance differences in the microbial taxa between the treatment groups

As the structure of the microbiota was markedly influenced by the trial, and additionally, given the antibiotic intervention in the first trial (Fig. 1), a differential analysis was carried out separately in each trial in order to define the differences between the farmhouses treated and not treated with the phage cocktail.

We performed an independent PcoA analysis by Trial. This analysis showed a separation by treatment after the second dose but this clustering disappeared towards the end of the experiment. This separation is clearer for trial 1 (Fig. 6), although a more compact clustering of all samples can be observed towards the last days of the experiment for both trials.

The differential abundance analysis among doses revealed four genera as significantly associated with the treatment; the taxa *Campylobacter*, *Helicobacter*, *Rikenellaceae* and *Butyricimonas* (Fig. 7). *Campylobacter* in the first trial appeared between the first and second dose in both control and treated samples, however, the abundance in treated farmhouses slowly increases its abundance in contrast to the abrupt increase of the control groups during the second dose with a subsequent decrease, leading to a final convergence (for treatments and controls) in abundance at the end of the experiment. Interestingly, opposing to *Campylobacter*'s under-representation in treated samples, the closely related *Helicobacter* seems to increase its abundance. *Butyricimonas* abundance increased significantly respect to the control after the second and third doses in trial 1 (ANOVA,  $p < 0.005$ ). Similarly, *Rikenellaceae* increased its abundance after the second doses and is significantly different, compared to the control group (Fig. 7A).

Abundance patterns of particular taxa displayed similarities in both trials. In both cases, *Rikenellaceae* was present at a higher abundance in treated chickens compared to the control in the last week of the experiment. Even though the tendency to increase abundance over time is observed for treatment groups in both trials, trial 2 showed overall lower abundances than trial 1, as seen for *Campylobacter* and

*Helicobacter* (Fig. 7B). In addition to these taxa, the genera *Parasutterella* that was not detected in trial 1, increased its abundance over time in trial 2.

Given the differential abundance of *Helicobacter* seen on the treatment groups and since some species of the genera *Helicobacter* are considered human and animal pathogens, a further characterization of the ASVs assigned to *Helicobacter* was performed. These ASVs were aligned with the collection of *Helicobacter* 16S rRNA gene sequences deposited in the NCBI Refseq collection. After the bioinformatics analysis, these ASVs were identified as *Helicobacter pullorum* (Supplementary material Fig. 4).

## Enterobacteriaceae abundance Analysis

Throughout the analysis implemented for *Salmonella*, one ASV from 32 was clustered in the clade that grouped *Salmonella enterica* subsp. *enterica* and was separated from the clades that grouped *Salmonella bongori* and *Escherichia coli*-*Shigella* (Supplementary material Fig. 5). Thus, we established that this ASV corresponds to *Salmonella* sp. The others ASVs assigned to Enterobacteriaceae family were grouped in the *E. coli*-*Shigella* clade.

The abundance of the *Salmonella*-related ASV across samples was rather low, in a range of  $2.5 \times 10^{-5}$  to  $6.8 \times 10^{-4}$ , and close to our estimated confidence detection limit of the method ( $1 \times 10^{-4}$ ). Moreover, the ASV was detected in a small number of samples ( $n = 22$ ). Thus, further inferences based on its abundance were discarded. In addition, no correlation was found with the abundance of this ASV and the molecular detection of *Salmonella* ( $p > 0.01$ ) (Supplementary material Fig. 6). This result indicates that, despite identifying specific regions to discriminate *Salmonella* from the other members of the Enterobacteriaceae family, due to its very low abundance it would be necessary to carry out a deeper sequencing in order to detect it within accurate detection levels.

We resorted to analyze patterns of the family Enterobacteriaceae due to the difficulties of detecting *Salmonella* sp. at accurate detection levels. This choice was justified based on two observations: (1) family Enterobacteriaceae showed a positive correlation with the molecular detection of the genus *Salmonella* (ANOVA,  $p < 0.001$ ) (Fig. 8A), and (2) a negative correlation with the detection of phages (ANOVA,  $p < 0.001$ ) was observed, meaning that the abundance of the family was lower in samples where phages were detected (Fig. 8B). Here the *Salmonella* and phage detection are referred to the molecular detection carried by the amplification of the specific nucleic acid sequences of *Salmonella* sp. and the phage tail fiber gene, respectively [34].

The abundance of Enterobacteriaceae throughout the days, discriminated by trials and treatments, is shown in Fig. 8. In trial 1 at the beginning of the experiment, the abundance of the family was lower in the farmhouses treated with the phage cocktail. This result was expected due to the antibiotic intervention that occurred in week 1 because the antibiotic used is active against several gram-negative bacteria. In treated farmhouses the low abundance of Enterobacteriaceae is maintained until the last week in the farm and also at the slaughterhouse. Control farmhouses also exhibited a significant reduction after the second dose (Fig. 8C). In trial 2 after the second dose, a high reduction of the Enterobacteriaceae

abundance is identified for both treated and non-treated houses. This abundance was lower than the results obtained in trial 1 and was also reflected in the samples taken at the slaughterhouse. Notably, the abundance of this group is increased before the second dose for treated houses in trial 2 (Fig. 8C). This increase might be due to a high mortality rate in the farmhouse 4 (a treated house) in this period of time that could lead a higher abundance of pathogens of this group reflecting in the abundance of Enterobacteriaceae (data shown in [33]).

## Discussion

The aim of this study was to determine the effect of the *Salmonella* phage cocktail SalmoFree® in the composition of the microbiota of the cecum of broilers. The study was performed supplying the phage cocktail to chickens reared with commercial purposes at a farm under production scale conditions. Our study also included the characterization of the dominant bacterial microbiota present at the grower stage of broilers in production conditions. Our results suggest a process of normal microbiota maturation characterized by a transition towards a higher diverse community. This observation was independent of the treatment applied, further demonstrating that phages do not affect the normal development of the microbiota.

Surprisingly, our results suggest that the behaviour of the microbiota in the period of the experiments (age 17–36 days), corresponding to the grower phase of the chickens, is similar to what have been previously reported in experimental chickens reared in a controlled environment [48, 49]. For instance, our observations support the stabilization dynamic of the microbiota at this developmental stage. Likewise, the observation of a microbial community similar in older chickens to the one observed at slaughter age was consolidated (Figs. 2 and 3). Additionally, the age of the animal was the variable that had the highest influence over the microbiota variation, in agreement with previous studies [48, 50]. This is a key evidence suggesting that microbiota approximations done under controlled environments do not differ largely from farming conditions.

Analyses of similarities among communities over time allowed us to identify two main moments of microbiota development at the last stage of the production cycle (PERMANOVA,  $P < 0,05$ ) (Fig. 3). The 3rd week, representing the first week of the experiment and the week just after the change to the grower diet, is where the community seems to be more uniform and with a significantly higher abundance of bacteria such as *Ruminococcaceae*, *Bacillales*, *Coprobacter*, *Hydrogenoanaerobacterium* and *Barnesiellaceae* (Fig. 5) compared to *Parasuterella* and *Flavobacteriaceae*. The other two weeks, the 4rd and 5th weeks, exhibit a higher variation in abundance and diversity, which could be attributed to the change in diet. During the weeks 4th and 5th the microbiota becomes populated by *Phascolarctobacterium*, *Desulfovibrionaceae*, *Megamonas*, *Odoribacter*, *Rikenellaceae* and *Alistipes*. These bacteria could represent biomarkers of microbiota maturation at these rearing conditions (altitude: 1230 m.s.n.m.; litter composition: ground; average of no. of chickens/m<sup>2</sup>: 13.86; average of area house in m<sup>2</sup>: 645.61). Nevertheless, further studies are necessary to confirm the generalization of our current results to other farms and conditions.

Analysis of the dynamics of the core microbiome identified members that were reported previously in the literature as being part of the most abundant genera in the microbiome of chicken cecum [48]. In one previous study, authors performed a comprehensive day-to-day microbiome analysis of the chicken cecum from day 3 to 35 using experimental chickens in a controlled environment. Authors identified the most abundant genera, also identified in our core analysis, as: *Escherichia*, *Shigella*, *Eisenbergiella*, *Ruminiclostridium*, *Flavonifractor*, *Anaerotruncus*, *Faecalibacterium*, *Lachnoclostridium*, *Megamonas*, *Intestinimonas*, *Shuttleworthia*, *Subdoligranulum*, *Tyzzereella*, *Lactobacillus*, *Blautia* and *Erysipelotrichaceae*, among others (Table 3).

The core composition of the microbiota identified key members at the last stage of the production cycle (Fig. 3) (Table 3). Those microorganisms may be responsible for important metabolic processes in the microbiota of broiler chickens. Among these, there are some important degraders of complex polysaccharides and producers of short chain fatty acids (SCFA). For instance, *Eisenbergiella*, *Lachnoclostridium* of the family *Lachnospiraceae* play an important role in the production of butyrate which is the preferred energy source for the gut epithelial cells [51]. Another butyrate producer found in the core was *Intestinimonas* [52]. *Megamonas* and *Bacteroides* were detected as well; these bacteria are known to produce propionate as the main end product of the degradation of complex plant polysaccharides. Although propionate is a less preferred energy source than butyrate, its production might represent an efficient balance between energy acquisition from available nutrients and sustained growth [50]. Other bacteria present in the core microbiome involved in producing SCFA were *Subdoligranulum*, *Faecalibacterium Alistipes*, *Coprobacter*, *Blautia* and *Butyricimonas* [53].

On the other hand, *Campylobacter*, *Helicobacter* and *Megamonas* are bacteria carrying hydrogenases, which have the potential to serve as hydrogen sinks that facilitate succinate production [54]. Succinate is an important metabolite in both host and microbial processes [55]. Meanwhile, the presence of *Oscillibacter*, a Clostridium cluster IV member, has been identified as an anaerobe producer of valerate and associated with diet-induced obesity [56]. Surprisingly, *Bifidobacterium*, a butyrate producer, was not detected while it has been reported consistently as a dominant member of the chicken microbiota [48–50].

Comparison analyses between treated and control farmhouses must be done with caution, because of the conditions and characteristics of the current trials (variation of temperature, humidity, feed composition; the antibiotic intervention, and phage's cross-contamination). Altogether, it seems that the treatment patterns visualized in trial 1 are not evident in trial 2. For example, in trial 1 the diversity seems to be higher in the control group, also in trial 1 the relative abundance of *Campylobacter* sp. is lower in the treatment group while in trial 2 this abundance is low in both experimental groups. We proposed that these dissimilarities might be an effect of the presence of phages due to cross-contamination starting at the beginning of the trial 2 in all treatments. This event could generate a bias in trial 2.

In this line, our data suggest a low trend of alpha diversity in treated farmhouses. Although an increase in microbial diversity in the gut has been linked to improved health in the elderly [57], a reduced alpha

diversity in samples subjected to phages has been reported previously as well as in trials with probiotics in chickens, where treated groups exhibit a lower diversity compared to non-treated groups [58]. An alternative explanation for the observed reduction of diversity could lie in the presence of opportunistic pathogens [59]. In some cases, the microbiota associated with disease is more diverse due to the presence of opportunistic pathogens that have the capacity to colonize this niche. Thus, the alpha diversity reduction could be attributed to a lower presence of opportunistic pathogens.

Differential analyses by treatment group determined that few taxa are significantly associated with the addition of phages, supporting the statement that phages are not affecting the structure of the microbiome. Particularly interesting is the evidence of the reduction of *Campylobacter* in treated farmhouses. *Campylobacter* is also considered an important food-borne pathogen associated to the consumption of poultry products and its importance in public health is substantial [60]. In addition, when this opportunistic pathogen is highly abundant in chickens, it has been demonstrated to cause damage to the gut [61]. The observed reduction of *Campylobacter* might suggest a beneficial modulation of phages while the reduction in *Salmonella* and pathogenic Enterobacteriaceae such as *Escherichia coli* represents a great added value to the application of SalmoFree® in broilers. Additionally, the presence of *Campylobacter jejuni* has been associated with a higher abundance of *E coli* not only in broilers [62] but also in humans [63]. It is important to highlight that the reduction of *Campylobacter* is likely occurring as a secondary effect of the phage lysis on *Salmonella*, since it is not expected that phages infect strains of this genera given the specificity of the cocktail towards *Salmonella* sp. strains. Furthermore, the correlated increase in *Helicobacter* abundance with the decrease in *Campylobacter* supports the proposal of a competitive dynamic between these two genera [62].

The increase of *Butyrivimonas* and *Rikenellaceae* following the phage treatment is noteworthy. These two genera are reported as beneficial bacteria in chickens due to their enrichment in samples treated with probiotics [58].

Since the relative abundance of *Salmonella* could not be obtained, the analysis of Enterobacteriaceae family is highly important to elucidate the performance of the phage cocktail. We consider this analysis useful since members of this family are phylogenetically closely related. Enterobacteria such as *E. coli* and *Salmonella enterica* are facultative anaerobic pathogens that occupy the same niche [64, 65], hence knowing the behaviour of the family will help us to understand what happened with *Salmonella*. Furthermore, the Enterobacteriaceae family has been reported as one consistent biomarker of poor gut health in poultry [66], as it has been negatively correlated with performance of production variables [67].

Regarding the Enterobacteriaceae analysis, our data confirmed that in the presence of the phages the abundance of this taxon is reduced. Although the reduction of this group was observed for both experimental groups in trial 1 and trial 2, it might be a consequence of the cross contamination of phages that occurred in control houses (Table 2). Apparently, the higher effect of phages is around the 4th week of the production cycle; this time point also corresponds to the moment where more microbial changes are occurring. This finding identified the importance of the second dose and suggests it as a relevant

dose in the regime proposed for SalmoFree®. The result that the reduction on the second trial was even better, let us to hypothesize that the reduction of the target could be higher with the application of phages by consecutive cycles.

Equally important is the maintenance of low abundance at slaughter. Hence, the application of phages is achieving its main objective that is to reduce the presence of *Salmonella* (an Enterobacteriaceae member) when chickens arrive in the slaughterhouse in order to impact directly the risk of food poisoning and reduce infection cases in humans.

## Conclusion

This study confirmed the safety of the SalmoFree® cocktail on the health and microbiome health in chickens. Its delivery to broilers did not affect the normal development of the microbiota structure. Compelling evidence was obtained on the SalmoFree® contribution to reduce the presence of pathogenic Enterobacteria including *Salmonella*. Additionally, our results suggest that the use of the phage cocktail might be proposed as a beneficial microbiota modulator improving host health. This differential effect could contribute to improve the chicken health and reduce pathogenic burden at the end of the production cycle of broilers. It is important to highlight that our results were obtained not via an experimental setup, but *in situ* at the production farm, giving a closer idea of what the real effect of the phages under commercial settings will be. These findings reinforce the knowledge that phages targeting *Salmonella* may constitute an efficient prevention measure to avoid food poisoning outbreaks associated with the pathogen and reduce the problematic widespread use of antibiotics in the poultry industry

## Abbreviations

### ASV

Amplicon sequence variants

### PCR

polymerase chain reaction.

## Declarations

### Acknowledgements

The authors thank Colciencias for funding the project “Uso de bacteriofagos nativos como alternativa para el control de la salmonelosis en Colombia” code 1204-569- 34190, contract 0845-2013; and for the doctoral student scholarship awarded to VC. Also, they would like to thank the School of Sciences, Universidad de los Andes, for funding the research projects codes INV-2017-25-1117, INV-2018-48-1342 and the research program of professor Martha Vives code INV-2018-50-1399. We acknowledge the High-Performance Computing Service at Universidad de los Andes at Bogotá, Colombia for providing HPC resources that have contributed to the research results reported within this paper. URL:

<http://hpc.uniandes.edu.co>. The authors also gratefully acknowledge the Universidad de los Andes and Instituto Tecnológico de Estudios Superiores de Monterrey for their financial support (project code P16.700099.001/02-04) and the vice-rector of research and creation for supporting de project INV-2019-87-1887. Authors thank Luis Chica for his support in bioinformatics.

### Availability of supporting data

Sequence files and metadata for all samples used in this study have been deposited at the European Nucleotide Archive (ENA) (<https://www.ebi.ac.uk/ena/>) under the study Accession No. PRJEB32104. A record of all statistical analysis is included as Additional file 5.

### Additional information

#### Competing interests

The authors Viviana Clavijo-Lopez and Martha Josefina Vives-Flórez declare a conflict of interest, as they are members of the spin-off company SciPhage S.A.S., which works for the development of phage therapy in Colombia.

#### Authors' contributions

VC conceived the study, collected samples, performed laboratory assays, analyzed the data and wrote the manuscript. TM performed laboratory assays. MV conceived and directed the study and resources. AR co-directed the study, conceived the study, resources and helped analyzing the data. All authors edited the manuscript and approved the final draft.

## References

1. Belizário JE, Faintuch J: **Microbiome and Gut Dysbiosis**. In *Metabolic Interaction in Infection*. Edited by Torrado RSE: Springer International Publishing; 2018
2. Turnbaugh PJ, Ley RE, Hamady M, Fraser-Liggett CM, Knight R, Gordon JI: **The human microbiome project**. *Nature* 2007, **449**:804-810.
3. Ley RE, Peterson DA, Gordon JI: **Ecological and evolutionary forces shaping microbial diversity in the human intestine**. *Cell* 2006, **124**:837-848.
4. Wang B, Yao M, Lv L, Ling Z, Li L: **The human microbiota in health and disease**. *Engineering* 2017, **3**:71-82.
5. Bäumlér AJ, Sperandio V: **Interactions between the microbiota and pathogenic bacteria in the gut**. *Nature* 2016, **535**:85.
6. Cameron EA, Sperandio V: **Frenemies: signaling and nutritional integration in pathogen-microbiota-host interactions**. *Cell host & microbe* 2015, **18**:275-284.

7. Pacheco AR, Sperandio V: **Enteric pathogens exploit the microbiota-generated nutritional environment of the gut.** *Microbiology spectrum* 2015, **3**.
8. Brisbin JT, Gong J, Sharif S: **Interactions between commensal bacteria and the gut-associated immune system of the chicken.** *Anim Health Res Rev* 2008, **9**:101-110.
9. Clavijo V, Flores MJV: **The gastrointestinal microbiome and its association with the control of pathogens in broiler chicken production: A review.** *Poultry science* 2017.
10. Teague K, Graham L, Dunn J, Cheng H, Anthony N, Latorre J, Menconi A, Wolfenden R, Wolfenden A, Mahaffey B: **In ovo evaluation of FloraMax®-B11 on Marek's disease HVT vaccine protective efficacy, hatchability, microbiota composition, morphometric analysis, and Salmonella enteritidis infection in broiler chickens.** *Poultry science* 2017, **96**:2074-2082.
11. Jacquier V, Nelson A, Jlali M, Rhayat L, Brinch K, Devillard E: **Bacillus subtilis 29784 induces a shift in broiler gut microbiome toward butyrate-producing bacteria and improves intestinal histomorphology and animal performance.** *Poultry science* 2019.
12. Oakley BB, Lillehoj HS, Kogut MH, Kim WK, Maurer JJ, Pedroso A, Lee MD, Collett SR, Johnson TJ, Cox NA: **The chicken gastrointestinal microbiome.** *FEMS Microbiol Lett* 2014, **360**:100-112.
13. Cieplak T, Soffer N, Sulakvelidze A, Nielsen DS: **A bacteriophage cocktail targeting Escherichia coli reduces E. coli in simulated gut conditions, while preserving a non-targeted representative commensal normal microbiota.** *Gut microbes* 2018:01-19.
14. Lan Y, Verstegen M, Tamminga S, Williams B: **The role of the commensal gut microbial community in broiler chickens.** *World's poultry science journal* 2005, **61**:95-104.
15. Allen HK, Stanton TB: **Altered Egos: Antibiotic Effects on Food Animal Microbiomes\***. *Annual review of microbiology* 2014, **68**:297-315.
16. Mancabelli L, Ferrario C, Milani C, Mangifesta M, Turrone F, Duranti S, Lugli GA, Viappiani A, Ossiprandi MC, van Sinderen D: **Insights into the biodiversity of the gut microbiota of broiler chickens.** *Environmental microbiology* 2016.
17. Danzeisen JL, Kim HB, Isaacson RE, Tu ZJ, Johnson TJ: **Modulations of the chicken cecal microbiome and metagenome in response to anticoccidial and growth promoter treatment.** *PloS one* 2011, **6**:e27949.
18. WHO: *Interventions for the control of non-typhoidal Salmonella spp. in beef and pork: meeting report and systematic review.* World Health Organization; 2016.
19. Barrow P, Huggins M, Lovell M, Simpson J: **Observations on the pathogenesis of experimental Salmonella typhimurium infection in chickens.** *Research in veterinary science* 1987, **42**:194-199.
20. Remus A, Hauschild L, Andretta I, Kipper M, Lehnen C, Sakomura N: **A meta-analysis of the feed intake and growth performance of broiler chickens challenged by bacteria.** *Poultry science* 2014, **93**:1149-1158.
21. Shivaprasad H: **Fowl typhoid and pullorum disease.** *Revue Scientifique et Technique-Office International des Epizooties* 2000, **19**:405-416.

22. Uzzau S, Brown DJ, Wallis T, Rubino S, Leori G, Bernard S, Casadesús J, Platt DJ, Olsen JE: **Host adapted serotypes of Salmonella enterica.** *Epidemiology & Infection* 2000, **125**:229-255.
23. Voidarou C, Vassos D, Rozos G, Alexopoulos A, Plessas S, Tsinas A, Skoufou M, Stavropoulou E, Bezirtzoglou E: **Microbial challenges of poultry meat production.** *Anaerobe* 2011, **17**:341-343.
24. Loc-Carrillo C, Abedon ST: **Pros and cons of phage therapy.** *Bacteriophage* 2011, **1**:111-114.
25. Paule A, Frezza D, Edeas M: **Microbiota and Phage Therapy: Future Challenges in Medicine.** *Medical Sciences* 2018, **6**:86.
26. Wernicki A, Nowaczek A, Urban-Chmiel R: **Bacteriophage therapy to combat bacterial infections in poultry.** *Virology journal* 2017, **14**:179.
27. Oliveira A, Sereno R, Nicolau A, Azeredo J: **In vivo toxicity study of phage lysate in chickens.** *British poultry science* 2009, **50**:558-563.
28. Ahmadi M, Karimi Torshizi MA, Rahimi S, Dennehy JJ: **Prophylactic Bacteriophage Administration More Effective than Post-infection Administration in Reducing Salmonella enterica serovar Enteritidis Shedding in Quail.** *Frontiers in microbiology* 2016, **7**:1253.
29. Fiorentin L, Vieira ND, Barioni WJ: **Oral treatment with bacteriophages reduces the concentration of Salmonella Enteritidis PT4 in caecal contents of broilers.** *Avian pathology* 2005, **34**:6.
30. Andreatti Filho R, Higgins J, Higgins S, Gaona G, Wolfenden A, Tellez G, Hargis B: **Ability of Bacteriophages Isolated from Different Sources to Reduce Salmonella enterica Serovar Enteritidis In Vitro and In Vivo.** *Poultry Science* 2007, **86**:6.
31. Andrews S: **FastQC: a quality control tool for high throughput sequence data.** 2010.
32. Carvalho C, Costa AR, Silva F, Oliveira A: **Bacteriophages and their derivatives for the treatment and control of food-producing animal infections.** *Critical reviews in microbiology* 2017, **43**:583-601.
33. Universidad-de-los-Andes: **Composition comprising bacteriophages for reducing, eliminating and/or preventing salmonella enteritidis, salmonella typhimurium and salmonella paratyphi b.** In <https://www.google.com/patents/WO2017089947A2?cl=en> (Andes UdL ed., vol. WO2017089947 A2. Colombia: Holguín-Moreno, Angela Victoria; Flores-Vives, Martha Josefina; Sanchez-Moreno, Ana Paula;; 2017.
34. Clavijo V, Baquero D, Hernandez S, Farfan JC, Arias J, Arévalo A, Donado-Godoy P, Vives-Flores M: **Phage cocktail SalmoFREE® reduces Salmonella on a commercial broiler farm.** Article accepted. *Poultry Science*; 2019.
35. Kutter E, Sulakvelidze A: *Bacteriophages: biology and applications.* CRC Press; 2004.
36. Caporaso JG, Kuczynski J, Stombaugh J, Bittinger K, Bushman FD, Costello EK, Fierer N, Pena AG, Goodrich JK, Gordon JI: **QIIME allows analysis of high-throughput community sequencing data.** *Nature methods* 2010, **7**:335.
37. Martin M: **Cutadapt removes adapter sequences from high-throughput sequencing reads.** *EMBnet journal* 2011, **17**:10-12.

38. Magoč T, Salzberg SL: **FLASH: fast length adjustment of short reads to improve genome assemblies.** *Bioinformatics* 2011, **27**:2957-2963.
39. Amir A, McDonald D, Navas-Molina JA, Kopylova E, Morton JT, Xu ZZ, Kightley EP, Thompson LR, Hyde ER, Gonzalez A: **Deblur rapidly resolves single-nucleotide community sequence patterns.** *MSystems* 2017, **2**:e00191-00116.
40. Pruesse E, Quast C, Knittel K, Fuchs BM, Ludwig W, Peplies J, Glöckner FO: **SILVA: a comprehensive online resource for quality checked and aligned ribosomal RNA sequence data compatible with ARB.** *Nucleic acids research* 2007, **35**:7188-7196.
41. McMurdie PJ, Holmes S: **phyloseq: an R package for reproducible interactive analysis and graphics of microbiome census data.** *PloS one* 2013, **8**:e61217.
42. Edgar RC: **MUSCLE: multiple sequence alignment with high accuracy and high throughput.** *Nucleic acids research* 2004, **32**:1792-1797.
43. Clamp M, Cuff J, Searle SM, Barton GJ: **The jalview java alignment editor.** *Bioinformatics* 2004, **20**:426-427.
44. Price MN, Dehal PS, Arkin AP: **FastTree 2—approximately maximum-likelihood trees for large alignments.** *PloS one* 2010, **5**:e9490.
45. Rambaut A, Drummond A: **FigTree: Tree figure drawing tool, version 1.2. 2.** *Institute of Evolutionary Biology, University of Edinburgh* 2008.
46. Oh JK, Pajarillo EAB, Chae JP, Kim IH, Yang DS, Kang D-K: **Effects of Bacillus subtilis CSL2 on the composition and functional diversity of the faecal microbiota of broiler chickens challenged with Salmonella Gallinarum.** *Journal of animal science and biotechnology* 2017, **8**:1.
47. Diaz Carrasco JM, Redondo EA, Pin Viso ND, Redondo LM, Farber MD, Fernández Miyakawa ME: **Tannins and bacitracin differentially modulate gut microbiota of broiler chickens.** *BioMed research international* 2018, **2018**.
48. Ijaz UZ, Sivaloganathan L, Mckenna A, Richmond A, Kelly C, Linton M, Stratakos AC, Lavery U, Elmi A, Wren BW: **Comprehensive longitudinal microbiome analysis of the chicken cecum reveals a shift from competitive to environmental drivers and a window of opportunity for Campylobacter.** *Frontiers in microbiology* 2018, **9**.
49. Videnska P, Sedlar K, Lukac M, Faldynova M, Gerzova L, Cejkova D, Sisak F, Rychlik I: **Succession and replacement of bacterial populations in the caecum of egg laying hens over their whole life.** *PLoS One* 2014, **9**:e115142.
50. Ocejo M, Oporto B, Hurtado A: **16S rRNA amplicon sequencing characterization of caecal microbiome composition of broilers and free-range slow-growing chickens throughout their productive lifespan.** *Sci Rep* 2019, **9**:2506.
51. Ríos-Covián D, Ruas-Madiedo P, Margolles A, Gueimonde M, de los Reyes-Gavilán CG, Salazar N: **Intestinal short chain fatty acids and their link with diet and human health.** *Frontiers in microbiology* 2016, **7**:185.

52. Kläring K, Hanske L, Bui N, Charrier C, Blaut M, Haller D, Plugge CM, Clavel T: **Intestinimonas butyriciproducens gen. nov., sp. nov., a butyrate-producing bacterium from the mouse intestine.** *Int J Syst Evol Microbiol* 2013, **63**:4606-4612.
53. Lund M, Bjerrum L, Pedersen K: **Quantification of Faecalibacterium prausnitzii- and Subdoligranulum variabile-like bacteria in the cecum of chickens by real-time PCR.** *Poult Sci* 2010, **89**:1217-1224.
54. Sergeant MJ, Constantinidou C, Cogan TA, Bedford MR, Penn CW, Pallen MJ: **Extensive microbial and functional diversity within the chicken cecal microbiome.** *PLoS One* 2014, **9**:e91941.
55. Connors J, Dawe N, Van Limbergen J: **The role of succinate in the regulation of intestinal inflammation.** *Nutrients* 2019, **11**:25.
56. Park SJ, Kim J, Lee JS, Rhee SK, Kim H: **Characterization of the fecal microbiome in different swine groups by high-throughput sequencing.** *Anaerobe* 2014, **28**:157-162.
57. Yan W, Sun C, Yuan J, Yang N: **Gut metagenomic analysis reveals prominent roles of Lactobacillus and cecal microbiota in chicken feed efficiency.** *Scientific reports* 2017, **7**:45308.
58. Mesa D, Lammel DR, Balsanelli E, Sena C, Nosedá MD, Caron LF, Cruz LM, Pedrosa FO, Souza EM: **Cecal Microbiota in Broilers Fed with Prebiotics.** *Frontiers in Genetics* 2017, **8**:153.
59. Bhalodi AA, van Engelen TS, Virk HS, Wiersinga WJ: **Impact of antimicrobial therapy on the gut microbiome.** *Journal of Antimicrobial Chemotherapy* 2019, **74**:i6-i15.
60. Silva J, Leite D, Fernandes M, Mena C, Gibbs PA, Teixeira P: **Campylobacter spp. as a foodborne pathogen: a review.** *Frontiers in microbiology* 2011, **2**:200.
61. Van Deun K, Pasmans F, Ducatelle R, Flahou B, Vissenberg K, Martel A, Van den Broeck W, Van Immerseel F, Haesebrouck F: **Colonization strategy of Campylobacter jejuni results in persistent infection of the chicken gut.** *Veterinary microbiology* 2008, **130**:285-297.
62. Kaakoush NO, Sodhi N, Chenu JW, Cox JM, Riordan SM, Mitchell HM: **The interplay between Campylobacter and Helicobacter species and other gastrointestinal microbiota of commercial broiler chickens.** *Gut Pathogens* 2014, **6**:18.
63. Bereswill S, Plickert R, Fischer A, Kühl A, Loddenkemper C, Batra A, Siegmund B, Göbel U, Heimesaat M: **What you eat is what you get: novel Campylobacter models in the quadrangle relationship between nutrition, obesity, microbiota and susceptibility to infection.** *European Journal of Microbiology and Immunology* 2011, **1**:237-248.
64. Rivera-Chávez F, Lopez CA, Bäumlér AJ: **Oxygen as a driver of gut dysbiosis.** *Free Radical Biology and Medicine* 2017, **105**:93-101.
65. Byndloss MX, Olsan EE, Rivera-Chávez F, Tiffany CR, Cevallos SA, Lokken KL, Torres TP, Byndloss AJ, Faber F, Gao Y: **Microbiota-activated PPAR- $\gamma$  signaling inhibits dysbiotic Enterobacteriaceae expansion.** *Science* 2017, **357**:570-575.
66. Ducatelle R, Goossens E, Meyer F, Eeckhaut V, Antonissen G, Haesebrouck F, Immerseel F: **Biomarkers for monitoring intestinal health in poultry: present status and future perspectives.** *Veterinary research* 2018, **49**:43.

67. Eeckhaut V, Wang J, Van Parys A, Haesebrouck F, Joossens M, Falony G, Raes J, Ducatelle R, Van Immerseel F: **The probiotic *Butyrivibrio parvulus* reduces feed conversion and protects from potentially harmful intestinal microorganisms and necrotic enteritis in broilers.** *Frontiers in microbiology* 2016, **7**:1416.

## Tables

**Table 1. *Salmonella* reduction throughout the production cycle of trials 1 and 2 for farmhouses treated with and without SalmoFree®. Data modified from Clavijo *et al.* [32].**

Farmhouse / Sampling point*	No of cloacal swaps <i>Salmonella</i> positive**										
	Trial I					Trial II					
	1b	1a	2b	2a	3b	3a	1b	1a	2b	2a	3b***
Control farmhouse 1	2	3	3	4	1	3	9	7	5	4	2
Control farmhouse 2	5	5	1	2	1	1	9	10	5	3	1
Phage-treated farmhouse 3	4	2	1	4	0	0	10	10	9	6	3
Phage-treated farmhouse 4	5	3	1	2	0	0	9	8	7	2	0

\* Nomenclature of the sampling point corresponds to the number of dose (1-3) followed by a letter indicating whether it was taken a day before (b) or after (a) the corresponding dose.

\*\* Total number of swaps sampled: n=5 in trial 1 and n=10 in trial 2.

\*\*\* Data for samples taken after dose 3 (3a) in the trial 2 are not shown due to the accidental loss of the samples.

**Table 2. Phage incidence throughout the production cycle of trials 1 and 2 for farmhouses treated with and without SalmoFree®.**

Farmhouse / Dose Day <sup>a</sup>	No. of samples positive for amplification of phages gene <sup>b</sup>							
	Trial I				Trial II			
	1b	1a	3b	3a	1b	1a	3b	3a
Control house No. 1	0	1	3	0	0	0	0	0
Control house No. 2	0	1	1	1	1	1	0	3
Treated house No. 3	0	2	5	3	0	4	1	4
Treated house No. 4	0	1	3	4	3	2	2	4

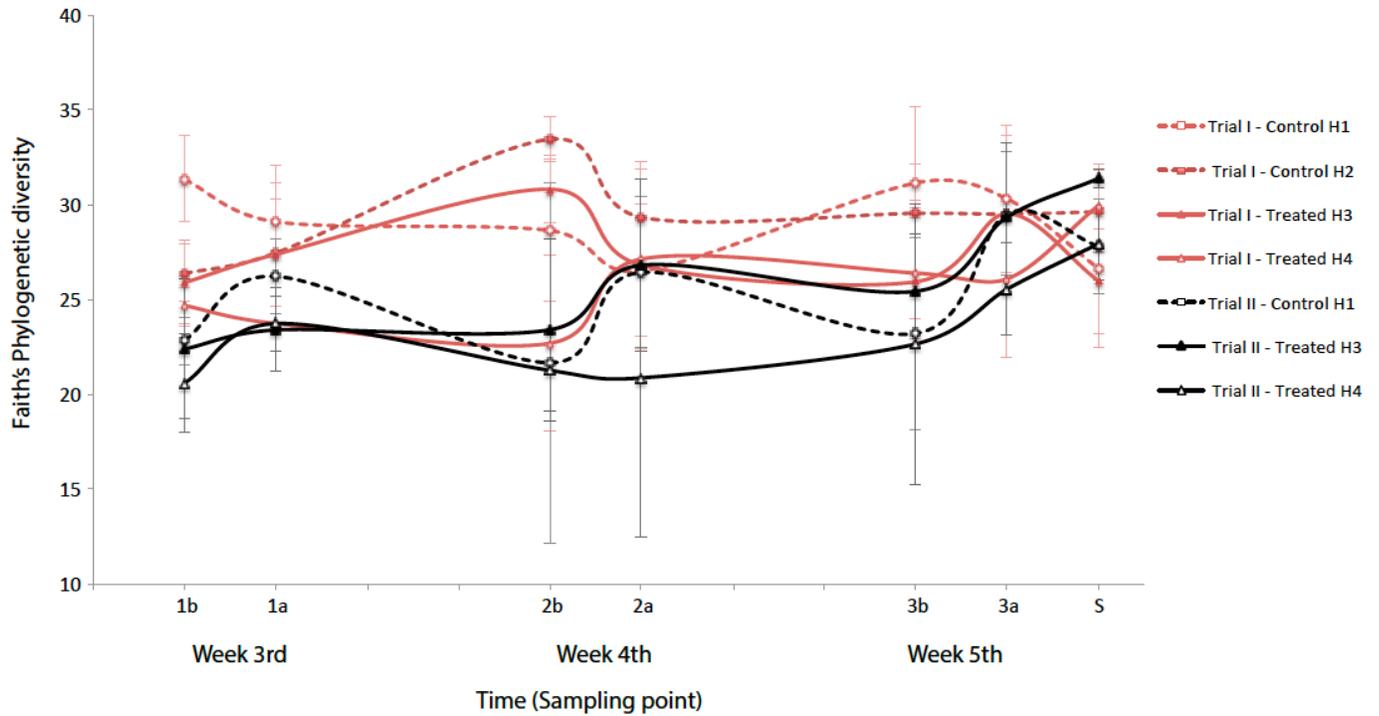
<sup>a</sup> Nomenclature of the dose day corresponds to the number of the dose followed by a letter indicating whether it was taken a day before (b) or after (a) the corresponding dose.

<sup>b</sup> No. of positive samples for amplification of the phage tail fiber protein gene of phages from the total samples where total sample size per time point is 5.

**Table 3. Core microbiome, at genus level, of the last three weeks of broiler's production cycle and at the slaughterhouse, for the two trials. Presence (+) of taxa is shown over time with their respective phylum. Green background indicates taxa that behave similarly in both cycles. In blue, patterns with variable behaviour for both trials are indicated. Brown indicates taxa that are part of the core microbiome (>50% of the samples) only in the first or second cycle. S shows the results at the slaughterhouse.**

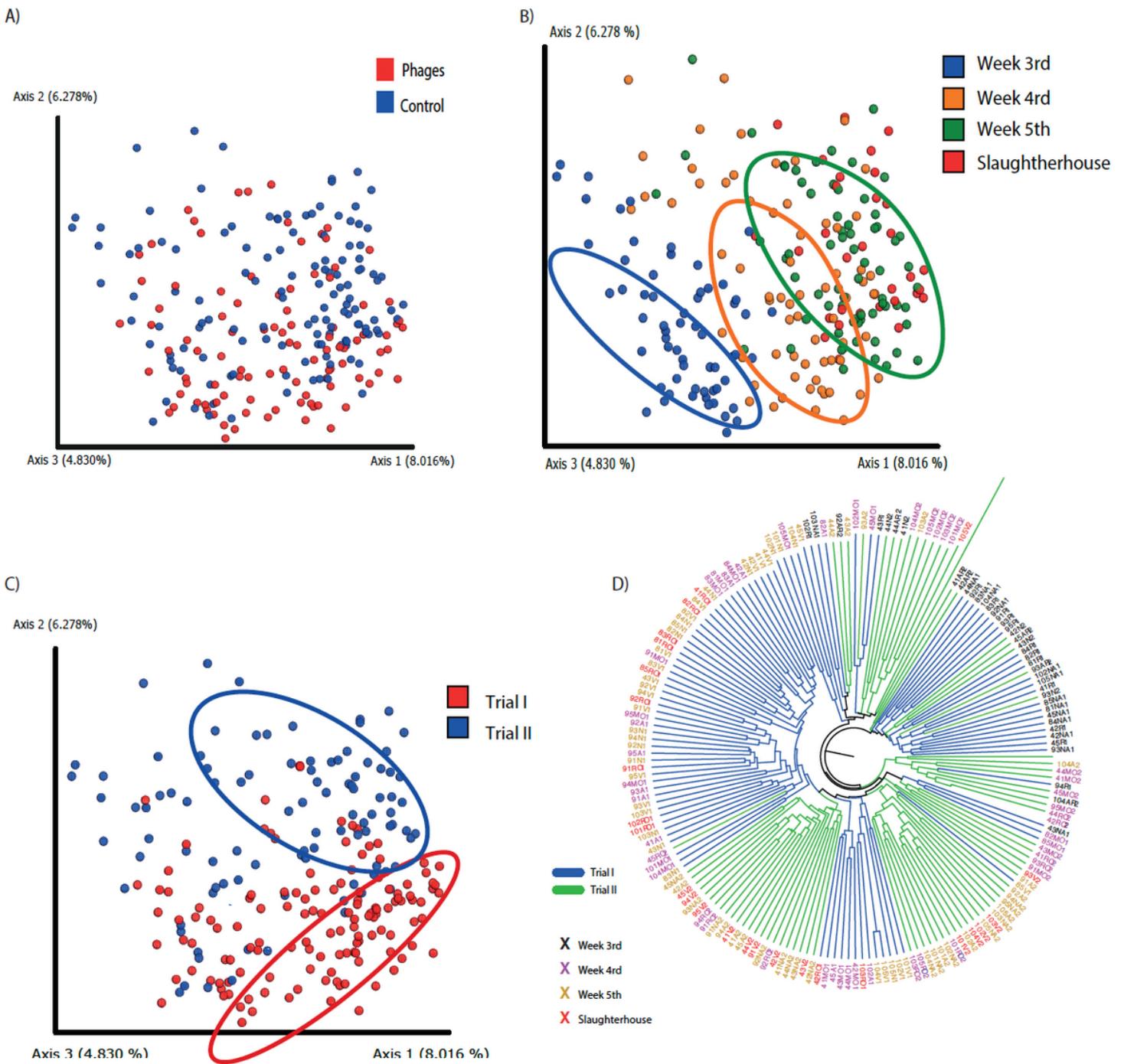
Taxa	Phylum	Trial I				Trial II			
		Week 3 <sup>rd</sup>	Week 4 <sup>th</sup>	Week 5 <sup>th</sup>	S <sup>a</sup>	Week 3 <sup>rd</sup>	Week 4 <sup>th</sup>	Week 5 <sup>th</sup>	S
<i>Bacteroides</i>	Bacteroidetes	+	+	+	+	+	+	+	+
<i>Eisenbergiella</i>	Firmicutes	+	+	+	+	+	+	+	+
<i>Escherichia-Shigella</i>	Proteobacteria	+	+	+	+	+	+	+	+
<i>Intestinimonas</i>	Firmicutes	+	+	+		+	+	+	+
<i>Lachnospiraceae</i>	Firmicutes	+	+	+	+	+	+	+	+
<i>Oscillibacter</i>	Firmicutes	+	+	+	+	+	+	+	+
<i>Ruminococcaceae</i>	Firmicutes	+	+	+	+	+	+	+	+
<i>Subdoligranulum</i>	Firmicutes	+	+	+	+	+	+	+	+
<i>Tyzzera</i>	Firmicutes	+	+	+	+	+	+	+	+
<i>Alistipes</i>	Bacteroidetes		+	+	+		+	+	+
<i>Bilophila</i>	Proteobacteria		+	+	+		+	+	+
<i>Campylobacter</i>	Proteobacteria		+	+	+		+	+	+
<i>Shuttleworthia</i>	Firmicutes	+		+	+	+		+	+
<i>Helicobacter</i>	Proteobacteria			+	+			+	+
<i>Barnesiella</i>	Bacteroidetes				+				+
<i>Clostridia</i>	Firmicutes	+	+	+	+	+		+	+
<i>Faecalibacterium</i>	Firmicutes	+	+	+	+	+		+	+
<i>Flavonifractor</i>	Firmicutes	+	+	+	+	+		+	+
<i>Ruminiclostridium</i>	Firmicutes	+	+	+	+	+		+	
<i>Lactobacillus</i>	Firmicutes	+	+		+	+	+	+	+
<i>Butyricimonas</i>	Bacteroidetes	+	+	+	+		+	+	+
<i>Phascolarctobacterium</i>	Firmicutes		+	+	+	+	+	+	+
<i>Negativibacillus</i>	Firmicutes	+	+	+	+	+			+
<i>Coprobacter</i>	Bacteroidetes	+	+			+			+
<i>Parabacteroides</i>	Bacteroidetes			+	+	+			+
<i>Erysipelotrichaceae</i>	Firmicutes	+	+	+	+				+





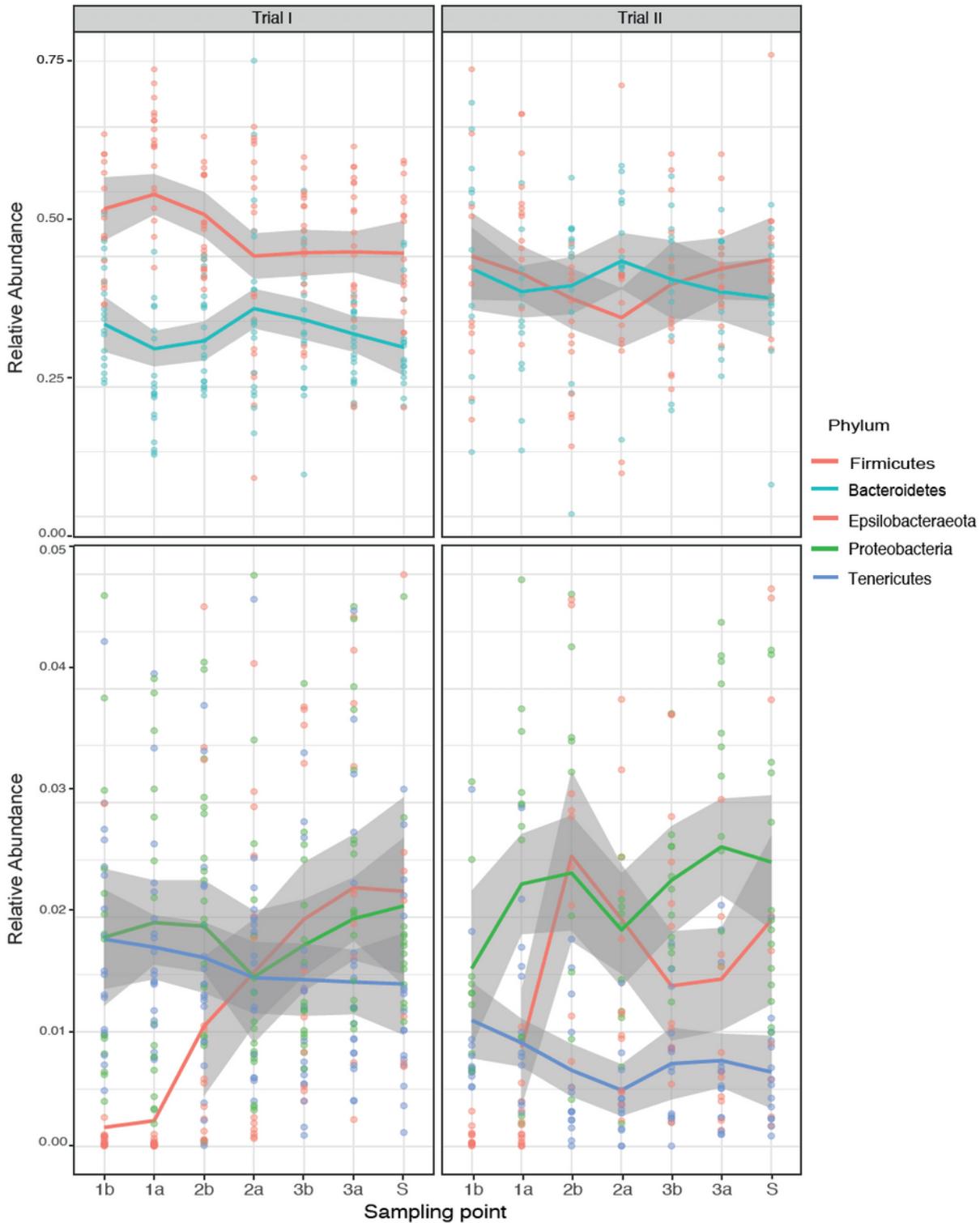
**Figure 2**

Alpha diversity using Faith phylogenetic diversity metric of samples throughout the experiment per farmhouse and trial. Red lines indicate trial 1, black lines indicate trial 2. Dotted lines are representing control farmhouses and solid lines are for SalmoFree® treated. The x-axis is indicating the time points for samples taken before (b) and after (a) the delivery of the treatments with their corresponding week of the cycle. The results at slaughterhouse are indicated (S). Positions on the x-axis are proportional to the time intervals between sampling. Error bars are representing the standard deviation of the mean of five cecum samples.



**Figure 3**

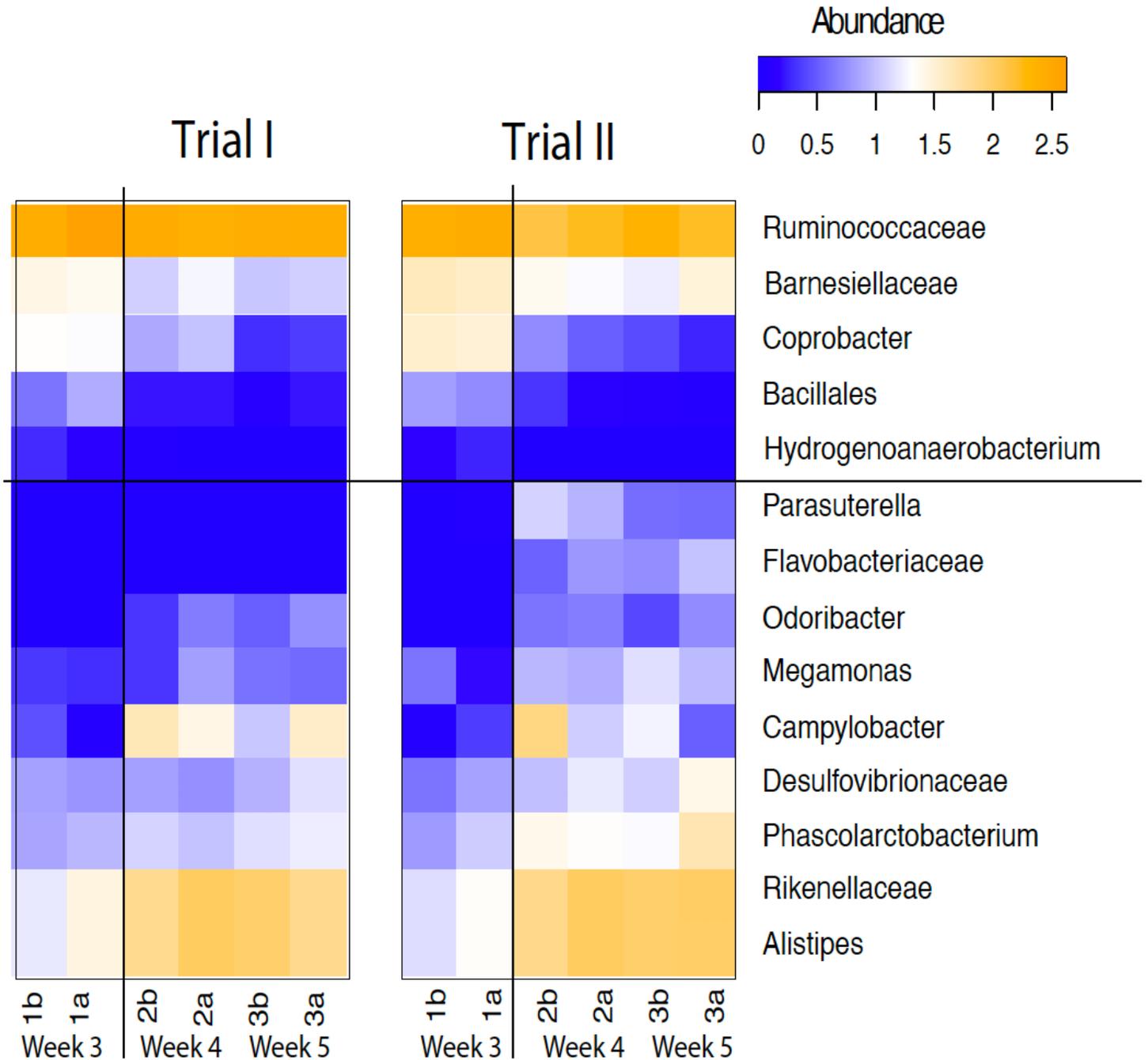
Similarity between the structure of bacterial communities found in cecum of broiler chicken based on Bray Curtis dissimilarity metric. A-C) Principal Coordinate Analysis (PCoA) where samples are colored by treatment (A) age of the animals (in weeks) (B) and by trial (C). Ellipses were manually overlaid to encompass the majority of the sample points of a given feature. D) UPGMA clustering colored by cycle and sampling week.



**Figure 4**

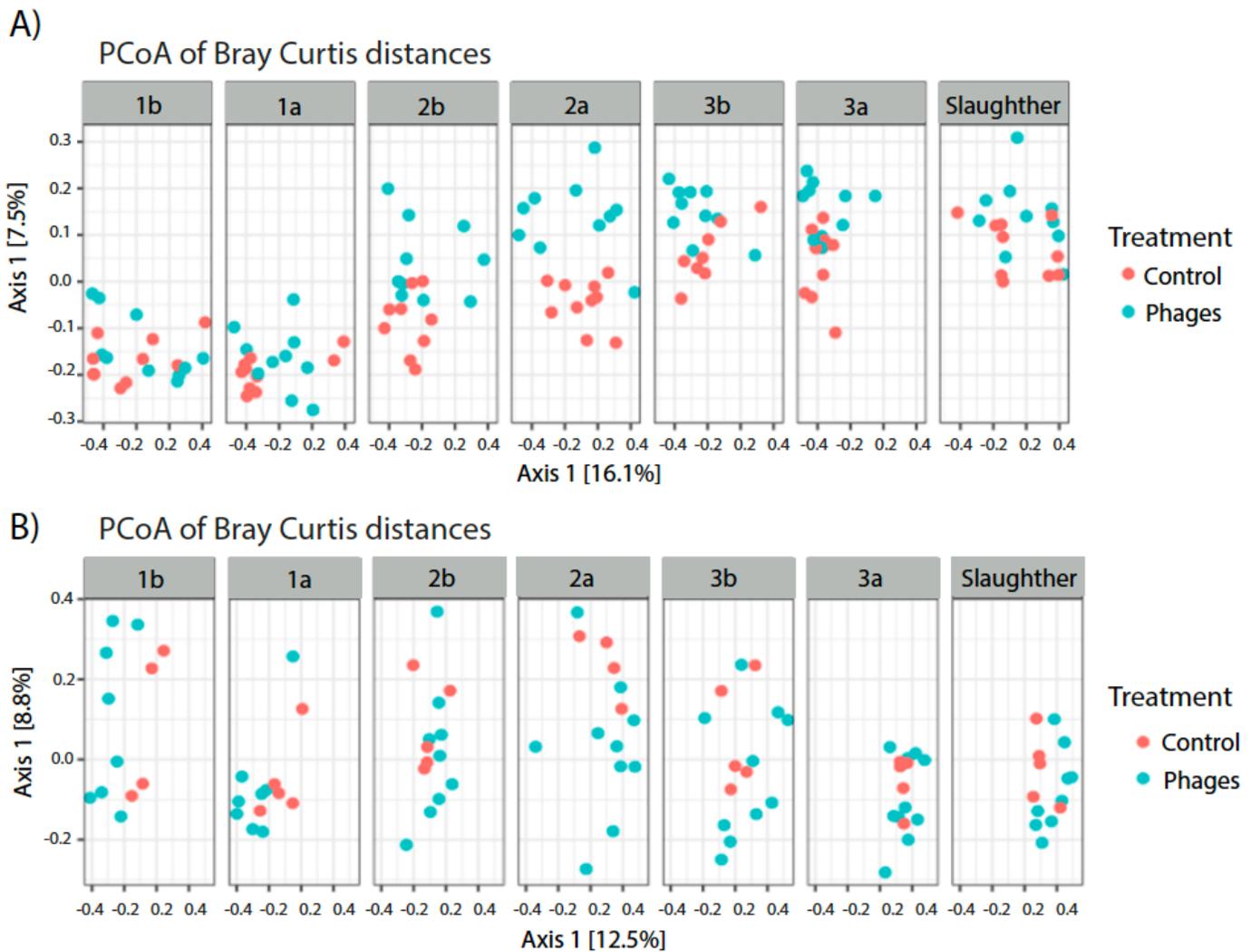
Relative abundance of dominant phyla identified in the microbiome of cecum of broilers. Results are presented discriminated by trial over time, shown as dose days. Sample days are indicated with the nomenclature of the number of dose followed by the letter a or b for samples taken before and after the delivery of the treatments, respectively. The results at slaughterhouse are indicated with (S). Upper and

lower panels are due to the range of abundance of Firmicutes and Bacteroidetes compared to other phyla. gray color is showing the standard deviation of the mean abundance.



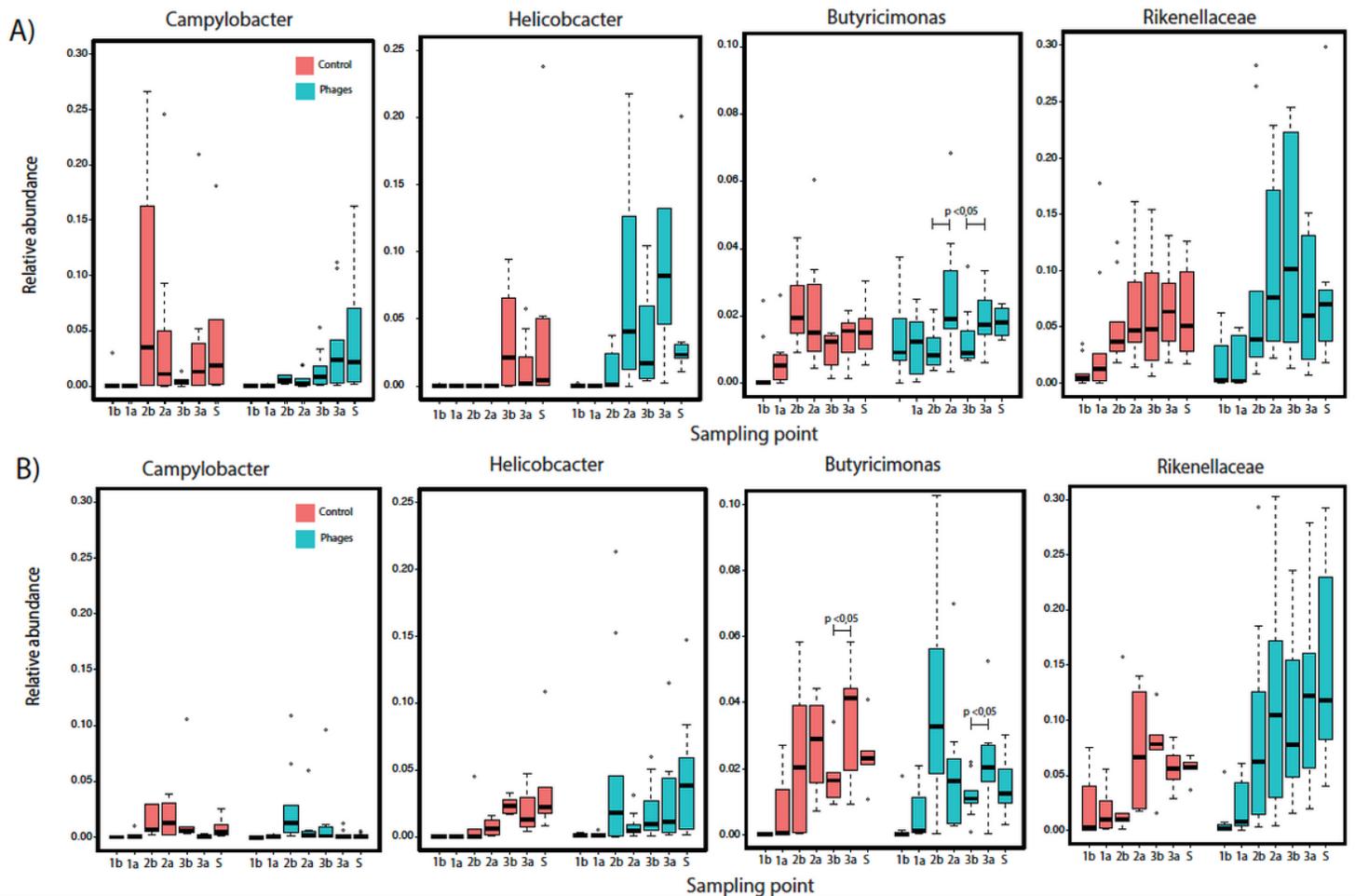
**Figure 5**

Heatmap built on average abundance values with bacterial taxa found to be significantly associated with the week of the production cycle. Results are presented discriminated by trial over time of the experiments. Nomenclature of the sample days as shown in Figure 1. Horizontal black line divides bacteria that decrease after the second week (upper) vs those that increase over time (lower rows). Vertical line marks the end of the 3rd week where most of the significant changes in abundance occur.



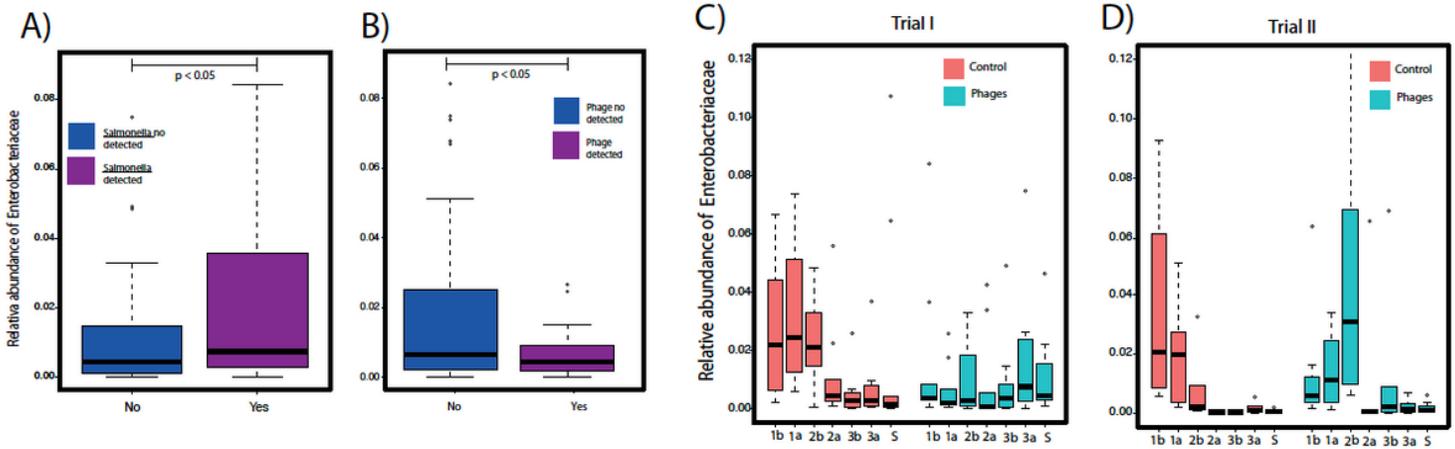
**Figure 6**

Principal Coordinate Analysis (PCoA) representing the similarity of bacterial communities found in cecum of broiler chicken treated with (phages) and without (controls) the bacteriophage cocktail, and measured using the Bray Curtis metric. Axis represent the coordinates of the samples in Axis 1 and 2 of the corresponding PCoA plots. Blocks of samples are divided by dose day and nomenclature is as described in Figure 1. Upper panel display trial 1 (A) and the lower panel trial 2 (B)



**Figure 7**

Relative abundance of Bacterial taxa significantly associated with the treatment. Results are presented discriminated by trial over time, shown as sampling point as in Figure 1. Results are presented by trial 1 (A) and 2 (B). Control groups are depicted in salmon while treated groups are shown in blue. The body of the box plot represent the first and third quartiles of the distribution and the median line of abundance of all ASV assigned to the correspond genera. The whiskers extend from the quartiles to the maximum or minimum data within 1.5 x interquartile range, with outliers beyond. ANOVA test  $p < 0,05$  show significant differences in microbial abundance of *Butyricimonas* after and before the dose of phages.



**Figure 8**

Relative abundance of the Enterobacteriaceae Family. A) and B) are boxplots displaying the abundance of the Enterobacteriaceae Family based on the detection of Salmonella and phages respectively. The data collapsed results for trial 1 and 2. ANOVA test  $p < 0,05$  show significant lower differences in microbial abundance of Enterobacteriaceae in samples where Salmonella is no detected and where phages are detected. C) and D) shown the abundance discriminated for trial 1 and trial 2 respectively, separated by treatment groups over time as in Figure 1. Salmon color are for control farmhouses and blue for phage treated farmhouses. The reduction of the controlled group is associated with the phage contamination that occurred.

## Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- [AdditionalFile1.pdf](#)
- [AdditionalFile2.pdf](#)
- [AdditionalFile3.docx](#)