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Interaction Between Bacteriophages and *Escherichia coli* in *Gallus gallus* Intestines

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Interaction Between Bacteriophages and *Escherichia coli* in *Gallus gallus* Intestines

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DEDICATORIA

A mi madre y mi padre, Martha y Galo, quienes con su amor me han guiado y apoyado en mi crecimiento personal y profesional. A mi gato, Coco, quien es la luz que alumbra cada nueva meta, acompañándome cada día.

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RESUMEN

El recambio de las poblaciones de *Escherichia coli* en el intestino sugiere que muchas cepas son únicas y se presentan solo una vez en cada individuo. Los factores que impulsan este recambio en las poblaciones intestinales de *E. coli* aún no se comprenden completamente. Se han propuesto las interacciones entre bacteriófagos y bacterias como posible factor del recambio. Este estudio investigó las interacciones entre cepas dominantes de *E. coli* y poblaciones de bacteriófagos aisladas de cinco pollos de engorde Cobb500 (*Gallus gallus*) durante tres semanas de muestreo (W1, W3 y W6). Se evaluó la sensibilidad de 75 cepas de *E. coli* a la infección por bacteriófagos mediante ensayos de doble capa, analizando cada cepa frente a extractos de bacteriófagos recolectados durante los periodos de muestreo. El número de cepas sensibles de *E. coli* variaron según el individuo y la semana de muestreo. No se observaron diferencias estadísticamente significativas en la sensibilidad de las cepas de *E. coli* a los bacteriófagos recolectados en la misma semana a lo largo de las tres semanas de muestreo. Se realizó un análisis metagenómico para evaluar el recambio de las poblaciones de bacteriófagos aisladas de las semanas W1 y W3, utilizando seis cepas seleccionadas de *E. coli* de la semana W1 como hospedadores para el enriquecimiento de bacteriófagos. Se identificaron veintisiete taxones de bacteriófagos, con una diversidad que varió según el individuo y la semana de muestreo. Nuestros resultados sugieren que los bacteriófagos no desempeñan un papel significativo en el recambio de las cepas dominantes de *E. coli* en el intestino. Sin embargo, la coexistencia de bacteriófagos con sus cepas hospedadoras sensibles, que son numéricamente dominantes, podría generar fluctuaciones en las poblaciones de bacteriófagos.

Palabras clave: *Escherichia coli*; Renovación; Cepa dominante; Pollos de engorde Cobb500 (*Gallus gallus*); Interacción bacteriófago-bacteria.

ABSTRACT

The turnover of *Escherichia coli* populations in the intestine suggests that strains are unique and occur only once in each individual. The factors driving this turnover in intestinal *E. coli* populations remain poorly understood. Bacteriophage-bacteria interactions have been proposed as a potential driver influencing this turnover. This study investigated the interaction between numerically dominant *E. coli* strains and bacteriophage populations isolated from five Cobb500 broilers (*Gallus gallus*) across three sampling weeks (W1, W3, and W6). We assessed the sensitivity of 75 *E. coli* strains to bacteriophage infection using double-layer assays, evaluating each strain against bacteriophage extracts collected during the sampling periods. Sensitive *E. coli* strains varied per individual and sampling week. No statistically significant differences were observed in sensitive *E. coli* strains by bacteriophages from the same week across the three sampling weeks. Metagenomic analysis was performed to evaluate the turnover of bacteriophage populations isolated from weeks W1 and W3, using six selected *E. coli* strains from W1 as hosts for bacteriophage enrichment. Twenty-seven bacteriophage taxonomic identifications were detected, with diversity varying per individual and sampling week. Our results suggest that bacteriophages may not play a significant role in driving the turnover of numerically dominant *E. coli* strains in the intestine. Nonetheless, the coexistence of bacteriophages with their sensitive host strains, which are numerically dominant, may drive fluctuations in bacteriophage populations.

Keywords: *Escherichia coli*; Turnover; Numerically dominant strain; Cobb500 broilers (*Gallus gallus*); Bacteriophage-bacteria interaction.

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PART I: GENERAL INTRODUCTION

The gastrointestinal tract (GIT) of humans and animals constitutes a complex ecosystem, hosting diverse microbial communities, including bacteria, viruses, fungi, archaea, and protozoa, collectively known as the gut microbiome (Guerin & Hill, 2020; Priya & Blekhman, 2019; Zuppi et al., 2022). The gut microbiome has evolved alongside its hosts over millions of years, contributing significant health benefits by being involved in essential metabolic and physiological processes (Guerin & Hill, 2020; Guinane & Cotter, 2013). Disruptions to the gut microbiome, characterized by alterations in microbial community composition, have been strongly associated with various diseases, from metabolic disorders to immune dysfunctions (Alkhalil, 2023; Guerin & Hill, 2020; Zuppi et al., 2022).

Longitudinal studies have reported the long-term stability of the gut human microbiota composition, while also investigating the significance of microbial turnover in maintaining health over time (Ghalayini et al., 2018; Johnson et al., 2008; Mehta et al., 2018). Yet, the underlying factors driving this turnover remain poorly understood (Priya & Blekhman, 2019). Therefore, comprehending the ecological and evolutionary dynamics of the gut microbiome is essential for a deeper understanding of its role in health maintenance and disease progression.

Escherichia coli in the gut microbiome

Escherichia coli is the facultative anaerobe bacterium predominantly found in the intestines of warm-blooded animals, representing around 0.1 - 1.21% of the gut microbiome (Han et al., 2024; Hu et al., 2022; Loayza et al., 2020) As one of the earliest gut colonizers after birth or hatching (Stromberg et al., 2017), *E. coli* is essential in shaping the initial microbial community (Moreira de Gouveia et al., 2024). *E. coli* resides within the mucus layer covering the epithelial

cells, from where it is periodically released along with degraded mucus components into the intestinal lumen and subsequently excreted in feces (Foster-Nyarko & Pallen, 2022).

The intestinal *E. coli* population is represented by strains distributed across seven phylogenetic groups: A, B1, B2, C, D, E, and F (Han et al., 2024). These strains vary not only in their abundance within the gut but also in the duration of their presence (Loayza et al., 2020). Strains that show a high degree of adaptation to specific intestinal regions tend to be long-term colonizers and are often defined as numerically dominant due to their majority in significant numbers within the microbial population (Calderón et al., 2022; Lautenbach et al., 2008; Loayza et al., 2020). To elucidate this dominance, single-gene long-read sequencing was employed, identifying 32 dominant *E. coli* amplicon sequence variants (ASVs) in fecal samples from 16 individuals (Hu et al., 2022).

Turnover in intestinal *E. coli* populations

The high genetic diversity of *E. coli* populations and the temporal variability of strains in the intestines of different host animals highlight a dynamic intestinal population (Barrera et al., 2019; Han et al., 2024; Loayza et al., 2020). Longitudinal studies in humans have enabled the analysis of the stability of these intestinal *E. coli* populations over time. In a cohort of eight healthy adults, slight stability and potential turnover in the clonal populations of the *Enterobacteriaceae* family were observed over approximately two years, with samples collected every two weeks (Martinson et al., 2019). The genomic diversity and population variability of resident *E. coli* were also observed in 30 children (aged 2 to 35 months) at three time points over six months (Richter et al., 2018). Likewise, numerically dominant *E. coli* turnover was observed in the intestines of 30

children (aged six months to four years) in less than three months, over one year (Calderón et al., 2022).

The turnover of *E. coli* populations in the intestine suggests that many of the strains encountered are unique and occur only once in each individual, with a potential turnover rate of 87.5% over one month (Han et al., 2024). This rapid turnover of intestinal strains provides valuable insights into the relationship between *E. coli* and human health by establishing a potential environment for the introduction of pathogenic strains (Barrera et al., 2019; Han et al., 2024; Loayza et al., 2020). Furthermore, this dynamic of constant turnover in the gut could select for pathogenic strains and phylogenetic groups of *E. coli* with antimicrobial resistance genes (ARGs) in their accessory genome—which makes up around 80% of the pangenome—suggesting a potential risk for disease development and challenges in treatment due to the spread of these strains (Calderón et al., 2022; Hu et al., 2022).

The factors related to the turnover in *E. coli* intestinal populations are still poorly understood. The transmission of *E. coli* strains between animals and humans through fecal material could represent one of the main factors contributing to this turnover (Amato et al., 2023; Anderson et al., 2006; Hedman et al., 2019; Johnson et al., 2008). However, it has been observed that strains transmitted by domestic animals do not thrive in the human GIT but are able to transmit ARGs via plasmids to dominant *E. coli* strains (Amato et al., 2023; Hedman et al., 2019; Loayza et al., 2020). Feeding, fecal flow in the intestine and environment, intestinal movements, antibiotic use, and antagonism between intestinal communities (bacteria-bacteriophage) are possible factors influencing *E. coli* turnover in the intestine (Anderson et al., 2006; Loayza et al., 2020; Richter et al., 2018; Zuppi et al., 2022).

Bacteriophages and their interaction with the host bacteria

Bacteriophages (phages), or viruses that infect bacteria, are the most abundant biological entities on Earth, with over 10^{31} virus-like particles (VLPs) found in every explored ecosystem (Sausset et al., 2020; Shkoporov et al., 2022; Tenorio-Carnalla et al., 2024). Structurally, they are composed of double-stranded or single-stranded DNA (dsDNA, ssDNA) or RNA (dsRNA, ssRNA) packaged within a protein shell called a capsid (pleomorphic, filamentous, or polyhedral in shape) (Alkhalil, 2023; Sausset et al., 2020). Moreover, they may or may not possess a tail and be enveloped in a lipid membrane (Alkhalil, 2023). Thus, phages have been classified according to the type of genetic material, the morphology of their capsid, and the presence or absence of an envelope and a tail (Sausset et al., 2020; Tenorio-Carnalla et al., 2024).

Bacteriophage-bacteria interactions are among the most frequent biological events. Upon infection of bacterial cells, phages can cause lysis or insertion of their genetic material into the bacterial genome (Cao et al., 2017; Shkoporov et al., 2022). According to their life cycle, phages can be virulent or temperate (Sausset et al., 2020). Virulent or lytic phages are those that, after recognizing and attaching to a specific bacterial receptor, inject their genome into the bacterial cell, replicate, and assemble viral particles (virions), which are released by phage-mediated lysis of the bacterial cell (Alkhalil, 2023; Cao et al., 2017). However, under conditions such as nutrient deprivation, phage replication can be temporarily halted, entering a state of pseudolysogeny (Davies et al., 2016; Sausset et al., 2020).

Conversely, lysogenic or temperate phages can integrate their genetic material into the bacterial genome, entering a prophage state where it is replicated together with the host genome (Alkhalil, 2023; Cao et al., 2017; Zuppi et al., 2022). The prophage can be stably maintained in the chromosomal or extrachromosomal genome of the host and transmitted to progeny (Shkoporov

et al., 2022). However, these viruses can enter a lytic phase when the host is under stress. A pathogenic strain can be infected by multiple prophages, as observed in *Clostridium difficile* strains, *Escherichia coli* O157:H7, and *Enterococcus faecalis* (Davies et al., 2016). The lysogenic cycle ends when prophage induction occurs in response to stressors or stimuli in the host cell, which promotes a lytic cycle with the production and release of new virions (Davies et al., 2016; Sausset et al., 2020; Zuppi et al., 2022).

Furthermore, a life cycle has been described in filamentous bacteriophages of the family *Inoviridae*, termed the chronic cycle (Shkoporov et al., 2022). This cycle is characterized by the entry of phage genetic material and its subsequent integration into the genome, as in the lysogenic cycle, with the continuous production of progeny that are released by extrusion without lysis of the bacterial cell (Sausset et al., 2020; Venturini et al., 2022). Like the pseudolysogeny state, the chronic cycle of bacteriophages is still poorly understood.

Bacteriophages in the gut microbiome

Bacteriophage communities within the intestine constitute the so-called virome or phageome. The advent of high-throughput metagenomic sequencing technologies has enabled the characterization of both the abundance ($\sim 10^9$ - 10^{10} VLPs per gram of feces) and the diversity ($\sim$200,000 distinct species identified) of intestinal bacteriophages (Cao et al., 2017). This diverse bacteriophage population is predominantly composed of viruses with icosahedral capsids and tails, classified within the dsDNA class *Caudoviricetes* and the ssDNA family *Myoviridae*, alongside filamentous ssDNA phages from the family *Inoviridae*. RNA viruses, by comparison, are relatively scarce in the intestinal environment, representing part of approximately 90% of the intestinal phage community that remains unclassified.

The composition of phages in the intestine is established shortly after birth, with viral richness and diversity reaching their peak during this early period (Cao et al., 2017). The relationship between viral and bacterial communities in neonates is characterized by a high bacteriophage diversity at birth, which subsequently shifts, marked by a decrease in viral populations and an increase in bacterial diversity by 2 – 3 years of age (Lim et al., 2015; Lou et al., 2024). Longitudinal studies in healthy adults have identified a homeostatic balance between bacteriophage and bacterial communities within the intestine, demonstrating the stability of viral populations over time, at least for one year (Shkoporov et al., 2019). Furthermore, these studies revealed notable inter-individual variability, with only a limited number of conserved bacteriophage species (e.g., crAss-like and Phix174 from *Microviridae* family) shared between individuals, and a predominance of lysogenic phages in the intestinal microbiota (20-50%) (Dikareva et al., 2023; Sausset et al., 2020; Shkoporov et al., 2019; Townsend et al., 2021; Wang et al., 2019).

However, the composition of phage within intestine can be affected by other factors, including diet (Minot et al., 2011) and gastrointestinal disorders such as *C. difficile* infections, inflammatory bowel diseases, ulcerative colitis, and Crohn's disease (Alkhalil, 2023). These changes in phage populations imply a potential ecological role of these entities within the gut, with the capacity to interact with and modulate both the host immune system and, more importantly, the gut microbiome (Zuppi et al., 2022).

Bacteriophages - bacteria interaction in the intestine

The interaction between bacteriophages and bacteria leads to a predator-prey dynamic (Alkhalil, 2023). Through the lytic cycle, bacteriophages exert selective pressure on the bacterial

population, eliminating susceptible bacteria while favoring those with mechanisms that confer resistance to viral infection (Zuppi et al., 2022). These resistant bacterial strains, in turn, evolve strategies to counteract phage-mediated predation. This results in an arms-race dynamic, where bacteria and phages co-evolve, with bacterial populations constantly selected for resistance and phages for enhanced infectivity (Alkhalil, 2023; Shkoporov et al., 2022). This ongoing interplay fosters a rapid turnover of microbial populations, thereby increasing bacterial and phage diversity within the gut (Fortuna et al., 2019).

Bacteriophage-bacteria interactions in GIT involve more complex population dynamics than the simple predator-prey relationship. These dynamics can include infections by lysogenic phages, also known as the piggy-back-the-winner dynamic (Davies et al., 2016; Dikareva et al., 2023; Tenorio-Carnalla et al., 2024). This interaction suggests a form of mutualism, wherein the infection of host bacteria by lysogenic phages leads to the integration of the prophage into the bacterial genome, conferring beneficial effects on the bacteria (Shkoporov et al., 2022). These benefits include enhanced fitness, improved colonization capacity, and competitive exclusion of foreign pathogens. Consequently, the prophage's survival is directly dependent on the host, as it replicates alongside the bacterial genome, ensuring its persistence within the intestinal population (Dikareva et al., 2023; Shkoporov et al., 2022).

It has been observed that population dynamics involving both antagonism and mutualism in phage-bacteria interactions are highly dependent on the environment in which they occur. In GIT, factors such as pH, bile salt concentrations, structural conformations, oxygen levels, nutrient availability, and the mucin layer influence these dynamics (Zuppi et al., 2022). Within the intestine, phages are distributed across the lumen and the mucin membrane, and their interaction with bacteria is determined by the gradient between these two communities (Barr et al., 2013).

The virus-to-microbe ratio (VMR) in the lumen and on the surface of the mucin layer (∼0.1–1:1) suggests a predominance of lysogenic infections in regions with high bacterial concentrations (Barr et al., 2013; Dikareva et al., 2023; Shkoporov & Hill, 2019; Zuppi et al., 2022). Lysogenic infections in these intestinal areas confer an advantage by promoting rapid bacterial proliferation and concurrent viral genome replication with the host (Barr et al., 2013; Shkoporov & Hill, 2019). At the same time, bacteria gain a competitive edge by integrating prophages carrying beneficial genes (piggy-back-the-winner). This dynamic further facilitates horizontal gene transfer (HGT) between bacterial strains and populations (Shkoporov et al., 2022).

Conversely, deeper within the mucin layer, a VMR of ∼20:1 suggests population dynamics dominated by lytic infections, characterized by an arms race, kill-the-winner strategies, and fluctuating selection dynamics (Barr et al., 2013). Nonetheless, despite the predominance of lysogenic phages suggested by the VMR at the mucosal surface and in the lumen, these regions have also identified strictly virulent phages from the *Microviridae* family (Shkoporov & Hill, 2019). The presence of these virulent phages, without causing substantial disruption to bacterial populations, can be explained by intrinsic factors of bacteria, such as their growth phase, receptor expression, as well as structural characteristics of the gut, where heterogeneous regions that act as a refuge for bacteria are abundant (Barr et al., 2013; Shkoporov & Hill, 2019).

Coevolution studies have provided valuable insights into phage-bacteria interactions, understanding them as an arms race, where coevolution between the two microbial communities drives the selection of resistant bacteria and more virulent phages (De Sordi et al., 2019; Koskella & Brockhurst, 2014). *In vitro* assays have focused on these population dynamics, often considering a single bacterial population exposed to a specific phage (Buckling & Rainey, 2002; Fortuna et al., 2019; Laanto et al., 2017). Nevertheless, as previously noted, the gut is a highly complex

ecosystem, composed of diverse microbial communities (De Sordi et al., 2019; Shkoporov & Hill, 2019). Therefore, understanding phage-bacteria interactions within the gut requires considering population dynamics at the community level, rather than in isolation (Koskella & Brockhurst, 2014; Lourenço et al., 2018).

Few experimental studies *in vitro* have investigated the pairwise interactions between phages and bacteria at the community level. For instance, it has been shown that the resistance of the plant pathogen bacterium *Pseudomonas syringae* to both a heterogeneous phage population and a single phage genotype evolved similarly, but with a significant fitness cost to maintaining resistance in a heterogeneous community (Koskella et al., 2012). Additionally, it was observed that a *P. aeruginosa* PAO1 strain evolved in the presence of two phages (PP7 and E79), exhibiting a decrease in growth rate compared to exposure to a single phage (Hosseinidoust et al., 2013).

Finally, *in vivo* studies in mice have allowed an approach to the complex dynamics of the gut microbiome (De Sordi et al., 2017; Reyes et al., 2013). In this way, these studies highlight population dynamics observed in the murine intestines where the role of an intermediate *E. coli* MEc1 strain is on facilitate the infection of the previously resistant *E. coli* MG1655 strain by phage P10 has been described (De Sordi et al., 2017). This mechanism, known as host jump, was driven by a single nucleotide polymorphism (SNP) in the genes *gp90* and *gp91* encoding the tail fiber. This mechanism could not be observed in *in vitro* assay with the same population (De Sordi et al., 2017, 2019).

PART II: SCIENTIFIC ARTICLE

Interaction Between Bacteriophages and *Escherichia coli* in *Gallus gallus* Intestines

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Abstract

The turnover of *Escherichia coli* populations in the intestine suggests that strains are unique and occur only once in each individual. The factors driving this turnover in intestinal *E. coli* populations remain poorly understood. Bacteriophage-bacteria interactions have been proposed as a potential driver influencing this turnover. This study investigated the interaction between numerically dominant *E. coli* strains and bacteriophage populations isolated from five Cobb500 broilers (*Gallus gallus*) across three sampling weeks (W1, W3, and W6). We assessed the sensitivity of 75 *E. coli* strains to bacteriophage infection using double-layer assays, evaluating each strain against bacteriophage extracts collected during the sampling periods. Sensitive *E. coli* strains varied per individual and sampling week. No statistically significant differences were observed in sensitive *E. coli* strains by bacteriophages from the same week across the three sampling weeks. Metagenomic analysis was performed to evaluate the turnover of bacteriophage populations isolated from weeks W1 and W3, using six selected *E. coli* strains from W1 as hosts for bacteriophage enrichment. Twenty-seven bacteriophage taxonomic identifications were detected, with diversity varying per individual and sampling week. Our results suggest that bacteriophages may not play a significant role in driving the turnover of numerically dominant *E. coli* strains in the intestine. Nonetheless, the coexistence of bacteriophages with their sensitive host strains, which are numerically dominant, may drive fluctuations in bacteriophage populations.

Keywords: *Escherichia coli*; Turnover; Numerically dominant strain; Cobb500 broilers (*Gallus gallus*); Bacteriophage-bacteria interaction.

Introduction

Escherichia coli is the facultative anaerobe bacterium predominantly found in the intestines of warm-blooded animals, representing around 0.1 - 1.21% of the gut microbiome (Han et al., 2024; Hu et al., 2022; Loayza et al., 2020). The intestinal *E. coli* population is represented by strains distributed across seven phylogenetic groups: A, B1, B2, C, D, E, and F (Han et al., 2024). Strains that show a high degree of adaptation to specific intestinal regions tend to be long-term colonizers and are often defined as numerically dominant (Calderón et al., 2022; Lautenbach et al., 2008; Loayza et al., 2020). To date, 32 dominant *E. coli* amplicon sequence variants (ASVs) were identified in fecal samples collected from 16 individuals (Hu et al., 2022).

The turnover of *E. coli* populations in the intestine suggests that many of the strains encountered are unique and occur only once in each individual, with a potential turnover rate of 87.5% over one month (Han et al., 2024). This constant turnover in the gut could select for pathogenic strains and phylogenetic groups of *E. coli* with antimicrobial resistance genes (ARGs) in their accessory genome—which makes up around 80% of the pangenome—suggesting a potential risk for disease development and challenges in treatment due to the spread of these strains (Calderón et al., 2022; Hu et al., 2022).

The factors related to the turnover in intestinal *E. coli* populations remain poorly understood. Nonetheless, transmission of *E. coli* strains between animals and humans through fecal material (Amato et al., 2023; Anderson et al., 2006; Hedman et al., 2019; Johnson et al., 2008), food, fecal flow in the intestine and environment, intestinal movements, antibiotic use, and antagonism between intestinal populations (bacteria-bacteriophage) are possible factors

influencing *E. coli* turnover in the intestine (Anderson et al., 2006; Loayza et al., 2020; Richter et al., 2018; Zuppi et al., 2022).

Bacteriophages (phages), or viruses that infect bacteria, are the most abundant biological entities on Earth (Sausset et al., 2020; Shkoporov et al., 2022). Bacteriophage-bacteria interactions are among the most frequent biological events. Upon infection of bacterial cells, bacteriophages can cause lysis or insertion of their genetic material into the bacterial genome (Cao et al., 2017; Shkoporov et al., 2022). In the gut, bacteriophage-bacterial interaction is environment-dependent and can involve both antagonism and mutualism dynamics.

In vitro studies have provided valuable insights into predator-prey dynamics, primarily focusing on the selection of resistant bacteria and bacteriophages capable of overcoming bacterial defense mechanisms (Buckling & Rainey, 2002; Fortuna et al., 2019; Laanto et al., 2017). Nonetheless, these studies typically follow a one-phage/one-bacterium approach, which does not account for the complexity of the GIT environment and its diverse microbial communities (De Sordi et al., 2019; Shkoporov & Hill, 2019).

Our study investigated the interaction between bacteriophages and *E. coli* isolated in Cobb500 broilers (*Gallus gallus*) intestines. In this way, we evaluated the interaction between bacteriophages and intestinal *E. coli* strains isolated from five Cobb500 broilers using across three sampling weeks (W1, W3, and W6). Additionally, metagenomic analysis was performed to assess turnover in the bacteriophage population isolated from two sampling weeks (W1 and W3), using six selected *E. coli* strains from W1 as hosts for bacteriophage enrichment.

Materials and methods

Study location

Five Cobb500 broilers were purchased from a local vendor in Pintag parish, Quito. The chickens were housed together for 46 days within a designated facility in Pintag parish, with the necessary temperature and humidity conditions (24 – 26°C and 50 – 60%, respectively). Each chicken was labeled for the study: chicken 1 (C1), chicken 2 (C2), chicken 3 (C3), chicken 4 (C4) and chicken 5 (C5). They were fed with water and ground corn/balanced meals (free of antibiotics).

Ethical considerations

This study protocol was approved by the Committee of Ethics in the Use of Animals in Research and Teaching at the Universidad San Francisco de Quito (no. 2023-03).

Sample collection

Fecal samples were collected from each chicken during three sampling times: week 1 (W1), week 3 (W3), and week 6 (W6), obtaining a total of 15 samples. Feces were collected in sterile plastic containers using a sterile spoon and labeled with the corresponding code. The samples were stored in a cooler (4°C) and transported to the laboratory of bacteriology at the Institute of Microbiology of the Universidad San Francisco de Quito (IM-USFQ).

E. coli isolation

Fecal samples were homogenized, inoculated directly onto MacConkey agar plates, and incubated at 37°C for 24 hours. To ensure the selection of numerically dominant *E. coli* strains in the intestine, five lactose fermentative colonies were obtained from each MacConkey plate (Lautenbach et al., 2008). Each colony was tested for β-D-glucuronidase activity using Chromocult

Agar. *E. coli* isolates were labeled with a letter representing the chicken of origin (C), followed by the number of strains (S) and the sampling week (W). The isolates were incubated in Brain Hearth Infusion (BHI) medium + glycerol (25%) and stored at -20°C and -80°C for further testing (Calderón et al., 2022).

One colony of an *E. coli* isolate was inoculated in 3 mL of Luria Bertani (LB) medium and incubated at 37°C overnight (approximately 18 - 24 hours). Then, 500 µL of the overnight culture (CFU/mL: 1,00E+09) were added to 4.5 mL of LB medium (1/10 dilution). The optical density (OD) was measured every 5 minutes for 1 to 2 hours at 600 nm until the strain reached exponential growth (OD_{600nm} 200 – 500) (Lin et al., 2010; Sezonov et al., 2007).

Enrichment of the bacteriophage population

To obtain the bacteriophage population in the fecal sample, 2 g of fecal sample was added to 18 mL of saline magnesium buffer (SM buffer) (1/10 dilution) (Larsen, 2021), homogenized, and centrifuged at 8000 *xg* for 10 minutes at 4°C. The supernatant was passed through a 0.22 µm filter and sterilized using a syringe. Then, 40 µL of chloroform was added to the final extract and homogenized. The bacteriophage was placed on 2 mL sterile microtubes and stored at 4°C (Osawa et al., 1981).

Bacteriophage extracts were tested with both *E. coli* isolates from the same chicken and laboratory *E. coli* strains (K12 MG1655, B, and TOP10) using 110 µL (colony forming units (CFU)/mL: 1.00E+09) (Sezonov et al., 2007; Tuttle et al., 2021) of exponential growing (OD_{600nm} 0.2 – 0.5) bacterial culture was mixed with 60 µL (plate-forming units (PFU)/mL: 5.21E+05 – 1.73E+06) (Table S1) of bacteriophage extract and then incubated at 37°C for 15 minutes. Then, 150 µL of the solution bacteria-bacteriophage was added to 3 mL of LB soft agar (0.5% agar),

gently homogenized, and spread into LB agar plates. The plates were incubated at 37°C for 24 hours. Plates, where plaques were observed, were established as a sensitive strain to the bacteriophage extract (Osawa et al., 1981).

Turnover of the bacteriophage population infecting specific hosts

To study variations in intestinal bacteriophage populations capable of infecting a single *E. coli* strain, we allowed the bacteriophages collected from different sampling weeks to infect a single *E. coli* isolate obtained from the same individual during the first week. As hosts, *E. coli* isolates selected were sensitive to bacteriophages from W1 and W3. Six isolates from W1 were chosen as hosts for the bacteriophage enrichment: two isolates from the C2 (C2S3W1 and C2S4W1), two isolates from the C3 (C3S4W1 and C3S5W1), one isolate from the C4 (C4S2W1) and one isolate from the C5 (C5S4W1). Briefly, 100 µL of bacteriophage extract (PFU/mL: 5.21E+05 – 1.73E+06) was added to 0.5 mL of host strains in exponential growth (OD_{600nm} 0.3) and incubated at 37°C for 20 minutes. This solution was added to 50 mL of supplemented LB medium (0.3% glycerol + 10 mM of $MgSO_4$) and incubated at 37°C and 200 rpm for 14 – 16 hours (Lee & Clark, 1997).

DNA extraction

Bacteriophage enriched cultures received 0.5 mL of chloroform and were shaken for 30 minutes at 200 rpm and 37°C. Bacterial cells were eliminated by centrifugation at 4000 *xg* and 37°C for 15 minutes. The supernatant was transferred to a clean tube, and 2.8 g of NaCl and 5 g of polyethylene glycol (PEG) 8000 were added, and contents were allowed to solubilize by gently shaking for 30 minutes at room temperature. Then, the supernatant was incubated on ice for 1 hour and centrifuged at 4000 *xg* for 15 min at 4°C to obtain the bacteriophage pellet. The supernatant was discarded, and the tube was left inverted on a paper towel for 15 min. The remaining PEG in

the tube was removed with a laboratory wipe (Lee & Clark, 1997). From the resulting pellet, DNA was extracted using the PureLink™ Microbiome DNA Purification Kit (A29790). The resulting DNA (100 µL) was quantified using a Qubit fluorometer and stored at -20°C.

Metagenomic sequencing

Considering the concentration of each sample, it was necessary to normalize the concentration before the library was prepared (Table S2). The homogenized samples, whose concentration was >2 ng/µL, were C2S3W1-PW1 (1/10 dilution), C2S4W1-PW1 (3/4 dilution), and C3S5W1-PW3 (3/4 dilution). The library preparation was performed using the Native Barcoding Kit 96 V14 (SQK-NBD114.96), according to the manufacturer's instructions. Five samples (C3S4W1/C4S2W1/C5S4W1 - PW1 and C2S3W1/C2S4W1 - PW3), whose concentration was <2 ng/µL, were divided into two barcodes that were then concatenated by bioinformatics analysis.

The resulting library was loaded on an Oxford GridION flowcell (ID: FAZ27480) and sequenced using MinKNOW (version 24.02.16) for 16 hours. Base-calling and quality control analysis were performed using Super-accurate base-calling and NanoPlot (version 1.38.1). The reads were filtered using Filtlong (version 0.2.1), removing reads below 1000 base pairs (bp). Genome assembly was performed using the Flye assembler (version 2.9-b1768), and the sequences were polished using medaka (GalaxyVersion 1.7.2+galaxy1).

Taxonomic identification and bacteriophage genome annotation were carried out by three different tools: Kraken2 (version Galaxy Version 2.1.3+galaxy1) using the Prebuilt Refseq indexes PlusPFP database, PHASTEST (version 3.0) (Wishart et al., 2023) and What the Phage (version 1.2.3) (Marquet et al., 2020). What the Phage uses nextflow (version 20.07.1) for working with

several tools related to taxonomic identification (VirFinder, PPR-Meta, VirSorter, MetaPhinder, DeepVirFinder, Sourmash, VIBRANT, VirNet, Phigaro, Virsorter2, and Seeker) and annotating bacteriophage genomes (prodigal, hmmer, chromomap). Furthermore, the genome completeness and quality score of the phage sequences were carried out by CheckV (version 1.0.3), which is integrated into the What the Phage multitool.

Statistical analysis

Statistical analysis was carried out using R (version 2024.04.2+764). Fisher test was performed to test the significant differences between the infection for bacteriophages to *E. coli* isolates, both from the same sampling week. p values <0.05 were considered significant.

Results

Sensitivity to Bacteriophage Infection in E. coli Strains

We isolated 75 *E. coli* strains and obtained 15 bacteriophage extracts from corresponding fecal samples. We selected isolates that were susceptible to bacteriophages in at least 2 different weeks. The susceptibility of these strains to bacteriophage-mediated infection was assessed by evaluating each strain against the bacteriophage extracts (both bacteriophages and bacteria from the same animal) collected during sampling weeks W1, W3, and W6.

Table 1. Sensitivity to bacteriophage infection in every strain from chickens.

Chicken	Strains tested	Sensitive strains
C1	15	4 (26.67)
C2	15	10 (66.67)
C3	15	6 (40)
C4	15	6 (40)
C5	15	9 (60)
Total	75	35 (46.67)

Notes: Values in parentheses are percentages.

Of the 75 *E. coli* strains, 35 (46.67%) were susceptible to lytic bacteriophage infection (Table 1). Sensitivity varied across the 15 strains from each chicken, with isolates from C2 (66.67%) and C5 (60%) exhibiting higher susceptibility than other individuals.

Regarding temporal variability, the sensitivity of the *E. coli* isolates to bacteriophage infection varied with each bacteriophage extract (Table 2): 33.33% (25/75 strains) to bacteriophages from W1, 36% (27/75 strains) to bacteriophages from W3, and 38.66% (29/75 strains) to bacteriophages from W6.

Table 2. Sensitivity to bacteriophage infection in every sampling, considering every individual.

Chicken	W1 (n=75)	W3 (n=75)	W6 (n=75)
C1	1	1	3
C2	7	10	9
C3	6	5	5
C4	5	4	4
C5	6	7	8
Total	25 (33.33)	27 (36)	29 (38.66)

Notes: Values in parentheses are percentages.

No statistically significant differences were observed in infection rates of *E. coli* strains by bacteriophages from the same week across the three sampling periods, nor in the cumulative data for all periods (Table 3). This indicates that lytic infections could not be influenced by the temporal alignment of bacteriophage and host strains in the gut.

Table 3. Sensitivity to bacteriophage infection considering sensitivity in the same week.

Bacteriophage extract	Strains tested	Sensitivity same week	p-value (<0.05)
W1	75	10	0.44
W3	75	9	1
W6	75	11	0.62
W (1+3+6)	225	30	0.38

Notes: Values in parentheses are percentages.

Additionally, laboratory *E. coli* strains (K12 MG1655, B, and TOP10) exhibited varying sensitivity to bacteriophage extracts from each chicken across all sampling weeks (Figure 1). While sensitivity to bacteriophage extracts from W1 and W3 remained relatively consistent, a significant shift was observed with W6 bacteriophage extracts, where most strains exhibited

resistance. This shift in wild-type and laboratory *E. coli* strains suggests a potential turnover of intestinal bacteriophage populations at each analyzed time point.

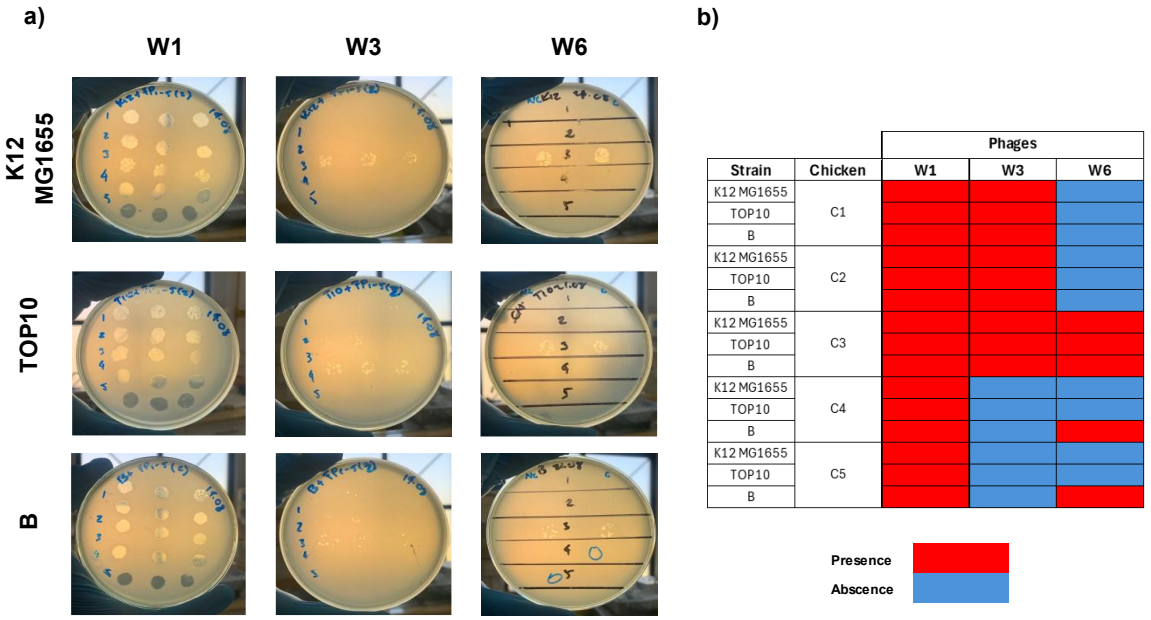

Figure 1. Bacteriophage sensitivity in laboratory *E. coli* strains K12 MG1655, TOP10, and B.

a) Double-layer plaque assays displaying the response of *E. coli* strains (row) to bacteriophage extracts over three sampling weeks (column). Each panel represents a different strain across the sampling times, showing clear zones of inhibition (plaques) where phage activity is present. b) Table summarizing the sensitivity (red) and resistance (blue) of the three *E. coli* strains to phages from five chickens (C1-C5) over three time points.

Metagenomic Analysis Reveals Bacteriophage Diversity

Initial data from the 17 metagenomic libraries comprised approximately 4.56 million reads, totaling approximately 4.78 gigabases (Gb). After quality analysis, 581,795 reads were retained, with an average length of 3,481.34 base pairs (bp) and a mean quality score of 17.20. Assembly of

these reads produced 1,377 contigs, with an average length of 100,906.90 bp. Among these, 144 contigs (10.45%) were identified as phage sequences, ranging in length from 2,412 to 167,471 bp across different samples.

Genome completeness assessment using CheckV classified the 144 contigs as follows: 59 contigs (41%) as high quality (HQ), 40 contigs (28%) as medium quality (MQ), and 45 contigs (31%) as low quality (LQ). The 144 phage contigs were assigned taxonomic classifications by Kraken2, PHASTEST, and What the Phage annotation tools. These taxonomic names indicate likely affiliations based on available genomic information rather than confirmed species identities. This analysis identified 27 taxonomic classifications whose, according to the NCBI Genome and Taxonomy databases, belonged to the viral class *Caudoviricetes*, encompassing both circular and linear dsDNA (National Center for Biotechnology Information, 2024) (Table S3).

Analysis of Bacteriophage Population in Chicken Hosts Reveals Strain and Temporal Variation

There was a difference in the diversity of bacteriophages that were able to cause lytic infection in the strains evaluated (Table 4). Specifically, strain C4S2W1 exhibited the highest diversity of bacteriophages at 66.67%, followed by strains C2S3W1 and C2S4W1, with 61.54% and 51.85%, respectively. In contrast, strains C3S4W1, C5S4W1, and C3S5W1 demonstrated lower diversity at 44.44%, 25.93%, and 14.81%, respectively.

Table 4. Bacteriophage frequency per host strain.

	C2S3W1	**C2S4W1**	**C3S4W1**	**C3S5W1**	**C4S2W1**	**C5S4W1**
Bacteriophage ID (n=27)	16 (61.54)	14 (51.85)	12 (44.44)	4 (14.81)	18 (66.67)	7 (25.93)

Note: Values in parentheses are percentages.

Three host strain-specific phage occurrences were identified: vb_EamM_Asesino was exclusively found in C2S4W1, while mEp460, BcepMu, and Nepra were uniquely identified in C4S2W1. C4S2W1 presented significant diversity between strains. Meanwhile, Sf6 and VB_EcoS-Golestan were also present across all evaluated strains (Table 5).

Table 5. Bacteriophage diversity per host strain.

Bacteriophage ID	C2S3W1	C2S4W1	C3S4W1	C3S5W1	C4S2W1	C5S4W1
Escherichia phage VB_EcoS-Golestan	+	+	+	+	+	+
Shigella phage Sf6	+	+	+	+	+	+
Erwinia phage vB_EamM_Asesino	+	-	-	-	-	-
Enterobacteria phage mEp460	-	-	-	-	+	-
Burkholderia phage BcepMu	-	-	-	-	+	-
Pectobacterium phage Nepra	-	-	-	-	+	-

Note: + represents presence, and – represents absence.

Since bacteriophage extracts were individual-specific, the number of bacteriophages found varied between the sampling weeks (Table 6). A higher number of bacteriophages capable of infecting strain C4S2W1 was detected in week 1 compared to week 3. Similarly, this pattern was observed for strains C3S4W1 and C5S4W1.

Table 6. Bacteriophage frequency in each host strain per sampling week.

Strains	W1	W3
C2S3W1	10 (37.04)	10 (37.04)
C2S4W1	10 (37.04)	13 (48.15)
C3S4W1	11 (40.74)	2 (7.41)
C3S5W1	3 (11.11)	3 (11.11)
C4S2W1	17 (62.96)	8 (29.63)
C5S4W1	6 (22.22)	4 (14.81)

Note: Values in parentheses are percentages.

In contrast, only strain C2S4W1 exhibited more phages identified at week 3. For strains C2S3W1 and C3S5W1, the number of bacteriophages identified was similar at both time points. Although phage counts were similar in these strains, bacteriophage diversity differed between the sampling times, with specific bacteriophages present only at one time sampling week (Table 7). This phenomenon was also observed in the bacteriophage cocktails associated with the other strains.

Table 7. Bacteriophage diversity in each host strain per sampling week (continue).

Bacteriophage ID	C2S3W1		C2S4W1		C3S4W1		C3S5W1		C4S2W1		C5S4W1	
	W1	W3	W1	W3	W1	W3	W1	W3	W1	W3	W1	W3
Enterobacteria phage cdtI	-	-	-	+	-	-	-	-	-	-	-	-
Enterobacteria phage P7	+	+	-	-	-	-	-	-	-	-	-	-
Enterobacteria phage P88	+	+	+	+	+	-	-	-	+	-	+	+
Enterobacteria phage Sf101	-	+	-	-	-	-	-	-	-	-	-	-
Enterobacteria phage SfI	+	+	+	+	+	-	-	-	+	+	-	-
Erwinia phage vB_EamM_Asesino	-	-	-	+	-	-	-	-	-	-	-	-
Escherichia phage 500465-1	+	-	-	-	-	-	-	-	-	-	+	-
Escherichia phage 500465-2	-	-	-	+	-	-	-	-	+	-	-	-
Escherichia phage DE3	-	-	-	+	-	-	-	-	-	-	-	-
Escherichia phage HK629	+	-	-	-	-	-	-	-	-	-	-	-
Escherichia phage phiV10	-	-	-	-	-	-	-	-	+	-	-	-
Escherichia phage RCS47	-	+	+	+	+	-	+	+	+	-	-	-
Escherichia phage vB_EcoM_Goslar	+	-	-	-	+	-	-	-	+	-	-	-
Escherichia phage vB_EcoS_ESCO41	-	+	+	+	+	-	-	-	+	-	+	-

Note: + represents presence, and – represents absence.

Table 7. Bacteriophage diversity in each host strain per sampling week.

Bacteriophage ID	C2S3W1		C2S4W1		C3S4W1		C3S5W1		C4S2W1		C5S4W1	
	W1	W3	W1	W3	W1	W3	W1	W3	W1	W3	W1	W3
Escherichia phage vB_EcoS-12210I	+	-	+	+	+	-	-	-	+	+	-	+
Escherichia phage VB_EcoS-Golestan	+	-	+	+	+	-	-	+	+	+	+	+
Klebsiella phage 4LV2017	-	+	+	+	-	+	-	-	+	-	-	-
Pectobacterium phage phiA41	-	+	-	-	+	-	-	-	-	-	-	-
Pectobacterium phage vB_PatP_CB1	-	+	+	+	+	-	+	-	-	-	-	-
Salmonella phage SJ46	+	-	-	-	-	-	-	-	+	+	+	-
Shigella phage Sf6	+	+	+	+	+	-	+	+	+	+	+	+
Escherichia phage 520873	-	-	-	-	-	-	-	-	+	+	-	-
Shigella phage SfIV	-	-	-	-	+	+	-	-	-	+	-	-
Stx2-converting phage 1717	-	-	+	-	-	-	-	-	+	+	-	-
Enterobacteria phage mEp460	-	-	-	-	-	-	-	-	+	-	-	-
Burkholderia phage BcepMu	-	-	-	-	-	-	-	-	+	-	-	-
Pectobacterium phage Nepra	-	-	-	-	-	-	-	-	+	-	-	-

Note: + represents presence, and – represents absence.

When considering the bacteriophages belonging to the cocktails of both weeks in the six strains evaluated, the number of bacteriophages found in week 1 (88.88%) was higher than the number of bacteriophages found in week 2 (74.07%) (Table 8).

Table 8. Bacteriophage frequency per sampling week.

	W1	**W3**
Bacteriophage ID (n=27)	24 (88.88)	20 (74.07)

Note: Values in parentheses are percentages.

Seventeen bacteriophages were consistently detected across both time points: P7, P88, Sf101, SfI, 500465-2, RCS47, vB_EcoS_ESCO41, vB_EcoS-12210I, VB_EcoS-Golestan, 4LV2017, phiA41, vB_PatP_CB1, SJ46, Sf6, 520873, SfIV, and 1717 (Table 9). Meanwhile, bacteriophages exclusively identified in one of the two evaluated weeks were observed: seven bacteriophages were unique to W1 (5004653-1, HK629, phiV10, mEp460, BcepMu, and Nepra), while three bacteriophages were exclusive to W3 (cdtI, vb_EamM_Asesino and DE3). Notably, Sf6 and VB_EcoS-Golestan bacteriophages were consistently identified across all the strains and sampling periods, highlighting their potential ecological significance within the bacteriophage populations in the gut.

Table 9. Bacteriophage diversity per sampling week.

Bacteriophage ID	W1	W3
Enterobacteria phage P7	+	+
Enterobacteria phage Sf101	+	+
Enterobacteria phage SfI	+	+
Escherichia phage 500465-2	+	+
Escherichia phage RCS47	+	+
Klebsiella phage 4LV2017	+	+
Pectobacterium phage phiA41	+	+
Pectobacterium phage vB_PatP_CB1	+	+
Salmonella phage SJ46	+	+
Escherichia phage 520873	+	+
Shigella phage SfIV	+	+
Stx2-converting phage 1717	+	+
Escherichia phage 500465-1	+	-
Escherichia phage HK629	+	-
Escherichia phage phiV10	+	-
Enterobacteria phage cdtI	-	+
Escherichia phage DE3	-	+
Enterobacteria phage P88	+	+
Escherichia phage vB_EcoM_Goslar	+	-
Escherichia phage vB_EcoS_ESCO41	+	+
Escherichia phage vB_EcoS-12210I	+	+
Escherichia phage VB_EcoS-Golestan	+	+
Shigella phage Sf6	+	+
Erwinia phage vB_EamM_Asesino	-	+
Enterobacteria phage mEp460	+	-
Burkholderia phage BcepMu	+	-
Pectobacterium phage Nepra	+	-

Note: + represents presence, and – represents absence.

Discussion

In this study, we investigated the interaction between numerically dominant *Escherichia coli* strains and bacteriophage populations across three temporalities, utilizing double-layer assays to determine the resistance/sensitivity of 75 dominant strains isolated from five chickens to phage infection. We observed that each of these strains exhibited a distinct phenotype of resistance or sensitivity in response to the phage extracts from the three sampling weeks (Table 1). Given that the chickens were housed together and no dietary changes were made, which could alter the gut microbiome, the phenotypic differences observed between strains may be attributed to the inter-individual variability of the *E. coli* population present in the intestine, a phenomenon previously reported in human gut microbiomes (Calderón et al., 2022; Han et al., 2024; Hu et al., 2022).

The phenotypic variation among the 75 dominant strains differed when considering their interaction with bacteriophage extracts from each of the three sampling weeks. Descriptive analyses (Table 2) revealed a shift in the proportion of sensitivity among the dominant strains across the three time points, with a slight increase in sensitivity observed after weeks 3 and 6. We observed that the bacteriophage population capable of infecting specific *E. coli* strains (coexisting in the same intestine) varied weekly (Buckling & Rainey, 2002; Fortuna et al., 2019; Laanto et al., 2017). Consequently, both populations *E. coli* and their associated bacteriophages appear to change over time (Calderón et al., 2022; Hu et al., 2022; Shkoporov et al., 2019).

It has been theorized that antagonistic bacteriophage-bacteria interactions over time alter the composition of the microbial population by reducing the prevalence of sensitive strains while maintaining diversity through the selection of resistant strains via lytic infections (Buckling & Rainey, 2002; Koskella & Brockhurst, 2014; Shkoporov & Hill, 2019). In our study, we observed

that bacteriophage populations capable of infecting a specific *E. coli* strain coexisted with sensitive strains. We found no differences in the infection rates between bacteriophages obtained in the same week as a specific *E. coli* isolate and those obtained in other weeks. These results suggest that bacteriophages may not play a significant role in driving the turnover of numerically dominant *E. coli* strains in the intestine. Nonetheless, the coexistence of bacteriophages with their sensitive host strains, which are numerically dominant, may drive fluctuations in viral populations (Connerton et al., 2004).

Our results suggest that the presence of numerically dominant *E. coli* strains exhibiting a variable resistance/sensitivity phenotype is likely determined by the interplay of these population dynamics within the intestine, where antagonistic interactions predominate (De Sordi et al., 2017; Lourenço et al., 2020; Reyes et al., 2013). It has been proposed when bacterial genotypes become more abundant in the intestine, bacteriophages selected for their increased infectivity range reduce the bacterial population (kill-the-winner/fluctuating-selection dynamic) without completely eliminating it and simultaneously selecting for strains with novel resistance mechanisms and more infective phages, thereby restarting the cycle (Barroso-Batista et al., 2014; Lourenço et al., 2016). Nonetheless, we observed that sensitive *E. coli* strains remained numerically dominant, which may indicate that the interaction of bacteriophages in the bacterial population may not be a major determinant for the *E. coli* population turnover. In addition, our results suggest that a very complex continuous process enables the coexistence of bacteria and bacteriophages or may lead to the potential extinction of some bacteriophages, as observed with SF1 phage infecting *Salmonella* strains within 30 days (L. Chen et al., 2024).

Finally, we assessed the turnover of the bacteriophage population extracted from two time points (W1 and W3), which were capable of infecting six *E. coli* host strains from a single time

point (W1). At the strain level, we observed that, across the 27 taxonomic identifications, each host strain was sensitive to lytic infection of a variable number of phages. Of the bacteriophages identified, only two were present in all the strains evaluated (Sf6 and VB_EcoS-Golestan), while the remaining bacteriophages varied between strains. A similar trend was observed when considering the bacteriophage extracts from both sampling weeks, with bacteriophage diversity differing between weeks. These findings suggest the constant turnover of bacteriophage populations at the bacterial species level and over time, highlighting the proliferation of bacteriophages in the presence of sensitive strains (Fortuna et al., 2019; Laanto et al., 2017).

Thus, our study highlights the constant turnover of bacteriophage population within the numerically dominant *E. coli* strains in the *Gallus gallus* intestine. This turnover suggests a persistent antagonistic dynamic that selects for bacteriophage genotypes capable of infecting diverse bacterial genotypes within the intestinal *E. coli* population. Nonetheless, this antagonism does not seem to affect the intestinal *E. coli* population by isolating numerically dominant strains with a variable resistance/sensitivity phenotype. In this way, our research underscores the need for more detailed *in vitro* and *in vivo* analyses that explore the interconnections between evolutionary dynamics within the complex intestinal environment.

Conclusions

Our study demonstrated that *E. coli* strains can maintain numerical dominance within the intestinal microbiota despite the presence of lytic bacteriophages capable of targeting them. Additionally, we observed that bacteriophage populations increase in abundance when susceptible bacterial strains are present, but do not drive the replacement of these strains. This suggests that bacteriophage-mediated strain displacement is not a primary mechanism of bacterial turnover in the chicken intestines. Evidence of turnover within the diverse intestinal bacteriophage population indicates dynamic interactions and continual renewal of bacteriophage populations at strain level. While bacteriophages proliferate in the presence of sensitive strains, they do not appear to affect the numerical dominance of these strains. This finding was unexpected because many reports indicate that bacteriophages are one of the main factors causing the turnover of bacterial strains in the intestine. These results provide important insights into the stability and dynamics of bacteriophage-bacteria interactions in the gut microbiome.

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SUPPLEMENTARY MATERIAL

Supplementary Table S1. Concentration (PFU/mL) of each bacteriophage extract per individual.

Bacteriophage extract	C1	C2	C3	C4	C5
W1	1,12E+06	1,01E+06	8,13E+05	1,12E+06	8,69E+05
W3	5,21E+05	1,48E+06	7,85E+05	1,73E+06	1,40E+06
W6	5,86E+05	1,37E+06	5,43E+05	8,36E+05	1,37E+06

Supplementary Table S2. DNA concentration (ng/µL) of each aliquot.

Bacteriophage extract	C2S3W1	C2S4W1	C3S4W1	C3S5W1	C4S2W1	C5S4W1
W1	59	4	1.65	2.94	1.14	1.01
W3	0.97	11.13	2.68	4.10	2.78	2.10

Supplementary Table S3. Features of the identified bacteriophages infecting the host strains (continue).

Bacteriophage ID	Taxonomy (class)	DNA	Life cycle	References
Enterobacteria phage cdtI	*Caudoviricetes*	dsDNA circular	Lysogenic	(Asakura et al., 2007)
Enterobacteria phage P7	*Caudoviricetes*	dsDNA linear	Lysogenic	(Billard-Pomares et al., 2014)
Enterobacteria phage P88	*Caudoviricetes*	dsDNA circular	Lysogenic	(M. Chen et al., 2017)
Enterobacteria phage Sf101	*Caudoviricetes*	dsDNA linear	Lysogenic	(Jakhetia et al., 2014)
Enterobacteria phage SfI	*Caudoviricetes*	dsDNA linear	Lysogenic	(Sun et al., 2013)
Erwinia phage vB_EamM_Asesino	*Caudoviricetes*	dsDNA linear	Lytic	(Sharma et al., 2018)
Escherichia phage 500465-1	*Caudoviricetes*	dsDNA linear	Lysogenic	(Mafakheri et al., 2022)
Escherichia phage 500465-2	*Caudoviricetes*	dsDNA linear	Lysogenic	(Vaughan et al., 2022)
Escherichia phage DE3	*Caudoviricetes*	dsDNA linear	Lysogenic	(Jeong et al., 2009)
Escherichia phage HK629	*Caudoviricetes*	dsDNA linear	Lysogenic	(Lai et al., 2018)
Escherichia phage phiV10	*Caudoviricetes*	dsDNA linear	Lysogenic	(Perry et al., 2009)
Escherichia phage RCS47	*Caudoviricetes*	dsDNA circular	Lysogenic	(Billard-Pomares et al., 2014)
Escherichia phage vB_EcoM_Goslar	*Caudoviricetes*	dsDNA linear	Lytic	(Prichard et al., 2023)
Escherichia phage vB_EcoS_ESCO41	*Caudoviricetes*	dsDNA linear	Lytic	(Nicolas et al., 2023)

Supplementary Table S3. Features of the identified bacteriophages infecting the host strains.

Bacteriophage ID	Taxonomy (class)	DNA	Life cycle	References
Escherichia phage vB_EcoS-12210I	*Caudoviricetes*	dsDNA linear	Lytic	(Nicolas et al., 2023)
Escherichia phage VB_EcoS-Golestan	*Caudoviricetes*	dsDNA linear	Lytic	(Yazdi et al., 2020)
Klebsiella phage 4LV2017	*Caudoviricetes*	dsDNA linear	Lytic	(Zurabov & Zhilenkov, 2021)
Pectobacterium phage phiA41	*Caudoviricetes*	dsDNA linear	Lytic	(Smolarska et al., 2018)
Pectobacterium phage vB_PatP_CB1	*Caudoviricetes*	dsDNA linear	Lytic	(Buttimer et al., 2018)
Salmonella phage SJ46	*Caudoviricetes*	dsDNA circular	Lysogenic	(Gabashvili et al., 2020)
Shigella phage Sf6	*Caudoviricetes*	dsDNA circular	Lysogenic	(Freiberg et al., 2003)
Escherichia phage 520873	*Caudoviricetes*	dsDNA linear	Lytic	(Nicolas et al., 2023)
Shigella phage SfIV	*Caudoviricetes*	dsDNA linear	Lysogenic	(Jakhetia et al., 2013)
Stx2-converting phage 1717	*Caudoviricetes*	dsDNA linear	Lysogenic	(Zhang et al., 2021)
Enterobacteria phage mEp460	*Caudoviricetes*	dsDNA linear	Lysogenic	(Langley et al., 2005)
Burkholderia phage BcepMu	*Caudoviricetes*	dsDNA linear	Lysogenic	(Naseri et al., 2022)
Pectobacterium phage Nepra	*Caudoviricetes*	dsDNA linear	Lytic	(Miroshnikov et al., 2021)