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Bacteriophage turnover in chicken intestines

Tesis en torno a una hipótesis o problema de investigación y su contrastación

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RESUMEN

En el presente trabajo, se investigó la relación entre el contaje de fagos en solución con el cambio en la población de *E. coli*. Se realizó un ensayo en placa para el contaje indirecto de UFP/ml de colifagos, utilizando 9 pollos pertenecientes a 3 linajes genéticos. Nuestros resultados podrían indicar que las diferencias más importantes en nuestra variable de respuesta se deben a diferencias fenotípicas en el hospedador, mas no en el linaje genético. Se observó que los contajes de fagos específicos para una cepa, disminuyen con el tiempo. Esto es congruente con estudios transversales realizados en humanos. Se requiere continuar con el estudio para obtener conclusiones más precisas sobre la dinámica poblacional de *E. coli* y también de los colifagos.

Palabras clave: *E. coli*, Bacteriófagos, población, UFP, colifagos

ABSTRACT

In the present work, the relationship between the phage count in solution and the change in the *E. coli* population was investigated. A plate assay was carried out for the indirect counting of PFU/ml of coliphages, using 9 chickens belonging to 3 genetic lines. Our results could indicate that the most important differences in our response variable are due to phenotypic differences in the host, but not in the genetic lineage. Strain-specific phage counts were observed, decrease over time. This is consistent with cross-sectional studies conducted in humans. It is necessary to continue with the study to obtain more precise conclusions about the population dynamics of *E. coli* and of coliphages.

Keywords: *E. coli,* Bacteriophages, population, UFP, coliphages

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JUSTIFICATION

E. coli is a commensal bacterium in warm-blooded vertebrates, and an important intestinal and extraintestinal pathogen, also one of the key organisms in fields such as molecular biology, genetics, and biochemistry (Teinallon, Skurnik, Picard, & Denamur, 2010). Study of commensal *E. coli* is important because ecological and evolutionary forces shape population structure and how this population relates to disease (Teinallon, Skurnik, Picard, & Denamur, 2010). Commensal intestinal microbiota has shown to be important for antibiotic resistance dissemination and virulence factors are present among commensal strains (Teinallon, Skurnik, Picard, & Denamur, 2010). Bacteriophages can be considered responsible for pathogen population control inside the gut (Muniesa & Jofre, 2014).

It is known that selective pressures promote increasing numbers of virulence and antibiotic resistance genes (Teinallon, Skurnik, Picard, & Denamur, 2010). The perfect combination for this scenario is the high bacterial density, and antibiotic exposure which is the selective pressure (Teinallon, Skurnik, Picard, & Denamur, 2010). Bacteriophages have regained interest as antimicrobial strategy in some pathogens such as: "*Escherichia coli, Staphylococcus aureus, Chlamydia trachomatis, Pseudomonas aeruginosa, and Helicobacter pylori*" (Nicastro, et al., 2016). Antimicrobial resistance (AMR) represents one of the most important threats for health, food security and development (Nabergoi, Modic, & Podgornik, 2018). Multidrug resistant *Enterobacteriaceae* isolated from humans can be acquired from domestic animals and related to the food industry. *E. coli* clones' diversity is constantly changing and challenges AMR research (Salinas, et al., 2019). As a consequence, there are higher medical bills, prolonged hospital stays and higher mortality rate (Nabergoi, Modic, & Podgornik, 2018). Also, bacteriophages have been implied in horizontal gene transfer between bacteria generating bacterial diversity for new niches adaptation, antibiotic resistance, toxins production and new

metabolism genes (Muniesa & Jofre, 2014). Relevance of phage investigation relies on different biotechnological applications such as bacteriophage-based therapeutics because of bacteriophages' genetic malleability and easy way of massive production (Nicastro, et al., 2016). Bacteriophages could represent an alternative to antibiotics for treatment of bacterial infections (Nabergoi, Modic, & Podgornik, 2018). Bacteriophages have already been used as antimicrobials in fields of veterinary medicine, agriculture, food industry and human medicine for clinical diagnostics and vaccine or therapeutic genes vehicle (Nabergoi, Modic, & Podgornik, 2018).

HYPOTHESIS

E. coli population genetic shifts could probably be attributed to changes in phage population counts.

LITERATURE REVIEW

E. coli commensal niches

Enterobacteriaceae is a diverse bacterial family which has an important role in disease and antimicrobial resistance (Martinson, et al., 2019). Within this family, *E. coli* is the member most common in the gut microbiota and feces of warm-blooded vertebrates. This microorganism is the first species to colonize hosts intestines during infancy, before colonization of anaerobes (Teinallon, Skurnik, Picard, & Denamur, 2010). This is a Gram-negative, non-sporulating facultative anaerobe microorganism found in large intestines, especially in the caecum and the colon in a concentration of 10^{10}-10^{11} cells per gram of intestinal contents in humans, nevertheless anaerobic bacteria outnumber *E. coli* in this habitat 100 to 10.000 times. In humans, its concentration stabilizes at 10^{8} CFU per gram after 2 years of age

and decreases during elderly (Teinallon, Skurnik, Picard, & Denamur, 2010). In water, its presence is used as a fecal contamination indicator together with total coliforms (Teinallon, Skurnik, Picard, & Denamur, 2010).

Prevalence in humans is near 90%, in wild mammals its only 56%, in birds its near 23% and in reptiles it's about 10% (Teinallon, Skurnik, Picard, & Denamur, 2010). *E. coli* metabolism has adapted to approximately seven mucus derived sugars whose concentrations in the intestine are low, and gluconate is considered the most important for *E. coli*'s growth (Teinallon, Skurnik, Picard, & Denamur, 2010). Also, growth is improved by the capacity to use microaerobic and aerobic respiration which helps anaerobic microorganisms removing O_2 from intestinal lumen, while anaerobic microorganisms help *E. coli* with the mucosal polysaccharide's degradation, this is a mutualistic relationship (Teinallon, Skurnik, Picard, & Denamur, 2010). Human gut provides a stable environment and protection for *E. coli* growth and development, and *E. coli* commensal strains avoid intestinal colonization by pathogens due to bacteriocins production and other mechanisms (Teinallon, Skurnik, Picard, & Denamur, 2010).

E. coli population structure studies

Population structure is defined as clonal composition of the population, understanding clonality as the core genome sequence similarity between isolates present in the population and consequently the presence of few SNPs. The first attempts to characterize the clonal composition of intestinal *E. coli* was to determine somatic O, Capsular K and flagellar H antigens (Teinallon, Skurnik, Picard, & Denamur, 2010); another approach was Multilocus Enzyme Electrophoresis (MLEE) (Teinallon, Skurnik, Picard, & Denamur, 2010). MLEE studies complemented by: biotyping, serotyping, outer-membrane protein electrophoretic analysis, random amplified polymorphic DNA and restriction fragment length polymorphism of ribosomal RNA gene regions, revealed that these markers were consistent (Teinallon,

Skurnik, Picard, & Denamur, 2010). After the introduction of DNA for molecular analysis, it was possible to define better the clonal structure, MLEE was replaced by Multi-Locus Sequence Typing (MLST) which allowed to group *E. coli* strains as clonal groups (Teinallon, Skurnik, Picard, & Denamur, 2010). The introduction of whole genome sequencing has allowed scientists to characterize 2000 genes (core genome) and to stablish those clonal strains can have different and variable accessory genome (Teinallon, Skurnik, Picard, & Denamur, 2010).

Intra host E. coli diversity

Studies showed that human hosts carry around 10 to 14 *E. coli* strains some of which are numerically predominant and account for half of the colonies isolated (Teinallon, Skurnik, Picard, & Denamur, 2010). Some of these strains are resident strains that can be carried for months, or years and transient strains can be carried for few days or weeks (Teinallon, Skurnik, Picard, & Denamur, 2010).

There is a different prevalence of phylogenetic groups: "In humans, strains of group A are predominant (40.5%), followed by B2 strains (25.5%), whereas B1 and D strains (17% each) are less common (these data were compiled from 1,117 subjects). In animals, a predominance of B1 strains (41%), followed by A (22%), B2 (21%), and, to a lesser extent, D (16%) strains is observed" (Teinallon, Skurnik, Picard, & Denamur, 2010). We must mention that only few strains have been shown to be host specific (Teinallon, Skurnik, Picard, & Denamur, 2010), There is no clear association patterns between MLST groups and their hosts, and their prevalence is variable (Teinallon, Skurnik, Picard, & Denamur, 2010). Factors shaping genetic structure of *E. coli* population can be divided in host characteristics and environmental factors such as animal domestication, hygiene, and diet, which leads to changes in proportion of B2 and A strains (Teinallon, Skurnik, Picard, & Denamur, 2010).

E. coli residency

There is a subset of strains highly stable recognized as resident bacteria (Martinson, et al., 2019). Gut colonization depends on different factors that include host genetics, diet, age and antimicrobial consumption and gut microbiome aspects such as strains already present and their metabolites produced. Residency is defined based on human transit time, understanding that those bacteria unable to colonize the gut will leave it. Human transit time has been defined as 0.7-4 days, and strain-level turnover in the gut is common over short time scales, as months (Martinson, et al., 2019). When studying residency in humans, there is a threshold of 14 days, knowing that OTUs (Operational Taxonomic Unit) and ASVs (Amplicon Sequence Variant) have already passed through the gut for 3 transit times. Microbiome is unique for each individual but has substantial fluctuation over time. Genome variation of gut strains could be relevant for some functional dynamics in human gut. (Martinson, et al., 2019).

Bacteriophages

Bacteriophages are the most abundant organisms on earth, composed of a DNA or RNA genome inside a protein coat (Nicastro, et al., 2016). These viruses are obligate parasites that can infect bacteria, found only in environments that contain their prey, because they require bacterial machinery for their reproduction. It is believed that phages outnumber bacterial population by almost 10 times (Weinbauer, 2004) and are present in the planet with an estimated number of 10^{31} (Dion, Oechslin, & Moineau, 2020). Bacteriophages are responsible for microbial communities' modulation, genetic diversity, and carbon recycling due to bacterial mortality (viral shunt) (Dion, Oechslin, & Moineau, 2020). Moreover, phages can affect physiology and metabolism of their hosts (Dion, Oechslin, & Moineau, 2020. Most of bacteriophages genome diversity is still uncharacterized and unknown sequences are still the majority (dark matter) (Dion, Oechslin, & Moineau, 2020). Genome sizes of phages range from

2.435 bp to >200 kb in jumbo phages and >540 kb in megaphages (Dion, Oechslin, & Moineau, 2020). Phage research has based on antibacterial potentials and phage therapy because of their potential in food, agriculture, biotechnology, and human health (Nicastro, et al., 2016). Moreover, they have been useful for the investigation and advances in phage biology and genetics (Nicastro, et al., 2016).

Bacteriophages Origin and Evolution

Bacteriophages have relatively small exceptionally nucleotide diverse genomes where mosaicism can be observed and makes difficult evolutionary relations understanding (Dion, Oechslin, & Moineau, 2020). Bacteriophages infecting the same host tend to group in genotypic clusters (Dion, Oechslin, & Moineau, 2020). Despite nucleotide diversity among genomes, protein structures are shared even when low sequence similarity (no aminoacidic and nucleic homologues) is present so we can find protein homologues among different viral families (Dion, Oechslin, & Moineau, 2020). There are 12 families and one unclassified group of bacteriophages according to NCBI based on ICTV classification with the majority of bacteriophages belonging to *Myoviridae* (17%) and *Podoviridae* (12%) families (Dion, Oechslin, & Moineau, 2020). Therefore, bacteriophages genomes show no synteny even in genes with associated functions that are dispersed (Dion, Oechslin, & Moineau, 2020).

There are two theories of phages origin, efforts to undermine absence of nucleotide and aminoacid level homology, gene exchange and high diversity (Dion, Oechslin, & Moineau, 2020). The first theory explains divergent evolution where phages share a common ancestor and architechture of structural proteins but there is no nucleotide homology due to divergence (Dion, Oechslin, & Moineau, 2020). Second theory explains convergent evolution where there is no common ancestor shared but bacteriophages share structures because of selective

pressures that determine these structures are optimal for virion formation (Dion, Oechslin, & Moineau, 2020).

Horizontal gene transfer is the major mediator of bacteriophages evolution (Dion, Oechslin, & Moineau, 2020). In bacteriophages, non-homologous recombination can occur randomly with an outcome of genes and gene blocks disruption (Dion, Oechslin, & Moineau, 2020). Due to natural selection, recombination sites are located at gene or gene block boundaries to avoid biological function disruption (Dion, Oechslin, & Moineau, 2020). Homologous recombination with high tolerance to sequence divergence occurs between related genome sequences, is the most frequent horizontal gene transfer mechanism among temperate phages and in several bacteriophages is responsible for gene shuffling (Dion, Oechslin, & Moineau, 2020). For virulent phages, homologous recombination is less crucial because most of the time, they fall into low gene content flux (Dion, Oechslin, & Moineau, 2020).

Gene exchange between bacteriophages require physical coexistence inside the cell, and coinfection seems to be common among bacterial populations which can even let to recombination even between ssRNA and ssDNA virus (Dion, Oechslin, & Moineau, 2020). Temperate phages play an important rol as viral sequence reservoirs due to integration in host genome (Dion, Oechslin, & Moineau, 2020).

Actual approaches to unentangle phage phylogeny is based on the use of networks, that show evidence of gene exchange due to connectivity between modules that seem to group bacteriophages according to ICTV family classification (Dion, Oechslin, & Moineau, 2020).

Bacteriophage Lifecycle

Phage adsorption starts when phage binds in a reversible way to one specific protein in cells surface. Furthermore, phage must bind in an irreversible way between one phage structure and the cell receptor. Therefore, cell wall must become penetrable, some factors that contribute to

this process are capsid or tail enzymes. This allows genetic material to traspass the cell wall and the membrane while the capsid remains extracellular. Genetic material inside of the cell has two possibilities: it can integrate to the host genome, or it can stay in the cytoplasm (Maslov & Sneppen, 2017). Either way, while the phage genome remains in the cytoplasm, this can be subject of gene expression, genome replication, capsid formation and phage assembly with genome packing inside capsids. The period before the formation of mature phages is called eclipse (Maslov & Sneppen, 2017). When phages "decide" for latency, there are no extracellular phages, so infection is undetectable during the time between adsorption and cell lysis. This latency lifecycle corresponds to the lysogenic cycle where both phages and host cells live and propagate. Throughout this phase, phages can replicate to inherit at least one copy of its DNA to daughter host cells or there can subsist as non-productive infection where phages replication can occur in tandem with host cell and no structural virions are produced (Nicastro, et al., 2016). Usually, phages remain in the hosts in a dormant stage called prophage and replication is carried out parallel to its host (Maslov & Sneppen, 2017). Integration into the host genome is the most common way to proceed during lysogeny and it requires an integrase, so temperate phages can switch between lytic and lysogenic stages in reaction to host response to stress or external danger signals (Nicastro, et al., 2016).

There is some time where the phage lysogenic decision is not accomplished, so this induces the lytic lifecycle. This process is called induction or derepression, because during lysogeny, phages produce proteins needed for the lysogenic cycle maintenance, and repressors necessary to prevent the lytic cycle proteins expression (Nicastro, et al., 2016). During the lytic lifecycle, lytic phages turn host metabolism towards reproduction of new phages and this process concludes with the cell lysis and infection of a new cell (Maslov & Sneppen, 2017). Burst size is the number of virions released per cell (Maslov & Sneppen, 2017). Cell lysis process can be

accomplished because of different molecules such as endolysins that can attack the cell wall and Holins that damage the plasma membrane. The fraction of viral infected cells can be in a range of 3.7 to 40% depending on the methods used to determine this percentages (Weinbauer, 2004). Viral induced mortality also ranges between 53% and 63% (Weinbauer, 2004).

Another possibility is to stablish a chronic infection, where phages are constantly being produced and reach extracellular environment due to budding or extrusion, so there is no cell lysis (Maslov & Sneppen, 2017). There is a chance to fulfill a carrier state where prophages are inside bacteria in a plasmid-like prophage. Similarly, phages can be doing pseudo lysogeny, where there is phage replication and assembly happening only in a portion of the population (Maslov & Sneppen, 2017). An alternative possibility for phages includes the expression of host restriction endonuclease, which produces the phage death and keeps the host alive; moreover, as result of the abortive infection system, both phage and host could die (Nicastro, et al., 2016).

Bacteriophages in Human Gut

Bacteriophage community in the human gut is dominated from members of *Caudovirales* order and *Microviridae* family, but most of phages remain unclassified (Dion, Oechslin, & Moineau, 2020). Transmission electron microscopy images showed inter-individual differences in bacteriophages morphologies and types (Dion, Oechslin, & Moineau, 2020). Human gut has different ecosystems because of its anatomic difference along the small and large intestine (Muniesa & Jofre, 2014). Phages replicate in places where its hosts are abundant, so bacteriophages population is also different along the gut. Bacteriophages present in fecal samples are probably representatives from bacteriophages present in the last large intestine portion where its hosts are abundant (Muniesa & Jofre, 2014). There are different theories about bacteriophage population dynamics, and information is controversially because there is the kill

winner model where depredation is the most important force and there are other theories where populations are more stable in time (Muniesa & Jofre, 2014). Kill the winner means that the high abundance of the bacterial host winner, makes it susceptible to the rapid depredation by its bacteriophage, becoming therefore the dominant type of phage in phages' population (Muniesa & Jofre, 2014). Theories involving stable bacteriophage populations implies bacteriophages replication as prophages, so this unable depredation as a driving force for bacteriophage population dynamics (Muniesa & Jofre, 2014). It is known that virus like particles (VLPs) are reported in gut or feces samples in $5x10^7$ to $1x10^{10}$ particles per gram (Muniesa & Jofre, 2014), although this can be an underestimation because of the complexity of the samples and the methods used to separate particles from stool. Fecal filtrates report 10^8 VLPs/mL but there are higher titers in gut mucosal biopsies (10^9 per biopsy) due to phages affinity for mucosal secretion binding and accumulation (Dion, Oechslin, & Moineau, 2020).

It is difficult to study bacteriophages diversity because there is no molecular marker shared among all bacteriophages and because most of the time, bacteriophages found in feces are brand new and there is no database information to compare with (Muniesa & Jofre, 2014). Also, there is no single gene or protein present in all phage genomes to let us build a tree based on shared genomic information (Dion, Oechslin, & Moineau, 2020). In feces, there have been identified 1200 viral genotypes vs the 800 bacterial spp. and it is known to be susceptible to almost 50 bacteriophage types (Muniesa & Jofre, 2014). Bacteriophages have also been implied in horizontal gene transfer scenarios, as bacterial DNA has been found in VLPs (Muniesa & Jofre, 2014).

Culture and genomic methods highlight temperate bacteriophages presence in human gut and their importance for bacterial DNA transport in human feces. Antibiotic exposure induces prophages to actively replicate in the bacterial cell, changing bacteriophages population and

increasing potentially transferable genes (Muniesa & Jofre, 2014). Virulence genes transference rate is still unknown as its unknown to what extent bacteriophages containing virulence genes can be considered a type of pathogen (Muniesa & Jofre, 2014). Antibiotic resistance genes in feces' bacteriophages are 10 times less than the counts found in bacterial populations, this genes are functional and can be transected (Muniesa & Jofre, 2014).

Bacteriophages counting methods

One of the easiest and most used ways of recovery is based on physical properties of bacteriophages, the concentration and ultracentrifugation, eliminates all cells present in the sample and other sample contaminants (Muniesa & Jofre, 2014). Some authors prefer to call the final product recovery Virus like particles instead of virus. Concentration can be performed also using membrane filters with pore size of 0.22 µm (Weinbauer, 2004).

Here we use the indirect viable counts, a method to assess the number of truly infective viruses of viable procaryotes (Maslov & Sneppen, 2017). With this methodology, results are obtained as Plaque Forming Units (PFU). Solid and semisolid agars are used , as a double layer assay where we can identify plaques result of bacteriophages mediated lysis in a bacterial lawn (Weinbauer, 2004). Another important method is the Most Probable Number (MPN), done in liquid culture media and observed as a result, changes in absorbance of the culture media (Weinbauer, 2004). Viable counts in natural environments represent only a small fraction of the total count (Maslov & Sneppen, 2017). "Since this requires the isolation of a host either on an agar plate or in liquid culture, and phages only infect a small range of host species, viable counts only represent a small fraction of the total counts" (Turko, et al., 2017) (Maslov & Sneppen, 2017). This limitation has led to the development of other host free methodologies.

Abundance can be estimated by transmission electron microscopy that gives information about morphology, also there is epifluorescence microscopy and flow cytometry as alternative

methodologies that use fluorescent dyes and the light dispersion and epifluorescence detection (Muniesa & Jofre, 2014). Electron microscopy is widely used for bacteriophages characterization because this allows sizing capsids and assessing morphological structures such as tails (Weinbauer, 2004). Negative staining of phages is performed using a mixture of two solutions. The first consists of 2% ammonium molybdate at pH 7.0 or 2% phosphotungstic acid; the second consists of 11% bacitracin in distilled water, or a 3% uranyl acetate solution. Formvar coated carbon grids are examined in a transmission electron microscope (Chibani-Chennoufi, et al., 2004). Problems associated with this technique are: “uneven collection, uneven staining, washing off of viruses, low detection limit as well as the lack of recognition of non-typical viruses” (Weinbauer, 2004). Also, large viruses can be confused with bacteria (Weinbauer, 2004). Electron microscopy gives lower count results (Weinbauer, 2004).

For epifluorescence and flow cytometry methods, bacteriophages can be marked using DAPI staining and other fluorochromes such as YOPRO-1, SYBRGreen I or SYBRGold (Weinbauer, 2004). One disadvantage with these techniques is the possibility to count DNA bounded to colloids because of the union of fluorophores to DNA (Weinbauer, 2004). Epifluorescence and flow cytometry gives comparable counts. We should mention that epifluorescence is mostly used for bacteriophage abundance estimation because of processing time, the possibility to apply during field work and lower costs (Weinbauer, 2004).

Another methodology to study bacteriophage populations in human feces is next generation sequencing. It is important to sequence all viral genomes present in one determined sample without any previous information about their identity. Studies of viral metagenomes avoid culture-based methods problems and single markers genes approach and asses the total viral nucleic acids isolated from environmental or patients’ sample (Dion, Oechslin, & Moineau, 2020). First human virome was published in 2003, since then, studies describe longitudinal and

interpersonal viral variations (Dion, Oechslin, & Moineau, 2020). This can give us information about presence and absence of viral particles in the sample but none about the susceptible hosts (Muniesa & Jofre, 2014). Metagenomics approach does not target viral DNA, but can give us some value information on phage sequences (Dion, Oechslin, & Moineau, 2020). Contigs with taxonomic attribution are ussually low (Dion, Oechslin, & Moineau, 2020). Analysis of CRISPR loci in bacterial hosts and its corresponding fragments in bacteriophages genome can give us an idea of non-cultured bacterial hosts (Muniesa & Jofre, 2014). Moreover, during metagenomics assembly, micro diverse phage populations can be discarded (Dion, Oechslin, & Moineau, 2020).

Bacteriophage therapy

Bacteriophages have been proposed since their discovery, for their use in bacterial infections. The World Health Organization (WHO) developed one study using bacteriophages against *Vibrio cholerae* in rural India in 1940 (Chibani-Chennoufi, et al., 2004), and ever since there has been interest in their use for therapeutics and industry. Seem to be important for intestinal homeostasis and consequently in host health, its known that disequilibrium is associated with sickness (Muniesa & Jofre, 2014). Bacteriophages play an important role in immunomodulation (Muniesa & Jofre, 2014) and these are useful to control infections as an alternative to antibiotic mediated treatments. Bacteriophages can be administrated to animals before sacrifice and to humans during infections and this has shown to regulate intestinal populations and pathogens of interest (Muniesa & Jofre, 2014). Bacteriophages are specific for certain bacterial populations, an interesting characteristic that helps us to leave the healthy microbiome components unaffected (Muniesa & Jofre, 2014). Moreover, bacteriophages can only replicate when there is the host presence, so replication can be auto limited (Muniesa & Jofre, 2014. One challenge for the therapeutical use of bacteriophages is the dose establishment as there is need

of a huge bacteriophage population to effectively infect bacteria and finally control the infection but when population is too big, this can interfere with other natural phage populations that have a specific activity related to host homeostasis (Muniesa & Jofre, 2014). Another challenge is the ability of bacteriophages to transduce bacterial genes, which can lead to an increase of antibiotic resistance and pathogen related genes between gut microbiome (Muniesa & Jofre, 2014). DNA fragments transduced by bacteriophages can size more than 100 kb, and include lineal chromosomes and mobile elements such as plasmids, transposons and insertion elements (Muniesa & Jofre, 2014). Nowadays, there is a preference for the use of non-lysogenic bacteriophages that are going to pack additional host DNA or to use Host's DNA to replicate their own (Muniesa & Jofre, 2014.

Bacteriophages' ability to inactivate and redirect cellular metabolism critical proteins is a key component to identify bacterial targets and discover new drugs (Muniesa & Jofre, 2014). Moreover, bacteriophages are implicated in cellular lysis through their lysins responsible for peptidoglycan degradation, proteins that can be used as antimicrobials more successful in gram positive bacteria but there are some examples of success in gram negative bacteria (Muniesa & Jofre, 2014).

One of the main problems that phage therapy undergoes is public opinion, as phages are seen as "enemies of life" (Nicastro, et al., 2016), likewise in the past, they have been tested and proven as unsuccessful antimicrobial agents. Other points to tackle are problems related to phage therapy including the necessity to improve and standardize scale-up and manufacturing process, improvement of drug delivery technologies and to study and improve phages that can evade mammalian immune system (Nicastro, et al., 2016).

Bacterial Growth rate effect on bacteriophage growth rate

Bacterial concentration and physiological host characteristics can influence bacteriophages propagation (Nabergoi, Modic, & Podgornik, 2018). Different culture media composition can also control bacterial growth rate. Latency period and burst size in bacteriophages depend on population sizes (Nabergoi, Modic, & Podgornik, 2018). An increase in bacterial growth can shorten eclipse and latency period while burst size increases (Nabergoi, Modic, & Podgornik, 2018). Higher dilution rate shortens latency period and increases burst size. Adsorption constant is higher in a shorter dilution rate (Nabergoi, Modic, & Podgornik, 2018). There is a direct correlation between bacterial growth rate and bacteriophage growth rate (Nabergoi, Modic, & Podgornik, 2018). In stationary phase, after infection, bacteriophages experiment a hibernation mode where infected cells produce bacteriophage enzymes but prevents phages assembly until there are enough nutrients in the culture (Nabergoi, Modic, & Podgornik, 2018). When fresh culture media is added, bacteriophage assembly starts and burst size increases proportionally to bacterial growth (Nabergoi, Modic, & Podgornik, 2018). Adsorption process is a physical process, because bacteriophages can adsorb even to death bacteria (Nabergoi, Modic, & Podgornik, 2018), this process depends on host physiological stage and culture conditions as well as temperature, pH, osmolarity, ionic force, divalent cations, adsorption cofactors (Nabergoi, Modic, & Podgornik, 2018). Literature shows that LPS in external membrane and external membrane proteins, such as protein C (OmpC) of *E. coli*, are relevant for bacteriophage adsorption, and its concentration determines bacteriophage adsorption rate, which increases during glucose and ammoniac limitation and anaerobic conditions (Nabergoi, Modic, & Podgornik, 2018). All described conjectures are still unknown if are specific for bacteriophage-host interaction or can be generalized for all bacteriophages and its hosts (Nabergoi, Modic, &

Podgornik, 2018). This understanding is important for bacteriophage cultures for any application (Nabergoi, Modic, & Podgornik, 2018).

Bacteriophage Impact in Bacterial Population

Since 1968, it was suggested that phages could have certain influence in bacterial communities, affecting number, types, and population growth time (Wiebe & Liston, 1968). Bacteria are continuously competing with phages (Maslov & Sneppen, 2017). Phage infections give rise to abrupt collapses on large bacterial populations (Maslov & Sneppen, 2017). It's known that *E. coli* populations suffer dramatic collapses in 4 or 5 log when they are exposed to phage infection (Maslov & Sneppen, 2017). Dominance structure of the population undergoes positive or negative selection according to changes in ecological and environmental variables (Turko, et al., 2017).

Red queen hypothesis states that species must undergo constant changes and still in the same place (Martín-Peciña & Osuna-Mascaró, 2018) meaning that antagonistic interactions will lead to evolution (Martín-Peciña & Osuna-Mascaró, 2018). Host-parasite interactions are marked by reciprocal adaptations (Martín-Peciña & Osuna-Mascaró, 2018). Epidemic parasitism promotes changes in genotype frequencies (Martín-Peciña & Osuna-Mascaró, 2018). Parasitism is important as a driver for evolution, disease, and virulence of human pathogens (Martín-Peciña & Osuna-Mascaró, 2018).

Higher prevalence of parasite epidemics strongly correlates with an increase of host's clonal turnover exhibited by oscillations in genotype frequencies in host and parasite due to negative dependent selection (Martín-Peciña & Osuna-Mascaró, 2018). Parasites are fast evolving and those infect the most common clones, this leads to appearance of less common or rare clones to be the new common clones, so common parasite genotypes are also negatively selected (Martín-Peciña & Osuna-Mascaró, 2018). For study of this coevolutionary scenarios, there is a

need of specific host-parasite interaction with genetic specificity in host defense and parasite infectivity (Turko, et al., 2017) and information of their reciprocal adaptation and interaction with environmental factors such as biomass and temperature (Martín-Peciña & Osuna-Mascaró, 2018). Interactions between hosts and parasites are dynamic and product of adaptation and counter-adaptation (Martín-Peciña & Osuna-Mascaró, 2018).

"Host genotypes should be infected disproportionally to their abundance. In particular, theory predicts that common host genotypes should be either over or under-infected, depending on the phase of the oscillatory cycle, whereas the actual time of a proportionate infection of common clones would be rather small" (Turko, et al., 2017). Parasites have also impose significant costs on infected individuals (Turko, et al., 2017). Parasite Infected clonal samples differ in clonal composition (Turko, et al., 2017). "Clonal diversity is a dynamic equilibrium between clonal emergence, clonal erosion, and population dominance structure" (Turko, et al., 2017). Clonal turnover is faster during epidemics because of parasite driven, negative frequencies selection (Turko, et al., 2017).

Kill the winner infection prevents the fastest growing organism from taking over the community (Maslov & Sneppen, 2017). Kill the winner classical view states that virulent phages reduce populations of their susceptible host to a low steady state level which is independent of hosts growth rate thus allowing multiple species per nutrient type (Maslov & Sneppen, 2017). Lotka-Volterra equations establish that bacterial ecosystems are in static equilibrium balanced by losses due to phage predation (Maslov & Sneppen, 2017). Bacterial exponential growth is interrupted when the population is huge by sudden collapses caused by phage infection and this bacteriophage predation causes negative frequency dependent selection (Maslov & Sneppen, 2017). "The number of co-existing bacterial species in the resulting ecosystem is determined exclusively by the parameters of phage predation, the topology of the phage-bacterial infection

network and the carrying capacity of the environment" (Maslov & Sneppen, 2017). The carrying capacity of the environment defines the size of the population in a particular ecosystem, depending on abiotic factors such as the availability of food, water, shelter, and mates. Species diversity cannot exceed the nutrient quantity.

This ideal state is not always the truth in microbial populations which are often outside the traditional steady state. In real world this simplified model is not applicable because microbial communities are open systems, meaning they are exposed to other external stresses such as new mutations and new invading virulent phages (Maslov & Sneppen, 2017).

Microbial systems are dynamic, exposed to changes in interaction rules and exposed to new species invasion (Maslov & Sneppen, 2017). There is a dynamic model interpretation of kill the winner principle where: "bacterial populations are characterized by periods of competitive exponential growth punctuated by rapid and severe collapses" (Maslov & Sneppen, 2017). Phage infection in this model is proportional to the population size (Maslov & Sneppen, 2017). In larger populations, when infection starts it can eliminate a big fraction of susceptible hosts, this outcomes in a severe collapse in the specific strain population (Maslov & Sneppen, 2017). Over long time periods, species population fluctuates between low and high population numbers and the total population number seems to be constant because of the carrying capacity of the environment (Maslov & Sneppen, 2017). All depends also on collapse frequencies; when the collapses are very rare, the strains that are the faster growing, will eventually become the only ones to survive (Maslov & Sneppen, 2017). When collapse rate is intermediate, this can allow more than one strain to coexist and leads the slowest strains to become extinct (Maslov & Sneppen, 2017). Besides, the smallest and slowest strains almost never collapse, not to mention that relative population of slowest strains can decrease not because of phage collapses,

but because it can be outnumbered by higher relative population sizes of faster growers (Maslov & Sneppen, 2017).

In the steady state, fastest growing strains are more likely to collapse, and population size fluctuates between small and huge (Maslov & Sneppen, 2017). Each collapse eliminates growth advantage, so there is an equilibrium between the excess growth rate with logarithmic population loss (Maslov & Sneppen, 2017). Growth rate of each strain depends inversely on resource competition (Maslov & Sneppen, 2017). When strains compete for the same niche (nutrient) as in our case, different *E. coli* strains are competing for the same carbon source, this limiting nutrient defines the carrying capacity of the environment (Maslov & Sneppen, 2017). Total bacterial population can reach the steady state given by the carrying capacity, consequently this value remains constant but relative proportion of each strain population changes vs. time because of population collapses and fitness differences between individuals (Maslov & Sneppen, 2017).

On the Kill the Winner fixed threshold model, collapses are independent on population relative size. Smaller population collapses are impact less to other populations relative sizes (Maslov & Sneppen, 2017). When largest populations collapse this ascent a scenario where two populations become closely in size and can fight between them for population dominance (Maslov & Sneppen, 2017).

OBJECTIVES

General Objective:

- Investigate the role of bacteriophages in the *E. coli* population avian intestines

Specific Objectives:

- Characterize changes in phage population in 9 chickens belonging to 3 genetic breeds.

- Characterize the *E. coli* population changes in susceptibility to different bacteriophages.

RESEARCH METHODOLOGY AND DESIGN

Chicken Sample Size

9 chicken of 11 days old belonging to 3 different lineages: Cobb 500, Malines, and Brahma (3 chicken per each strain) were selected. Each chicken was tagged with a number in chicken legs. Chicken were prepared during10 days before the study began without balanced meal (which has an antibiotic cocktail). Instead these were fed with chopped corn and common water until day 22 when sampling began and during the 6 weeks experiment.

Feces Sample collection

In the day 22, chicken were fed and then separated into individual cages with clean newspaper on the cage floor. They were left on that place until we noticed the feces were already on the newspaper. Subsequently, the chickens were returned to the shed. Feces were collected in individual sterile plastic containers using sterile spoon and labeled with chicken's number. All samples were stored in a cooler and taken to the lab facilities within a maximum time range of 2 hours.

E. coli isolation

Feces were homogenized using a sterile swab and then inoculated in one third of the petri dish with Chromocult® Coliform Agar by using a sterile loop, consequently, were striated by exhaustion. After 24 hours, we selected 5 colonies per petri dish belonging to typical *E. coli* strains whose phenotype is dark blue to violet colonies. Those were confirmed to be lactose fermenters with MacConkey Agar, where their phenotype was pink. The petri dishes were placed in an incubator at 37 ° C for 24 h. Each strain was frozen in 2 ml cryovials with 1 ml Difco™ Skim Milk, BD.

Phage isolation

The rest of the feces that were not used for the isolation of *E. coli*, were homogenized and 2 g of the sample were weighed and placed in 10 ml falcon tubes with 5 ml of Bacto Tryptic Soy Broth with 4.98 $x10^{-6}$ M of $MgSO_4$. The solution was homogenized using vortex mixer and 3 ml were taken using a 100 µl pipette with filter tips into two 1.5 ml Eppendorf microtubes. The solution was centrifuged at 8064 **g** for 10 minutes. Supernatants were taken with 3 ml sterile syringe. The syringe was adapted to a Nalgene filter with 0.22 µm membrane pore to eliminate remaining bacteria from the final solution, which was placed on new Eppendorf microtubes with screw caps and saved at 4°C until use.

Strain revival

Cryotubes with frozen strains were taken out of the freezer. A sterile swab was used to scrape the top of the ice from the skimmed milk medium in the cryotube and inoculated in Petri plates with TSB + 4.98 $x10^{-6}$ M $MgSO_4$ + 1.5% Bacto Agar. The petri dishes were placed in an incubator at 37 °C for 24 h.

Strain exponential growth.

Cultivation of each strain in exponential phase was obtained by inoculating a colony from the TSB medium 4.98 $x10^{-6}$ M $MgSO_4$ and placed in an incubator at 37 °C for 24 h.

Plaque Assay

Solid medium was prepared in sterile petri dishes with TSB + 0.6 mg/L MgSO4 + 1.5% Bacto Agar. Semi-solid medium was prepared with TSB + 0.6 mg/L MgSO4 + 0.6% Bacto Agar, dispensed 5 ml in each test tube with screw caps and maintained at 4 °C until use. At first, 100 µl from the bacteria in exponential growth with 50 µl of the phage solution were incubated for 15 min at 35 °C. For the double layer method, the semi-solid medium was heated in 500 ml

beakers filled with 100 ml of water for 2 min in the microwave until it was completely dissolved and allowed to cool to a touch-tolerable temperature. When this happened, the 150 µl of solution phage-bacteria, was transferred from the microtube to the test tubes with semisolid medium. This was lightly mixed until a homogeneous solution was obtained and settled on the solid agar. Plates were left to settle for about 30 min and then incubated at 35 °C for 24 hours. All assays were done with in duplicate. As control, we used *E. coli* strain ATCC 25922 to test against all bacteriophages cocktails to determine if bacteriophages were present in the sample.

Statistical Analysis.

The number of plaques was counted and averaged among the duplicates. The objective of this work was to determine the treatment or test had a significant effect on the results obtained in the different combinations of phage and bacteria cocktail. For this reason, an initial attempt was made to check the assumptions of normal distribution, equality of variances and independence of the residuals to be able to perform ANOVA. On the other hand, after several transformations to normalize the data, the objective was not achieved, so non-parametric analysis had to be used. The chi-square test was carried out using the option Cross tabulation and Chi-square for Raw data (categorical variables) also we confirmed the preliminary results using hypothesis test option and chi square test for association. Finally, we performed Kruskal Wallis test to determine the difference between means. All statistics were carried out on Software Minitab 19.

RESULTS

From the 406 *E. coli* isolates analyzed during the 6 weeks assay (5 strains per chicken and per week), 96 (23.64%) were observed to be susceptible to bacteriophages present in the feces. From now on we will describe only the *E. coli* strains which were susceptible to bacteriophages. Of these 96 strains, 47 (51.65%) showed higher viral titers in the fecal sample obtained at the same time; 19 (20.88%) showed higher viral titers in fecal sample obtained 2 weeks after and 25 (27,47%) showed higher titers in fecal samples obtained 2 weeks before (Table 1)

Table 1: *Number and percentage of positive plaques on isolated strains.*

Treatment	**Number of *E. coli* strains showing plaques**	**Percentage of positive plaques (%)**
Same Week	47	51.65
Two Weeks After	19	20.88
Two weeks Before	25	27.47
Total	**91**	100.00

In table 1, the highest percentage of positive plaques corresponds to phages from the same week. The second highest percentage corresponds for two weeks before, but there is a little difference between the two weeks before and two weeks after treatment (6.59%). To determine which factor was directly affecting our response variable, statistical analysis was carried out (Appendix 1-26).

Table 2: *Number and percentage of positive plaques on E. coli ATCC25922.*

	Chicken								
Week	1	2	3	4	5	6	7	8	9
1	uncountable	uncountable	uncountable	uncountable	uncountable	uncountable	uncountable	uncountable	uncountable
2	uncountable	uncountable	uncountable	uncountable	uncountable	uncountable	uncountable	uncountable	uncountable
3	uncountable	uncountable	uncountable	uncountable	uncountable	uncountable	uncountable	uncountable	uncountable
4	uncountable	uncountable	uncountable	uncountable	uncountable	uncountable	uncountable	uncountable	uncountable
5	uncountable	uncountable	uncountable	uncountable	uncountable	uncountable	uncountable	uncountable	uncountable
6	uncountable	uncountable	uncountable	uncountable	uncountable	uncountable	uncountable	uncountable	uncountable

For *E.coli* ATCC 25922, the number of plaques in each plate with 10 µl of bacteriophage cocktail was higher than 300 FPU per plate along the six weeks, so the plates were determined as uncountable. This is important for us to validate that our lack of data during plate counting method was due to bacteriophage resistance rather than to bacteriophage absence on the bacteriophages cocktail samples.

DISCUSSION

We studied the sensitivity of the intestinal *E. coli* to bacteriophages present in the intestine along 6 weeks. We found a large abundance of bacteriophages, which were able to attack *E. coli,* in chicken intestines, however most intestinal strains were far more resistant to most co-occurring bacteriophages, than a reference collection *E. coli* strain ATCC 25922. These results are in agreement with recent reports indicating that bacterial strains in their natural environment contain many mechanisms that confer resistance to bacteriophage infection (Hussain, et al., 2021). Our data also showed that most of the lytic infections were seen on

numerically dominant isolates from the same week as the bacteriophage extracts (Table 1) and the phage counts declined after 2 weeks (Appendix 2-10), this agrees with previous observations (Chibani-Chennoufi, et al., 2004) which showed that prevalence of specific strains of bacteriophages in *E. coli* in humans tends to decline overtime. Likewise, the present study also evidenced that the numbers of phage-sensitive strains were also declining during time as most strains obtained in the following weeks showed different phage resistance patterns. These data are also in agreement with the observation of multiple transient *E. coli* strains in the intestine (Richter, et al., 2018; Chen, et al., 2021) and the concept that the continual predator-prey dynamics promote microbial diversity in the intestine (De Sordi, et al., 2019).

It's known that *E. coli* populations suffer dramatic collapses (4 or 5 log) when they are exposed to phage infection (Maslov & Sneppen, 2017). Bacteriophages could infect numerically dominant *E. coli* clones, affecting their population and allowing less dominant clones to arise (Martín-Peciña & Osuna-Mascaró, 2018), also common clones can be under infected (Turko, et al., 2017).

Our study is in contraposition to previous studies done in humans in which they did not find any "cyclic changes in phage and bacterial abundance as expected in Lotka-Volterra predator–prey relationships or episodes of outgrowth of bacterial species followed by blooms of their phage as in "kill-the-winner" dynamics" (Minot , et al., 2011). According to kill the winner fixed threshold model, population collapses are independent of their size and more than one population can have similar sizes and continuously compete for dominance (Maslov & Sneppen, 2017). In the dynamic model interpretation of this theory, there are episodes of exponential growth and rapid severe collapses proportional to population sizes (Maslov & Sneppen, 2017).

Red queen dynamics is probably involved in this process as bacteriophage can gain ability to infect new strains while bacteriophage sensitive bacteria can gain resistance to a specific bacteriophage (de Sordi, et al., 2019; Hussain et al., 2021). Finally, it is worth to mention that bacteriophages seem to be daily acquired as they are transient in ingested food (Minot , et al., 2011).

Our results showed significative differences between chicken PFUs count (Appendix 26). It is known that virome shows high inter-individual variation, and phenotypical individual variation may account for the differences (Liang & Bushman, 2021; Minot, et. al., 2011). The gut virome is individual specific and stable over time (Dion, Oechslin, & Moineau, 2020). Some factors also affecting virome are anatomical site, diet, age, geographical distribution of the sampled individual and health status of the individual (Liang & Bushman, 2021). Studies showed that clonal turnover is strongly correlated with viral parasitism than another environmental factor (Turko, et al., 2017). Parasites are known to maintain host genetic diversity, and this can explain why natural populations of asexual organisms are often very diverse (Turko, et al., 2017). There can be rapid changes during early life (Dion, Oechslin, & Moineau, 2020).

There is a long path before we can completely understand the role of bacteriophages in the gut microbiome. For this purpose, there is a need to identify unknown bacteriophages DNA in human feces, associated with non-culturable bacterial hosts, and to identify susceptible non culturable bacterial hosts from samples by metagenomic approaches (Muniesa & Jofre, 2014). *"E. coli* is by far the most dominant species in *Enterobacteriaceae* family from healthy humans" (Martinson, et al., 2019), which means coliphages are also common. Comparisons between human and animal strains are difficult as their prevalence of *E. coli* phylogroups seems to be different (Teinallon , Skurnik, Picard, & Denamur, 2010). We used chickens as a model

to explain changes in bacterial population and correlate these results with bacteriophages count. It would be important to replicate this experiment in healthy humans' stool to try to unentangle and determine if the same phenomenon is seen in humans' bacteriophages and *E. coli* population.

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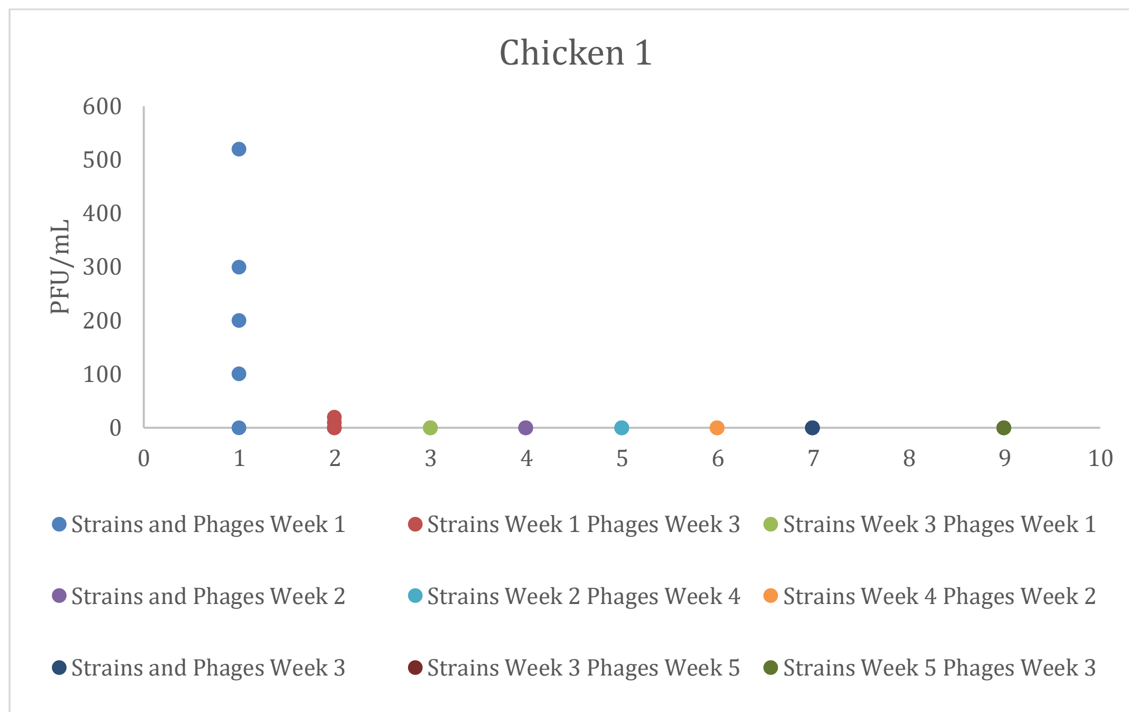
APPENDIX 1: NUMBER AND PERCENTAGE OF POSITIVE PLAQUES FROM EACH CHICKEN

Chicken	**Number of positive plaques**	**Percentage of positive plaques (%)**
1	8	8,79
2	9	9,89
3	10	10,99
4	0	0,00
5	5	5,49
6	11	12,09
7	19	20,88
8	16	17,58
9	13	14,29
Total	**91**	100,00

From the table, we can extract that the highest percentage of positive plaques were shown in the chicken number 7. To observe from a general perspective, the changes in the size of the phage population, quantified in 9 different tests, a Dotplot was carried out from which it can be seen in general, the tests in which a phage count was obtained.

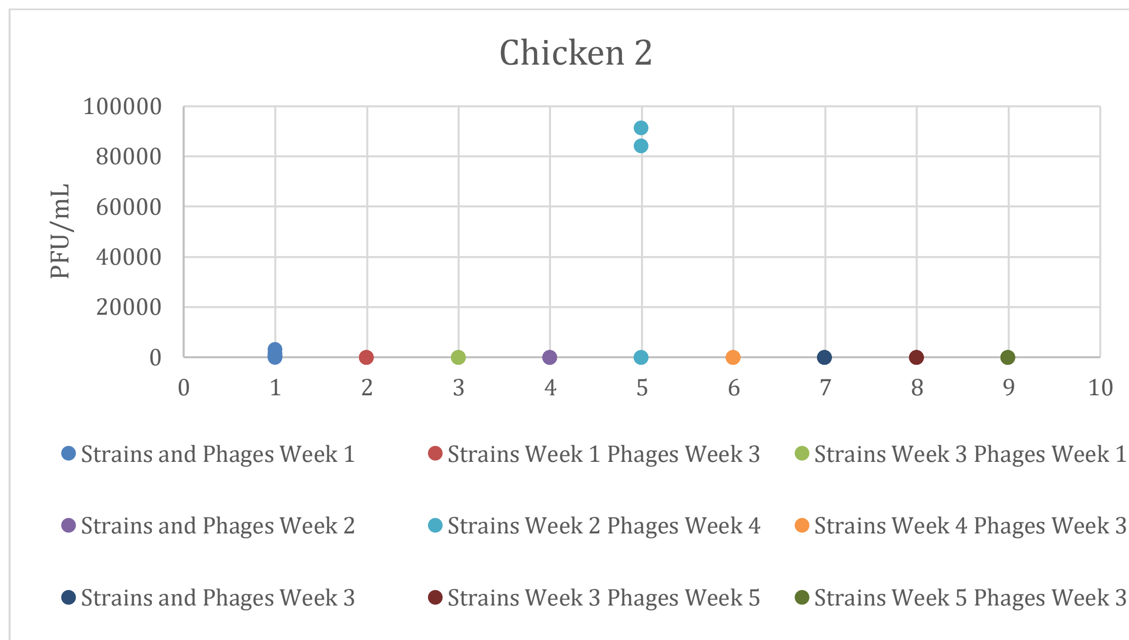
APPENDIX 2. DOTPLOT OF THE PFUS / ML CHICKEN 1



PFU/ml were quantified in 9 different tests carried out on the feces from chicken 1. Graph was made with Microsoft Office Excel.

In the case of chicken 1, it can be observed that there is a peak in PFU / ml of phages against isolates from week 1. Additionally, we can infer that after 2 weeks a certain number of phages remain in solution and can be active against the strains of the first week. No more PFU / ml peaks were observed in the other tests performed.

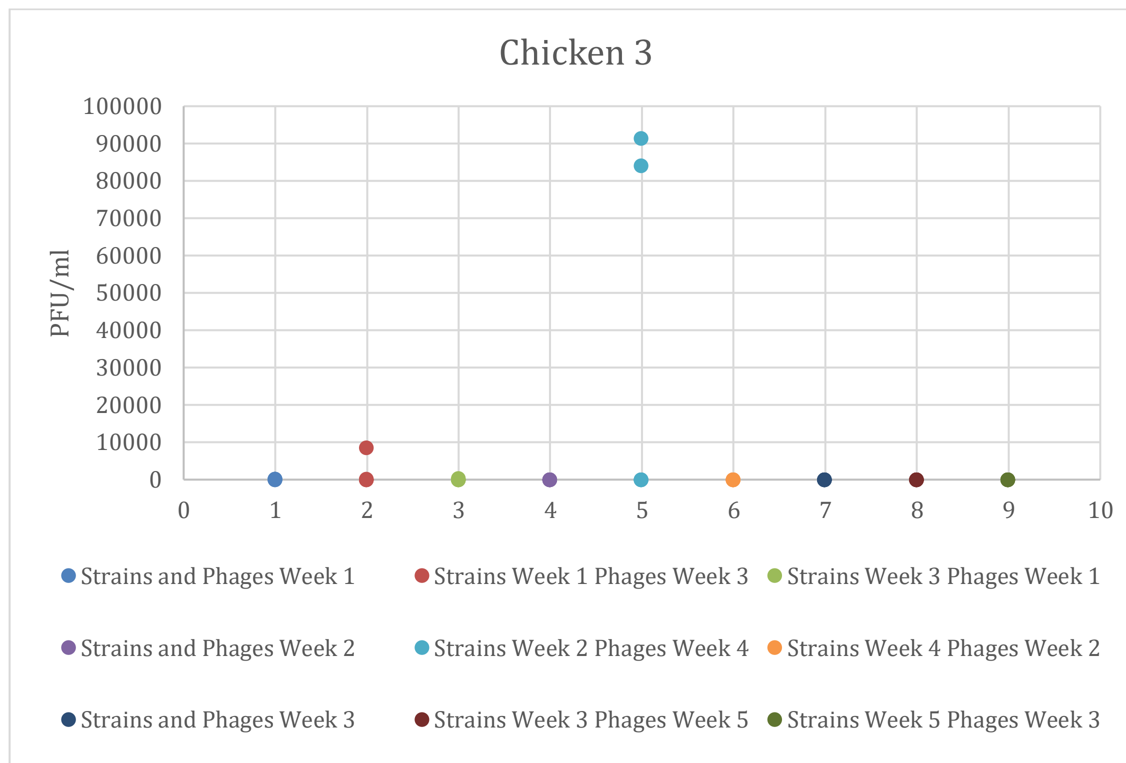
APPENDIX 3. DOTPLOT OF THE PFU / ML CHICKEN 2



PFU / ml quantified in 9 different tests carried out on the feces from chicken 2. Graph was made with Microsoft Office Excel.

In chicken 2, there is only one phage peak in the treatment including the strains from week 2 with the phages from week 4. No other peaks were observed in the other tests performed.

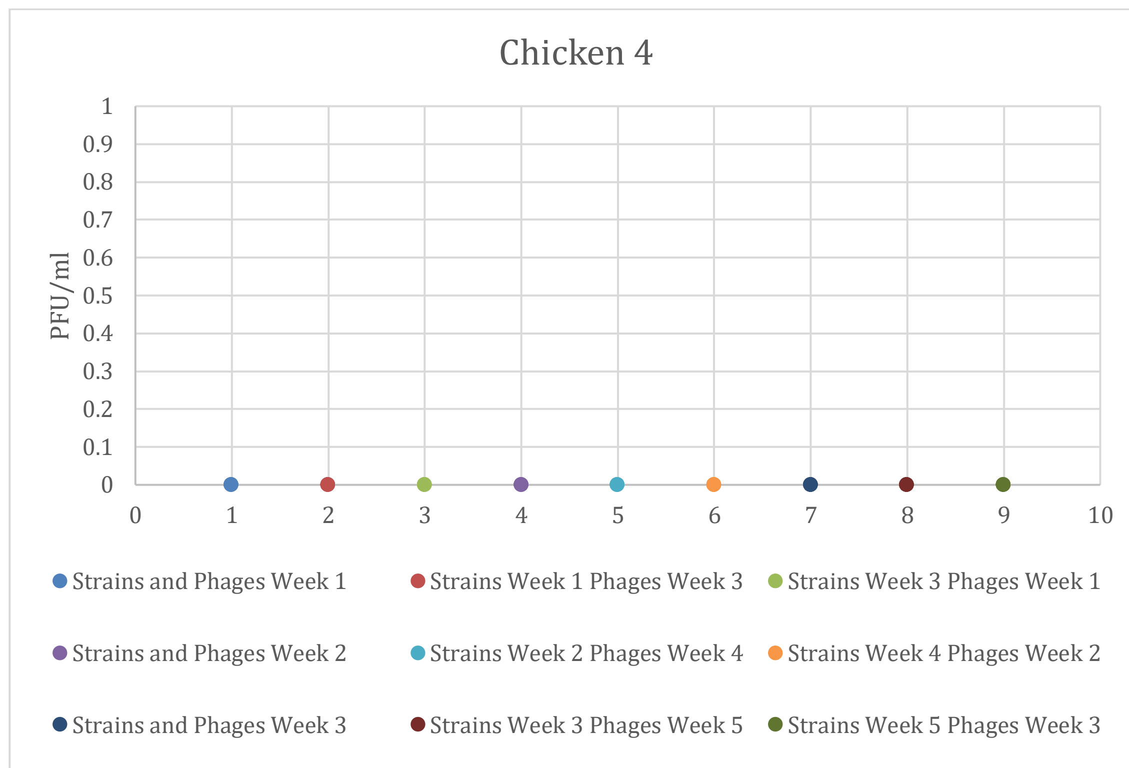
APPENDIX 4. DOTPLOT OF THE PFU/ML CHICKEN 4



PFUs / ml quantified in 9 different tests carried out on the feces from chicken 3. Graph was made with Microsoft Office Excel.

In chicken number 3, 2 peaks were observed. The first peak corresponds to the test involving strains from week 1 with the phages from week 3. The second peak corresponds to the test with the strains from week 2 and the phages from week 4. Even 2 weeks after isolating the strains from the chicken intestine, there is still a population of bacteriophages against these strains.

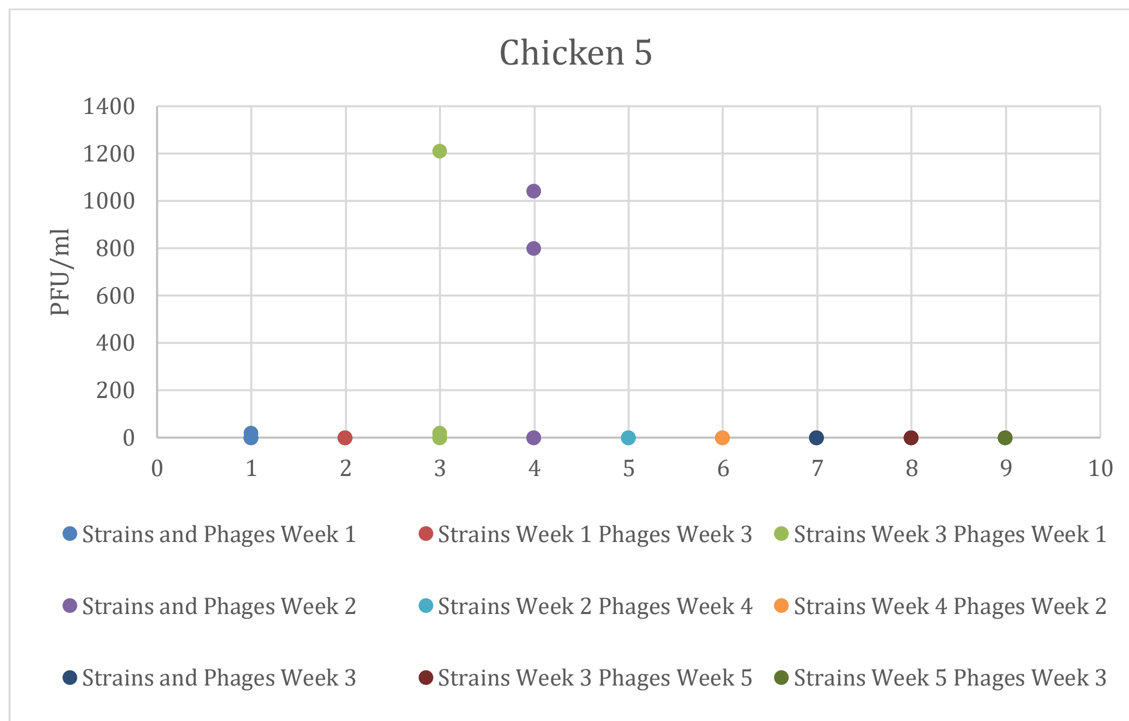
APPENDIX 5. DOTPLOT OF THE PFU/ML CHICKEN 4



PFUs / ml quantified in 9 different tests carried out on the feces from chicken 4. Graph was made with Microsoft Office Excel.

In the case of chicken 4, no bacteriophages were obtained against the isolated strains. This is not to say that there were no phages in the solution.

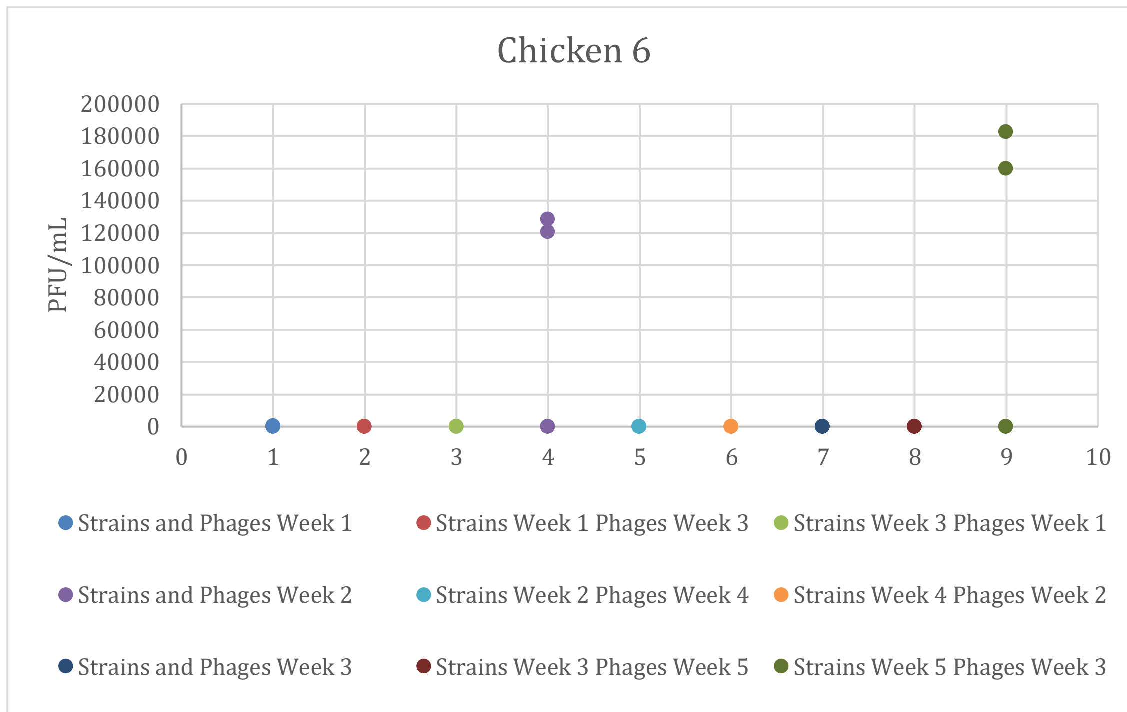
APPENDIX 6. DOTPLOT OF THE PFU/ML CHICKEN 5



PFUs / ml quantified in 9 different tests carried out on the feces from chicken 5. Graph was made with Microsoft Office Excel.

In chicken 5, one peak is observed in the test carried out with the strains of week 3 with the phages of week 1. Additionally, there is a peak in the test carried out with the strains and phages of week 2. The phage population against the isolated strains was active from the 1st week.

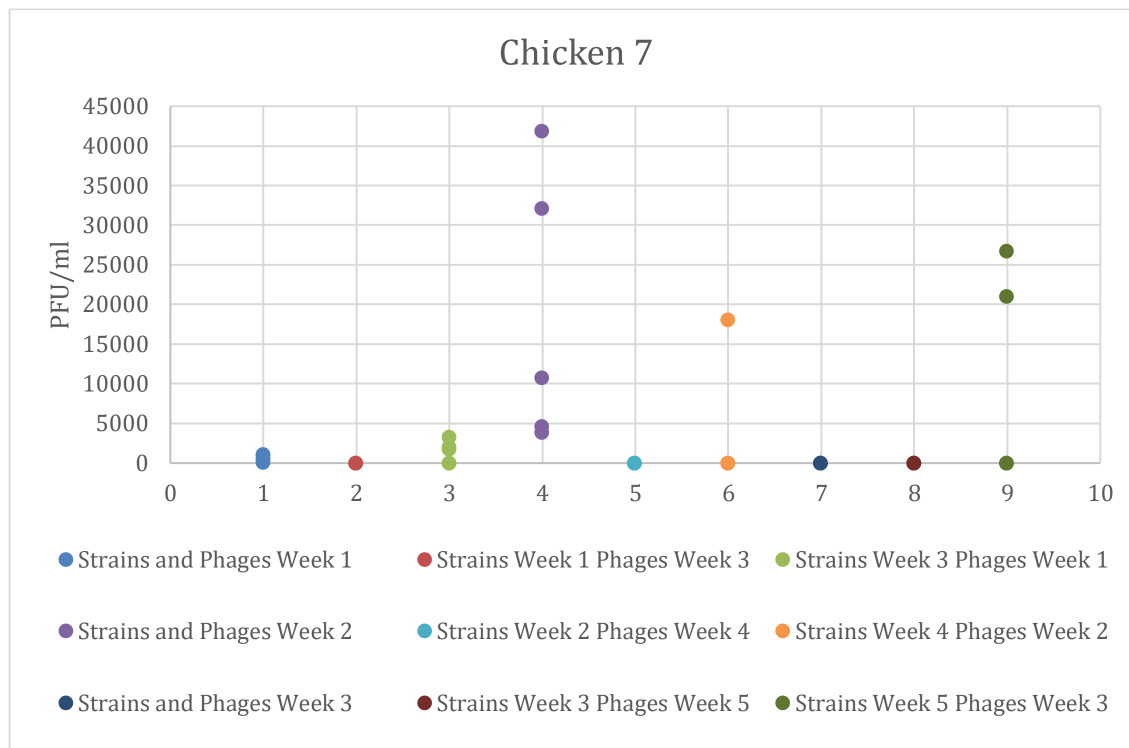
APPENDIX 7. DOTPLOT OF THE PFU/ML CHICKEN 6



PFUs / ml quantified in 9 different tests carried out on the feces from chicken 6. Graph was made with Microsoft Office Excel.

For chicken 6, two peaks are observed, the first one among the strains of week 3 with the phages of week 5 and the second with the strains of week 5 and the phages of week 3, this peak is higher in PFUs/ml. In this case we can infer that the bacteriophage population fluctuated between week 2 and 5. The bacteriophage population remains active against isolates for approximately 2 weeks.

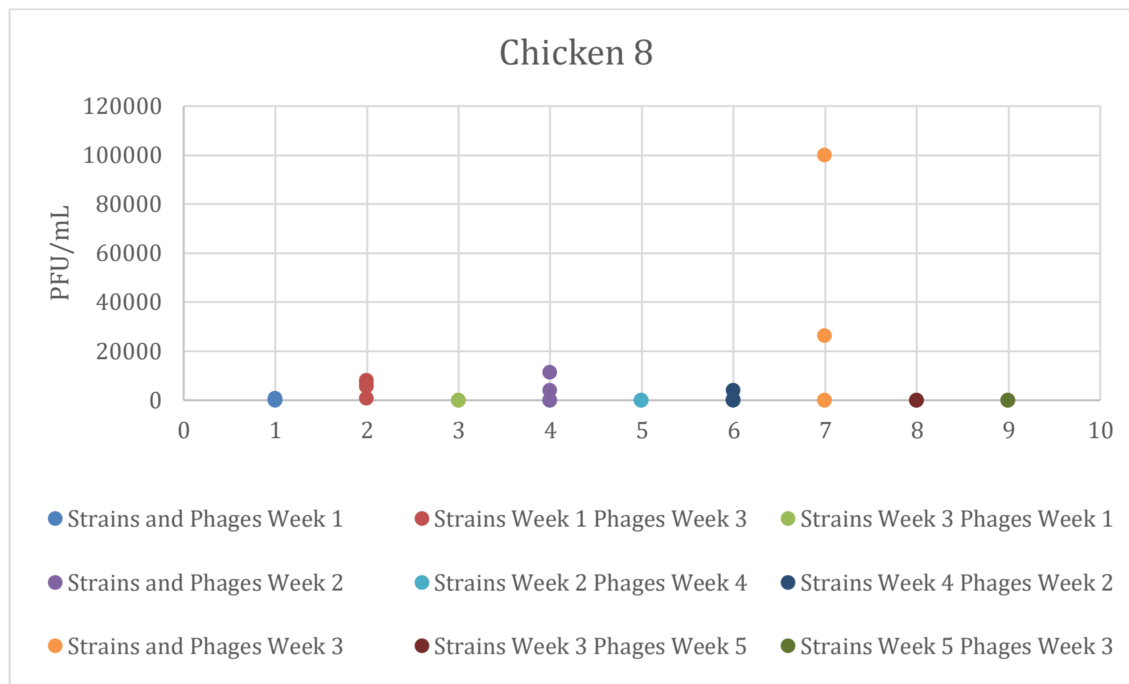
APPENDIX 8. DOTPLOT OF THE PFU/ML CHICKEN 7



PFUs / ml quantified in 9 different tests carried out on the feces from chicken 7. Graph was made with Microsoft Office Excel.

For chicken 7, it is observed that there was a quantifiable population of phages from week 1. Also, it was possible to quantify bacteriophages in 5 of the 9 tests carried out. The bacteriophages from week 1 were active against the strains isolated from week 3. It is also observed that there was the highest peak in the bacteriophage population count at week 2 and it was also active against the strains from week 4. Finally, phages from week 3 were active against strains from week 5 too.

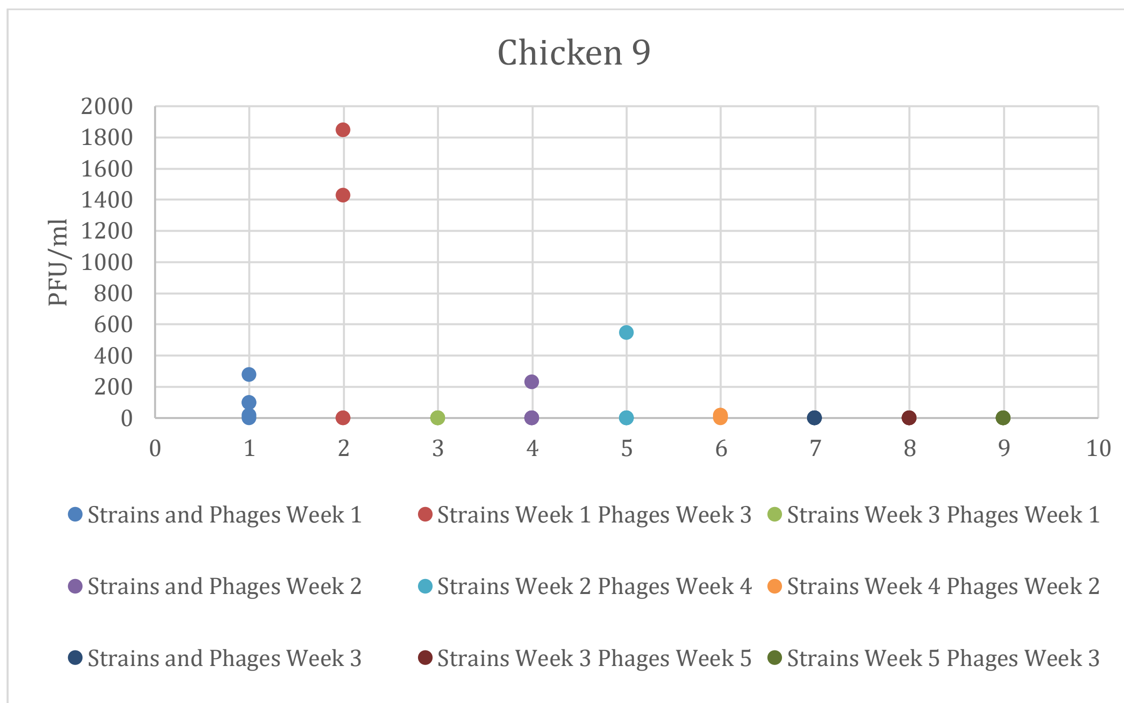
APPENDIX 9. DOTPLOT OF THE PFU/ML CHICKEN 8



PFUs / ml quantified in 9 different tests carried out on the feces from chicken 8. Graph was made with Microsoft Office Excel.

In chicken 8, there is a quantifiable number of phages since week 2, and there are three peaks of quantifiable PFUs, two of these peaks correspond to week 2 and 3 with a phage treatment of the same week. On the other hand, the highest peak was obtained in the combination of phages and strains from week 3.

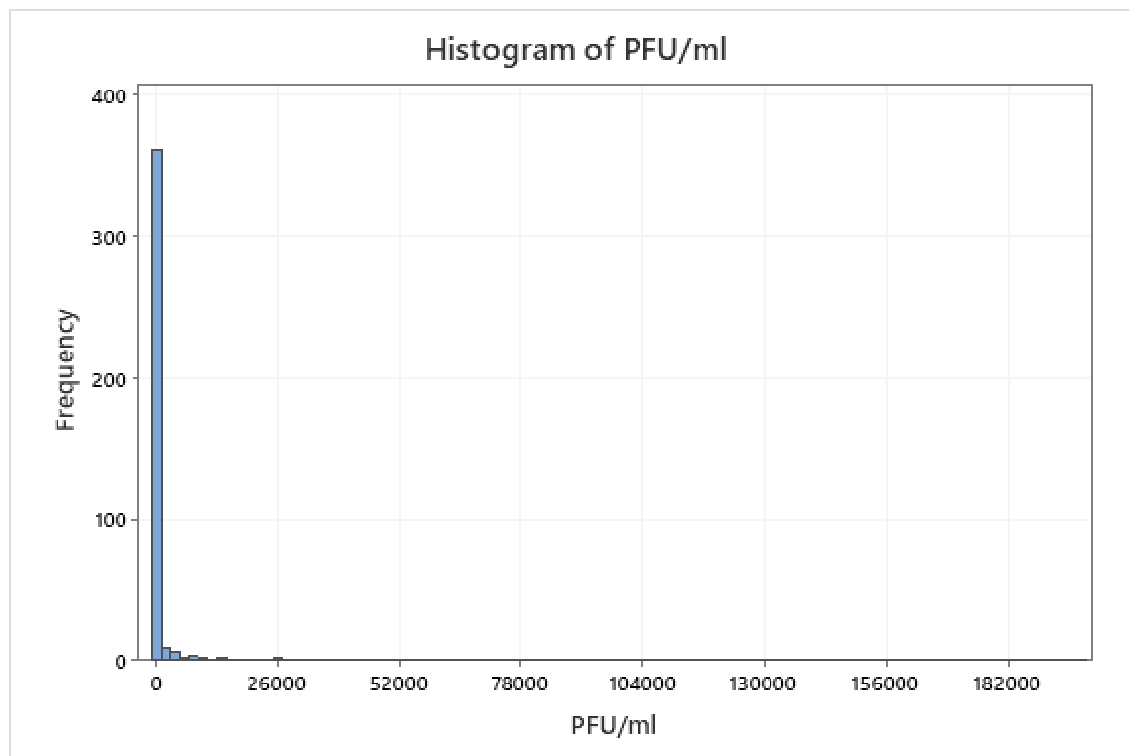
APPENDIX 10. DOTPLOT OF THE PFU/ML CHICKEN 9



PFUs / ml quantified in 9 different tests carried out on the feces from chicken 9. Graph was made with Microsoft Office Excel.

In chicken 9, phages were quantified since the first week. The highest peak of PFU/ml was quantified in strains from week 1 with phages from week 3. Two of the treatments with quantified phages correspond to week 1 and 2 with phages from the same week. On the other hand, other 2 peaks correspond to the strains of week 1 and 2 with the phages of 2 weeks later.

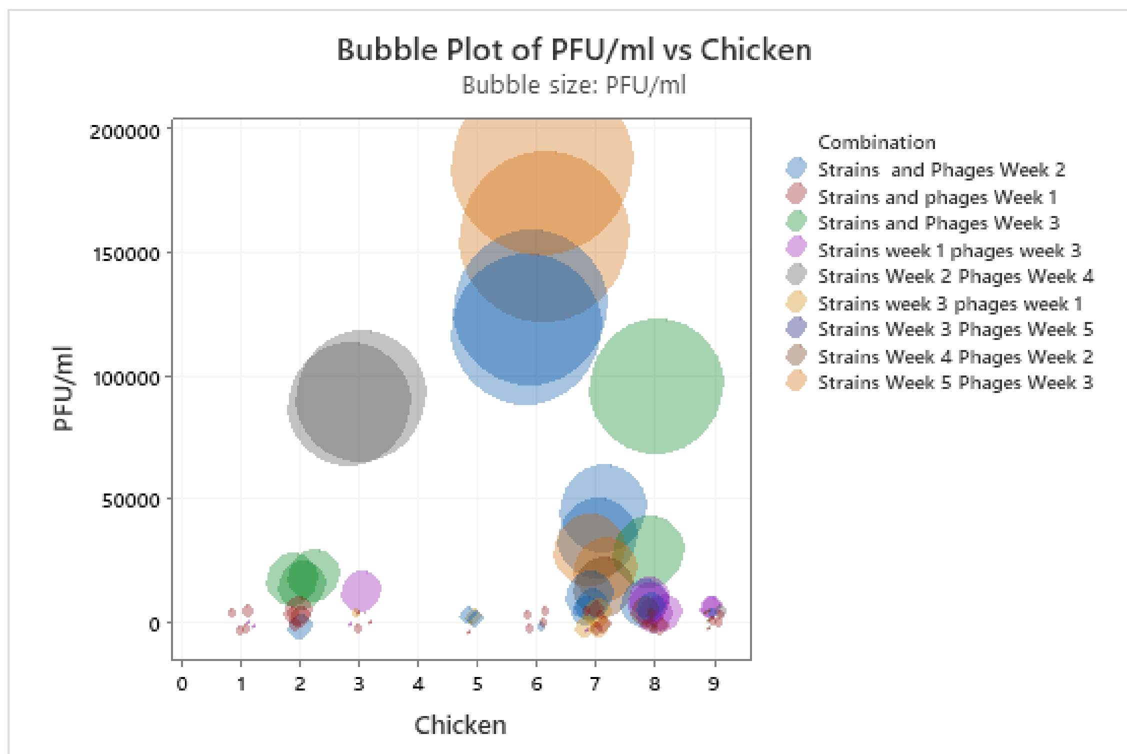
APPENDIX 11. HISTOGRAM OF PFU/ML



PFU/ ml to observe the frequency and distribution of the data in 100 intervals. The graph was made using Minitab 19 software.

Most of the results obtained in all the tests correspond to a frequency of zero, all other counts have a much lower frequency.

APPENDIX 12. BUBBLE PLOT OF PFU/ML VS. CHICKEN NUMBER.

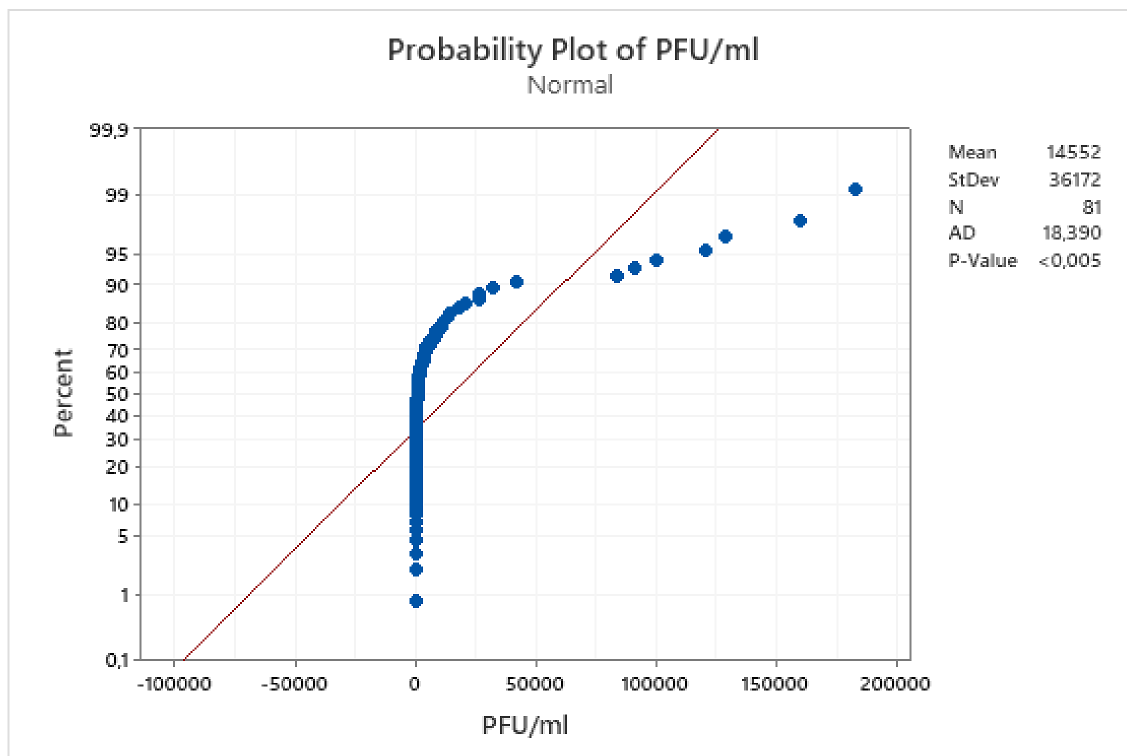


Each color represents different Phages and strains combination. The graph was made using Minitab 19 software.

There are bubbles in 8 of the 9 chickens. The largest bubbles are found in chicken 6 and correspond to the combination of strains and phages from week 2 and strains from week 5 with phages from week 3. Other 2 important bubbles are also observed in chicken 3, corresponding to the assay with strains from week 2 and phages from week 4; and in chicken 8 with strains from week 1 and phages from week 3.

The following tests were performed with the aim of verifying if the data meet the assumptions to perform the ANOVA test to determine which variables have a significant impact on the PFU / ml counts.

APPENDIX 13. PROBABILITY PLOT OF PFU/ML.



The graph presents the results obtained for the normality test. The null hypothesis of the test states that the residuals follow a normal distribution. On the other hand, the alternative hypothesis states that the residuals do not follow a normal distribution. We can see that the P value (0.005) obtained is less than the significance value of 0.05, so the null hypothesis is rejected, and it is concluded that the data do not follow a normal distribution. Different transformations were carried out to be able to normalize the data. The other graphs obtained are not shown, but there was no significant change in the P value and we were not able to normalize data. The graph was made using Minitab 19 software.

APPENDIX 14. TEST OF EQUALITY OF VARIANCES USING BONFERRONI CONFIDENCE INTERVALS FOR STANDARD DEVIATIONS.

95% Bonferroni Confidence Intervals for Standard Deviations

Combination Chicken Phage	Chicken	N	StDev	CI
Strains and Phages Week 2	2	1	*	(*; *)
Strains and Phages Week 2	5	2	169,7	(*; *)
Strains and Phages Week 2	6	3	71972,7	(*; *)
Strains and Phages Week 2	7	5	17308,6	(3620,47; 215275)
Strains and Phages Week 2	8	2	5289,2	(*; *)
Strains and Phages Week 2	9	1	*	(*; *)
Strains and phages Week 1	1	4	179,6	(2,72; 51525)
Strains and phages Week 1	2	4	1254,0	(16,16; 422211)
Strains and phages Week 1	3	4	43,9	(1,10; 7584)
Strains and phages Week 1	5	1	*	(*; *)
Strains and phages Week 1	6	4	150,0	(6,25; 15628)
Strains and phages Week 1	7	5	414,7	(81,23; 5509)
Strains and phages Week 1	8	3	300,0	(*; *)
Strains and phages Week 1	9	4	110,0	(1,17; 44860)
Strains and Phages Week 3	2	3	1967,8	(*; *)
Strains and Phages Week 3	8	2	52071,3	(*; *)
Strains week 1 phages week 3	1	2	7,1	(*; *)
Strains week 1 phages week 3	3	2	5922,7	(*; *)
Strains week 1 phages week 3	7	1	*	(*; *)
Strains week 1 phages week 3	8	5	2831,5	(215,00; 97016)
Strains week 1 phages week 3	9	2	297,0	(*; *)
Strains Week 2 Phages Week 4	3	2	5098,2	(*; *)
Strains Week 2 Phages Week 4	9	1	*	(*; *)
Strains week 3 phages week 1	3	1	*	(*; *)
Strains week 3 phages week 1	5	2	841,5	(*; *)
Strains week 3 phages week 1	7	3	801,6	(*; *)
Strains Week 4 Phages Week 2	7	1	*	(*; *)
Strains Week 4 Phages Week 2	8	3	2291,6	(*; *)
Strains Week 4 Phages Week 2	9	4	5,2	(0,18; 657)
Strains Week 5 Phages Week 3	6	2	16037,2	(*; *)
Strains Week 5 Phages Week 3	7	2	4037,6	(*; *)

Individual confidence level = 99,7917%

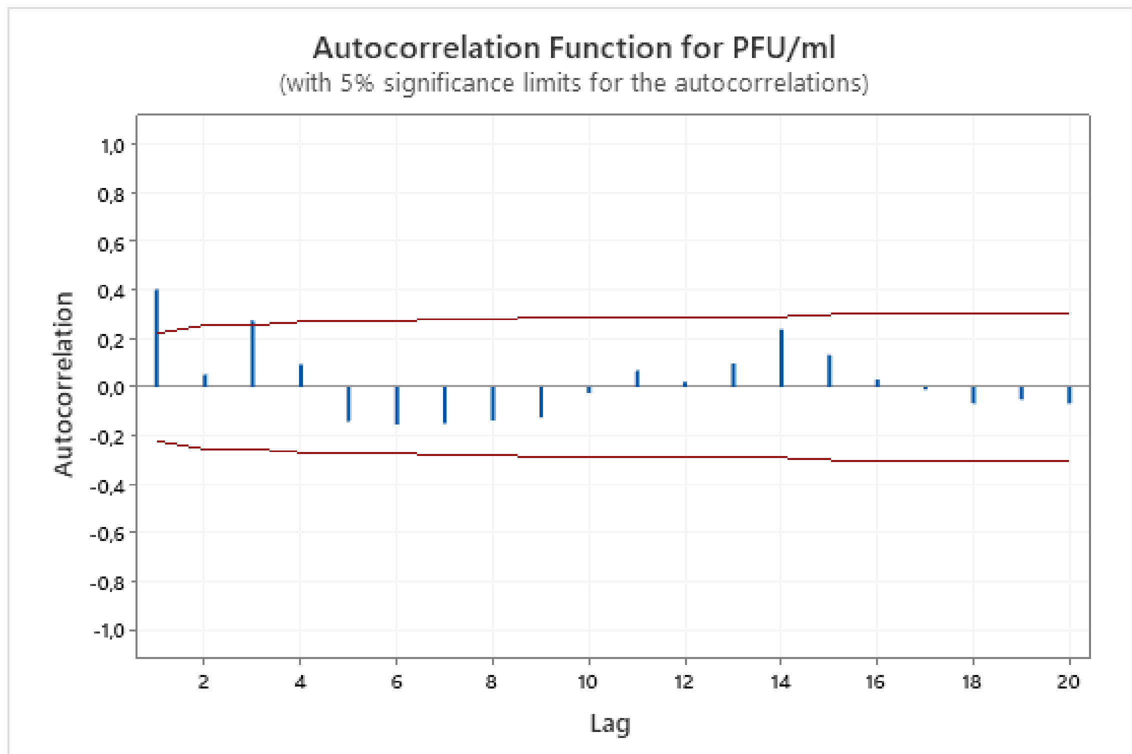
Tests

Method	Test Statistic	P-Value
Multiple comparisons	—	0,000
Levene	1,74	0,052

Samples are omitted from the tests if their standard deviations are 0 or missing.

For this test, null hypothesis establishes that there is equality of variances for the residuals between the treatments. On the other hand, the alternative hypothesis establishes that there is no equality of variances for the treatment residuals. This test yields 2 statistics, the multiple comparisons or Barlets statistic cannot be used in our data because it is for a normal distribution. Additionally, the Levene statistic cannot be used either because the data do not come from a continuous distribution. We can conclude that the test for equality of variances did not gave us a straightforward result. The graph was made using Minitab 19 software.

APPENDIX 15. RESIDUAL INDEPENDENCE TEST.



This test allows calculating the autocorrelation coefficients of the residuals and graphs them within the limits. Since there are calculated coefficients that exceed the limits, it can be concluded that there is no independence of the residuals. The graph was made using Minitab 19 software.

Since none of the 3 assumptions to perform ANOVA are met, a non-parametric analysis is carried out to make a comparison between factors and find if there is an association between the different variables tested.

The chosen test is Chi square to compare variances. The null hypothesis in this test establishes that the variables are independent therefore, there is no association between the variables. The alternative hypothesis states that the variables are not independent so, there is an association between the variables and the variables are dependent.

APPENDIX 16. TABULATED STATISTICS FOR GENETIC LINE BY PHAGE COMBINATION.

Rows: Phage Combination   Columns: Genetic Lineage

	Brahma	Cobb 500	Malines	All
2 weeks after	31740	183856	0	215596
	63879	42466	109251	
	-127,2	686,1	-330,5	
2 weeks before	77429	320	344510	422259
	125111	83173	213976	
	-134,8	-287,3	282,2	
Same Week	240070	47995	252790	540855
	160249	106532	274073	
	199,4	-179,3	-40,7	
All	349239	232171	597300	1178710

Cell Contents

Graph obtained from Minitab option for Cross tabulation and Chi-square for factors Phage combination and Genetic Lineage obtained using Minitab 19 software.

For this test it was sought to establish whether the combination between phages and strains has an interaction between the genetic lineages. The description of the phage pool is based on the collection time of the phages used to confront the strains. The data obtained was unified in 3 categories. The “2 weeks after category” implies that the strains were confronted with the phage cocktail obtained in 2 subsequent weeks. “The 2 weeks before” category implies that the strains were confronted with the phage cocktail obtained 2 weeks earlier. Finally, we find the data obtained in the strains that were tested against the phage cocktail of the same week. In the table, it is observed that most of the differences between observed and expected is less than zero, so most data are presided by the negative sign. Additionally, we can observe that there is a zero in the data corresponding to the Malines genetic line of chickens.

APPENDIX 17. CHI SQUARE TEST FOR GENETIC LINE BY PHAGE COMBINATION.

Chi-Square Test

	Chi-Square	DF	P-Value
Pearson	850090,642	4	0,000
Likelihood Ratio	821417,319	4	0,000

The table shows the degrees of freedom and the P values. Because the P value is less than the significance 0.05, the null hypothesis is rejected; therefore, it can be concluded that the variables combination of phages and genetic line are associated. Table obtained using Minitab 19 software.

Parallel to this, the Chi square association test was performed for the variables genetic line and phage combination.

APPENDIX 18. CHI-SQUARE TEST FOR ASSOCIATION: GENETIC LINE BY PHAGE COMBINATION.

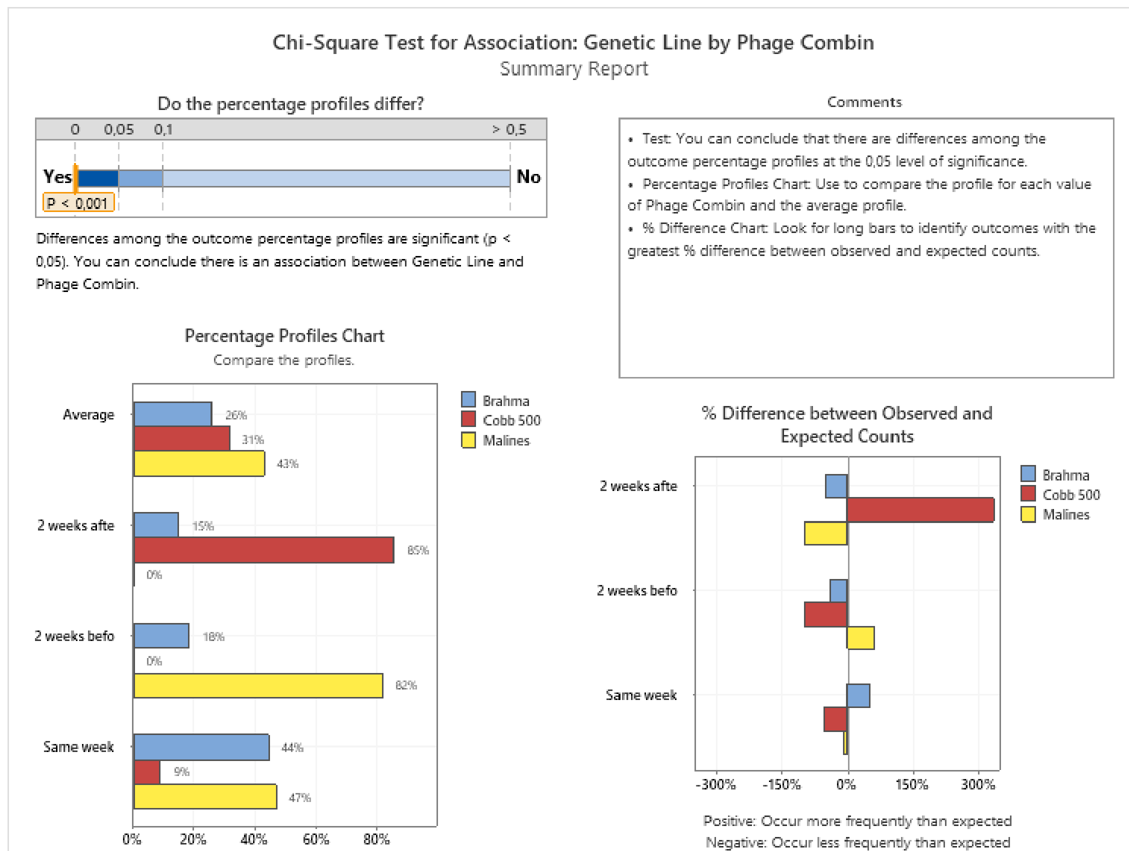
Chi-Square Test for Association: Genetic Line by Phage Combin

Diagnostic Report

Observed and Expected Counts

	2 weeks afte		2 weeks befo		Same week	
	Obs	Exp	Obs	Exp	Obs	Exp
Brahma	31740	63879	77429	125111	240070	160249
Cobb 500	183856	42466	320	83173	47995	106532
Malines	0	109251	344510	213976	252790	274073
Total	215596		422259		540855	

Expected counts should be at least 2 to ensure the validity of the p-value for the test.



Chi-Square Test for Association: Genetic Line by Phage Combin
Report Card

Check	Status	Description
Validity of Test	✓	All samples are large enough to obtain sufficient expected counts. The p-value for the test should be accurate.

In the association test, the summary table with the observed and expected data is observed. When we look at the graph of the percentage difference between observed and expected counts, the longest bar corresponds to the Cobb 500 breed chicken, with a phage treatment 2 weeks later. On the other hand, in the percentage profiles graph, the Malines breed had a higher average percentage. Additionally, the second highest percentage after the Cobb 500 chickens corresponds to the Malines chickens, with a phage treatment from the previous 2 weeks.

It is concluded that the chi-square association test does obtain valid data, since the sample size is large enough and therefore the p-value does have an accurate result. The graph was made using Minitab 19 software

After this, the same analysis was carried out, to see the association between factors, but now we wanted to differentiate each chicken.

APPENDIX 19. TABULATED STATISTICS FROM MINITAB OPTION FOR CROSS TABULATION AND CHI-SQUARE FOR FACTORS PHAGE COMBINATION AND CHICKEN

Rows: Phage Combination   Columns: Chicken

	1	2	3	5	6	7	8	9
2 weeks after	30	0	183826	0	0	10	27900	3830
	210	8533	33722	565	108686	30903	32132	843
	-12,43	-92,38	817,40	-23,77	-329,68	-175,74	-23,61	102,88
2 weeks before	0	0	320	1230	343280	72865	4515	49
	412	16713	66047	1107	212869	60526	62934	1651
	-20,30	-129,28	-255,75	3,70	282,66	50,15	-232,87	-39,43
Same Week	1120	46654	221	1860	250930	96080	143260	730
	528	21407	84597	1418	272655	77526	80609	2115
	25,79	172,55	-290,10	11,74	-41,61	66,64	220,67	-30,11
All	1150	46654	184367	3090	594210	168955	175675	4609

	All
2 weeks after	215596
2 weeks before	422259
Same Week	540855

In the summary table, as the categories increased, the number of zeros obtained for the observed data also increased and that these are mainly found in the assays that included phages 2 weeks later and 2 weeks earlier. Zero values were not obtained in the tests carried out with the phages of the same week. When relating the observed values with the expected ones, negative values are obtained, indicating that the observed values were lower than those expected. Graph obtained using Minitab 19 software.

APPENDIX 20. CHI SQUARE TEST FOR FACTORS PHAGE COMBINATION AND CHICKEN

Chi-Square Test

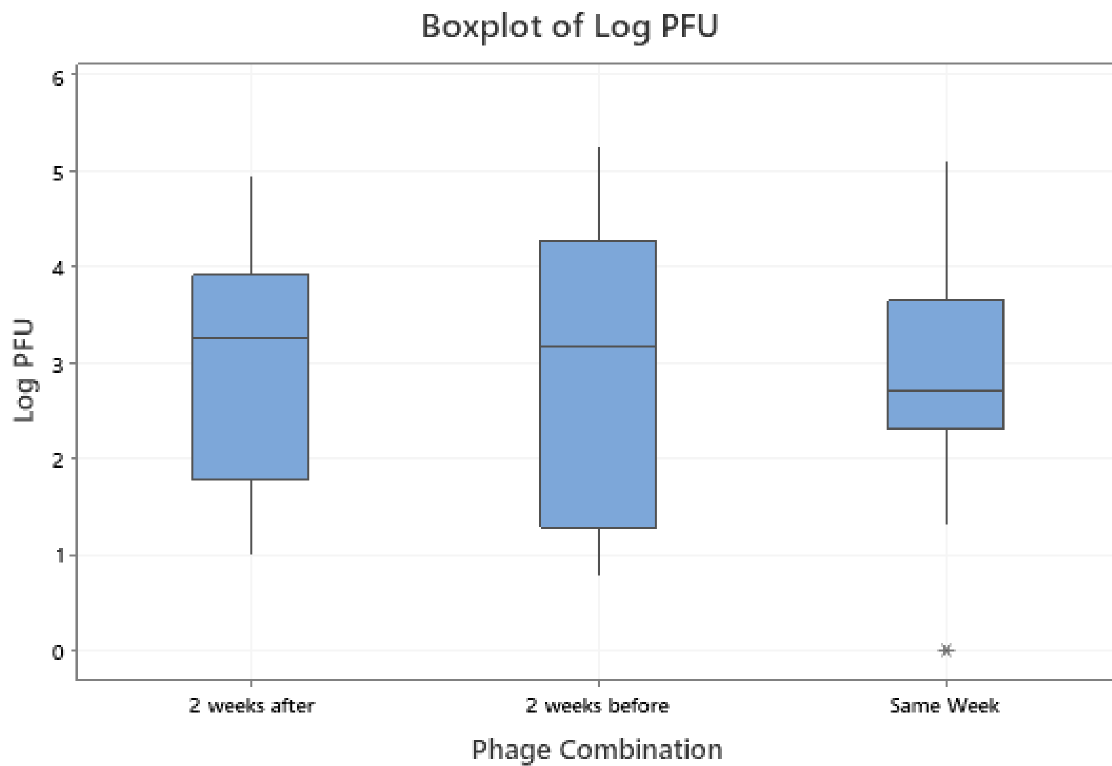
	Chi-Square	DF	P-Value
Pearson	1219346,413	14	0,000
Likelihood Ratio	1190275,561	14	0,000

The table shows that the degrees of freedom increased from 4 in the previous evaluation to 14 in the current one. Again, the P values are less than the significance of 0.05, so we can conclude that the studied factors are associated to affect the value of the response variable obtained in PFU / ml. The null hypothesis is rejected, so there is a significant difference in the response variable depending on the factors studied.

The chi-square association test could not be carried out for the association of the variables combination of phages and chicken number, because the software only allows up to 6 categories of columns and in the case of our test, they are obtained 9 chickens. By using the same data as in the previous test, we can conclude that the results of the current test are equally reliable and that the obtained P-value correctly shows the significance, since we have a sample large enough to conclude about the results in such a general way test obtained using Minitab 19 software.

To determine which factor contributed the most to the observed difference, we proceeded to perform the non-parametric Kruskal-Wallis test to determine the difference between means in data that have a similar distribution.

Appendix 21: Boxplot of Log PFU vs Phage combination



We can conclude that although the interquartile range of the boxes is not similar, the value of the means of the data and the whiskers is similar for this factor so if the Kruskal-Wallis test can be performed.

APPENDIX 22. KRUSKAL-WALLIS TEST FOR RESPONSE VARIABLE PFU/ML VERSUS PHAGE COMBINATION

Descriptive Statistics

Phage Combination	N	Median	Mean Rank	Z-Value
2 weeks after	15	1850	44,4	0,61
2 weeks before	18	1485	40,6	-0,07
Same Week	48	500	40,1	-0,42
Overall	81		41,0	

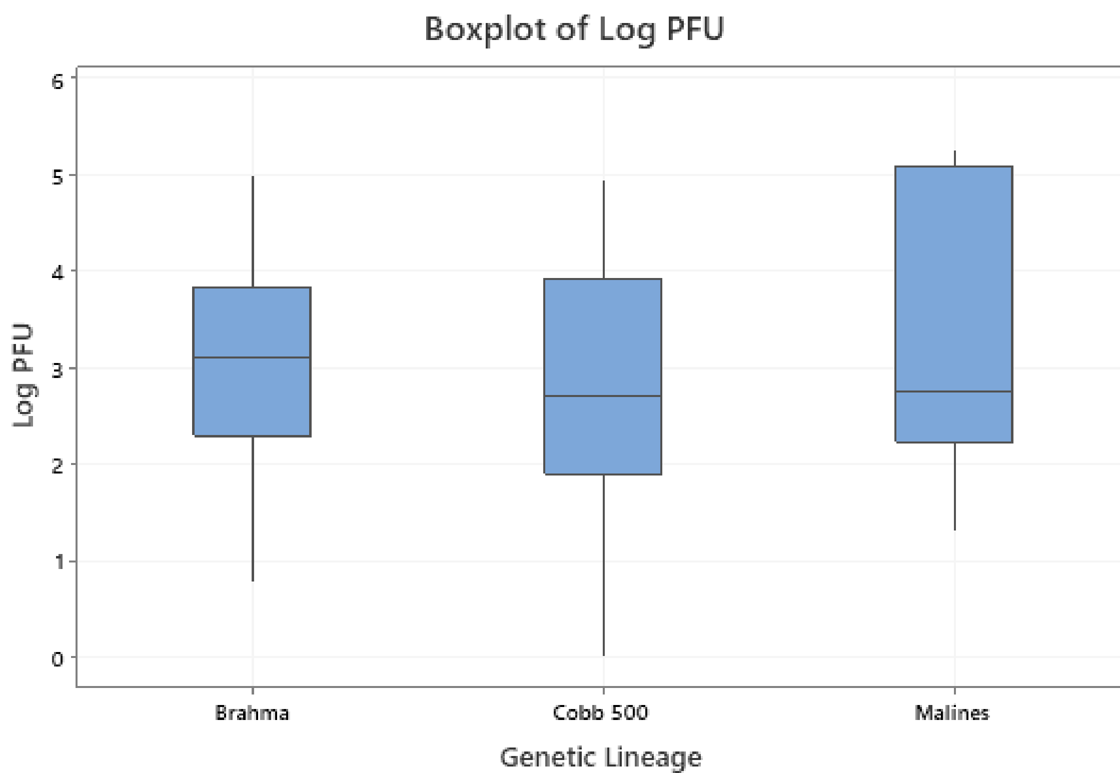
Test

Null hypothesis H_0: All medians are equal
Alternative hypothesis H_1: At least one median is different

Method	DF	H-Value	P-Value
Not adjusted for ties	2	0,38	0,825
Adjusted for ties	2	0,38	0,825

The P value is not less than the significance value 0.05 so there is no significant evidence to reject the null hypothesis so with respect to this value, we cannot say that there is a difference in the value of the means. Test obtained using Minitab 19 software

APPENDIX 23: BOXPLOT OF LOG PFU VERSUS GENETIC LINEAGE



This graph is like the previous one, with different interquartile range and similar value of the means of the data and the whiskers. For this factor the Kruskal-Wallis test can be performed.

APPENDIX 24: KRUSKAL WALLIS TEST FOR RESPONSE VARIABLE PFU/ML VERSUS GENETIC LINEAGE

Descriptive Statistics

Genetic Lineage	N	Median	Mean Rank	Z-Value
Brahma	44	1265	42,5	0,64
Cobb 500	23	500	36,8	-1,01
Malines	14	600	43,0	0,35
Overall	81		41,0	

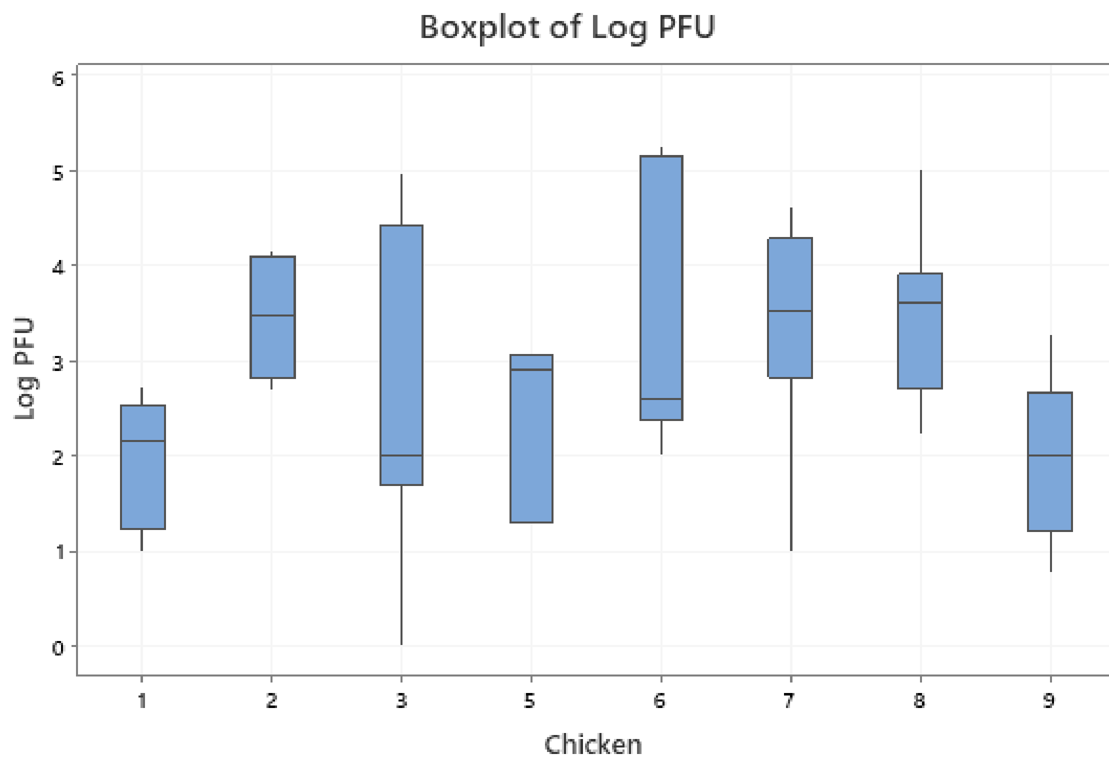
Test

Null hypothesis	H_0: All medians are equal
Alternative hypothesis	H_1: At least one median is different

Method	DF	H-Value	P-Value
Not adjusted for ties	2	1,01	0,602
Adjusted for ties	2	1,02	0,602

There is no significant evidence to reject the null hypothesis according to the P value larger than the significance value of 0.05 so there is no evidence to stablish a difference in the means value. Test obtained using Minitab 19 software

APPENDIX 25: **BOXPLOT OF LOG PFU VS. CHICKEN.**



In this graph is evident that interquartile ranges, means value and wishers value is different between each chicken.

APPENDIX 26: **KRUSKAL WALLIS TEST FOR RESPONSE VARIABLE PFU/ML VERSUS CHICKEN**

Descriptive Statistics

Chicken	N	Median	Mean Rank	Z-Value
1	6	150	20,4	-2,23
2	8	2942	52,8	1,49
3	9	100	33,6	-1,00
5	5	800	29,9	-1,09
6	9	400	50,3	1,25
7	17	3260	50,9	1,94
8	15	4151	50,3	1,69
9	12	100	21,1	-3,17
Overall	81		41,0	

Test

Null hypothesis H_0: All medians are equal

Alternative hypothesis H_1: At least one median is different

Method	DF	H-Value	P-Value
Not adjusted for ties	7	23,86	0,001
Adjusted for ties	7	23,88	0,001

The P value in this case, if it is less than the alpha significance level of 0.05 so there is significant evidence to reject the null hypothesis and therefore, we can say that at least one of the means is different.