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Bacteriophage application for *Salmonella* control in poultry and its implications on their microbiota and metabolome

TESIS DOCTORAL

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DOCTORAL THESIS

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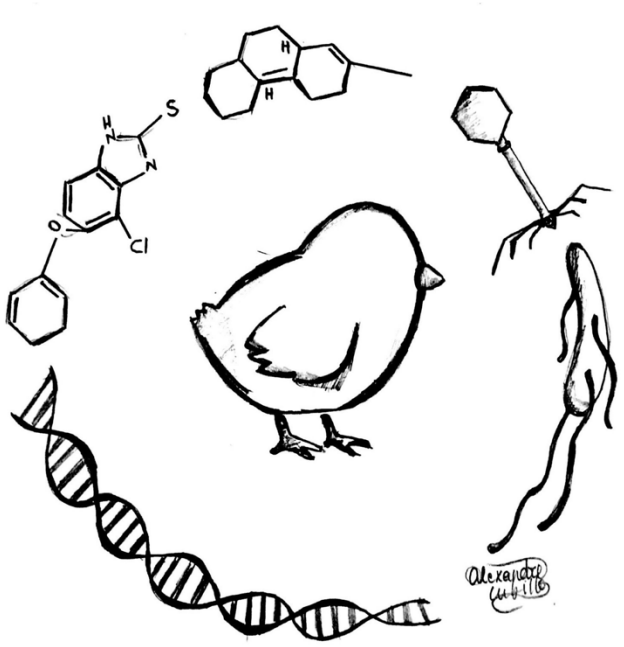

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La Dr. DÑA. CLARA MARÍN ORENGA, como Directora de la Tesis Doctoral realizada por la Doctoranda Dña. LAURA LORENZO REBENAQUE, titulada "**Bacteriophage application for *Salmonella* control in poultry and its implications on their microbiota and metabolome**", autoriza la presentación de la citada Tesis Doctoral, puesto que reúne las condiciones necesarias para su defensa.

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A Mamá, Papá y Sara

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La tesis doctoral titulada "**Bacteriophage application for *Salmonella* control in poultry and its implications on their microbiota and metabolome"** es un compendio de estudios que incluye los siguientes trabajos:

1) **Laura Lorenzo-Rebenaque**, Danish J Malik, Pablo Catalá-Gregori, Clara Marin, Sandra Sevilla-Navarro. *In Vitro and In Vivo Gastrointestinal Survival of Non-Encapsulated and Microencapsulated Salmonella Bacteriophages: Implications for Bacteriophage Therapy in Poultry;* **Pharmaceuticals**, 2021, 14(5), 434.
   Impact factor: 4.286, Q1 (Pharmaceutical Science).

2) **Laura Lorenzo-Rebenaque**, Danish J Malik, Pablo Catalá-Gregori, Clara Marin, Sandra Sevilla-Navarro. *Gastrointestinal Dynamics of Non-Encapsulated and Microencapsulated Salmonella Bacteriophages in Broiler Production;* **Animals**, 2022, 12(2), 144.
   Impact factor: 2.752, Q1 (Veterinary Science).

3) **Laura Lorenzo-Rebenaque**, Cristina Casto-Rebollo, Gianfranco Diretto, Sarah Frusciante, Juan Carlos Rodríguez, María-Paz Ventero, Carmen Molina-Pardines, Santiago Vega, Clara Marin, Francisco Marco-Jiménez. *Examining the effects of Salmonella phage on the caecal microbiota and metabolome features in Salmonella-free broilers;* **Frontiers in Genetics**, *Under review.*

4) **Laura Lorenzo-Rebenaque**, Danish J Malik, Pablo Catalá-Gregori, Jan Torres, Clara Marin, Sandra Sevilla-Navarro. *Microencapsulated Bacteriophages Incorporated in Feed for Salmonella Control in Broilers;* **Veterinary Microbiology**, 2022, 274, 109579.
   Impact factor: 3.246, Q1 (Veterinary Science).

5) *Bacteriophage therapy against Salmonella in broilers modulates the caecal microbiota and metabolome with no biological significance in broilers.*

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Abstract / Resumen

Abstract

Non-typhoidal *Salmonella* spp. has been recognized as one of the most important zoonotic pathogens worldwide, and the second most commonly reported zoonotic pathogen in the European Union, being poultry derived products the main source of human infection. The implementation of strict National *Salmonella* Control Programs in Europe, which have entailed high investments in biosecurity and preventive vaccination of animals by the poultry sector, has led to a considerable decrease in its prevalence at the field level. Nevertheless, the European Food Safety Authority reported the bacteria as the main cause of foodborne outbreaks in the European Union. In this context, it is mandatory to carry out innovative, cost-effective, and eco-friendly measures at the field level to complement the strict biosecurity and management measures in place. Among the most promising tools for *Salmonella* control at the field level are included bacteriophages (or phages). Phages are viruses that specifically infect bacterial cells, with high specificity towards the target bacterium. Moreover, its *en mass* application could allow cost-effective and practical use, directly reaching the intestine, the predilected site for *Salmonella* colonization, for quick and effective intervention. However, little is known about the phage application impact on the rest of the gut microbiota. In this sense, new studies suggest that phage application may affects the gastrointestinal ecology homeostasis.

Therefore, the general objective of this doctoral thesis was to apply bacteriophages for *Salmonella* control in broiler production, focusing on their effect on intestinal health, by means of genomic sequencing and metabolomic study. To achieve this goal, two different parts were performed.

The first part of this study is entitled "Bacteriophage gastrointestinal dynamics in *Salmonella*-free broilers and its influence on microbiota and metabolome" and is divided into three experimental trials. In the first experiment, the objective was to determine the best phage delivery format (encapsulated or not) to assess *in vitro* its survival throughout the simulated gastrointestinal tract of poultry. Based on these results, in the second experiment, the different phage delivery formats were applied *in vivo* in *Salmonella*-free one-day chicks to assess *in vivo* its dynamics throughout the gastrointestinal tract of day-old chicks. Finally, in the third experiment, the phage delivery format with the best results

obtained in previous experiments (L100) it has been used in different moments of the rearing period (1, 2, 3, 4, 5, and 6 weeks) to assess under rearing conditions its dynamics throughout the gastrointestinal tract of *Salmonella*-free broilers and the best moment of phage application, as well as its effect on the intestinal microbiota and metabolome.

For these purposes, in the first experiment, phage FGS011 was encapsulated in two different pH-responsive formulations using polymers Eudragit® L100 (L100), and Eudragit® S100 (S100) using the process of spray drying. The release of the encapsulated phages, and the survival of non-encapsulated (FP) and encapsulated (L100 and S100) phages under simulated gastrointestinal conditions were evaluated. The results showed that FP was likely to be inactivated upon exposure to simulated proventriculus-gizzard pH, meanwhile, its encapsulation significantly improved phage survival with S100 remaining encapsulated until the end of the gut.

Then, in the second experiment, FP, L100, and S100 were orally administered to day-old chicks for 24 hours, an age where chickens are highly susceptible to infection because of their immature immune system. For this purpose, twenty day-old chicks were divided into four groups: group 1 received FP *via* drinking water, group 2 L100 *via* feed, group 3 S100 *via* feed, and group 4 did not receive any phage (control group). Then, 24 hours after phage administration, animals were slaughtered, and phage enumeration of the different gastrointestinal tract sections was performed. The results showed that on day-old chicks no statistically significant differences were observed in the phage concentrations across the gastrointestinal tract for either the FP, L100, or S100 given to chicks, except in the gut, where L100 showed the best survival results.

Finally, in the third experiment, chickens were reared under commercial conditions, and phages (FP and L100) were administered to them for 24 hours at different moments of the rearing period. Then, phage administration effects on the intestinal microbiota and metabolome were assessed. Thus, ninety day-old-chicks were housed in a experimental room. Each week of rearing, 15 birds were randomly selected and moved to another experimental room and randomly divided into three groups: group 1 received FP *via* drinking water, group 2 L100 *via* feed, and group 3 did not receive any phage (control group). Then, 24 hours after phage administration, animals were slaughtered, phage enumeration of the different gastrointestinal tract sections was performed, and cecal content was taken for microbiota and metabolome analysis. To investigate the microbiota

and metabolome composition, 16S rRNA sequencing analysis and LC-HRMS-based metabolomics analyses were performed, respectively. In each of the six weeks of application, the crop displayed the highest phage concentration for both phage delivery methods. The L100-based encapsulation protected the phages from the harsh environmental conditions in the Proventriculus-Gizzard in each of the administration moments which may help to reach the delivery of high phage doses to the caecum. Microbiota results of the caecal content showed a high level of similarity (beta diversity) but revealed a significant change in alpha diversity between broilers with FP and control. Moreover, phages affected only a few genera of the microbiota's structure, regardless of the group. Among these, a significant increase in *Streptococcus* and *Sellimonas* in FP and *Lactobacillus*, *Anaeroplasma,* and *Clostridia_vadinBB60_group* were found in L100. Despite few genera were significantly affected, a substantial number of metabolites, especially in FP, were significantly altered (64 and 14 in FP and L100 groups, respectively).

The second part of this study is entitled "Bacteriophage dynamics in *Salmonella*-infected broilers and its influence on microbiota and metabolome" and consists of one experimental trial. Based on the previous results obtained, the phage with the best survival results (L100) was confronted with *Salmonella* in order to assess its efficacy as a control tool and its influence on the microbiota, metabolome, and chicken growing.

For this purpose, one hundred day-old chicks were placed into 2 groups of 50 animals each. Group 1 (control group) was challenged with *Salmonella* and Group 2 (Φ-treated group) was challenged with *Salmonella* and received for the first 21 days feed supplemented L100. To assess *Salmonella* colonization, excretion, and diffusion, caecum samples, cloacal swabs, and boot swabs were taken weekly. *Salmonella* detection was based on ISO 6579-1:2017 (Annex D). Moreover, caecum content and serum of 4, 5, and 6-weeks-old broilers were taken for microbiota and metabolome analysis. *Salmonella* colonization was significantly reduced in most of the rearing period, meanwhile, the excretion was significantly reduced on the 2nd, 4th, and 5th week of rearing. Moreover, *Salmonella* contamination of the farm environment was eliminated at the end of the cycle. Microbiota results showed a high level of similarity (alpha and beta diversity) between Φ-treated broilers and control. The results showed that the phage treatment affected several genera of the microbiota's structure (30 % of the identified genera). Moreover,

the metabolomics analyses revealed a substantial number of altered metabolites in the caecum, nevertheless, serum metabolites were minimum altered.

The main results obtained from this doctoral thesis include that significant differences were observed between phage delivery results of *in vitro* studies compared with *in vivo* results. Furthermore, in one-day-old chicks there were no statistically significant differences between phage delivered along the gastrointestinal tract for the encapsulated and non-encapsulated phage (the gut being the exception). Thus, phage encapsulation with L100 and S100 resulted in delivery of phage in day-old chicks with no adverse reactions observed in the animals. Moreover, when the phages were administered at different moments in the production cycle, the encapsulation with L100, especially when administered at the beginning and at the end of the cycle, could ensure targeted delivery of high titres of phages to the caecum affording encapsulated phages protection from the harsh environmental conditions found in the Proventriculus-Gizzard. Moreover, the fact that more encapsulated phages were found in the crop and caecum, known sites of high *Salmonella* colonization, makes encapsulation of phages a promising tool to control the bacteria at the field level. On the other hand, the easy dissemination of the phages through faeces may also facilitate the control of the bacterium in the farm environment. In addition, preventive therapy with phages minimally alters the intestinal microbiota but significantly impacts their metabolites, regardless of the route of administration. Further studies are needed to asses evaluated the beneficial effects of encapsulation of phages using L100 formulation to control the bacteria in the field during the rearing period and understand the potential interplay between differentially abundant bacterial species, and significantly altered metabolites to clarify phage treatment implications.

In this sense, *S*. Enteritidis flock contamination may be markedly curtailed through L100 encapsulated phage application as a feed additive in the starter diet during rearing. A reduction in *Salmonella* colonization and excretion was noted with complete elimination

of bacteria recorded from the environment at the end of the rearing period. However, higher phage doses, improved delivery protocols and/or combination with other strategies may be necessary to achieve total elimination of *Salmonella* from the animals. Finally, the application of *Salmonella* phages under production conditions modulates the cecal microbiome and metabolome profiles in broilers. However, the response in blood serum metabolites and growth performance suggests that the phage modulation seems have no biological significance. Further studies are required to assess whether such a shift implies that *Salmonella* phages shift the microbiota composition, which promotes the change in metabolic profile, or whether the phages are actively involved in metabolite changes. These results aim to provide important insights into the use of phages as a preventative and biocontrol strategy against *Salmonella* infection from farm-to-fork.

Resumen

Salmonella spp. no tifoidea ha sido reconocida como uno de los patógenos zoonóticos más importantes a nivel mundial, y el segundo patógeno zoonótico más común en la Unión Europea, siendo los productos derivados de las aves de corral la principal fuente de infección humana. La aplicación de estrictos Programas Nacionales de Control de *Salmonella* en Europa, a través de elevadas inversiones por parte del sector avícola en bioseguridad y vacunación preventiva de los animales, ha llevado a una considerable disminución de su prevalencia a nivel de campo. Sin embargo, la Autoridad Europea de Seguridad Alimentaria destaca de que la bacteria continúa siendo la principal causa de brotes de origen alimentario en la Unión Europea. En este contexto, es necesario llevar a cabo medidas innovadoras, rentables y ecológicas a nivel de campo para complementar las estrictas medidas de bioseguridad y de manejo aplicadas en las explotaciones. Una de las herramientas más prometedoras para el control de la *Salmonella* a nivel de campo son los bacteriófagos (o fagos). Los fagos son virus que infectan específicamente a las bacterias, que presentan una gran especificidad frente la bacteria objetivo. Además, su aplicación en masa podría permitir una aplicación rentable y práctica, llegando directamente al intestino, el lugar preferente para la colonización de *Salmonella*, para una intervención rápida y eficaz. Sin embargo, se sabe poco sobre el impacto de la aplicación de fagos en el resto de la microbiota intestinal. En este sentido, los nuevos estudios sugieren que la aplicación de fagos puede afectar a la homeostasis de la ecología gastrointestinal.

Por ello, el objetivo general de esta tesis doctoral es estudiar el efecto de la aplicación de bacteriófagos para el control de *Salmonella* en la producción de pollos de engorde, centrándose en su efecto sobre la salud intestinal, mediante la secuenciación genómica y el estudio metabolómico. Para lograr este objetivo, se realizaron dos bloques diferentes.

El primer bloque, denominado "Dinámica gastrointestinal de los bacteriófagos en pollos de engorde libres de *Salmonella* y su influencia en la microbiota y el metaboloma", se divide en tres experimentales. En el primer experimento, el objetivo fue determinar el mejor formato de administración de fagos (encapsulados o no) para evaluar *in vitro* su supervivencia a lo largo del tracto gastrointestinal simulado de las aves de corral. A partir

de estos resultados, en el segundo experimento, los diferentes formatos de administración de fagos se aplicaron *in vivo* en pollitos de un día libres de *Salmonella*, ya que es el momento de mayor riesgo de infección debido a que poseen un sistema inmunitario poco desarrollado, para evaluar *in vivo* su dinámica a lo largo del tracto gastrointestinal de los pollitos. En base a estos resultados, en el tercer experimento se utilizó el formato de administración de fagos con mejores resultados (L100) en diferentes momentos del periodo de crecimiento (1, 2, 3, 4, 5 y 6 semanas) para evaluar *in vivo* su dinámica a lo largo del tracto gastrointestinal de los pollos de engorde libres de *Salmonella* y el mejor momento de aplicación de los fagos, así como su efecto sobre la microbiota y el metaboloma intestinal.

Para ello, en el primer experimento, el fago FGS011 se encapsuló en dos formulaciones diferentes sensibles al pH utilizando los polímeros Eudragit® L100 (L100), y Eudragit® S100 (S100) mediante el proceso de secado por pulverización. Se evaluó la liberación de los fagos encapsulados y la supervivencia de los fagos no encapsulados (FP) y encapsulados (L100 y S100) en condiciones gastrointestinales simuladas. Los resultados mostraron que FP se inactiva tras la exposición al pH del proventrículo-molleja, mientras que su encapsulación mejoró significativamente la supervivencia de los fagos, permaneciendo S100 encapsulado hasta el final del intestino.

Para contrastar los resultados *in vitro* con las condiciones *in vivo*, en el segundo experimento se administraron por vía oral FP, L100 y S100 a pollitos de un día durante 24 horas. Se dividieron veinte pollitos de un día en cuatro grupos: al grupo 1 se le administró FP a través del agua de bebida, al grupo 2 L100 a través del alimento, al grupo 3 S100 a través del alimento y el grupo 4 no recibió ningún fago (grupo de control). A continuación, 24 horas después de la administración del fago, se sacrificaron los animales y se realizó el recuento de fagos en las diferentes secciones del tracto gastrointestinal. Los resultados mostraron que en los pollitos de un día no se observaron diferencias estadísticamente significativas en las concentraciones de fagos a lo largo del tracto gastrointestinal, excepto en el intestino, donde el L100 mostró los mejores resultados.

Para contrastar los resultados de los pollitos de un día con las condiciones comerciales (seis semanas de periodo de cría) y su efecto sobre la microbiota intestinal y el metaboloma, en el tercer experimento, se administró FP y L100 por vía oral a los pollos

durante 24 horas en diferentes momentos del periodo de cría. Noventa pollitos de un día se alojaron en una sala de cría. Cada semana del periodo de cría, se trasladaron 15 aves a otra sala (sala experimental) y se dividieron aleatoriamente en tres grupos: el grupo 1 recibió FP a través del agua de bebida, el grupo 2 L100 a través del pienso y el grupo 3 no recibió ningún fago (grupo de control). A continuación, tras 24 horas de administración, se realizó la enumeración de los fagos en las diferentes secciones del tracto gastrointestinal y se tomó el contenido cecal para el análisis de la microbiota y el metaboloma. Para investigar la composición de la microbiota y el metaboloma, se realizaron análisis de secuenciación del 16S ARNr y análisis metabolómicos LC-HRMS, respectivamente. En las diferentes edades en las que se administraron los bacteriófagos, las mayores concentraciones de bacteriófago se encontraron en el buche de los animales. A lo largo del ciclo productivo, se demostró que la microencapsulación permitía la llegada de los bacteriófagos al intestino y el ciego de los animales. La encapsulación basada en L100 protegió a los fagos de las duras condiciones ambientales en el proventrículo-molleja en cada uno de los momentos de administración, lo que puede asegurar la llegada de altas dosis de fagos al ciego. Los resultados de la microbiota del contenido cecal mostraron un alto nivel de similitud (diversidad beta) pero revelaron un cambio significativo en la diversidad alfa entre los pollos de engorde a los que se les administró FP y el grupo control. Además, los fagos sólo afectaron a unos pocos géneros de la estructura de la microbiota, independientemente de la vía de administración. Entre ellos, se encontró un aumento significativo de *Streptococcus* y *Sellimonas* en FP y *Lactobacillus*, Anaeroplasma y Clostridia_vadinBB60_group en L100. A pesar de que pocos géneros se vieron significativamente afectados, un número elevado de metabolitos, especialmente en FP, se vieron significativamente alterados (64 y 14 en FP y L100, respectivamente).

El segundo bloque, denominado "Dinámica de los bacteriófagos en pollos de engorde infectados por *Salmonella* y su influencia en la microbiota y el metaboloma", consiste en un ensayo experimental. A partir de los resultados de la dinámica de los fagos en el tracto gastrointestinal de los pollos de engorde, el fago con mejores resultados (L100) se enfrentó a *Salmonella* para conocer su eficacia como herramienta de control y su influencia en la microbiota, el metaboloma y el crecimiento de los animales.

Para ello, se alojaron 100 pollitos de un día de edad en 2 grupos de 50 animales cada uno. El grupo 1 (grupo de control) fue desafiado con *Salmonella* y el grupo 2 (grupo tratado con fago) fue desafiado con *Salmonella* y recibió durante los primeros 21 días pienso suplementado con L100. Para evaluar la colonización, excreción y difusión de *Salmonella*, se tomaron semanalmente muestras de ciego, hisopos cloacales y muestras de calzas ambientales. Semanalmente se pesaron a los animales. La detección de *Salmonella* se llevó a cabo siguiendo la norma ISO 6579-1:2017 (Anexo D). Además, se tomó el contenido cecal y el suero de pollos de 4, 5 y 6 semanas de edad para el análisis de la microbiota y el metaboloma. La colonización de *Salmonella* se redujo significativamente durante la mayor parte del ciclo productivo, mientras que la excreción se redujo significativamente en la segunda, cuarta y quinta semana. Además, la presencia de la bacteria en el ambiente de la nave se eliminó al final del ciclo. No se obtuvieron diferencias significativas entre los pesos de los animales. Los resultados de la microbiota mostraron un alto nivel de similitud (diversidad alfa y beta) entre los pollos de engorde tratados con fago y el control. Los resultados mostraron que el tratamiento con fagos afectó a varios géneros de la estructura de la microbiota (30 % de los géneros identificados). Además, los análisis metabolómicos revelaron un número sustancial de metabolitos alterados en el ciego, sin embargo, los metabolitos del suero se alteraron mínimamente.

En conclusión, los principales resultados obtenidos de esta tesis doctoral incluyen que se observaron diferencias significativas entre los resultados de la administración de fagos de los estudios *in vitro* en comparación con los resultados *in vivo*. Además, en los pollitos de un día no se obtuvieron diferencias estadísticamente significativas entre la administración de fagos a lo largo del tracto gastrointestinal para los fagos encapsulados y los no encapsulados (siendo el intestino la excepción). Así pues, la administración de fagos encapsulados con L100 y S100 en pollitos de un día fue administrada con éxito sin que se observaran reacciones adversas en los animales. Además, cuando los fagos se administraron en diferentes momentos del ciclo productivo, la encapsulación con L100, pudo asegurar la entrega selectiva de altos títulos de fagos en el ciego, especialmente cuando se administró al principio y al final del ciclo, al proporcionarles protección contra las duras condiciones ambientales a las que se encuentran en el proventrículo-molleja. Además, el hecho de que se encontraran más fagos encapsulados en el buche y en el ciego, lugares predilectos para la alta colonización de *Salmonella*, hace que la encapsulación de

fagos sea una herramienta prometedora para controlar la bacteria a nivel de campo. Por otra parte, la fácil diseminación de los fagos a través de las heces también puede facilitar el control de la bacteria en el entorno de la granja. Además, la terapia preventiva con fagos altera mínimamente la microbiota intestinal pero afecta significativamente a sus metabolitos, independientemente de la vía de administración. En este sentido, se necesitan más estudios para evaluar los efectos beneficiosos de la encapsulación de fagos con L100 para controlar la bacteria en el campo durante el periodo de producción y poder comprender la posible interacción entre las especies bacterianas diferencialmente abundantes y los metabolitos significativamente alterados para aclarar las implicaciones del tratamiento con fagos.

En este sentido, la infección de *Salmonella* Enteritidis puede reducirse notablemente en el lote de aves mediante la aplicación de fagos encapsulados con L100, administrados como aditivo en la dieta de arranque durante el ciclo productivo. Se observó una reducción de la colonización y la excreción de *Salmonella*, con una eliminación completa de la bacteria en el entorno de la nave final del periodo productivo. Sin embargo, pueden ser necesarias dosis más altas de fagos, protocolos de administración mejorados y/o la combinación con otras estrategias para poder lograr la eliminación total de Salmonella en los animales. Por último, la aplicación de fagos de *Salmonella* en condiciones de producción modula el microbioma cecal y los perfiles del metaboloma en los pollos de engorde infectados con la bacteria. Sin embargo, el efecto en los metabolitos del suero sanguíneo y el rendimiento del crecimiento sugieren que la modulación de los fagos parece no tener un impacto biológico en los animales. En este sentido, se necesitan más estudios para evaluar si este cambio observado en el ciego de los animales implica que los fagos de *Salmonella* cambian la composición de la microbiota, lo que promueve el cambio en el perfil metabólico, o si los fagos están activamente involucrados en los cambios de los metabolitos. Estos resultados pretenden aportar importantes conocimientos sobre el uso de fagos como estrategia preventiva y de biocontrol contra la infección por *Salmonella* desde la granja hasta el tenedor.

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Abbreviatures

%	Percentage	**EU**	European Union
16S rRNA	16S ribosomal RNA	**FAO**	Food and Agriculture Origanization of the United
ALR	Additive log-ratio	**FP**	Free phage
AMR	Antimicrobial Resistance	**FTICR**	Fourier Transform Ion Cyclotron Resonance
APCI	Atmospheric Pressure Chemical Ionization	**g**	Grams
API	Identification system for Enterobacteriaceae and other Gram-negative	**GC**	Gas Chromatography
APPI	Atmospheric Pressure Photoionization	**GIT**	Gastrointestinal Tract
ASVs	Amplicon Sequence Variants	**GLM**	General Linear Model
BPW	Buffered Peptone Water	**h**	Hours
CDC	Centers for Disease Control and Prevention	**HCl**	Hydrochloric Acid
CE	Capillary Electrophoresis	**HEPA**	High-Efficiency Particulate Air
CECAV	Poultry Quality and Animal Nutrition Centre of the Valencia	**HPD95%**	Highest posterior density region at 95% of probability
CFU	Colony-Forming Unit	**HRMS**	High-resolution Mass Spectrometry
CI	Chemical Ionization	**ISO**	International Organisation for Standardisation
CITA	Centre for Research and Animal Technology	**IVIA**	Valencian Institute for Agrarian Research
D	Median of the marginal posterior distribution	**Kg**	Kilograms
DNA	Deoxyribonucleic Acid	**L100**	Bacteriocphage encapsulated with the
E.	*Escherichia*	**LB**	Luria Bertani
ECDC	European Centre for Disease Prevention and Control	**LC**	Liquid Chromatography
EFSA	European Food Safety Authority	**LIT**	Linear Ion Trap
EI	Electron Impact	**Log**	Logarithms
ESI	Electrospray Ionization	**M**	Molar
EC	European Comission	**m/z**	Mass-to-charge ratio

m2	Square meter	**Q**	Quadrupole
MALDI	Matrix Assisted Laser Desorption Ionization	**QIT**	Quadrupole Ion Trap
MDR	Multidrug Resistant	**S100**	Bacteriocphage encapsulated with the
mg	Milligram	**sd**	Standard deviation
mL	Mililiters	**SMRT**	Single Molecule Real-Time
mm	Millimetre	**spp.**	Species
MS	Mass Spectrometry	**TLR**	Toll-like Receptors
NaOH	Sodium Hydroxide	**TOF**	Time of Flight
NGS	Next-Generation Sequencing	**UV**	Ultraviolet Radiation
nm	Nanometer	**v/v**	Volume per volume
NMDR	National Metabolomics Data Repository	**VER**	Balance error rate
NMR	Nuclear Magnetic Resonance	**VIP**	Variable importance prediction
NSCP	National *Salmonella* Control Program	**vs**	Vesus
NTS	Non-Typhoidal *Salmonella*	**WGS**	Whole Genome Sequencing
ºC	Celsius degrees	**WHO**	World Health Organization
OD	Optical density	**x g**	Relative Centrifugal Force
P	Probability value	**XLD**	Xylose Lysine Deoxycholate agar
P0	Probability of the difference	**QTOF**	Quadrupole Time of Flight
pb	Base pair	**RNA**	Ribonucleic Acid
PCR	Polymerase Chain Reaction	**rRNA**	Ribosomal Ribonucleic Acid
PLS-DA	Partial least square-discriminant analysis	***S.***	*Salmonella*
PRR	Pathogen Recognition Receptors	**µg**	Micrograms
PV	Proventriculus	**µL**	Microliters

Chapter I. General Introduction

1.1 Poultry meat sector

1.1.1 Poultry meat production worldwide

The last decades have witnessed intensified poultry production in response to the growing global population demand for affordable, healthier, and high-quality animal protein (OECD/FAO, 2022). Thus, the world poultry meat production soared from 9 to 133 million tons from 1961 to 2020 (OECD/FAO, 2022). Currently, poultry meat represents 40 % of the global meat production, being the United States responsible for the largest share of the total world poultry meat production, with 17 % of global output, followed by China, Brazil, Russian Federation, and the European Union (FAO, 2020; MAPA, 2022). Moreover, it is expected to account for 52 % of the global growth in meat production over the coming decade (OECD/FAO, 2022). These facts have been a result of the high production efficiency that poultry production presents, and the absence of cultural, and religious restrictions in its consumption (Nhung et al., 2017; FAO, 2020).

1.1.2 Poultry meat production in Europe

The European Union (**EU**) has become one of the world's largest poultry products producers (OECD and FAO, 2018; EC, 2022a). Indeed, European poultry meat production has exceeded 13 million tons (Eurostat, 2022). This success of poultry farming has been translated into a 4.7 % for poultry meat of the € 414.1 billion of the total EU agricultural output in 2020, and even has been expected to continue increasing, accompanied by the focus on farm-animal welfare (Van Horne and Achterbosch, 2008; OECD and FAO, 2018; Van Horne and Bondt, 2018; Augère-Granier, 2019)

In the case of poultry meat production, six leading producers of poultry meat produce 70 % of the EU's broiler meat (Augère-Granier, 2019). Poland has been responsible for the largest share of the total EU production, followed by the United Kingdom, France, Spain, Germany, and Italy (**Figure 1**) (Augère-Granier, 2019; ECb, 2022). Although broiler meat is dominant (83 %), turkey, and duck, account for a significant share of total production (14 and 3 %, respectively) (ECb, 2022). In this sense, chicken is the second most consumed meat at over 24 kg per capita (Augère-Granier, 2019).

In Spain, poultry meat production is approximately € 2,499 million, representing 5.0 % of the Final Agricultural Production, and 12.2% of the Final Livestock Production (MAPA, 2022). The country has a total of 4,989 production broiler farms throughout the

territory, that produced 1.3 million tons of meat (MAPA, 2022). About 70 % of this Spanish poultry meat production has come from just four regions: Andalusia, Catalonia, Galicia, and the Community of Valencia (**Figure 1**) (MAPA, 2022). As the European countries, most poultry meat in Spain comes from broiler (81.7 %), followed by turkey meat, which represents about 13.8 % of total production (MAPA, 2022).

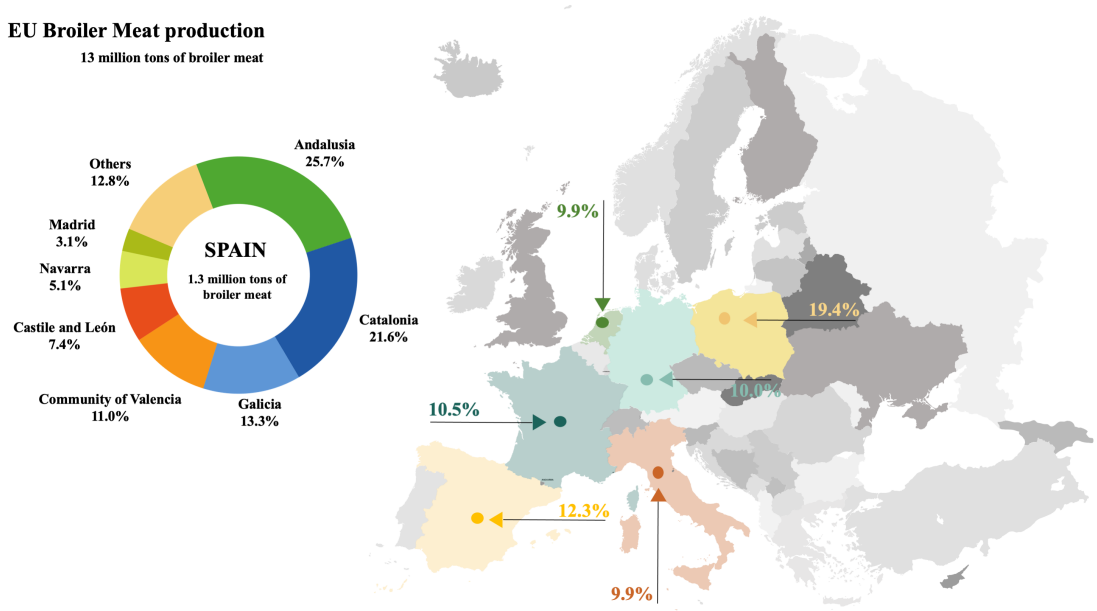

Figure 1. European and Spanish leading poultry meat producers in 2021 (Percentage share of EU members, based on tonnes of carcass weight). Adapted from MAPA (2022).

1.1.3 Evolution of poultry production systems

Since the "Neolithic revolution", animal husbandry has been in continuous changed to face societal challenges and according to the way in which people lived (Scanes, 2018). The domestication of the red junglefowl (*Gallus gallus*), probably before 6000 BC in Southeast Asia, marked the beginning of the use of chickens, first for their use in rituals or to proclaim the hour of dawn, and later for their bred for both its meat and egg (Hata et al., 2021). Throughout its history, crossbreeding between indigenous and commercial chickens has been performed to develop breeds that could be adapted to different challenging conditions (heat stress, humidity, diseases), and increase its production (Hata et al., 2021). The characteristics of poultry production have meant that it has been considered one of the most important agri-food industries in the world, and it is expected to remain (Mottet and Tempio, 2017).

From mid- 20th century onwards, the majority of chicken production has been intensive, optimizing the performance, throughout the breed selections, shorter generation times, less feed conversion ratios, and highest densities (Gilbert et al., 2015; Alders et al., 2018; Albrecht et al., 2019). This intensification responds to the demands of a growing and more affluent population, for animal-derived products (Gilbert et al., 2015). However, chicken intensive production, which takes place mainly in high-income countries, contrasts with the extensive production mainly by family-based smallholder farms in low-income countries, and the co-existence of extensive backyard production with intensive farming in transition economies (Gilbert et al., 2015). Intensified poultry production has mainly constituted by huge sophisticated national or international companies, which are generally highly integrated, that market millions of poultry carcasses annually (McMillin et al., 2012). Nevertheless, this type of production system may entail health risks. The concentration of a large number of animals, and the environmental disturbances (temperature, air recycling) facilitate the acquisition, evolution, and transmission of diseases (Gilbert et al., 2015). As a consequence, intensive poultry production requires the application of strict biosecurity measures. Moreover, public awareness regarding animal welfare, antimicrobial resistance, and environmental health has led to the adaptation of intensive production to more sustainable farming productions. There is a wide diversity in this type of production, since independent farmers sell their products themselves only to the local market, and farmers in a company (Souillard et al., 2019).

This adaptation to consumer demands has been carried out by the improvement of biosecurity, vaccination protocols, and livestock farming systems, and the use of more resistant and rustic slow-growing breeds, but attempting not to diminish the profitability of broiler farms (Sassi et al., 2016; Clavijo and Flórez, 2018a; El-Deek and El-Sabrout, 2019). These efforts carried out by the poultry are materialised in EU and Spanish legislation. The European Commission (**EC**) has developed different regulations to control animal welfare (EC, 2007), and Spain has followed European instructions, establishing the Animal Protection and Welfare Code (BOE, 2022). Nevertheless, this adaptation to more sustainable production systems has some unprecedented challenges, such as exposure to adverse weather conditions, cross-infections with wild animals, exposure to predators, and risk of endoparasites and other infections (Elson, 2015; Maes et al., 2021).

1.1.4 Impact of poultry production on public health

Globally, diarrhoeal disease has been estimated as the leading cause of death among all ages, with a disproportionate impact on young children (Troeger et al., 2017). One of the principal causes of this disease has been foodborne hazards, with approximately 550 million cases of illness each year (WHO, 2015). In this sense, the European Food Safety Authority (**EFSA**) and the European Centre for Disease Prevention and Control (**ECDC**) reported poultry products as the most frequent causative foods of foodborne illness and outbreaks in the EU (EFSA and ECDC, 2015).

In fact, poultry products have been considered the source of pathogens such as *Campylobacter*, *Salmonella*, *Escherichia coli*, *Listeria monocytogenes, Clostridium perfringens, Yersinia enterocolitica, Staphylococcus aureus,* as well as *Bacillus cereus* (Bremner and Johnston, 1996; Heredia and García, 2018). However, *Campylobacter* and non-typhoidal *Salmonella* remain the leading causative agents of foodborne illnesses in European countries (Rautelin and Hänninen, 2000; CDC, 2019; EFSA and ECDC, 2021). Being *Salmonella* the most common cause of foodborne outbreaks in Europe.

In this sense, the potential for human exposure to *Salmonella via* the poultry production chain has been increased by the ever-growing consumption of poultry products (Clemente et al., 2014). Prevention and control are crucial in the intensification of poultry farming systems, where the high throughput of animal husbandry presents a high risk of developing and transmitting zoonotic agents (Borda-Molina et al., 2018).

1.2 *Salmonella* in poultry

1.2.1 General aspects of *Salmonella*

1.2.1.1 Placing the disease in the current context

The first historical references related to *Salmonella* come from ancient physicians, such as Hippocrates, the father of medicine (about 460 to 377 B.C.), and Galen (about 200 to 130 B.C.), who described enteric fevers as a common symptom of prolonged fever, gastroenteritis, and partial unconsciousness (Lancaster, 1990; Dawoud et al., 2017) (Lancaster, 1990). But it was not until the 17th century when the disease was studied in more detail. At this time, enteric fever was differentiated from others, and typhoid fever

was distinguished from typhus by Thomas Willis in his *Treatise of Fevers* (Lancaster, 1990; Dawoud et al., 2017).

During the 19th century, the disease was described frequently, and a great number of scientists tried to discover its nature (Lancaster, 1990; Dawoud et al., 2017). In the middle of that century, William Budd hypothesized that typhoid fever could be transmitted by an unknown agent through contaminated water, introducing the concept of fecal-oral transmission (Moorhead, 2002; Dawoud et al., 2017). In 1880, Karl Joseph Eberth reported the first observation of the causal agent from typhoid victims (Dawoud et al., 2017). Nevertheless, it was Georg Gaffky, who four years later isolated the bacterium, and named *Eberthella typhosa,* currently known as *Salmonella* Typhi (Dawoud et al., 2017). In the following years, Salmon and Smith isolated *Bacillus cholerasuis*, now called *Salmonella enterica* subsp. *enterica* serovar Choleraesuis, from pigs diagnosed with the "hog cholera" (Cosby et al., 2015; Dawoud et al., 2017). Salmon was credited with the discovery, and the organism was named after him, although Smith was the first to identify it (Nair and Johny, 2019). In the late 1880s, the first confirmed case connecting the consumption of food with a human salmonellosis outbreak was carried out by Gäertner, though the isolation of *Bacterium enteritidis*, currently known as *Salmonella* Enteritidis (Dawoud et al., 2017). Indeed, since the 1950s, poultry products play the main role in *Salmonella*-associated foodborne outbreaks, moreover, since the mid-1980s have been considered an important public health threat worldwide (Nair and Johny, 2019).

Figure 2. Thomas Willis, William Budd, Karl Joseph Eberth, Georg Gaffy, Daniel Elmer Salmon and Theobald Smith. Obtained from: www.adc.bmj.com; www.timetoast.com; and www.wikimedia.org.

1.2.1.2 General characteristics

Bacteria of the genus *Salmonella* are a Gram-negative, non-spore-forming, and motile bacillus belonging to the *Enterobacteriaceae* family (**Figure 3**) (Barrow, 2000; Octavia and Lan, 2013). Members of this genus are oxidase-negative and catalase-positive. The organism is non-encapsulated, and varies from 0.5 to 1.5 μm in width, and 2.0 to 5.0 μm in length (Jajere, 2019).

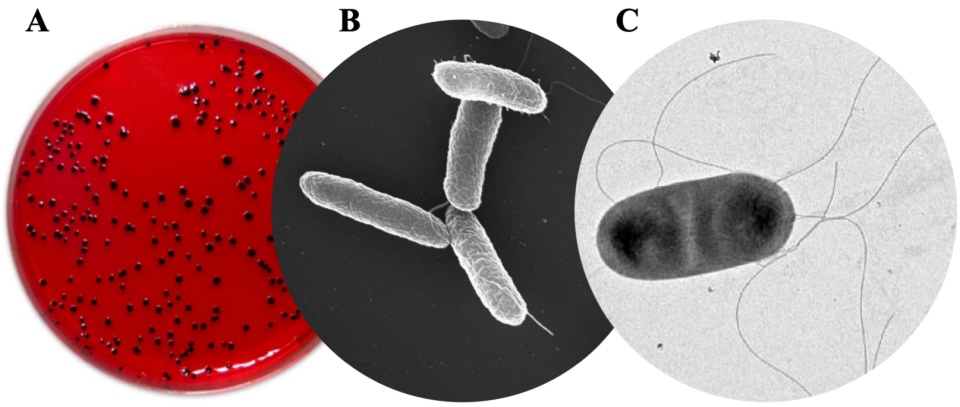

Figure 3. Colonies of *Salmonella* in Xylose-Lysine-Desoxycholate Agar (A), scanning electron micrograph (B), and transmission electron microscopy micrographs of *Salmonella* (C). Obtained from www.cnb.csic.es; https://hotcore.info/act/kareff-10079.html.

Generally, this genus is motile, except for *Salmonella* Gallinarum and *Salmonella* Pullorum, conferred by peritrichous flagella (Barrow, 2000). They grow between 8 and 45ºC, with an optimum growing temperature of approximately 37ºC. In addition, *Salmonella* is able to remain viable but not grow at a temperature between 0 and 5 ºC (Cosby et al., 2015). Although the optimal pH range enabling the growth of *Salmonella* is from 6.5 to 7.5, they are able to grow in a media with a pH range of 4.5 to 9.0. Moreover, *Salmonella* grows at water activity above 0.93, and resists drying, but is sensitive to gamma irradiation and organic acids (Cosby et al., 2015).

The genus *Salmonella* has three major antigens, with applications in the identification, and diagnosis, the Flagellar antigen or H; the Somatic antigen or O; and the Surface antigen or Vi, found only in a few serovars (Cosby et al., 2015).

1.2.1.3 Nomenclature

Salmonella is classified into two species, *Salmonella* enterica and *Salmonella* bongori. However, only *Salmonella* enterica, which includes 2,579 serovars, is strongly linked to human toxi-infections through the consumption of food of animal origin (Andino and Hanning, 2015; Gut et al., 2018). This specie has been further divided into six subspecies, *Salmonella enterica* subsp. *enterica* (subspecies I), *Salmonella enterica* subsp. *salamae* (subspecies II), *Salmonella enterica* subsp. *arizonae* (subspecies IIIa), *Salmonella enterica* subsp. *diarizonae* (subspecies IIIb), *Salmonella enterica* subsp. *houtenae* (subspecies IV), and *Salmonella enterica* subsp. *indica* (subspecies VI) (Dawoud et al., 2017).

The genus *Salmonella* is associated with gastrointestinal tract of the animals (Cosby et al., 2015), thus, the bacterium is generally classified into typhoidal *Salmonella* and non-Typhoidal *Salmonella* (**NTS**) based on host specificity and infectious nature (Winter et al., 2010; Feasey et al., 2012). The NTS serovars are non-host adapted with a wide range of vertebrate hosts, whereas typhoidal *Salmonella* serovars are highly adapted to humans, being their exclusive reservoir (Winter et al., 2010; Feasey et al., 2012; Gal-Mor et al., 2014). In addition, while infections by typhoid fever are common in developing and under-developed countries, NTS infections develop (Gal-Mor et al., 2014).

1.2.2 Epidemiology in humans

1.2.2.1 Public health significance of *Salmonella*

Public health implications of *Salmonella* infections have evolved over more than a century (Nair and Johny, 2019). Indeed, since the mid-20th, this bacterium has been recognized as one of the most important zoonotic pathogens worldwide (Nair and Johny, 2019). The outbreaks still occur in both developed, developing, and under-developed countries, highlighting the human morbidity and mortality contributions of *Salmonella* in the human population (Cosby et al., 2015). Furthermore, salmonellosis due to NTS has been one of the main causes of foodborne illness, with an overall impact on human health estimated at 93.8 million sick and 155,000 deaths each year worldwide (Bula-Rudas et al., 2015). Moreover, it is assumed that only 1 in 7 cases is diagnosed (Majowicz et al., 2010; Andino and Hanning, 2015; Gut et al., 2018). Although different serotypes have been associated with salmonellosis, *Salmonella* Enteritidis (***S.* Enteritidis**) has been the

most common serotype of *Salmonella* that cause food-borne illness in humans worldwide (Dawoud et al., 2017), as well as in the EU (EFSA and ECDC, 2021).

In Europe, the latest data published in 2020, revealed a total of 52,702 cases of human salmonellosis, of which 6.7 % were diagnosed in Spain, only behind *Campylobacter* (**Figure 4**) (EFSA and ECDC, 2021). Nevertheless, this prevalence could be underestimated due to the impact of COVID-19 on the surveillance/reporting of human cases (EFSA and ECDC, 2021). The sources of *Salmonella* infection are relatively diverse, but the consumption of poultry products, such as eggs and undercooked chicken meat, is considered the main source of human infection (EFSA and ECDC, 2021). Given this situation, *Salmonella* continues to be one of the main concerns, both for public health, and for livestock.

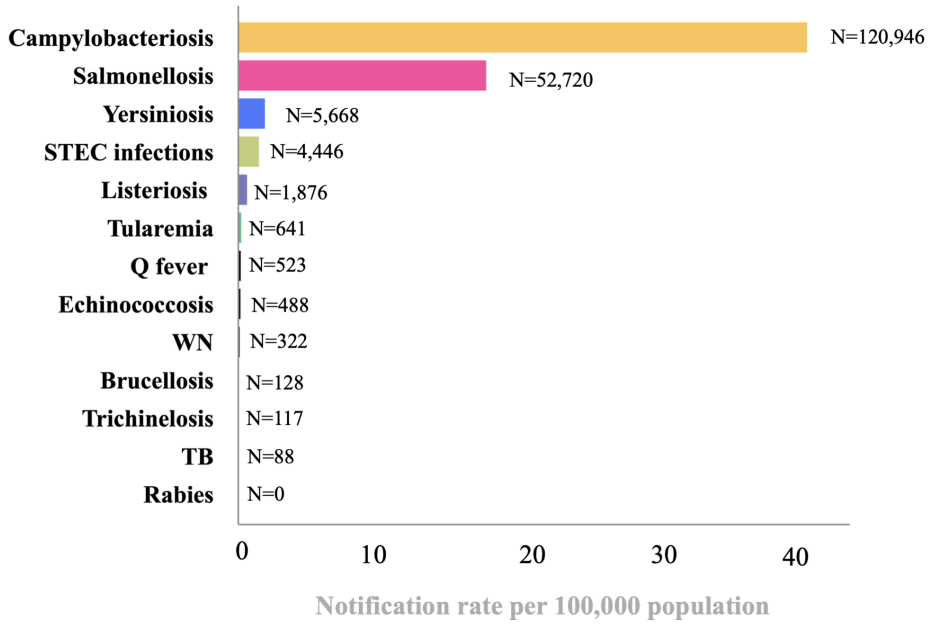

Figure 4. Reported hospitalisations and case fatalities due to main zoonoses in confirmed human cases in the European Union in 2020. The total number of confirmed cases is indicated at the end of each bar. WN: West Nile virus infection; TB: Tuberculosis due to *Mycobacterium bovis* and *Mycobacterium caprae*. Adapted from EFSA and ECDC (2021).

1.2.2.2 Human clinical aspects

Salmonella infection causes a wide range of severity illnesses, depending on the serotype, and the human host´s health status (Eng et al., 2015). The illness in humans could be induced by an oral dose of 10^4 to 10^6 colony-forming units (**CFU**) of the bacterium, however, an infective dose 10^1 to 10^2 CFU may be sufficient (Cosby et al., 2015). After

that, the human *Salmonella* infection initially involves the attachment and colonization of the intestinal epithelium of the small intestine, and the specialized microfold cells overlying Peyer's patches (Liu et al., 1988; Gal-Mor et al., 2014; Cosby et al., 2015). After 12 to 72 hours of the incubation period, the infection results in a week of acute gastroenteritis with diarrhoea, abdominal pain, nausea, and vomiting (Gal-Mor et al., 2014; Cosby et al., 2015; Nair and Johny, 2019). Although these symptoms usually are self-limiting, the infection could be more severe in immunocompetent, children and older patients (Cosby et al., 2015; Nair and Johny, 2019). In these cases, the infection could progress to septicemia, and even death (Cosby et al., 2015). Also, *Salmonella* infections could lead to chronic conditions, such as reactive arthritis, Reiter's syndrome, or ankylosing spondylitis (Cosby et al., 2015).

The currently employed treatment in *Salmonella* infections generally involves intravenous fluid therapy. However, antimicrobial therapy could be required in severe cases or risk groups, considering that antibiotic therapy could prolong the excretion of NTS (Gal-Mor et al., 2014). The recommended antimicrobial treatment of *Salmonella* infections includes fluoroquinolones as a first choice, third-generation cephalosporins as second-line treatment, and macrolides as the choice against both nalidixic acid resistant and multidrug-resistant (**MDR**) NTS strains (Gal-Mor et al., 2014; Cosby et al., 2015). In this sense, during the last decades, it has been reported an increase of *Salmonella* antibiotic-resistant strains isolates, that could result in severe illness with long periods of hospitalization and high medical costs (Nair and Johny, 2019).

1.2.3 Epidemiology in poultry production

Since the end of 19th century, *Salmonella* was recognised as a zoonotic pathogen, being poultry products the main source of food poisoning in humans (Dawoud et al., 2017). From farm to table, poultry could be colonized by *Salmonella* through horizontal or vertical transmission, and act as a constant source of infection (Liljebjelke et al., 2005; Chlebicz and Śliżewska, 2018). In poultry, *Salmonella* vertical transmission is crucial. In this sense, the bacterium could lead to a persistent infection in birds, and is also located in the ovary (Zamora-Sanabria and Alvarado, 2017). Thus, if *Salmonella* is present in a breeding flock, it could be transmitted to the progeny by transovarial infection (Wray et al., 1999; Zamora-Sanabria and Alvarado, 2017). Furthermore, the shells contamination from parent flocks *via* fecal transmission leading to a "pseudo-vertical transmission" of

Salmonella has been described (Wray et al., 1999; EFSA, 2009). Infection of *Salmonella* during the laying period leads to the production of contaminated eggs, that could arrive at the market, or, if these are fertile and are hatched, results in extensive infection of the progeny (Dawoud et al., 2017; Wigley and Barrow, 2017). Nevertheless, its high presence in the poultry flocks has been achieved not only by the vertical transmission of the pathogen from breeder flocks but also by the horizontal transmission that could take place in the housing facilities (Nair and Johny, 2019).

Salmonella horizontal transmission is a significant source in commercial poultry flocks, due to it is extremely widespread and persistent in the environment (Zamora-Sanabria and Alvarado, 2017). Thus, there are numerous risk factors for *Salmonella* dissemination and contamination in the poultry flock, being feco-oral transmission the main route of the infection (Shah et al., 2017). First, several authors reported the day-old chicks infected with *Salmonella* as a major *Salmonella* contamination risk factor (Cox et al., 1990; Rose et al., 1999; Marin et al., 2009). They are able to transmit the bacterium from the hatching, loading, and transport to the farm (Cox et al., 1990; Cason et al., 1993; Marin et al., 2011). Day-old chicks could be infected with less than five cells of *Salmonella,* being a critical moment for the chicks as its immune system is still immature, facilitating the rapid colonization and multiplication of the bacterium, compromising the entire production period (Bailey, 1987; Marin and Lainez, 2009; Hashemzadeh et al., 2010; Koutsoumanis et al., 2019; Groves et al., 2021). In addition, the lack of proper cleaning and disinfection protocols between consecutive flocks has been suggested as an important risk in *Salmonella* transmission (Wray et al., 1999; Davies and Breslin, 2003; Marin et al., 2009, 2011). In this sense, the presence of contaminated vectors, especially rodents, is an important source of recontamination of houses after these procedures (Davies and Breslin, 2003; Carrique-Mas et al., 2009; Marin et al., 2011). Furthermore, environmental contamination sources such as feed, litter, or water could be considered important risk factors (Bailey et al., 2001; Heyndrickx et al., 2002; Marin et al., 2011). Moreover, it has also been demonstrated that biofilm formation has been an important survival factor for *Salmonella* in poultry farms (Marin et al., 2009). Additional potential environmental sources, such as farm workers, that could spread the bacteria between flocks *via* clothes, boots, and transport crates, are a key factor in *Salmonella* persistence and recontamination of the flocks (Rose et al., 1999; Marin et al., 2011)

Once the bacteria are present in the flock, some variables that could affect the chicken colonization, such as age and genetic susceptibility of the chickens, the animal health status, diet, environmental and physiological stressors, level of exposure to the pathogen, and *Salmonella* serovar and strain (Foley et al., 2011; Cosby et al., 2015). One of the most important factors is the age of the birds, the lack of mature gut microbiota in day-old chicks, leads to a very low dose of *Salmonella* infection, such as 10 cells, and the susceptibility decrease with the age (Cosby et al., 2015). After that, another factor, such as the pH of the gastrointestinal tract could affect chicken colonization (Cosby et al., 2015). Usually, *Salmonella* encounters the acidic environment of the crop, with a pH between 4.5 to 5, that is maintained and controlled by the bacteria of the genus *Lactobacillus* (Cosby et al., 2015). Nevertheless, a decrease in the *Lactobacillus* population could produce an increase in the pH of the crop up to 6.3, leading to a more suitable environment for the survival of *Salmonella* (Cosby et al., 2015).

Subsequently, *Salmonella* could colonize the gastrointestinal tract, preferentially the lower ileum, caeca, and cloaca, and within 4 hours post-infection, it is able to invade the intestinal epithelium and localizes in the submucosa (Shah et al., 2017). Nevertheless, a third factor could affect chicken colonization, the dose and strain of *Salmonella* (Cosby et al., 2015). In this sense, higher levels of *Salmonella,* such as 10^4 to 10^5 CFU, could be more likely to colonize than lowers, and the efficiency of the colonization depends on the *Salmonella* serotype (Cosby et al., 2015).

Chickens usually are colonized without clinical signs, remaining as asymptomatic carriers until the age of slaughter, being the detection and elimination of carriers more difficult (Kogut and Arsenault, 2017). In addition, contamination of *Salmonella* poultry flocks during transport to the slaughterhouse has been reported as an important source of *Salmonella* contamination, due to the high-stress conditions to with the animals are subjected, such as crowding, motion, temperature fluctuations, and feed and water deprivation (Heyndrickx et al., 2002; Slader et al., 2002; Marin and Lainez, 2009). This situation leads to a disturbance of intestinal functions, with increations in the spreading of intestinal bacteria, favoring the contamination risk in the slaughterhouse (Mulder, 1995; Corry et al., 2002; Marin and Lainez, 2009). Also, when a positive flock enters in the slaughterhouse colonized with *Salmonella*, gut content spillage could result in contamination of the carcasses during the defeathering operations, and also the

slaughterhouse environment and consequently subsequently processed flocks, that finally leads to a contaminated carcass on the market (Marin et al., 2022).

1.2.4 The immunobiology of avian salmonellosis

Immune responses to *Salmonella* depend on the host species and the infecting *Salmonella* serotype (van Immerseel et al., 2005).

The gastrointestinal tract (**GIT**) plays an important role in the innate immunity that controls bacterial infections, especially the physical barriers, such as mucosal surfaces; and others, such as the acid environment of the gizzard and proventriculus, antimicrobial secretions, and mucociliary clearance (Wigley, 2013). Subsequently, innate immune activation takes place through the pattern recognition of the pathogen recognition receptors (**PRR**) bacteria (Meade et al., 2009; Keestra et al., 2010; Wigley, 2013). This recognition takes place through receptors such as Toll-like receptors (**TLR**), particularly by TLR-2, TLR-4, TLR-5, and TLR-21, that recognise the peptidoglycans, lipopolysaccharides, flagellins, and unmethylated CpG DNA, respectively, commonly found in bacteria (Meade et al., 2009; Keestra et al., 2010; Wigley, 2013).

Salmonella usually infects chickens *via* the fecal-oral route with the spread from the GIT (Barrow et al., 2012; Wigley, 2014). On the basis of the experimental studies related to poultry immunology, *Salmonella* host-restricted avian-adapted serovars, *Salmonella* Gallinarum and *Salmonella* Pullorum, lack flagella and thereby evade the recognition, allowing the evasion of immunity and the establishment of systemic infection, which may result in the death of the bird (van Immerseel et al., 2005; Chappell et al., 2009; Wigley, 2013). In contrast, non-host-specific *Salmonella* enterica serovars elicit a strong inflammatory response to invasion that is largely mediated by recognition of flagellin through TLR-5. This recognition leads to the expression of pro-inflammatory cytokines and the CXCL chemokines that activates innate immunity (Wigley, 2013).

The result of activation of innate immunity is an influx of heterophils, polymorphonuclear cells, and macrophages to the intestine that may results in inflammation, usually mild in the chicken, but also leads to immune activation and largely restricts infection to the gut (Wigley, 2013, 2014). However, the heterophil response does not have a significant protective response against the bacteria and a number of the infecting *Salmonella* are able to persist within macrophages by evading most of the antimicrobial mechanisms,

surviving and replicating within them, playing a crucial role in pathogenesis (Kogut and Santin, 2019). Other innate factors include the antimicrobial peptides, particularly β-Defensins termed gallinacins in the chicken, that are produced by a range of cells and tissues in response to *Salmonella* infection, or the increased expression of mucins, that are likely to play a role in the epithelial barrier maintenance.

The interaction between the intestinal microbiota and the host's innate immune system results in a specific immune response (Pan and Yu, 2013). Within this specific immune response are B-lymphocytes and T-lymphocytes, which give rise to humoral (antibody-mediated) and cellular (cell-mediated) immune responses, respectively (Pan and Yu, 2013). Infection with *Salmonella* elicits both antibody and cellular responses that can be detected from around a week post-infection (Wigley, 2013). Nevertheless, it has been reported a degree of regulation of the immune response, that allows *Salmonella* to persist within the gut for several weeks without the disease to the chicken in a tolerant stage (Wigley, 2014).

1.2.5 Laboratory isolation and detection methods: traditional, official, and other techniques

The most commonly used technique for *Salmonella* diagnostic is bacteriological isolation and the subsequent confirmation by biochemical and serological tests (Mousing et al., 1997). Based on this, the International Organisation for Standardisation (**ISO**) stablished in 2002 the official standard method for the detection, isolation, and enumeration of *Salmonella*, the ISO 6579:2002, and resulted in a new version of this method in 2017 with the ISO 6579:2017.

This standard method is divided into two parts. Part one (ISO 6579-1:2017), described the detection and isolation of *Salmonella*; and part 2 (ISO 6579-2:2017), described the standard for bacteria enumeration. The bacteriological isolation of *Salmonella* requires four stages, which includes the pre-enrichment in a non-selective liquid medium (e.g. Buffered Peptone Water), followed by the enrichment in the selective medium Modified Semi-Solid Rappaport Vassiliadis, the plating-out and identification that allows us to characterize *Salmonella* against other enterobacteria, and finally the confirmation with a biochemical and serological test (e.g. API-E20). In addition, phenotyping methods, such as serotyping and phage typing, have been required for the *Salmonella* serovars differentiation.

Although these traditional methods are the gold standard, they are generally labor-intensive and time-consuming, requiring at least 4 to 6 days of a confirmatory result, therefore, and show poor specificity and sensitivity for low-level contamination samples, increasing the risk of transmitting the bacteria (Gwida and Al-Ashmawy, 2014; Franco-Duarte et al., 2019). Despite these phenotypic and biochemical methods continuing to hold a place in the laboratories, molecular-based techniques have provided new points of view into bacterial identification and typing (Franco-Duarte et al., 2019).

For more than 20 years, these molecular techniques have been used to detect and differentiate *Salmonella* serotypes (Zanetti et al., 2019), that are relying on the polymerase chain reaction (**PCR**) (Fernández-Cuenca, 2004). This technology is based on a DNA polymerase that is used to amplify a piece of DNA by *in vitro* enzymatic replication and seems to offer the greatest potential for rapid, specific, and sensitive assays; ranging from the relatively simple DNA amplification-based approaches towards the more complex methods based on restriction fragment analysis, targeted gene and whole genome sequencing (**WGS**) (Franco-Duarte et al., 2019). Last decades, continuous improvements have advanced sequencing technologies with the objective that WGS methods have become more practical and suitable for the identification of micro-organisms, such as *Salmonella* (Ricke et al., 2018; Franco-Duarte et al., 2019). For this reason, making molecular technologies more user-friendly and cost-effective for routine diagnosis to determine the status of the flocks have gained great attention to make available to the food industry (Ricke et al., 2018).

1.2.6 Control in the European Union

The presence of *Salmonella* in poultry primary production is of paramount importance, due to it is considered a risk factor for the presence of *Salmonella* in the food web (Messens et al., 2013). To protect human health against NTS infections, food legislation purposes for the poultry production chain have been implemented in the EU.

During the 1950s, several food outbreaks in Sweden, related to animal products contaminated by *Salmonella*, leads to an alarm in Public Health (Lundbeck et al., 1955). As a result, Sweden was the first country in Europe to initiate a National *Salmonella* Control Program (**NSCP**), applying specific measures for the control of this zoonosis in pigs, poultry, and ruminants (Wierup et al., 1995). The objective of the Swedish program

was to market products for human consumption that were free of *Salmonella*, and it was based on the prevention of contamination by this bacterium *via* the food production chain (Wierup et al., 1995).

At the end of the 20th century, Directive 92/117/EC, for protection against specified zoonoses and specified zoonotic agents in animals and products of animal origin, included the monitoring, prevention, and control of *Salmonella* infections in *Gallus gallus* breeding flocks, and products for poultry feed. This Directive laid down the specific minimum measures to control *S.* Enteritidis and *Salmonella* Typhimurium (***S.* Typhimurium**) in breeding stocks (Voss, 2007). Following the Directive, the Member States started working on national plans to control and prevent the introduction of *Salmonella* at the farm level. Nevertheless, in 2003 Directive 2003/99/EC on the monitoring of zoonoses and zoonotic agents repealed Directive 92/117/EC, due to the need to take stricter measures to control food-borne zoonotic pathogens, and the need to collect data (Boqvist et al., 2018). The same year, Regulation (EC) No 2160/2003 on the control of zoonoses has been implemented and has been formed as an inseparable unit with Directive 2003/99/EC. In this sense, EU regulations have set targets for the reduction of specific serotypes in the target population, selected due to their public health significance. For breeding hens, laying hens, broilers, breeding and fattening turkeys the target serotypes have been *S.* Enteritidis and *S.* Typhimurium including monophasic variant, while for breeding hens, *Salmonella* Hadar, *Salmonella* Virchow, and *Salmonella* Infantis, have been also controlled (Messens et al., 2013; Alvarez et al., 2020).

To address this need, the Regulation established the mandatory preparation of NCPs that must include monitoring schemes to the determination of the prevalence of *Salmonella* in the target population, and control measures as foreseen by EU legislation, nevertheless, each Member Estate could add further sampling methods if considered necessary based on the specific epidemiological situation (Messens et al., 2013). Thus, since 2008, NCPs for the control of *Salmonella* in commercial scale flocks have been implemented in the EU, and in Spain have been transposed by Royal Decree 1940/2004 of 27 September on the monitoring of zoonoses and zoonotic agents (Martelli et al., 2017). This monitoring responsibility of the food business operators (auto-control checks) and includes the periodical testing of the target flocks, being every two weeks for breeding flocks of *Gallus gallus*, every 15 weeks for flocks of laying hens, and birds leaving for slaughter for flocks

of broilers and turkeys (Alvarez et al., 2020). Moreover, depending on commercial farm size, the official veterinary services perform annual controls (official controls)(Alvarez et al., 2020).

1.3 Antimicrobial resistance in poultry

1.3.1 Tackling the antibiotic resistance problem in poultry

Since Alexander Fleming discovered penicillin (Bornstein, 1940), antibiotics have meant a revolution in humans´, as well as animals´ health, due to the prevention, protection, and control of the infections that have conferred (Grant et al., 2017; Florez-Cuadrado et al., 2018). Antibiotics are products of natural, synthetic, or semi-synthetic origin that kill or inhibit the growth of microorganisms (Florez-Cuadrado et al., 2018; Jajere, 2019). Nevertheless, the extensive use or misuse of antibiotics in humans and animals has led to the evolutionary emergence of bacterial antimicrobial resistance (**AMR**), which threatens antibiotic efficiency in combating bacterial infections (van Vuuren, 2003; Cosby et al., 2015). As a result, commonly used antibiotics have become ineffective for the treatment of a wide variety of bacterial diseases, increasing public concern and scientific interest (van Vuuren, 2003; Khurana et al., 2017; EFSA and ECDC, 2018). Indeed, the World Health Organization considered the AMR one of the most important health threats in the 21st century, wich could cause 10 million deaths a year by 2050, ahead of other diseases such as cancer (O´Neill, 2014; WHO, 2019). In this sense, the use of antibiotics in food-producing animals could contribute to the development and propagation of AMR bacteria, and these bacteria could reach humans through the food chain and the environment (WHO, 2017; Florez-Cuadrado et al., 2018; Kumar et al., 2019). Owing to the public health risk, the non-therapeutic use of antibiotics in animal production has been banned since 1986, first in Sweden, and was followed by entire the European Union under the “One Health” principle (Founou et al., 2016). Furthermore, in the EU all countries have developed regulatory actions addressing the AMR threat in zoonotic and animal pathogens (European Court of Auditors, 2019).

Antibiotics in poultry have also been used since the 1940s, however, it was not until the 1960s that their commercial employment was widespread in Europe, with concerns about the development of resistance dating back to 1969 (Florez-Cuadrado et al., 2018; Yang et al., 2019). Against this background, different routes have been suggested for the AMR development and dissemination into the different stages of poultry production, such as

vertical transmission or horizontal gene transfer from an environmental source at the breeder level, hatchery and production farm level (Yang et al., 2019; Karunarathna et al., 2020; Marin et al., 2020).

Bacteria could counteract the actions of antibiotics through different mechanisms, such as enzyme modification, alteration of the target binding sites, active efflux pumps, or decreased permeability of bacterial membrane (Agyare et al., 2019). This expression of AMR towards by bacteria could either be intrinsic or acquired (Florez-Cuadrado et al., 2018; Agyare et al., 2019). Intrinsic resistance means that all isolates belonging to the same species are resistant to an antimicrobial, due to inherent properties within the bacteria chromosome such as mutations in genes and chromosomally inducible enzyme production, that naturally prevents access to the antimicrobial to its target (Florez-Cuadrado et al., 2018; Agyare et al., 2019). Whereas acquired resistance refers to the horizontal transfer of mobile genetic elements from other bacteria and/or the transmission of resistance genes from the environment (Agyare et al., 2019). In this sense, the ease transmission of resistance genes plays an important role in the spread of AMR among strains (van Vuuren, 2003; Yang et al., 2019). This fact has implications for the increase of AMR between both virulent strains and normal bacterial microbiota, providing a reservoir of AMR bacteria that could be transferred to humans through the food chain (van Vuuren, 2003; Yang et al., 2019). Specially, the development of MDR, defined as resistance to more than three classes of antimicrobials, in zoonotic pathogens like *Salmonella* has been a growing serious concern (Kumar et al., 2019).

1.3.2 *Salmonella* antimicrobial resistance

The first incidence of antibiotic resistance to *Salmonella* was reported in the early 1960s, with the resistance to chloramphenicol (Jajere, 2019). Since then, the isolation frequency of bacteria resistant to one or more antibiotics has increased globally, with implications of therapeutic failure in cases of life-threatening disease in human, and veterinary medicine (Threlfall et al., 2003; Münch et al., 2012; O 'Neill, 2014; EFSA, 2019; Jajere, 2019). In this sense, *Salmonella* has been included in the World Health Organization priority list of 12 antibiotic-resistant bacteria (Tacconelli et al., 2018).

In the EU, more than 62.70 % of *Salmonella* isolated from broilers showed resistance to at least one antimicrobial, and approximately 41.80 % showed MDR (EFSA and ECDC,

2020). High prevalence of resistant *Salmonella* to ciprofloxacin, nalidixic acid, sulfamethoxazole, and tetracycline. Moreover, AMR was evident in 71.70 % of the broiler carcasses (EFSA and ECDC, 2020).

1.3.2 Alternatives in poultry production

The emergence of AMR bacteria in the food chain is a growing public health problem worldwide (van Vuuren, 2003). Indeed, since the 1980s no new class of antibiotics has become available (Gigante and Atterbury, 2019). This situation leads to the need for the develop of alternative strategies for fighting these bacteria (Gigante and Atterbury, 2019). In poultry, current approaches related to coordinated multidisciplinary strategies that aim to develop new antibiotic alternatives combined with improvements in management practices (FDA, 2013). In this sense, the combination of biosecurity measures, as well as vaccination programs, with food additives such as probiotics, prebiotics, symbiotics, organic acids, plant extracts, and bacteriophages could play a fundamental role, not only in the prevention of pathogens such as *Salmonella*, but also in the production parameters, thus complying with market demands (**Figure 5**).

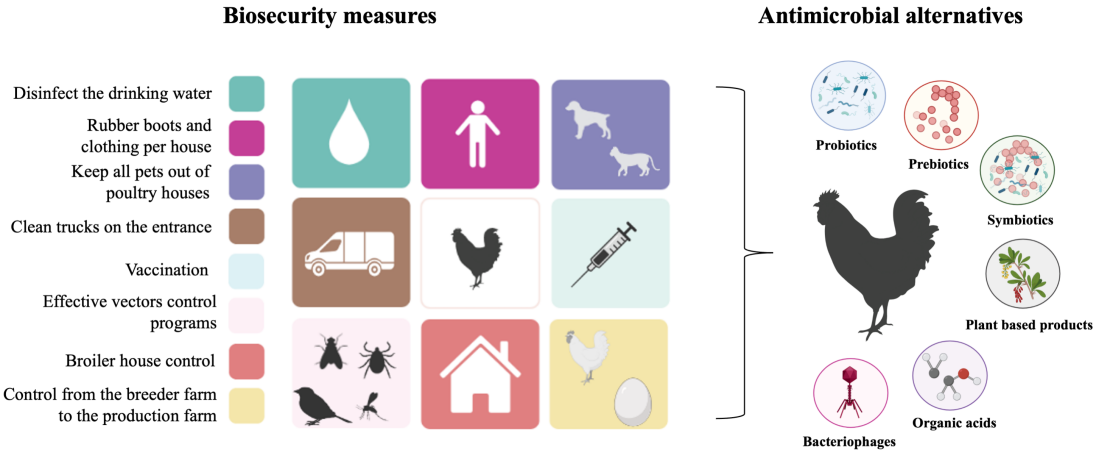

Figure 5. Combination of biosecurity measures with antimicrobial alternatives in poultry production. Adapted from www.cladan.com.

Probiotics are live microorganisms that improve the intestinal health of the host and animal performance, when administered at an adequate dosage (Gut et al., 2018). The administration of probiotic strains develops specific skills in the host. To do this, probiotics exclude or reduce the adherence of pathogens to the GIT by different

mechanisms, such as competitive exclusion, the production of antibiotic molecules (hydrogen peroxide and bacteriocins), and/or stimulating the immune system (Borda-Molina et al., 2018; Gut et al., 2018; Khan and Chousalkar, 2020). In addition, probiotics enhance enzyme activity (Borda-Molina et al., 2018), improve epithelial cell function (Gut et al., 2018), ultrastructure the intestinal mucosa (Borda-Molina et al., 2018), and produce host angiogenesis (Gut et al., 2018). This results in increased assimilation of nutrients and growth (Vandeplas et al., 2010; Borda-Molina et al., 2018).

Prebiotics are non-digestible oligosaccharides that selectively promote the growth and/or the activity of beneficial microbiota (Vandeplas et al., 2010). In addition, prebiotics can increase the utilization of nutrients through intestinal function improvement (Kridtayopas et al., 2019). The mechanisms of action of prebiotics include the production of antimicrobial substances, modulation of the host immune system, and improvement of intestinal morphology (Borda-Molina et al., 2018; Khan and Chousalkar, 2020). Lactose, lactulose, and lactosucrose are natural disaccharides, or products of isomerization, that have prebiotic effects in birds (Vandeplas et al., 2010).

Symbiotics are the combination of prebiotic substrates and probiotic strains, that have been a synergistic effect on the fecal microbiota of experimental animals (Vandeplas et al., 2010). Such mixtures improve the implantation and survival of beneficial bacteria supplemented in the gut (Borda-Molina et al., 2018). Previous research results have indicated that the combination of probiotics and prebiotics has a synergistic effect on the fecal microbiota of experimental birds (Gmeiner et al., 2000; Liong and Shah, 2006; Vandeplas et al., 2010).

Organic acids, including short-chain fatty acids, such as butyric, propionic, and acetic acids; and medium-chain fatty acids emulsion such as caproic, caprylic, capric, and lauric acids, could exert bacteriostatic or bactericidal effects against gram-negative bacteria by entering the bacterial cell and lowering bacterial pH levels. This decrease in pH in the crop and gizzard results in the establishment of lactic acid-producing bacteria, such as *Lactobacillus* and *Bifidobacterium* (Gibson and Wang, 1994; Zhu and Joerger, 2003; Borda-Molina et al., 2018), that aims to reduce the colonization by other bacteria like *Salmonella* and *Campylobacter* (Borda-Molina et al., 2018).

Plant-based products are another alternative whose interest is increasing due to their antioxidant and antimicrobial characteristics and the enhanced digestibility that could produce (Borda-Molina et al., 2018). Historically, plants have served in many cultures as a source of dietary supplements for reducing spoilage and promoting growth and food preservatives. These products have been based on the stimulation of endogenous enzymes, nitrogen absorption, and the inhibition of odor and ammonia (Borda-Molina et al., 2018). Plants are capable of synthesizing molecules as a defense mechanism against predation by microorganisms and insects, with biological effects, including anti-inflammatory, antimicrobial, anticarcinogenic, cardioprotective, and neuroprotective properties (Arsi et al., 2019). Compounds such as caprylic acid, trans-cinnamaldehyde, carvacrol, and eugenol have shown effectiveness in controlling pathogens in poultry (Arsi et al., 2019).

Finally, the use of **bacteriophages** is a possible method to achieve *Salmonella* control in poultry farms that have gained importance in recent years (Toro et al., 2005; Atterbury et al., 2007; Sevilla-Navarro et al., 2018, 2020).

1.4 Bacteriophages in poultry

1.4.1 General aspects

Bacteriophages (or phages) are the viruses that specifically infect bacterial cells (**Figure 6**) (Wernicki et al., 2017; Żbikowska et al., 2020). They are known as natural parasites or parasitoids of bacteria because they lack cellular structures and enzyme systems necessary for food uptake, protein synthesis, or construction of new bacteriophage particles, needing the bacterial cell to replicate (Toro et al., 2005; Wernicki et al., 2017; Żbikowska et al., 2020). Phages are highly specific, self-replicating, self-limiting, well tolerated, and accessible from multiple sources (Yin et al., 2021; Ruvalcaba-Gómez et al., 2022). Phages are ubiquitously distributed in nature, with an estimated total 10^{31} phage particles in the biosphere, 10-times more than the estimated number of bacteria cells, making them the most abundant biological entities on Earth (Keen, 2015; Batinovic et al., 2019; Żbikowska et al., 2020). These viruses are usually distributed based on the presence of their host bacteria, including water, soil, air, plants, humans, and other animals, and therefore are consumed by people (Clokie et al., 2011; Żbikowska et al., 2020). Indeed, phages have been recognized as the main component of the microbiota, even in human microbiota, known as the phageome, and dominating the gut virome

(Żbikowska et al., 2020). They are capable of adhering to the mucosal surfaces, reducing bacterial colonization and pathology, and providing a non–host-derived layer of immunity (Barr et al., 2013; Krut and Bekeredjian-Ding, 2018).

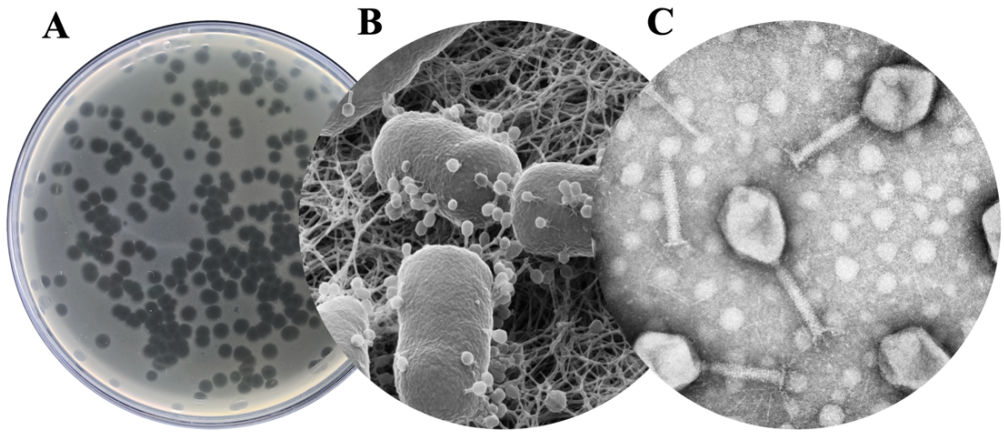

Figure 6. Plaques of *Salmonella* phage in LB Agar (A), scanning helium ion microscope phage image (B), and scanning electron microscope phage electron micrograph (C). Obtained from www.sciencephoto.com; www.sciencedirect.com.

Thus, it is now widely accepted that phages could control the composition and diversity of the microbiome. Its potential applications of this kind of human medicine have been attempted to implement in preventive and therapeutic veterinary medicine procedures (Gigante and Attebury, 2019). It´s potential as a biocontrol tool has been considered a promising approach in both pre-harvest and post-harvest settings, due to its ability to infect a narrow number of species, serotypes, or strains (Wernicki et al., 2017; Batinovic et al., 2019). In this sense, the effect of the phages in the prevention or treatment of diseases, and even in the growth promotion, could offer new opportunities in the food industry that need to be studied (Wernicki et al., 2017; Svircev et al., 2018; Batinovic et al., 2019). Nevertheless, just as bacteria may become resistant to antibiotics they could develop resistance to phages by a variety of mechanisms, such as spontaneous mutations, restriction/ modification systems, and adaptive immunity *via* the CRISPR-Cas system, among others (Oechslin, 2018; Svircev et al., 2018). However, the emergence of phage-resistant bacteria to one phage does not necessarily result in resistance to others, and even the phage could acquire phage receptors from lysed sensitive cells and lysed the resistant bacteria (Svircev et al., 2018; Gigante and Atterbury, 2019).

1.4.2 Brief history of bacteriophage discovery and research.

The early observation of the phage activity was carried out by Ernest Hankin in 1986, who published the French article "The bactericidal action of waters of Jumna and Ganges on the cholera microbe" where he described a biological principle with antibacterial properties that could destroy cultures of cholera-inducing bacteria (Abedon et al., 2011; Wittebole et al., 2014). In 1915, Frederick Twort observed bacteria-free regions in bacterial laws (Trudil, 2015; Domingo-Calap and Delgado-Martínez, 2018). However, he was unaware of what kind of substance produced those "glassy and transparent" spots (Keen, 2015; Domingo-Calap and Delgado-Martínez, 2018), and propose three hypotheses of this bacteriolytic agent: a manifestation of the bacteria life cycle, an enzyme produced by the bacteria themselves or, an "ultra-microscopic virus" (Keen, 2015). Two years later, Félix d'Herelle independently described a microbe that showed an antagonistic effect against Shiga bacillus while he was studying patients suffering bacillary dysentery (Wittebole et al., 2014; Wei et al., 2019). Moreover, d'Herelle recognized the nature of this phenomenon as a result of viral parasitic action on bacteria and he dubbed this new type of virus "bacteriophage" (Keen, 2015; Trudil, 2015). D'Hérelle and his assistant George Eliava founded the George Eliava Institute of Bacteriophages, Microbiology, and Virology in Tbilisi in 1923, which has remained in continuous use since then (Trudil, 2015; Domingo-Calap and Delgado-Martínez, 2018). Indeed, they provided combinations of different phages to soldiers to treat wounds, gangrene, and diseases during the Second World War (Domingo-Calap and Delgado-Martínez, 2018). Nevertheless, the lack of proper quality controls, reproducible results, and the discovery of antibiotics produced a decrease of the phage interest in Western countries, only being used in Eastern countries, especially in the Soviet Union (Wei et al., 2019). In fact, in these countries, phage therapy was used to treat bacterial infections, and d'Herelle provided the first studies in animals, when he proposed the use of bacteriophages to control *Salmonella* in calves (Trudil, 2015). The rise of antibiotic resistance over the last decade has led investigators to re-consider this approach, a century after their discovery, and take a fresh look at phage therapy as a potentially viable treatment of bacterial disease (Trudil, 2015; Domingo-Calap and Delgado-Martínez, 2018).

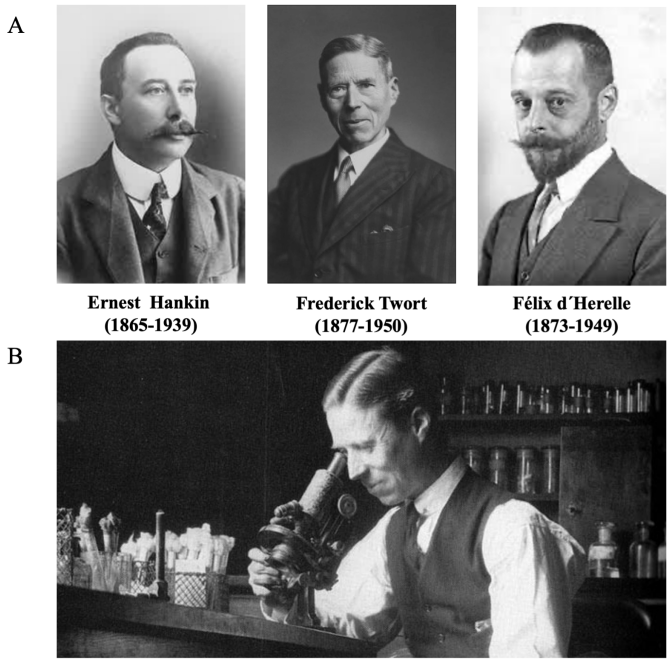

Figure 7. (A) Ernest Hankin, Frederick Twort and Félix d'Herelle. (B) Frederick Twort at the bench in the lab of the brown institution. Obtained from www.microbiologysociety.org: www. wikipedia.org.

1.4.3 General characteristics

Phages have relatively simple structures, with a variable morphology based on a tail and an icosahedral head that encapsulate a DNA or RNA genome (Barton et al., 2011; Ye et al., 2019). Moreover, they are up to 1,000 times smaller than the average bacterium (20 to 200 nm) and kept stable in thermal conditions from 30 to 60 ºC, and pH ranges from 3 to 13. The current criterion of the taxonomy of bacteriophages is based mainly on morphology and nucleic acid (Wernicki et al., 2017). Indeed, the current Report on Virus Classification and Taxon Nomenclature classified them into many orders, 15 families, 204 genera, and 873 species. The vast majority of known phages have been belonged to the tailed order *Caudovirales* (about 96 %), which is divided into three families: *Siphoviridae* (61 %), *Myoviridae* (25 %), and *Podoviridae* (14 %), wich are classified based on their tail morphology (Barton et al., 2011; Wernicki et al., 2017; Żbikowska et al., 2020). In terms of the basis of capsid symmetry phages are distinguished in isometrics and helical, presenting the majority of phages with a broad range of isometric heads (Żbikowska et al., 2020). In addition, phages could be divided into three groups based on nucleic acid: those containing DNA in the form of a double helix, those with a single strand of DNA, and phages containing RNA (Żbikowska et al., 2020).

Furthermore, bacteriophages could be divided based on their interactions with bacteria and their life cycle into two types: lytic and lysogenic (Żbikowska et al., 2020). The lytic cycle involves the adsorption, penetration, replication of nucleic acids, formation of virions, and the killing of the bacterium by lysis, characteristic of virulent phages and usually vary within 20-40 min to 1-2 hours; meanwhile, the lysogenic cycle, release the integration of the phage genetic material into the bacterial genome and replication as part of them, leading to in the appearance of a prophage, that under abnormal environmental conditions, it could turn to a lytic cycle (Wernicki et al., 2017; Żbikowska et al., 2020) (**Figure 8**).

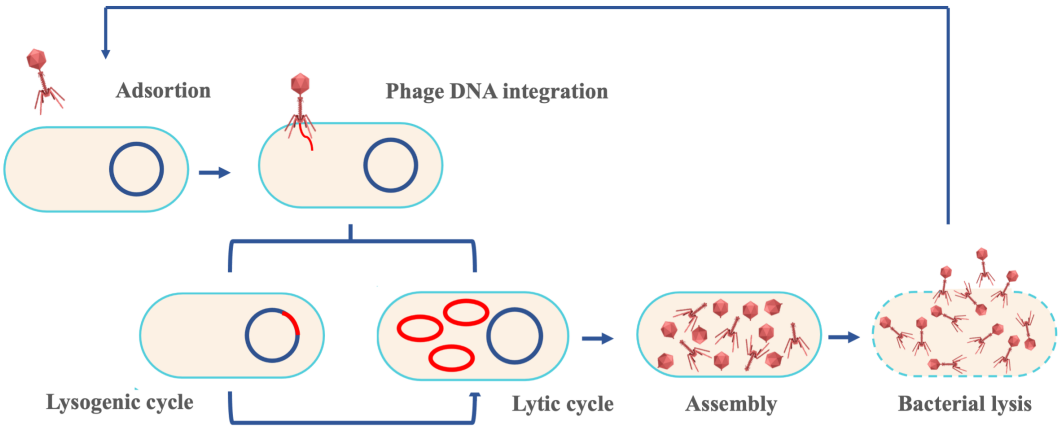

Figure 8. Schematic representation of the bacteriophage replication cycle. Based on their life cycles, bacteriophages can be divided into two types based on their life cycles: lytic and lysogenic phages. The lytic cycle entails insertion and absorption of the bacteriophage genetic material into the bacteria cell and the replication to make multiple copies of the bacteriophage virus and lyse the bacteria wall. The lysogenic cycle entails the integration of the phage genetic material into the nuclear chromosome of the bacteria after the insertion and absorption. Under certain conditions lysogenic phages could initiate a lysis cycle, while others will remain lysogenic.

1.4.4 Application of bacteriophages in poultry production

Bacteriophages have gained great attention in food animal production to enhance animal health and control the transmission of zoonotic pathogens and AMR *via* de food chain (Svircev et al., 2018). Integrated production systems, such as poultry, are ideal for phage application since their control of the entire production chain allows their application throughout the whole chain of the process (Gigante and Atterbury, 2019). Indeed, several commercial phages against *Salmonella* in the poultry industry (Bafasal®, Biotector®S, SalmoFreshTM, SalmoPro®, SalmonelexTM (PhageGuard), PhageGuard STM,

BacWashTM, and SalmoFREE®) are available. It must take into account that different variables could affect the effectiveness of bacteriophages employed in biocontrol procedures, in particular the lytic title, the type, and period of application (Werniki et al., 2017). Moreover, the lack of phage stability upon exposure to acidic conditions (e.g. gastric acidity), the activity of enzymes and other digestive compounds (e.g. bile), and the relatively short residence times in the intestinal tract may limit the efficacy of phages that were administered orally (Sabouri et al., 2016; Dec et al., 2020). To overcome these limitations, its co-administration with antacids (Koo et al., 2001; Sabouri et al., 2016) or its encapsulation (Rastogi et al., 2016; Malik et al., 2017; Malik, 2021) has been used. In this sense, encapsulation provides protection from gastric acidity and could release high doses of the phages at the target site, incorporating approaches for burst release and/or sustained release; improving the efficacy of the orally administered phages (Ly-Chatain, 2014; Hussain et al., 2015; Rastogi et al., 2016). Furthermore, the effectiveness of phage therapy against infections depends on a number of factors, including the type of the infection, the phage application form, and the quantitative and qualitative composition of the phage dose (Dec et al., 2020). Thus, it is important to develop strategies in which phage therapy is applied at the most effective time, in order to avoid economic losses.

Another advantage that phages have been presented is the easy and cost-effective isolation (Sevilla-Navarro et al., 2018). Moreover, one of the most characteristic properties of bacteriophages is their usually narrow and limited spectrum of infection, so they should not trigger negative effects on the host microbiota (Clavijo and Flórez, 2018), however, new studies begin to shed light on the impact of phage therapy on gastrointestinal ecology (Paule et al., 2018; Hsu et al., 2019; Fazzino et al., 2020; Clavijo et al., 2022). Different studies have demonstrated that phages induce changes in the microbiome, although these do not appear to be of biological significance or impact in productive parameters (Sarker et al., 2012; McCallin et al., 2013; Galtier et al., 2016; Sarker et al., 2016; Sarker et al., 2017; Clavijo et al., 2022). In this sense, studies with a broader scope are necessary for the implementation of adequate protocols in the administration of bacteriophages in poultry farms as a routine treatment and to improve the understanding of this application response in multi-species systems, in order to obtain safe, innocuous, high quality and cost-effective poultry products for the consumer (Wernicki et al., 2017; Nabil et al., 2018; Fazzino et al., 2020).

1.5 Poultry microbiome in the "omics" sciences era

1.5.1 Defining the microbiome landscape

1.5.1.1 Key factors that shape the chicken gut microbiome

The microbiota is defined as the microbial community which shares a space of the human and animal body, and includes commensal, symbiotic, and pathogenic microorganisms (Clavijo and Flórez, 2018; Sutton and Hill, 2019). The chicken gastrointestinal tract is a complex environment containing billions of bacteria, viruses, protozoa, and fungi, referred as the "gut microbiome" (Roto et al., 2015). Of them, bacteria are the most characterized, making up the vast majority of the genomic sequences and biomass (Roto et al., 2015; Sutton and Hill, 2019). The composition and function of gut microorganisms could vary thought the life depending on factors intrinsic and extrinsic factors such as age, location in the gastrointestinal tract, and diet (Shang et al., 2018). In this regard, it is essential to maintain the balance of the gut microbiome of poultry, as its vast metabolic capacity, it has been considered a "superorganism" that directly impacts the health of the host by the modulation of physiological functions such as nutrition, metabolism, and immunity (Brisbin et al., 2008; Jankowski et al., 2009; Wei et al., 2013; Clavijo and Flórez, 2018; Carrasco et al., 2019). Importantly, the gut not only harbors microbial and epithelial cells, but also entails a continuous influx of molecules (Rojo et al., 2017). Indeed, it has been shown the possible relationship between the intestinal microbiota and the brain through the brain-intestinal-microbiota-enteric axis, a bi-directional communication system based on neural, endocrine, and immunological mechanisms (Rhee et al., 2009; Paule et al., 2018; Mahmood and Guo, 2020). In this sense, the dynamic crosstalk host-microbe leads to the production and transformation of an expanding number of molecules in the gut that could act as signals in its interplay and influence the host metabolism through their cognate receptors (Lee et al., 2022). Therefore, the scientific community emphasizes that we are in the golden age of microbiota (Oakley et al., 2014b).

1.5.1.2 Acquisition, maturation, and diversity of the chicken gut microbiota

The gastrointestinal tract of the chickens begins at the esophagus and continues in the crop, proventriculus, gizzard, gut (duodenum, jejunum, ileum, and caeca), and finally the colon, and cloaca (Yadav and Jha, 2019). Moreover, compared to mammals, it is shorter relative to their body length, with short retention times, which is going to result in a

microbiota that requires unique adaptations to establish and proliferate (Clavijo and Flórez, 2018a; Yadav and Jha, 2019). Furthermore, each organ performs different nutritional functions as well as the microorganisms present in them, leading to a significant difference in the taxonomic composition of the different organs, so although they are strongly interconnected, they could be considered different ecosystems (Van Der Wielen et al., 2002; Clavijo and Flórez, 2018). Indeed, the cecum plays a pivotal role in the chicken's physiology, as a complex system that harbors the most microbial communities' diversification with a direct impact on host health and productivity (Roto et al., 2015). The important biological role of the cecum in poultry is based on its slow passage rate, where contents may be retained here as long as 35 hours, meanwhile, the gut transit from mouth to the ileum is approximately 3 hours (Roto et al., 2015).

The initial colonization of the chickens GIT could occurs naturally by microorganisms that pass through the pores of the eggshell, being 99% of the embryo microbiota is inherited from the maternal cloaca and/or oviduct (Roto et al., 2016; Carrasco et al., 2019; Lee et al., 2019). Additionally, the faecal and/or environmental contaminants attached to eggshells also colonized the gastrointestinal tract of the newly hatched chicks from the hatchery, transportation vehicle, and the farm (Lee et al., 2019; Mahmood and Guo, 2020). Moreover, the maternal antibodies supplied through the yolk, that protect against pathogenic bacteria, could modulate the establishment of the intestinal microbiota (Mahmood and Guo, 2020). At first days of age, the gastrointestinal tract becomes successively colonized by specific bacterial species, belonging to the *Enterobacteriaceae* family, and approximately from a week of age by the *Firmicutes* family, probably through contact with microorganisms coming from the rearing environment, food, and water (Ballou et al., 2016a; Carrasco et al., 2019). For this purpose, the host immune response is suppressed towards the microorganism, to establish a symbiotic relationship, with a cost-to-benefit ratio for the host (Mahmood and Guo, 2020).

After the initial colonization, a succession of microorganisms is observed in which the diversity of species and the complexity of the structure of the microbiota population, increases as the birds grow, and finally, the microbiota reaches a state of maturation and stabilizes (Carrasco et al., 2019). This process normally occurs around 3 weeks of age in poultry, enriched with complex microbial communities including bacteria, fungi, archaea, protozoa, and viruses (Wei et al., 2013; Oakley et al., 2014b; Johnson et al., 2018; Shang

et al., 2018; Carrasco et al., 2019; Mahmood and Guo, 2020). Indeed, it has been suggested that the total number of bacteria in the gastrointestinal tract overwhelmingly outnumber the eukaryotic cells of the host body (Yadav and Jha, 2019). Furthermore, the bacteria in the host could be divided into three types: dominant bacteria, when the population is higher than 10^6 CFU/g sample; subdominant bacteria, when is between 10^3 to 10^6 CFU/g sample; and residual bacteria, when is lower than 10^3CFU/g sample (Burel and Valat, 2009; Yadav and Jha, 2019). Moreover, bacterial growth has been influenced by the variation of the physicochemical conditions and substrate availability throughout the gastrointestinal tract, which promote or hamper the colonization of certain species (Lustri et al., 2017). It has been estimated that of the 12 phyla of bacteria, *Firmicutes* has been the most predominant phylum that accounted for almost 70% of all the bacterial sequences, followed by *Bacteroidetes*, with the around 12%, and *Proteobacteria*, with the approximately 9 % (Wei et al., 2013; Mahmood and Guo, 2020). Overall, the microbiota in chickens varies according to diverse factors leading to a different profile of taxonomic composition in reported studies (Clavijo and Flórez, 2018). Moreover, any alteration of this balance could initiate a cascade of reactions that lead to inflammation of the gut, altering the entire process of digestion, absorption, and metabolism of nutrients, affecting productive performance (Mahmood and Guo, 2020).

1.5.2 Meeting the poultry microbiome

The past two decades have been witness to a flare-up of data derived from the development and consolidation of 'omics' science and bioinformatics (Boja et al., 2014). Omics sciences are high-throughput biochemical assays that have emerged with the goal of analyzing comprehensively and simultaneously the components of a living organism (Evans, 2000; Conesa and Beck, 2019). The term **'omics'** derives from the Greek suffix '-ome', which means "whole", "all" or "complete" (Chakraborty et al., 2022). The goal of the omics sciences is the characterization of the biological molecules that translate into structure, function, and life processes, providing a holistic view of the biological system (Scharf, 2015).

Since 1920, when the German scientist Hans Winkler used the word "genome" to describe all the material on the chromosomes in a sperm or egg in his book "*Verbreitung und Ursache der Parthenogenesis im Pflanzen- und Tierreiche*", different technologies have been performing paradigm shifts in various research areas of biological sciences

(Lederberg and Mccray, 2001). Thought the genomic revolution carried out by the Human Genome Project has been aroused the need for novel sophisticated approaches that help in the understanding of biological processes. These new life sciences approaches should combine molecular biology at different organizational levels with bioinformatics, to obtain a complete picture that shed light on the biological processes at systems levels (Sánchez-Vidaña et al., 2017). This postgenomic era was a pivotal stage for the overwhelming emergence of omics studies in the new paradigm of biological research, establishing the beginning of the systems biology era. In the last two decades several omics tools have been developed to collect and analyse high-throughput data on proteins (proteomics), mRNA transcripts (transcriptomics), gene sequences (genomics), microbial diversity (metagenomics), epigenetic regulation of gene expression (epigenomics), metabolic profile (metabolomics), etc., of a particular cell, tissue, organ, or whole organism at a specific time point.

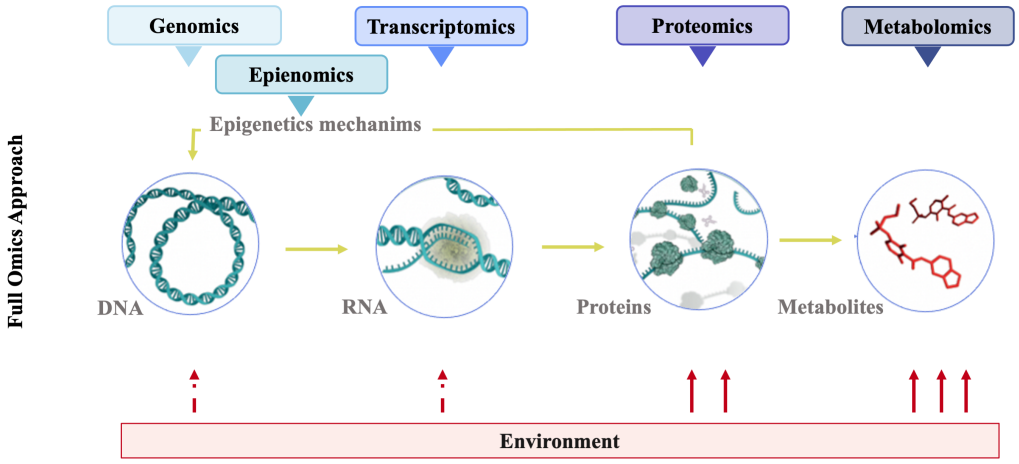

Figure 9. Schematic representation of omics approach. Adapted from Bedia (2018).

Increasingly studies have been conducted to insight chicken caecal consequences on surrounding environments, nutritional management, or treatments (Montoro-Dasi et al., 2020, 2021; Mohebodini et al., 2021; Memon et al., 2022). These have revealed that regardless of the presence or absence of pathogens monitoring gastrointestinal ecosystems and understanding the complex and diverse microbial interactions and composition is paramount (Pan and Yu, 2013; Clavijo and Flórez, 2018). Nevertheless, few studies have shown how phage therapy, one of the most promising antimicrobial

therapies, impacts not only on the pathogen but also on the animal (Hsu et al., 2019; Zhao et al., 2022).

1.5.2.1 Methods used to study the microbiota

Metagenomics is the science that studies the genetic material from a community of organisms (Chakraborty et al., 2022). The typical metagenomics experiments have been involved in (i) the isolation of 16S ribosomal RNA (**16S rRNA**) gene, which could answer "who´s there?"; and (ii) shotgun metagenomics sequencing, that performs an unrestricted sequencing of all DNA of the sample, which could answer "what microbes can do?". Profiling microbial communities using 16S rRNA genes is a straightforward method to study their taxonomic composition (Peterson et al., 2021). Nevertheless, it has limitations, such as the conservation of the target gene or the amplicon size product (Peterson et al., 2021).

Poultry gut microbiota composition studies began in 1901 (Roto et al., 2015), but comprehensive surveys based on culture-dependent techniques were not carried out until the 1970s (Zhu et al., 2002). In this sense, it has been estimated that as little as 20% of the entire gut microbiota was easily cultured, limiting their knowledge (Oakley et al., 2014b). Thus, researchers began to develop culture-independent techniques, like genome-sequencing technologies, that have led to a revolution in the discovery and characterization of microorganisms (Zhu et al., 2002; Wensel et al., 2022). Studies on the gut microbiome generally involve four steps: (1) DNA or RNA extraction, (2) selection of hypervariable regions, DNA amplification and generation of DNA libraries, (3) sequencing, and (4) bioinformatics data analysis. For DNA sequencing, the Sanger method has been the dominant approach for the past 30 years. Nevertheless, this technique only sequences a single DNA fragment at a time, that led to the need for newer sequencing technologies that allowed the massively-parallel sequencing (Voelkerding et al., 2009). The first decade of the 21st century brought forth the increasing availability of second and subsequent-generation high-throughput sequencing platforms, that could be used to investigate microbiomes at unprecedented phylogenetic depth (Yeoman et al., 2012; Shang et al., 2018). The high-throughput sequencing approaches or next-generation sequencing (**NGS**) are a powerful tools to study the biological and ecological role of gut microbiota, which are mainly based on amplifying the small subunits of the 16S ribosomal gene from Bacteria and Archaea, the 18S rRNA gene from eukaryotic cells,

and the nuclear ribosomal internal transcribed spacer regions from Fungi (Borda-Molina et al., 2018; Shang et al., 2018). Among NGS platforms, the usually used systems in the chicken gut microbiome and metagenomic research have been the Illumina HiSeq and MiSeq instruments, nevertheless, these platforms have been presented limitations such as the short-read assembly and high cost (Shang et al., 2018). Due to these limitations, the third-generation sequencing platforms for long-read single molecule, such as single molecule real-time (SMRT) by Pacific Bioscience and nanopore sequencing by Oxford Nanopore Technologies (Shang et al., 2018), have been arising.

The development of techniques for the study of bacterial genomic material triggered molecular approaches based on the sequences of bacterial 16S rRNA genes, that improved the understanding of the chicken gut microbiota composition, diversity, and function (Yeoman et al., 2012; Shang et al., 2018). However, the microbial community could stablish mutualistic or antagonistic relationships, performing a defined function with ecological implications that could change in a stability disruption (Batinovic et al., 2019). Because of that, the use of metatranscriptomics and metabolomics, and their combination have become powerful tools to identify the microbial populations activities carried out in the gut and how they respond to the variations of the community structures (Borda-Molina et al., 2018). Moreover, these variations of the community structures were mainly investigated with a focus on the influence of pathogenic species, even their control under production conditions, and how the changes could influence the performance of the broilers (Borda-Molina et al., 2018).

1.5.2.2 Methods used to study the metabolome

Metabolomics is the science that studies the metabolome. The metabolome consists of the complete set of substrates, intermediates, and final small-molecules (metabolites) within a biological system or systems (Nalbantoglu, 2019; Dufour-Rainfray et al., 2020). This medium to high-throughput technology allows the automatically identification, quantification, and characterization of hundreds to thousands of metabolites simultaneously (Liang et al., 2015). Metabolites have been considered as the “canaries” of the genome, as canaries for coalminers served as sensitive detectors of toxic gases in coal mines, and metabolites could be considered as sensitive indicators of alterations in the genome (Goldansaz et al., 2017). In this sense, metabolomics is considered the omic science that is closest the phenotype reflection, because metabolites are the result of the

complex interactions that take place inside the cell, the genome, and the exposures occurring outside the cell or organism, the environment (Goldansaz et al., 2017; Nalbantoglu, 2019). Metabolomics covers a wide variety of metabolites (<1 kDa) with diverse chemical structures, such as amino acids, lipids, vitamins, organic acids, peptides, carbohydrates, nucleotides, etc., that could act as metabolic intermediates, hormones, signalling molecules and/or secondary metabolites (Agin et al., 2016; Nalbantoglu, 2019). These metabolites may originate from endogenous interactions of the host's own cells or may also be from exogenous sources (such as microbiota, feed, drugs, etc.) (Kosmides et al., 2013).

Metabolome studies could be divided into two different approaches: untargeted and targeted metabolomics (Agin et al., 2016). The untargeted metabolomics (or global approach) allows for a global scanning (detection and relative quantitation) of the metabolites in a sample (Agin et al., 2016; Schrimpe-Rutledge et al., 2016). This approach covers the collection and measurement of as many metabolites as possible without bias (Agin et al., 2016; Schrimpe-Rutledge et al., 2016). In untargeted metabolomics, there is no prior knowledge of the identification of the assessed metabolites and may include further identification of those metabolites and to provide hypotheses that can be further studied with targeted approaches (Agin et al., 2016; Schrimpe-Rutledge et al., 2016). In contrast, targeted metabolomics (or validation-based approach) has the aim of measuring well-defined groups of metabolites, typically focusing on one or more related pathways of interest testing (Schrimpe-Rutledge et al., 2016; Nalbantoglu, 2019). This approach could allow the validation of untargeted analysis and hypothesis testing (Schrimpe-Rutledge et al., 2016; Nalbantoglu, 2019).

Metabolomics involves several steps. First, the extraction phase of the metabolites of the sample to obtain a stable extract that reflects the levels of endogenous metabolism. It is not a standardized method, but it must have rapid quenching of the sample, to stop the enzyme and chemical activities and prevent changes in metabolite levels, and the use of different solvents that allow specific extraction for each type of tissue (Alseekh et al., 2021). After sample preparation and extraction, there is metabolite quantification. For metabolomics experiments, there are two main analytical techniques: nuclear magnetic resonance (**NMR**)-based and mass spectrometry (**MS**)-based approaches (Azad and Shulaev, 2019). Indeed, NMR and MS could be both supplementary and complementary

to one another. MS and NMR offer multifaceted approaches bellow the different techniques that allowed the detection, identification, and measure of metabolites (Nagana Gowda and Djukovic, 2014). Currently, MS-based methods have become a leading technology for small molecule analyses, due to their highly sensitive detection, quantitation, and structure elucidation of upwards of several hundred metabolites in a single measurement (Nagana Gowda and Djukovic, 2014). Moreover, over the last years, as a result of a wide adoption of high-resolution mass spectrometry (**HRMS**), substantial development in metabolite profiling has been fuel (Azad and Shulaev, 2019). MS is often combined with different methods of chromatographic separation prior to the metabolite quantification: liquid chromatography (**LC**), gas chromatography (**GC**) and capillary electrophoresis (**CE**) (Schrimpe-Rutledge et al., 2016; Azad and Shulaev, 2019). The chromatographic separation has the objective of separating the molecules in mixture from each other while moving with the aid of a mobile phase (liquid mobile phase in LC, gas mobile phase in GC) (Coskun, 2016). After the chromatographic separation, the MS performs the separation of the particles (atoms, molecules, and clusters), based on the differences in the ratios of their charges to their respective masses (mass/charge; m/z), and determine the molecular weight of the particles (Murayama et al., 2009). To do this, samples are pumped through MS capillary to obtain a separation in the gaseous phase of the positive or negative electrically charged ions (Nalbantoglu, 2019). Ionization is one of the most critical steps, due to its influence on the capability to detect and quantify a metabolite (Nagana Gowda and Djukovic, 2014). In metabolomics, the most common ionization techniques are electrospray ionization (**ESI**) and electron impact (**EI**) ionization, chemical ionization (**CI**), atmospheric pressure chemical ionization (**APCI**) and atmospheric pressure photoionization (**APPI**), and matrix assisted laser desorption ionization (**MALDI**) (Nagana Gowda and Djukovic, 2014; Nalbantoglu, 2019). After the ionization of the molecules, the mass-to-charge (m/z) ratios of fragment ions are detected by MS (Nalbantoglu, 2019). There are different mass analyzers: quadrupole (**Q**), linear ion trap (**LIT**), quadrupole ion trap (**QIT**), time of flight (**TOF**), quadrupole time of flight (**QTOF**), Fourier transform ion cyclotron resonance (**FTICR**), and Orbitrap (Nagana Gowda and Djukovic, 2014). Metabolite raw data generated by MS has to be processed in specialized software to their correct biological interpretation the data and identify the metabolite of interest (Nalbantoglu, 2019). Processing of the raw data required multiple steps that include background spectral filtering, correction of the retention times, peak assignment, detection, alignment, and normalization (Nalbantoglu, 2019). After that, the

data obtained must be normalized, and compounds identified and classified with statistical analyses (Nalbantoglu, 2019).

1.5.3 Applications of microbiota and metabolome in poultry

1.5.3.1 Relationship between microbiota and foodborne pathogens

The gastrointestinal microbiota plays an integral barrier against pathogenic microorganisms, detoxifying and modulating the immune system (Brisbin et al., 2008; Pan and Yu, 2013; Clavijo and Flórez, 2018; Carrasco et al., 2019). The key to achieving a balance between the pathogenic and beneficial microbiota lies in understanding the complex interaction between the host and its gut microbiota (**Figure 10**) (Mahmood and Guo, 2020). In this sense, the gut microbiota established a protective layer that sheltered the chicken from colonization by pathogenic bacteria, through the association with the intestinal epithelial surface of the enterocyte, out-competing the pathogenic bacteria for space and nutrients, in turn resulting in enhanced bird growth (Roto et al., 2015; Mahmood and Guo, 2020).

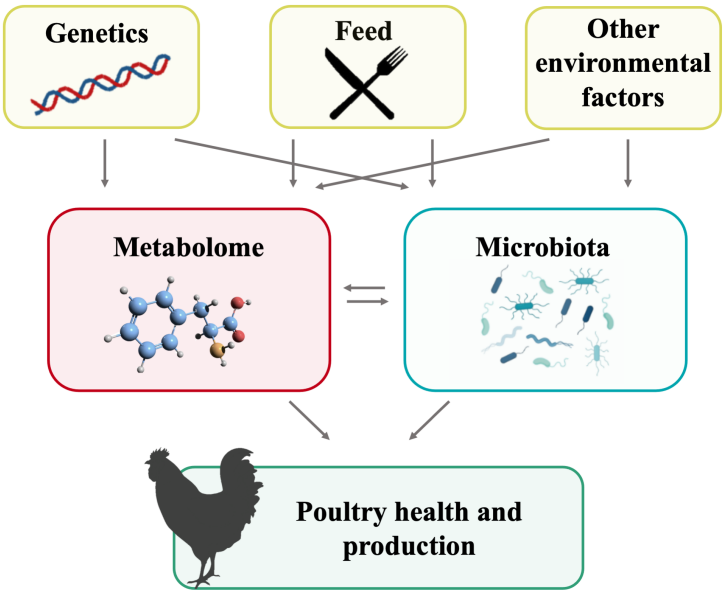

Figure 10. Schematic representation of two-way interactions between the metabolome and gut microbiota. Both the metabolome and microbiome are influenced by genetics, feed, and other environmental factors. Both also impact the risk of poultry disease. Adapted from Lee-Sarwar et al. (2020).

The presence of pathogenic bacteria in the broiler chicken microbiota has implications for animal health, as well as human health. In the chicken microbiota it has been reported different taxa that could cause illness in humans, such as *Campylobacter*, *Salmonella*, *Escherichia coli*, and *Clostridium perfringens* (Oakley et al., 2014a). In this sense, *Salmonella* is highly adapted to the gastrointestinal tract of birds, without producing any symptoms in them, so the bird is considered a reservoir of the bacteria (Vikram et al., 2012; Clavijo and Flórez, 2018; Nabil et al., 2018).

1.5.3.2 Applications of the omics technologies in poultry

The goal of poultry production is to achieve increased productivity to fulfill consumers demands while ensuring the health and food security and wellbeing of the chickens. Microbiota and metabolomics offer powerful analytical tools that could address many important questions in animal production, such as the impact on the accurate selection of therapies for improved health.

One advantage of profiling metabolites is gaining a more holistic understanding of the impact of metabolism on systemic health by monitoring the collection of metabolites present in a cell, a tissue, an organ, or an organism, as well as on matrices such as feaces, caecal content, blood, soil, etc (Weston et al., 2015; Chakraborty et al., 2022). In this sense, the development of the metabolomics approaches could allow the elucidation of this complex disease and potentially also improve its understanding and management (Aldars-García et al., 2021). These include papers demonstrating how metabolomics can be used to profile meat quality (Sawano et al., 2020; Taniguchi et al., 2020), feed efficiency (Wang and Kadarmideen, 2020), animal selection (Chakraborty et al., 2022), and ascertain other important economic or breeding traits associated with livestock (Goldansaz et al., 2017). However, metabolic profiles are not limited to endogenous processes but also contain metabolites originating from exogenous sources such as feed, drugs, or microbiota. The gut microbiota metabolic cross-talks represent an increasingly studied major factor in the individual's health or pathogenic status (Agin et al., 2016).

1.6 Study cornerstone

Poultry production is considered one of the most important agriculture-based industry worldwide (OECD/FAO, 2022). However, the development of this production must be accompanied of higher biosecurity measures to prevent the spread of pathogens, such as

Salmonella (Nair and Johny, 2019). This bacterium continues to be one of the main concerns, both for public health, and for livestock, being poultry products the main source of the infection (EFSA and ECDC, 2021).

The combination of biosecurity measures, as well as vaccination programs, with food additives alternatives to antibiotics could play a fundamental role in the control of *Salmonella*. In this sense, bacteriophages have been considered an innovative preharvest strategy in poultry production (Alali and Hofacre, 2016; Ruvalcaba-Gómez et al., 2022). However, its important to highlight that the administration method needs to be practical from a commercial point-of-view, and also it must take into account its impact on the gut microbiome homeostasis (Thanki et al., 2021; Clavijo et al., 2022). In this sense, gut microbiota plays a key role in vital metabolic functions, with a great impact on host health and performance (Tang et al., 2019; Chen et al., 2019).

In this context, the following chapters were designed to assess the effect of bacteriophage application for *Salmonella* control in broiler production, focusing their effect on intestinal health, by means of genomic sequencing and metabolomic study.

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Chapter II. Objectives

The general objective of this doctoral thesis was to apply bacteriophages for *Salmonella* control in broiler production, focusing on their effect on intestinal health, by means of genomic sequencing and metabolomic study. To achieve this goal, two different parts were performed.

Firstly, the objective of the Part I was to assess the bacteriophage gastrointestinal dynamics in *Salmonella*-free broilers and its influence on microbiota and metabolome. For this purpose, the following specific objectives were proposed:

1. To assess the release of the encapsulated bacteriophages and the survival of *Salmonella* phage FGS011 (non-encapsulated and microencapsulated) through the chicken's GIT under *in vitro* and *in vivo* conditions after oral administration to one-day-old chicks.

2. To assess the effect of the phage intervention over a six-week production cycle and to compare microencapsulated and non-encapsulated phages and the spatial and temporal dynamics of the phage delivery along the GIT during the chicken rearing period.

3. To investigate the interaction of phages with the animal. Specifically, we compare the caecal microbiome and metabolome after a *Salmonella* phage challenge in *Salmonella*-free broilers, evaluating the role of the phage administration route.

Secondly, the objective of the Part II was to assess the bacteriophage dynamics in *Salmonella*-infected broilers and its influence on microbiota and metabolome. For this purpose, the following specific objectives were proposed:

1. To evaluate the application of microencapsulated phages delivered in animal feed during the six week broiler production cycle as a strategy to control *Salmonella* in the animals and in the environment.

2. To investigate the influence of *Salmonella* phage on host physiology through modulation of the microbiota and the cecal metabolome in late-stage broiler rearing.

Chapter III. Experimental chapters

Part I

3.1 Bacteriophage gastrointestinal dynamics in *Salmonella*-free broilers and its influence on microbiota and metabolome

3.1.1 *In vitro* and *in vivo* gastrointestinal survival of non-encapsulated and microencapsulated *Salmonella* bacteriophages: implications for bacteriophage therapy in poultry

An adapted version of this chapter has been published with the reference:

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3.1.1.1 Abstract

Therapeutic use of bacteriophages is recognised a viable method to control *Salmonella*. Microencapsulation of phages in oral dosage forms may protect phages from inherent challenges of the gastrointestinal tract in chickens. Therefore, the main objective of this study was to assess the survival of *Salmonella* phage FGS011 (non-encapsulated and microencapsulated) through the gastrointestinal tract under *in vitro* as well as *in vivo* conditions after oral administration to one-day-old chicks. To this end, the phage FGS011 was encapsulated in two different pH-responsive formulations using polymers Eudragit® L100, and Eudragit® S100 using the process of spray drying. Phages encapsulated in either of the two formulations were able to survive exposure to the proventriculus-gizzard *in vitro* conditions whereas free phages did not. Moreover, phages formulated in polymer Eudragit® S100 would be better suited to deliver phage to the caeca in chickens. In the *in vivo* assay no statistically, significant differences were observed in the phage concentrations across the gastrointestinal tract for either the free phage or the encapsulated phage given to chicks. This suggested that the pH of the proventriculus/gizzard in young chicks is not sufficiently acidic enough to cause differential phage titre reductions thereby allowing free phage survival *in vivo*.

3.1.1.2 Introduction

Non-typhoidal *Salmonella* spp. has been recognized as one of the most important zoonotic pathogens worldwide (WHO, 2015, 2019). Latest data reported by the World Health Organization estimated 78 million annual cases of foodborne illness worldwide, of which 59,000 resulted in death (WHO, 2015). By the same token, in the EU, the latest data published in 2019, revealed more than 90,000 cases of human salmonellosis (EFSA and ECDC, 2019). *Salmonella* sources in human infection are relatively diverse, however, poultry is considered a major reservoir (Andino and Hanning, 2015; Shah et al., 2017b; EFSA and ECDC, 2019; Koutsoumanis et al., 2019). Due to this situation, since 2008, NSCPs in accordance with Regulation (EC) No 2160/2003 have been implemented in the EU (EP, 2003; Vandeplas et al., 2010). These measures have resulted in significant *Salmonella* prevalence reduction in poultry flocks throughout Europe (EFSA and ECDC, 2017). However, some *Salmonella* serovars related to food-borne outbreaks are still present in some poultry farms, due to their ability to survive and grow in the GIT of chickens and/or farm environment (Carrique-Mas et al., 2009; Walker et al., 2018; Koutsoumanis et al., 2019; Davies and Wales, 2019). When *Salmonella* comes into contact with the bird and reaches the GIT, its ability to rapidly colonize and multiply results in long-term bacterial excretion in faeces especially in one-day-old chicks (Barrow et al., 2004; Marin and Lainez, 2009; Foley et al., 2013; Berry and Wells, 2016). *Salmonella* almost colonizes every part of the chicken GIT, nevertheless, the pH of the GIT could affect the bacteria colonization (Cosby et al., 2015). *Salmonella* encounters the acidic environment of the crop, with a pH of 5, that is maintained and controlled by the bacteria of the genus *Lactobacillus* (Cosby et al., 2015). Subsequently, *Salmonella* survives the low pH of the proventriculus and gizzard (pH 2.5), and colonize the gut (pH from 5.5 to 8), with highest predilection potential noticed in the caeca (Ravindran, 2013; Shah et al., 2017a; Nair and Johny, 2019). Thus, innovative and cost-effective techniques are needed suitable for deployment under field conditions to control the bacteria in the poultry sector (Barrow et al., 2004; Sevilla-Navarro et al., 2018; Clavijo et al., 2019).

In this sense, the use of bacteriophages has garnered increasing interest as a possible method to achieve *Salmonella* control in poultry farms in recent years, due to its high degree of specificity against the bacteria (Toro et al., 2005; Atterbury et al., 2007; Wernicki et al., 2017; Sevilla-Navarro et al., 2018, 2020a). Among the different routes of

phage therapy administration, the oral route is likely to be the most applicable in humans and animals (Zelasko et al., 2017; Otero et al., 2019; Romero-Calle et al., 2019). The lack of phage stability upon exposure to acidic conditions (e.g. in the proventriculus/gizzard) and the relatively short residence times in the intestinal tract may limit the efficacy of orally delivered phages (Sabouri et al., 2016). These limitations can however be overcome, such as through the co-administration of antacids (Koo et al., 2001; Sabouri et al., 2016) or through encapsulation of the phages (Rastogi et al., 2016; Malik et al., 2017; Malik, 2021). Encapsulation provides protection from gastric acidity and could release high doses of the bacteriophages at the caeca, the predilected *Salmonella* site, incorporating approaches for burst release and/or sustained release; improving the efficacy of the orally administered phages (Ly-Chatain, 2014; Hussain et al., 2015; Rastogi et al., 2016). Nevertheless, there is a need for better understanding of the stability and viability of the non-encapsulated and microencapsulated phages after being orally administered (Ma et al., 2016; Dąbrowska, 2018; Vinner et al., 2019). Therefore, the main objective of this study was to assess the release of the encapsulated bacteriophages and the survival of *Salmonella* phage FGS011 (non-encapsulated and microencapsulated) through the chicken's GIT under *in vitro* and *in vivo* conditions after oral administration to one-day-old chicks.

3.1.1.3 Materials and Methods

All the animals were handled according to the principles of animal care published by Spanish Royal Decree 53/2013 (Spain, 2013).

3.1.1.3.1 Bacteriophage Origin

Bacteriophage FGS011 used in this study was isolated by Sevilla-Navarro et al. (2020b). It was isolated from faeces samples from commercial poultry farms (broilers and layers) in Eastern Spain in the Poultry Quality and Animal Nutrition Centre of the Valencia Region (**CECAV**). The bacteriophage was stored at 4 °C until use. This phage was selected for this study due to its high lytic activity against its propagating strain and the wide lytic host range against *Salmonella* strains isolated from poultry farms. The bacterial strain used for phage isolation and amplification was *Salmonella* Senftenberg (***S.* Senftenberg**), a field strain isolated from poultry farms during the NSCP (NSCP, 2020) and selected from the collection repository of CECAV.

3.1.1.3.1.1 Bacteriophage characterization assay

3.1.1.3.1.2 Bacteriophage lytic spectrum

In order to assess the lytic spectrum of the phage, the sensitivity of 13 field and reference bacterial strains including ten *Salmonella* serovars, one *Escherichia coli* strain, one *Citrobacter freundi* strain and one *Pseudomonas aeruginosa* strain were determined by spot test using the double agar method (Sevilla-Navarro et al., 2020b). *Salmonella* serovars selected for bacteriophage lytic spectrum were those more prevalent in European poultry farms (*S.* Enteritidis, *S.* Typhimurium, monophasic *S.* Typhimurium, *S.* Infantis, *S.* Virchow, *S.* Kentucky, *S.* Ohio, *S.* Senftemberg, *S*. Indiana and *S.* Havana). 200 µL of a log-phase culture of the bacterial suspensions in LB (Luria Bertani, VWR Chemicals, Barcelona, Spain) at an optical density (OD) 600 nm of 0.2 (~10^8 CFU/mL) was added to 5 mL of LB agar (LB with 0.6% agar) tempered to 45 °C and poured onto previously prepared and dried LB basal agar (with 1.6% agar). The plates were dried in a laminar flow hood for 15 min. Subsequently, 10 µL of the phage FGS011 (10^{10} PFU/mL) suspension was spotted onto the surface of the double layer agar. The resulting plates were incubated overnight at 37 °C, and subsequently checked for the phage plaque formation on the bacterial lawns (Sevilla-Navarro et al., 2020b).

3.1.1.3.1.3 Bacteriophage thermal and pH stability

For thermal-stability testing, tubes with 100 µL of FGS011 (10^{10} PFU/mL) were mixed with 900 µL pre-heated sterile LB broth and were kept in a water bath ranging from 30 to 80 °C for 30 min and 60 min (Ahmadi et al., 2016). In addition, phage stability at −20 °C in a standard refrigerator, and at −80 °C in an ultra-low temperature freezer, was also evaluated for 24 hours (Krasowska et al., 2015). For pH-stability testing, 100 µL of FGS011 (10^{10} PFU/mL) aliquots were mixed in a series of tubes containing 900 µL of sterile BPW (Buffered Peptone Water, VWR Chemicals, Barcelona, Spain) with varying pH values ranging from pH 2 to pH 13 (adjusted using NaOH or HCl) and incubated for 2 h at 37 °C (Ahmadi et al., 2016; Huang et al., 2018). After the incubation, ten-fold serial dilutions of the samples were carried out with LB and were plated by the spot test method and incubated 24 hours at 37 °C. Bacteriophage titration was performed per triplicate. The rates of phage pH/thermal stability were determined calculated with the formula: phage stability rate (%) = phage concentration (PFU/mL) under certain condition / initial

phage concentration added (PFU/mL) × 100 % (Huang et al., 2018). These experiments were performed three times.

3.1.1.3.1.4 Bacteriophage inactivation with Ultraviolet radiation

To quantify phage inactivation by ultraviolet radiation (**UV**), 1 mL of FGS011 (10^{10} PFU/mL) was exposed to UV irradiation in glass petri dishes at room temperature for 24 hours (Kim et al., 2018). A 15-watt, low-pressure mercury germicidal lamp (U.V ESTERIL, J.P. SELECTA s.a.) with a sharp emission maximum at 230 nm was used as the UV source for irradiation of the bacteriophage. Samples were taken at different time intervals, at 5, 30, 60 min, and 24 hours. Then, ten-fold serial dilutions of the samples were plated by the spot test method described above and incubated 24 hours at 37 °C. Bacteriophage titration was performed per triplicate. The rate of phage UV radiation inactivation was calculated with the stability rate (%) formula described above. This experiment was performed three times.

3.1.1.3.2 Bacteriophage Encapsulation

Encapsulation was performed according to Malik et al. (2021). For this study, two anionic polymers Eudragit® L100, and Eudragit® S100 were used for the phage encapsulation. Eudragit® L100 dissolves at pH 6 and greater, while Eudragit® Sl00 is less soluble than Eudragit® Ll00 and dissolves at a pH of 7 or greater (Kislalioglu et al., 1991). During this study, the bacteriophage FGS011 was evaluated without encapsulation as free phage (**FP**), and encapsulated with the polymers Eudragit® L100 (**L100**) and Eudragit® S100 (**S100**).

Eudragit L100 and S100 were kindly supplied by Evonik Germany. D-(+)-Trehalose dihydrate was purchased from Fisher Scientific (Loughborough, UK). Solutions containing different excipient (Eudragit S100, L100 with added trehalose) amounts were dissolved in 500 mL of deionised distilled water (dH_2O). The ratio of S100 and L100 to trehalose was 2:1 and total solids content 12g per 100ml of solution. In order to dissolve Eudragit, the pH of the water was changed to alkaline (pH 12) *via* addition of 4 M NaOH (Fisher Scientific, UK) to allow polymer dissolution, followed by pH adjustment to pH 7 using 0.1 M HCl prior to addition of trehalose powder, its dissolution, and then addition of bacteriophages to the solution. For each formulation, typically 10 % (*v*/*v*) high-titre phage (~10^{10} PFU/mL) was added to the solution, yielding phage titres of ~10^9 PFU/mL

in the final formulations. The phage-containing solutions were spray-dried using a commercially available Labplant spray-dryer SD-06 (Labplant, UK Limited), which is a co-current dryer with a pneumatic atomiser and a cylindrical drying chamber of dimensions 215 mm outer diameter and 420 mm height. The air exit stream was passed through a high-efficiency particulate air (**HEPA**) filter prior to discharge. The diameter of the atomization nozzle used throughout the work was 0.5 mm with the measured feed liquid flow rate at 280 $mL \cdot h^{-1}$ and a drying gas air flow rate of ~20 $L \cdot s^{-1}$. The air inlet temperatures were set at 100 °C resulting in corresponding air outlet temperatures of 60 ± 2 °C respectively. The outlet temperature is only indicative of the highest temperature the phages could be exposed to as dry powders in the collection bottle; temperature in the collection bottle varied between 40 and 60 °C.

3.1.1.3.3 *In vitro* evaluation of the release of bacteriophage under different GIT conditions (Experiment 1)

To assess the release of the encapsulated bacteriophage (L100 and S100) along the GIT section, an *in vitro* assay was performed. For this, the amount of phages that remained encapsulated throughout the simulated GIT was analysed. The GIT conditions of the crop, proventriculus-gizzard, duodenum, ileum, jejunum, and caeca were simulated *in vitro* according to Ravindran (2013) (**Figure 11**).

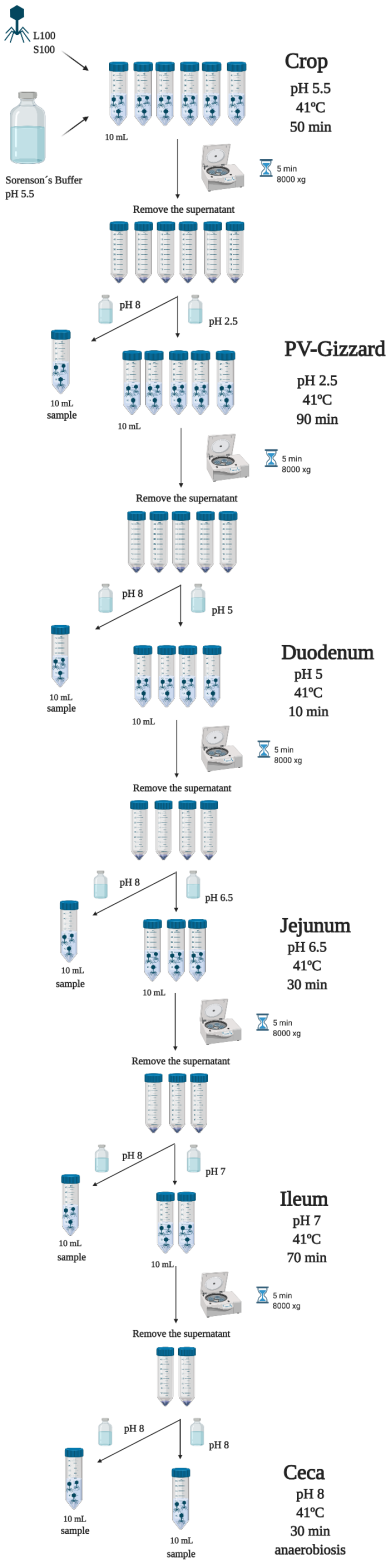

Figure 11. Scheme of the *in vitro* assay simulating the broiler GIT to assess the encapsulation maintenance (L100 and S100) along the GIT. The transit-time, temperature and pH of the crop (pH 5.5), proventriculus-gizzard (pH 2.5), duodenum (pH 5), ileum (pH 6.5), jejunum (pH 7), and caeca (pH 8 in anaerobiosis) were simulated. L100: encapsulated phage with the polymer Eudragit® L100; S100: encapsulated phage with the polymer Eudragit® S100. Created with BioRender.com.

Firstly, to mimic the crop conditions, 10 mL aliquots of Sorenson's Buffer pH 5.5 was added to six falcon tubes for each of the encapsulated phages (L100 and S100). After that, an initial inoculum of 10^{10} PFU of each encapsulated phage (L100 and S100) was added into each respective tube. Samples were then incubated, with shaking, at 41 °C for 50 min. At the end of the incubation, all the tubes were centrifuged to sediment the phage containing capsules for 5 min at 8,000 x g and the supernatant was removed. In one tube for each phage, the capsule pellet was resuspended with 10 mL of Sorenson´s buffer at pH 8, and was vortexed and left for 10 min to allow the capsules to dissolve. After that, 100 µL of aliquot were taken for phage enumeration. From the remaining tubes, the pellets were resuspended in 10 mL of Sorenson's Buffer with adjusted to pH 2.5 with 1 M HCl to mimic the proventriculus and gizzard conditions. The samples were incubated for another 90 min. After the incubation period, all tubes were centrifuged, and the procedure was repeated as described above. Hereafter, the process was repeated with 10 mL of Sorenson´s buffer at pH 5 for 10 min, followed by pH 6.5 for 30 min, and pH 7 for 70 min, to mimic the duodenum, ileum, and jejunum conditions, respectively. Finally, caeca conditions were mimicked using 10 mL of Sorenson´s buffer at pH 8 for 30 min under anaerobic conditions obtained with AnaeroGen Gas pack (Oxoid AN0035A) in anaerobic jars. Ten-fold serial dilutions of all the aliquots were carried out with LB and were plated by the spot test method and incubated for 24 hours at 37 °C. Bacteriophage titration was performed per triplicate.

3.1.1.3.4 *In vitro* evaluation of bacteriophages titres along the GIT (Experiment 2)

To assess the survival of the phages (FP, L100 and S100) across the GIT an *in vitro* assay was performed (Ma et al., 2016). For this, the amount of released phage that survived throughout the simulated GIT was analysed. The phages (FP, L100 and S100) were passed sequentially through each simulated GIT section according to Ravindran (2013). To this end, the following stages were carried out (**Figure 12**).

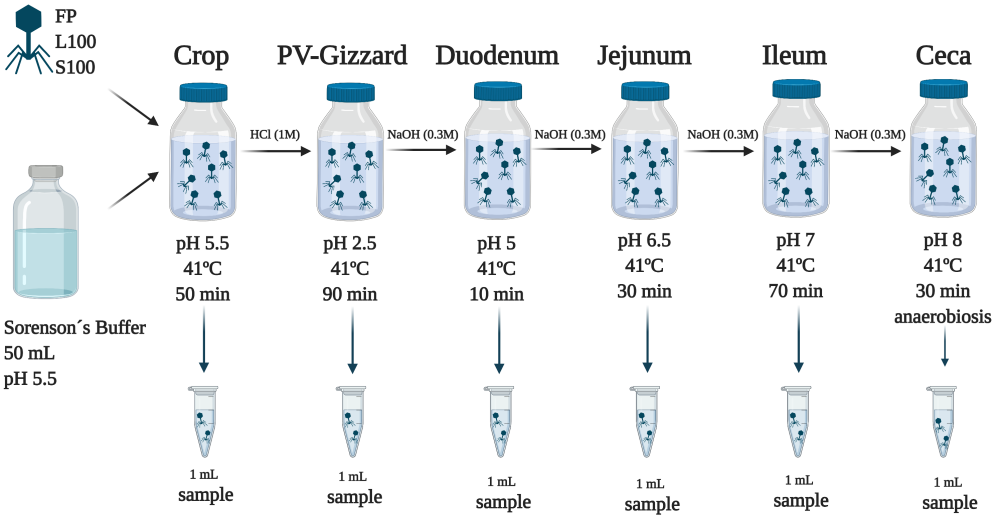

Figure 12. Scheme of the *in vitro* assay simulating the broiler to assess the release and survival of microencapsulated phage and the survival of non-encapsulated phages through the different pH, times and temperature conditions. The pH, transit-time and temperature of the crop (pH 5.5), proventriculus-gizzard (pH 2.5), duodenum (pH 5), ileum (pH 6.5), jejunum (pH 7), and caeca (pH 8 in anaerobiosis), were simulated. FP: free BP, L100: encapsulated phage with the polymer Eudragit® L100; S100: encapsulated phage with the polymer Eudragit® S100. Created with BioRender.com.

Firstly, to mimic the crop conditions, 50 mL of Sorenson's Buffer (pH 5.5) was inoculated with 10^{10} PFU of FP or 10^{10} PFU of encapsulated L100 and S100. Samples were then incubated, with shaking, at 41 °C for 50 min. At the end of the incubation period, 1 mL of aliquot was taken for phage enumeration. Then, to mimic the proventriculus and gizzard conditions, HCl (1 M) was added to each tube to adjust solution pH to pH 2.5 and the incubation continued for another 90 min. After this additional incubation period, 1 mL of aliquot was taken for phage enumeration and the procedure was repeated by adjusting the solution pH to pH 5 (using 0.3 M NaOH) for 10 min, followed by pH 6.5 for 30 min, and pH 7 for 70 min, to mimic the duodenum, ileum, and jejunum conditions, respectively. Finally, the caeca conditions were mimicked with pH 8 for 30 min under anaerobic conditions obtained with AnaeroGen Gas pack (Oxoid AN0035A) in anaerobic jars. At the end of each incubation, 1 mL of aliquot was taken for phage enumeration. The samples were centrifugated for 5 min at 8,000 x g and the supernatant was taken. Ten-fold serial dilutions of the supernatants were carried out with LB and were plated by the spot test method and incubated 24 hours at 37 ºC. Bacteriophage titration was performed per triplicate.

3.1.1.3.5 *In vivo* study of bacteriophages survival along GIT (Experiment 3)

An *in vivo* study of phages (FP, L100 and S100) survival along the GIT after oral administration of phages to one-day-old chicks was carried out. Faecal shedding of phage was also measured. Twenty one-day-old *Salmonella* free chicks (Ross) were randomly divided into four groups of five birds (**Figure 13**).

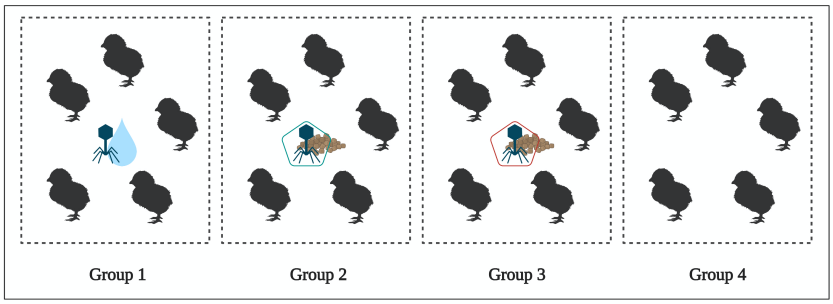

Figure 13. Experimental design of phage application in one-day-old chicks. Group 1 received the FP *via* drinking water; group 2 received L100 *via* feed; group 3 received S100 *via* feed; and group 4 did not receive bacteriophage (control group). Created with BioRender.com

To assess the survival of the phages through the GIT, group 1 received 100 mL of 10^6 PFU/mL of FP *via* drinking water, group 2 received 100 g of 10^6 PFU/g of L100 *via* feed, group 3 received 100 g of 10^6 PFU/g of S100 *via* feed, and group 4 did not receive any phage (control group). Then, 24 hours after phage administration, animals from each experimental group (n = 5/group) were taken and the GIT was removed and processed for phage enumeration. The following sections were processed from each animal: crop, proventriculus, gizzard, gut, and caeca. Samples of each section were collected under sterile conditions. The material used was sterile and it was changed between groups. Between samples the material was sterilised with alcohol and fire (Marcó et al., 2019).

Moreover, to assess fecal shedding, at least 10 g of faeces were taken from each experimental group. A total of 104 samples were weighed and homogenized in 1:10 mL of LB broth medium, centrifuged for 5 min at 16,000 x g, and the supernatant was filtered through a 0.45 µm membrane. Afterwards, 100 µL of each dilution was transferred to an empty well and ten-fold serial dilutions were performed using sterile dilution buffer (LB). Then, 10 µL of each dilution with 200 µL of the bacterial host suspension was mixed with 5 mL of LB 0.6 % top agar layer and placed over a 1.6 % LB agar bottom layer. Plates were incubated overnight at 37 °C. Bacteriophage titration was performed per triplicate.

3.1.1.3.6 Statistical Analysis

Concentrations (PFU/mL) of phage were converted to Log10 (PFU/mL) and then averaged. A General Linear Model (**GLM**) was used to evaluate the release of encapsulated phage (L100 and S100) along the *in vitro* GIT conditions. GLM was used to evaluate the survival of the released phages (L100 and S100) and non-encapsulated phage along the *in vitro* GIT conditions. As a fixed effect were included the gastrointestinal localization tract simulated (crop, proventriculus-gizzard, duodenum, ileum, jejunum and caeca), the initial phage concentration, and the phage (FP, L100 and S100). GLM was used to compare the activity of the phage (FP, L100 and S100) *in vivo*, including as a fixed effect, the gastrointestinal localization (crop, pro-ventriculus, gizzard, gut, caeca and faeces) and the phage (FP, L100 and S100). A *p-value* < 0.05 was considered to indicate a statistically significant difference. All statistical analyses were carried out using SPSS 16.0 software.

3.1.1.4 Results

3.1.1.4.1 Bacteriophage characterization assay

3.1.1.4.1.1 Bacteriophage lytic spectrum

The lytic host range against a panel of 13 strains indicated that phage FGS011 was able to lyse seven strains of *Salmonella* and one *Citrobacter* strain (**Table 1**).

Table 1. The lytic spectrum of FGS011 against 13 bacterial strains from 4 genera

	Strain	Source/Reference	Lysis by phage FGS011
mST:	*Salmonella* Enteritidis	CCM160	+
	Salmonella Typhimurium	CCM157	+
	mST	CCM188	+
	Salmonella Virchow	CAECAV	-
	Salmonella Ohio	CAECAV	-
	Salmonella Kentucky	CAECAV	-
	Salmonella Infantis	CAECAV	+
	Salmonella Senftemberg	CAECAV	+
	Salmonella Indiana	CAECAV	+
	Salmonella Havana	CAECAV	+
	Escherichia coli	CCM099	-
	Citrobacter freundi	CCM091	+
	Pseudomonas aeruginosa	CCM054	-

mST:monophasic *Salmonella* Typhimurium

hours of incubation (**Figure 14A**). However, FGS011 titers declined when exposed to acidic pH less than pH 3 or alkaline pH greater than 12. The phage was rapidly inactivated at low pH 2 and high pH 13 (**Figure 14A**). Thermal stability tests indicated that phage FGS011 was relatively heat stable for up to 60 min at temperatures between 30 to 60 °C, however, the viable bacteriophage concentration decreased upon exposure to temperatures between 70 to 80 °C (**Figure 14B**). Bacteriophage FGS011 also survived upon undergoing a freeze-thaw cycle at -80 °C.

3.1.1.4.1.3 Bacteriophage inactivation with UV radiation

The viability of phage FGS011 was maintained for up to one hour of exposure to UV irradiation, nevertheless, no viable phage was measurable after 24 hours of exposure (**Figure 14C**).

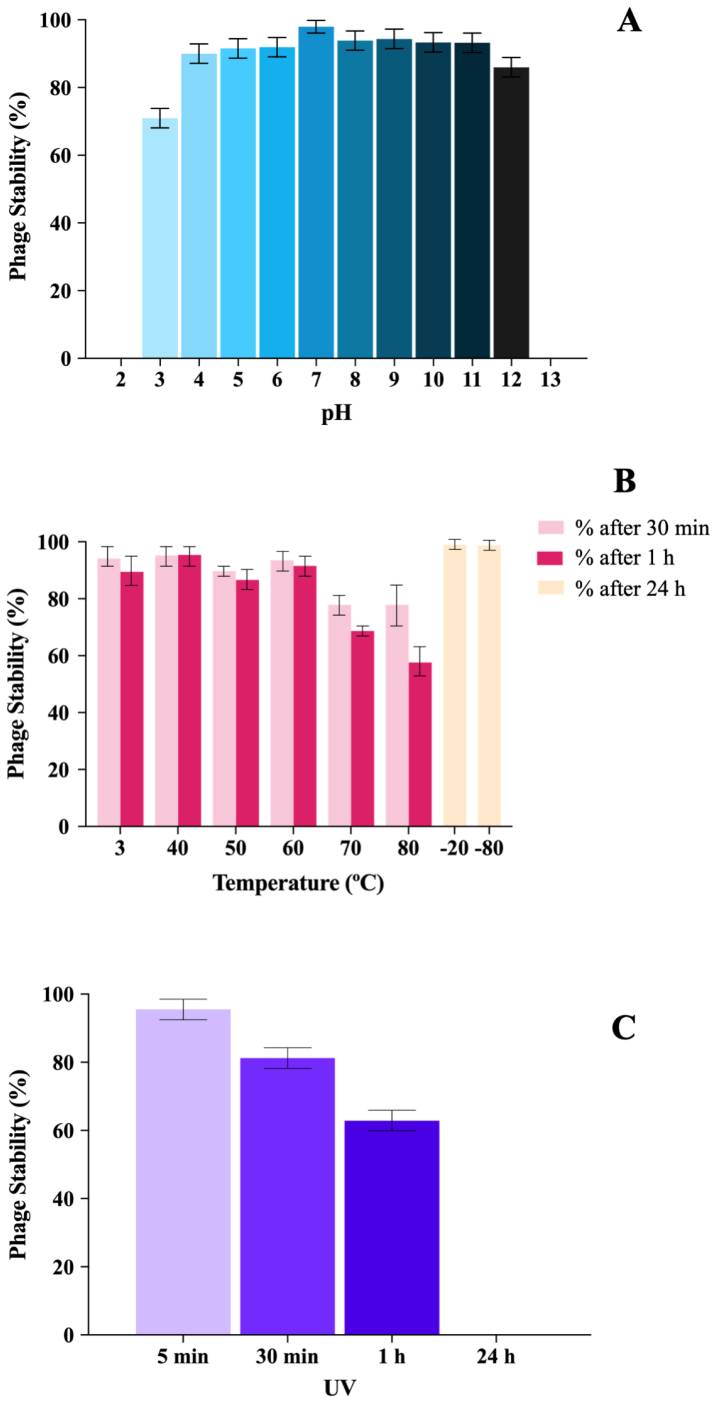

Figure 14. Stability of FGS011 exposed to different stress conditions. (A) pH stability of FGS011; (B) thermal stability of FGS011; and (C) stability upon exposure to UV radiation of FGS011. Data reported are means ± standard error of three independent trials. Error bars show standard error.

3.1.1.4.2 *In vitro* evaluation of the release of bacteriophages under different GIT conditions (Experiment 1)

Protection of encapsulation from GIT pH stress and phage release under different GIT pH conditions was evaluated for FGS011 encapsulated in formulations L100 and S100 simulating *in vitro* GIT conditions. All results represented in these experiments are with regard to the starting concentration of phage used in the experiment (10^{10} PFU). Bacteriophage encapsulated in L100 was released in the crop due to the pH 5.5 (*p-value* < 0.05) (**Figure 15A**). However, phages encapsulated in S100 did not release in the crop (pH 5.5), proventriculus-gizzard (pH 2.5) and the duodenum (pH 5) (**Figure 15B**). Bacteriophage release was noted in the jejunum (pH 6.5), ileum (pH 7) and any residual encapsulated phage in the caeca (pH 8), indicating a statistically significant decrease along the lower GIT (*p-value* < 0.05) (**Figure 15**).

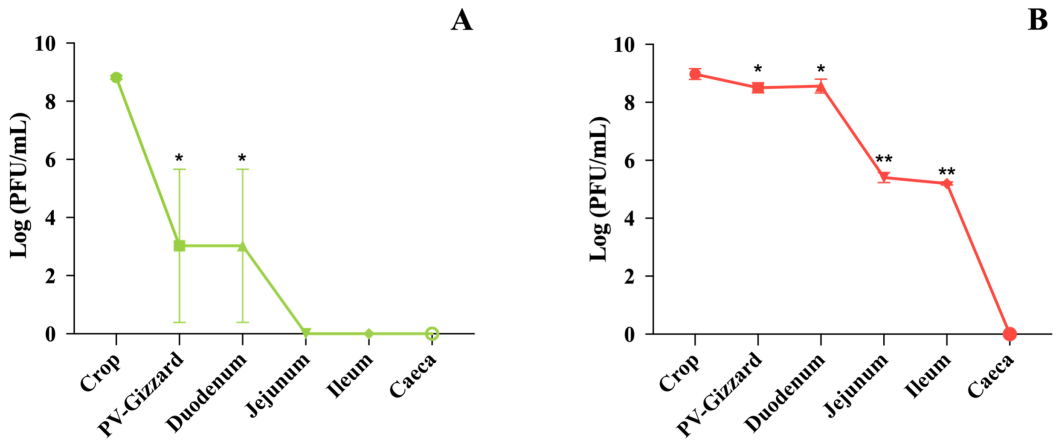

Figure 15. Amount of phages (Log UFP/mL) that remains encapsulated with the polymer Eudragit® L100 (A) and Eudragit® S100 (B) *in vitro* under simulated transit times and pH conditions of the crop (50 min at pH 5.5), proventriculus-gizzard (90 min at pH 2.5), duodenum (10 min at pH 5), jejunum (30 min at pH 6.5), ileum (70 min at pH 7), and caeca under anaerobic conditions (30 min at pH 8). Values are presented in Log10 (PFU/mL). L100: encapsulated phage with the polymer Eudragit® L100; S100: encapsulated bacteriophage with the polymer Eudragit® S100; PV: Proventriculus. Values shown are means ± standard deviations. Error bars show one standard deviation. The statistically significant differences in the count of phage that remain encapsulated throughout the simulated GIT with respect to the initial phage administered was represented as *, *p-value* < 0.001; and **, *p-value* < 0.000.

3.1.1.4.3 *In vitro* evaluation of bacteriophage titres along the GIT (Experiment 2)

The survival of the bacteriophages (FP, L100 and S100) across the GIT was evaluated *in vitro* by passing the phages sequentially through each simulated GIT section (**Figure 3**).

All results represented in these experiments are with regard to the starting concentration of phages used in the experiment (10^{10} PFU). The results showed that FP were inactivated upon exposure to proventriculus-gizzard conditions, and viable phages were not detected again in the following stages downstream of the proventriculus-gizzard. For L100, significant differences were observed along the simulated GIT sections (*p-value* < 0.05). Bacteriophage released from L100 were detected in the crop, but no phage was detected in the neutralized supernatant of the simulated proventriculus-gizzard section. Thereafter, phage detection in the supernatant increased in the duodenum and phage titres were maintained in the jejunum through to the caeca (*p-value* > 0.05). For S100, the highest concentration of phage was detected in the crop (*p-value* < 0.05). No viable phage was detected in the proventriculus-gizzard. The concentration of phages in the supernatant increased in the duodenum and jejunum and was highest in the ileum with a 0.5 log decrease observed in the caeca (*p-value* < 0.05).

Moreover, statistically significant differences were observed among FP, L100 and S100 across the different controlled GIT conditions (pH, transit-time, and temperature) (**Figure 16**).

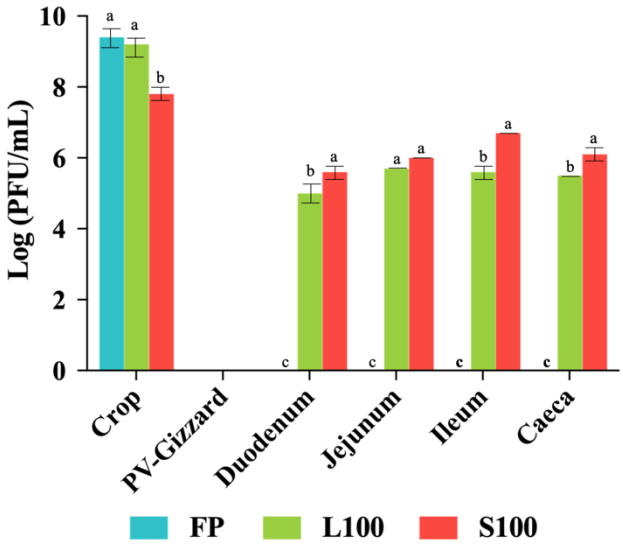

Figure 16. Amount of phages (Log UFP/mL) that remains in the supernatant whilst transiting across the simulated chicken GIT. FP: free phage; L100: encapsulated phage with the polymer Eudragit® L100; S100: encapsulated phage with the polymer Eudragit® S100; PV: Proventriculus. Error bars show one standard deviation. [a,b,c]: different letters indicate statistically significant differences between groups in the count of bacteriophages that released and survives throughout the simulated GIT at *p-value* < 0.05.

3.1.1.4.4 *In vivo* study of bacteriophage survival along GIT (Experiment 3)

The distribution of FP, L100 and S100 within the GIT of one-day-old chicks administered phages either in drinking water or in feed was determined after 24 hours in the absence of Salmonella host. After 24 hours, all the bacteriophages were present in all of the GIT sections (crop, proventriculus, gizzard, gut and caeca) of the treated groups. The control group was negative for the presence of phages in any of the samples analyzed.

From each free/released FGS011 (FP, L100 and S100), no statistically significant differences of phage concentration were observed through the different GIT sections (*p-value* > 0.05). For the different GIT sections, phages counts, are summarized in **Table 2**.

Table 2. *In vivo* comparation of bacteriophage treatments (FP, L100 and S100) within each GIT section.

	FP Log10 (PFU/g content)	L100 Log10 (PFU/g content)	S100 Log10 (PFU/g content)
Crop	4.9 ± 0.4	6.3 ± 0.2	5.6 ± 0.3
Proventriculus	3.8 ± 0.4	5.7 ± 0.9	4.7 ± 0.7
Gizzard	4.5 ± 0.2	4.9 ± 0.5	4.6 ± 0.4
Gut	4.7 ± 0.2^{b}	5.9 ± 0.5^{a}	4.6 ± 0.5^{b}
Caeca	5.2 ± 0.6	5.9 ± 0.2	4.7 ± 0.5

FP: free phage; L100: encapsulated phage with the polymer Eudragit® L100; S100: encapsulated phage with the polymer Eudragit® S100. Values shown are means ± standard error. ^{a,b} Different superscripts within each row indicates significant differences between means at *p-value* < 0.05.

On the other hand, statistically significant differences were shown between bacteriophages in the gut, being L100 the phage who showed the highest concentration (*p-value* < 0.05). However, no statistically significant differences were observed in the crop, proventriculus, gizzard and caeca (*p-value* > 0.05) (**Table 2**).

For faecal samples, statistical differences were found in phage counts among the different groups (*p-value* < 0.05). The highest phage counts were in faeces of chicks fed L100 (6.4 Log10 PFU/g), followed by S100 (6.1 Log10 PFU/g) and finally FP (5.8 Log 10 PFU/g) (*p-value* = 0.000).

3.1.1.5 Discussion

The survival of *Salmonella* phage FGS011 (non-encapsulated and microencapsulated) through the GIT under *in vitro* and *in vivo* conditions after oral administration to day-old chicks was assessed. This is the first study to report the dynamics of the *Salmonella* phage

encapsulated with the anionic polymers Eudragit® L100 and Eudragit® S100 through the chicken GIT.

Despite the NSCP, *Salmonella* continues to be a threat pathogen to the poultry sector and a potential risk from farm to fork (Sevilla-Navarro et al., 2020b). Therefore, prevention and control are required throughout the poultry chain (Sevilla-Navarro et al., 2018). The negative economic impact of *Salmonella* for the poultry sector, the effects on human health, increasing antimicrobial resistance and the absence of effective alternatives for controlling *Salmonella* are drivers for exploration of the potential of phage therapy as a more effective biocontrol tool, reducing economic loses in the poultry industry and reducing the risk of food-borne diseases (Wernicki et al., 2017). Oral phage therapy has been used as a tool for *Salmonella* control in the poultry industry (Ahmadi et al., 2016; Adhikari et al., 2017; Nabil et al., 2018). Nevertheless, inherent challenges, such as the GIT environmental conditions, has led to divergent phage therapy results (Ma et al., 2016; Malik et al., 2017). Although is this study both phage forms reached the caeca, previous studies reported a reduction in phage titres *in vivo* and suggested that this may be due to phage inactivation attributed to the acidic environment of the chicken's gizzard. This may especially be the case for sensitive phage that do not survive low acidic pH exposure (Ma et al., 2016; Malik et al., 2017). Previous studies have therefore highlighted the need for encapsulated phages that allow high titres of phage to be delivered to the cecum which is the most likely site of Salmonella colonization (Colom et al., 2015; Ma et al., 2016). The encapsulation of phages and their controlled release may help in ensuring that the *in situ* phage concentration remains at a therapeutically effective level thereby allowing phage to amplify once the bacteria concentration increases to levels sufficient for *in situ* phage amplification (Malik et al., 2017). The *in vitro* results of this study demonstrated that FP are likely to be inactivated upon exposure to proventriculus-gizzard pH (Figure 3). Whereas encapsulation of bacteriophages significantly improved phage survival with S100 remaining encapsulated until the end of gut (Figures 2 and 3). Encapsulation would therefore ensure that high phage concentration would be delivered to the caeca (Figure 3). Therapeutically effective levels reaching the intended *Salmonella* infection site, such as the lower intestine including caeca may improve phage therapy outcomes (Malik et al., 2017; Vinner et al., 2019). The current results indeed demonstrated that phage non-encapsulated and microencapsulated appeared in the faeces, showing its high capacity of dissemination in the environment (Sevilla-Navarro et al., 2018). This matter will allow

not only the control of the bacteria in the environment but also will limit the reinfection of the animals (Sevilla-Navarro et al., 2018). The reduction of faecal *Salmonella* shedding controlled by the NSCP, will entail an important economic reduction for the poultry sector (EP, 2003; Sevilla-Navarro et al., 2018).

Several complex external factors could influence the treatment success *in vivo*, such as the rapid clearance of the phage by active or passive host immunity, interactions of phages with the intestinal mucosa and with other gut microbiota (Bull and Gill, 2014; Colom et al., 2017; Dąbrowska, 2019). The *in vivo* results obtained after phage administration in one-day-old chicks demonstrated that encapsulated and non-encapsulated phage could survive through the GIT and were excreted in the faeces (Table 2). This fact may be explained due to the higher pH of the gizzard in the young animals (4.3 pH), which would allow not only the survival of the encapsulated phage, but also the survival of the FP (unpublished data) (Smith and Shaw, 1987). These results are in agreement with those obtained by Ma et al. (2016), who also observed that although FP did not survive gastric fluid conditions *in vitro*, in day-old chicks phages were able to survive throughout the GIT, without no notable differences between free phages titres compared with the encapsulated form. Other authors have highlighted the possibility that for mature chicken GIT where the pH is lower, FP could not survive the gastric passage, and were not found to effectively reduce *Salmonella* numbers in the chicken intestine (Chibani-Chennoufi et al., 2004; Ma et al., 2008), compromising phage therapy outcomes in older animals (Smith and Shaw, 1987; Sabouri et al., 2016).

Regarding the survival of phages formulated in different encapsulated forms administered to one-day-old chicks, slightly higher concentrations of phage encapsulated in L100 were found to be delivered to the intestine compared with the S100 formulation (5.9 Log 10 and 4.6 Log 10 PFU, respectively). This may be due to lower amounts of phage released in the caeca of day-old chicks from S100 which requires higher pH levels to release phages (Cheng et al., 2004; Asghar et al., 2009). Bacteriophage therapy has previously been used to control *Salmonella* in poultry farms (Toro et al., 2005; Atterbury et al., 2007; Wernicki et al., 2017; Sevilla-Navarro et al., 2018, 2020a), there is however, lack of available data at field level (Hussain et al., 2015). Further studies are therefore needed to study the phage dynamics in the GIT throughout the six-week rearing period of broilers to assess the best way to incorporate phage in animal feed as well as the best intervention moment to prevent spread of *Salmonella* in chicken farms.

3.1.1.6 Conclusions

Significant differences were observed between phage delivery results of *in vitro* studies compared with *in vivo* results. In one-day-old chicks there were no statistically significant differences between phage delivered along the GIT for the encapsulated and non-encapsulated phage (the gut being the exception, but differences were small here too). Encapsulation of the phage using the polymers Eudragit® L100 and Eudragit® S100 resulted in delivery of phage in day-old chicks with no adverse reactions observed in the animals. Further studies are needed to better understand the dynamics of the encapsulated phage released during transit through the GIT of the chickens during the entire production cycle.

3.1.1.7 References

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3.1.2 Gastrointestinal dynamics of non-encapsulated and microencapsulated *Salmonella* bacteriophages in broiler production

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Laura Lorenzo-Rebenaque[1], Danish J Malik[2], Pablo Catalá-Gregori[1,3], Clara Marin[1*], and Sandra Sevilla-Navarro[1,3]. Gastrointestinal Dynamics of Non-Encapsulated and Microencapsulated *Salmonella* Bacteriophages in Broiler Production. Animals. 2022;12(2):144. doi: 10.3390/ani12020144.

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3.1.2.1 Abstract

Bacteriophage therapy has been considered a promising tool to control *Salmonella* in poultry. Nevertheless, changes in gastrointestinal tract environmental conditions throughout the production cycle could compromise the efficacy of phage *via* oral administration. The main objectives of this study were to assess the optimal timing of the phage administration over a 42-day production cycle and to compare microencapsulated and non-encapsulated phages and the spatial and temporal dynamics of the phage delivery along the GIT. Phage FGS011 was encapsulated in the pH-responsive polymer Eudragit® L100 using the process of spray drying. At different weeks of the chicken rearing period, 15 broilers were divided in 3 groups. Over a period of 24 h, group 1 received non-encapsulated phages (delivered through drinking water), group 2 received microencapsulated phages (incorporated in animal feed), and group 3 did not receive any phages. Microencapsulation was shown to enable delivery of the bacteriophages to the animal gut and cecum throughout the animal rearing period. During the six weeks of application, the crop displayed the highest phage concentration for both phage delivery methods. The L100 based encapsulation protected the phages from the harsh environmental conditions in the Proventriculus-Gizzard which may help in delivery of high phage doses to the cecum. Future *Salmonella* challenge studies are necessary to demonstrate benefits of microencapsulation of phages using L100 formulation on phage therapy in field studies during the rearing period.

3.1.2.2 Introduction

Nontyphoidal *Salmonella* is considered one of the main causes of foodborne outbreaks; it is responsible for around 70 million worldwide cases of human illness and 58 thousand deaths each year (WHO, 2015; Dawoud et al., 2017). In the European Union, despite strict measures carried out by the NSCP in the poultry sector, new cases continue to emerge every year (EP, 2003; Vandeplas et al., 2010; EFSA and ECDC, 2021). When the bacteria come into contact with the birds, *Salmonella* colonizes the GIT and spreads to the environment through faeces (Nair and Johny, 2019). For this reason, *Salmonella* colonization is particularly important at three points of the production cycle: During the first week of rearing when the immune system of the animals is still immature (Marin and Lainez, 2009), the mid-cycle (around 4 weeks old), when *Salmonella* sampling control takes place at farm level (EP, 2003; Montoro-Dasi et al., 2020), and at the end of the production cycle (around 6 weeks old), just before the transport of the animals to the slaughterhouse (Montoro-Dasi et al., 2020). Thus, the development of effective management strategies, including improved biosecurity measures, vaccination, use of organic acids and prebiotics to improve animal gut health and use of bacteriophages could all help to control the bacteria at farm level, while maintaining animal health and welfare (Ahmadi et al., 2016).

In poultry, bacteriophages are increasingly considered as a potential viable method to enhance animal health and *Salmonella* control through the food chain (Wernicki et al., 2017; Svircev et al., 2018). Bacteriophages are viruses whose life cycle is strictly associated with prokaryotic cells (Wernicki et al., 2017; Żbikowska et al., 2020). Their ubiquitous nature, specificity, prevalence in the biosphere, and low inherent toxicity, makes them a safe, natural, and sustainable technology as specific narrow-spectrum antimicrobials (Wernicki et al., 2017; Żbikowska et al., 2020). A number of different studies have assessed efficient and cost-effective administration routes and the timing of phage application to improve *Salmonella* control in livestock (Borie et al., 2008; Gigante and Atterbury, 2019). Phage therapy (Atterbury et al., 2007; Borie et al., 2008; Nabil et al., 2018) is considered safe and especially useful against antibiotic-resistant bacteria (H et al., 2021). Despite several trials with bacteriophages reporting success in the reduction of *Salmonella* at field level (Ahmadi et al., 2016), more research into its effectiveness under commercial conditions is still needed in the poultry sector (Clavijo et al., 2019). Oral administration of phages has previously shown to successfully treat GIT and

systemic infections, however, the effects may be transient and age dependent. In addition, the dosing interval may be a critical factor for the successful implementation of phage therapy (Ryan et al., 2011). The possibility of phage administration *via* incorporation in feed or through drinking water would make phage therapy suitable for treatment *en masse*, overcoming a major limiting factor for large scale poultry (Reynaud et al., 1992). Nevertheless, changes in GIT conditions throughout the production cycle could compromise the efficacy of the orally administered phages, leading to variable efficacy outcomes (Stanford et al., 2010; Colom et al., 2017; Lorenzo-Rebenaque et al., 2021). In this sense, controlled-release formulation technology has gained interest due to the capability of delivering therapeutics at the target site where they are needed to control the pathogen (Widjaja et al., 2018). Controlled release dosage forms could delay the release of the drug substance in the first stretch of the GIT (crop, PV, and gizzard), reaching the gut and the cecum (Widjaja et al., 2018; Lorenzo-Rebenaque et al., 2021). This way, high doses of phages will reach the target site where *Salmonella* mainly colonizes (the caecum) improving the effectiveness of phage therapy.

The main objectives of this study were to assess the effect of the phage intervention over a six-week production cycle and to compare microencapsulated and non-encapsulated phages and the spatial and temporal dynamics of the bacteriophage delivery along the GIT during the chicken rearing period.

3.1.2.3 Materials and Methods

The *in vivo* study was carried out in accordance with the recommendations of European Commission (2010/63/CE and 2007/526/CE) and the Spanish legislation (RD 53/2013) (Spain, 2013). Protocols were designed to comply with the European policy on the "3 Rs" (Replace, Reduce and Refine) in animal experimentation.

3.1.2.3.1 Bacteriophage Origin and encapsulation

Bacteriophage FGS011 used in this study was isolated by Sevilla-Navarro et al. (2020) and characterized by Lorenzo-Rebenaque et al. (2021). Phage FGS011 was propagated on *S.* Senftenberg, obtained from poultry farms during the NSCP (NSCP, 2020) in the CAECAV. During this study, the phage FGS011 was evaluated as FP and L100. Encapsulation was performed according to Malik (2021) and Lorenzo-Rebenaque et al. (2021). For this study, the anionic polymer Eudragit® L100 was used for the phage

encapsulation. The polymer Eudragit® L100 is insoluble in acid medium, dissolving at a pH 6 and greater (Kislalioglu et al., 1991).

Commercially available Eudragit polymer L100 has been specifically designed for enteric delivery applications with the aim of protecting therapeutics from gastric acidity and allowing controlled release of therapeutics utilising a pH-dependent trigger mechanism. L100 is a copolymer of methacrylic acid and methyl methacrylate with different amounts of carboxylic acid residues providing differences in pH dissolution characteristics, the ratio of free carboxyl groups to ester groups is 1:1.

In order to dissolve Eudragit® L100, the pH of the water was changed to alkaline (pH 12) *via* addition of 4 M NaOH (Fisher Scientific, Hampshire, UK) to allow polymer dissolution, followed by pH adjustment to pH 7 using 0.1 M HCl prior to addition of trehalose powder (Fisher Scientific, Hampshire, UK), its dissolution, and further addition of bacteriophages to the solution. Typically 10% (v/v) high-titre phage (~10^{10} PFU/mL) was added to the solution, yielding phage titres of ~10^{9} PFU/mL in the final formulation. The phage-containing solutions were spray-dried using a commercially available Labplant spray-dryer SD-06 (Labplant, UK Limited), which is a co-current dryer with a pneumatic atomiser and a cylindrical drying chamber of dimensions 215 mm outer diameter and 420 mm height. The diameter of the atomization nozzle used throughout the work was 0.5 mm with the measured feed liquid flow rate at 280 $mL \cdot h^{-1}$ and a drying gas air flow rate of ~20 $L \cdot s^{-1}$. The air inlet temperatures were set at 100 °C resulting in corresponding air outlet temperatures of 60 ± 2 °C respectively.

3.1.2.3.2 Experimental design

The study was performed in an experimental poultry house (A) in the Centre for Animal Research and Technology (CITA, IVIA, Segorbe, Spain). A total of 90 one-day-old *Salmonella* free chicks (Ross, males), provided from the same hatchery, were housed to simulate real production conditions. The house was supplied with wood shavings as bedding material, programmable electrical lights, automated electric heating and forced ventilation. The environmental temperature was gradually reduced from 32 °C on arrival day to 19 °C at 39 days post hatch (Montoro-Dasi et al., 2020). The birds received drinking water and were fed *ad libitum*. Two different age commercial diets were offered to the animals, a pelleted starter diet from arrival until 21 days post hatch (*Camperbroiler*

iniciación, Alimentación Animal Nanta, Spain) and pelleted grower diet from 21 days post hatch to the slaughter day (*Pollos crecimiento G, Alimentación Animal Nanta, Spain*).

Once per week, 15 birds were moved to another house (B) and randomly divided into 3 pens separate by walls in groups of 5 birds (group 1, 2 and 3). Subsequently, each treatment group was challenged with a single dose of the microencapsulated and non-encapsulated bacteriophages, and after 24 hours, animals of each experimental (n = 5/group) were slaughtered and sampled. Group 1 received FP at a concentration of 10^8 PFU/mL *via* drinking water, group 2 received L100 at a dose of 10^8 PFU/g *via* feed, and group 3 did not receive any phage (control group) (**Figure 17**). Samples were collected from the upper and lower GIT. From the upper GIT samples from the crop (to assess the percentage of phages entering in the animals) and from the PV-gizzard (to assess acid segments in the phage release) were taken. Concerning the lower GIT, samples from the gut (duodenum, jejunum, ileum and colon) and the cecum (target segment for *Salmonella* colonization) were taken. Moreover, to assess fecal shedding, at least 10 g of faeces were taken from each experimental group.

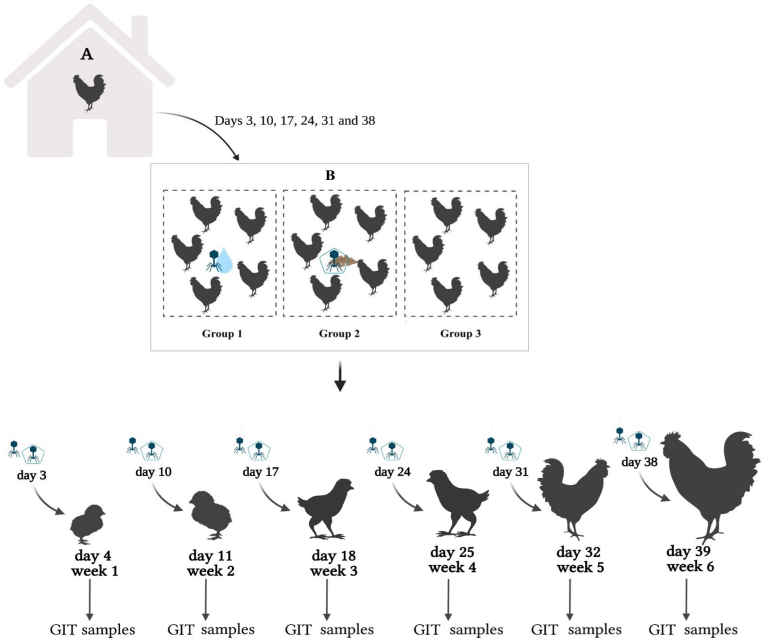

Figure 17. Experimental design of phage application and sampling throughout the entire production cycle. A: control poultry house; B: experimental poultry house; GIT: gastrointestinal tract. Group 1 received the FP *via* drinking water; group 2 received L100 *via* feed; group 3 was the control group. Created with BioRender.com.

3.1.2.3.3 Processing of GIT samples

The GIT and faeces samples were analyzed according to Lorenzo-Rebenaque et al. (2021). Briefly, samples were weighed and emulsified individually in LB broth supplemented with salts (Luria Bertani, VWR Chemicals, Barcelona, Spain) at 1:10 (w/v). The samples were centrifuged at 16,000 x g for 5 min and filtered through 0.45 µm. The phage concentration was measured in each sample using the *spot test* by the double overlay agar plaque assay method. Thus, ten-fold serial dilutions were performed using sterile dilution buffer (LB), these were spotted onto the surface of bacterial lawns. For this purpose, 200 µL of a log-phase culture of the bacterial suspensions in LB, at an optical density (OD) 600 nm of 0.2 (~10^8 CFU/mL) was added to 5 mL of molten LB agar (LB with 0.6 %(w/v) agar) tempered set at 45 ºC and poured onto previously prepared and dried LB basal agar (with 1.6 % (w/v) agar). Plates were incubated overnight at 37 °C. Bacteriophage titration was performed per triplicate.

3.1.2.3.4 Statistical Analysis

Concentrations (PFU/mL) of bacteriophage were converted to Log10 (PFU/mL) (Gao and Martos, 2019). A Univariate General Linear Model was used to access and compare the dynamics of the phage (FP and L100) along the GIT including as fixed-effects factors the gastrointestinal localization (crop, PV-gizzard, gut and caeca), the application week (weeks 1 to 6), and the phage form (FP and L100), and as random-effect factor for the different replicates. A *p-value* < 0.05 was considered indicative of a statistically significant difference. Statistical analyses were performed using SPSS 16.0 software package (SPSS Inc., Chicago, IL, USA).

3.1.2.4.1 Results

No adverse clinical signs in animals were observed during the entire experiment. The performance parameters (body weight, feed intake, daily gain and feed conversion ratio) obtained were in accordance with breeding standards (Ross, 2020). No statistically significant differences were observed between the treatment groups and the control group.

3.1.2.4.1.1 Bacteriophage gastrointestinal dynamics in chickens according to the week and form of phage application

3.1.2.4.1.1.1 Bacteriophage concentration in the first section of the GIT (crop and PV-gizzard)

Statistically significant differences in phage concentrations were found between the organs (crop and PV-gizzard), the phage delivery method (FP and L100) and the application week (1 to 6) (*p-value* < 0.05). In the crop, statistically significant differences were found between different weeks. The highest phage recovery for both delivery methods was at the end of the production cycle (week 5 and week 6). Regarding the concentration of phage recovered at each point of sampling, the concentrations of FP obtained were significantly lower than for the microencapsulated phages regardless of the week of application (*p-value* < 0.05) (**Figure 18A**). In the PV-gizzard, the free/encapsulated bacteriophages counts were lower than those compared to the crop (*p-value* < 0.05). Significantly higher concentrations of phages were recovered from PV-gizzard for the microencapsulated phage L100 compared with free phage treated animals, regardless of the application week (*p-value* < 0.05), except in the sixth week (*p-value* < 0.05) (**Figure 18B**).

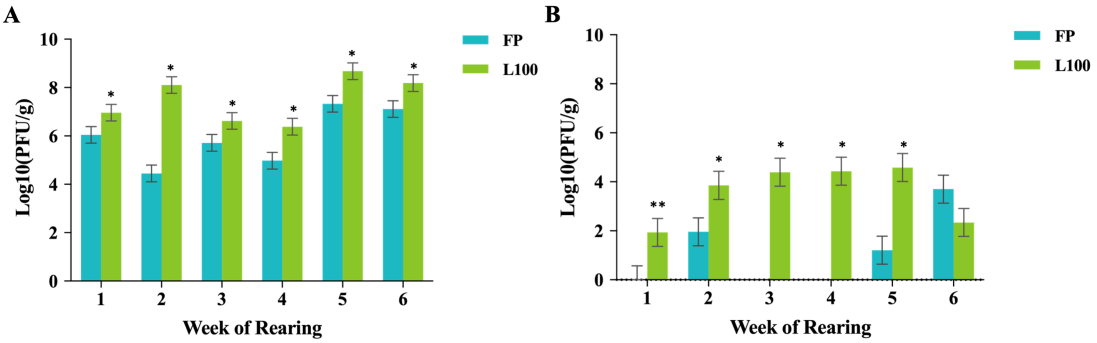

Figure 18. Concentration of bacteriophages recovered following administration depending on the week of application and organ analyzed: (A) the crop, (B) the PV-gizzard. Values are presented as Log10 (PFU/g). Error bars show one standard deviation. The statistically significant differences between groups comparing phage delivery method have been represented as * (*p-value* < 0.05) and ** (*p-value* < 0.001).

3.1.2.4.1.1.2 Bacteriophage concentration in the gut and caeca

Statistically significant phage concentration differences were observed in the gut and caeca (FP and L100) during the treatment period (*p-value* = 0.000). Phages were recovered from animals in the FP treated group throughout the treatment period except for the 1st application week. Bacteriophages concentrations were observed to increase

over time for both delivery methods as the treatment period progressed. Higher counts of phages were measured at the end of the production cycle (**Figure 19, A and B**).

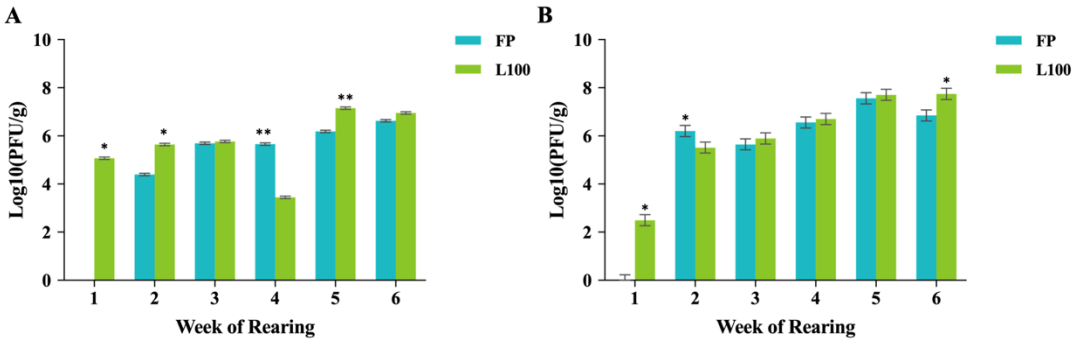

Figure 19. Concentration of the BPs recovered following administration depending on the week of application and organ analyzed: (A) the gut and (B) the cecum. Values are presented as Log10 (PFU/g). The statistically significant differences between groups comparing phage delivery method have been represented as * (*p-value* < 0.05) and ** (*p-value* < 0.001).

3.1.2.4.1.2 Fecal phage excretion profile

Higher concentrations of phages were measured in faeces for the encapsulated form (*p-value* < 0.05) when comparing the different bacteriophages delivery methods (**Figure 20**). The exceptions were for the fourth and last week of the rearing.

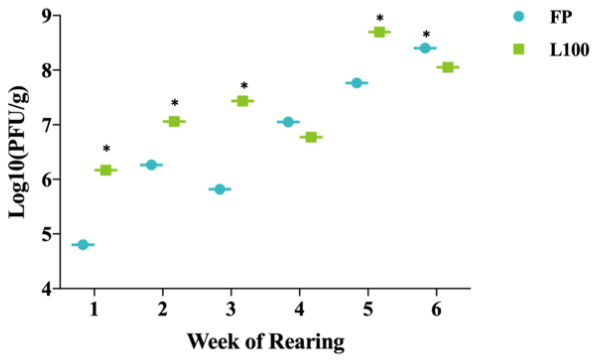

Figure 20. Concentration of the phages found in faeces depending on the week of application. Values are presented as Log10 (PFU/g). FP: free phage; L100: encapsulated bacteriophage with the polymer Eudragit® L100. The statistically significant differences between groups comparing phage delivery method have been represented as * (*p-value* < 0.05).

3.1.2.5 Discussion

Bacteriophage therapy is considered a promising tool to control *Salmonella* in poultry (Wernicki et al., 2017; Svircev et al., 2018). The easy implementation, host-specificity and cost-effectiveness of phage therapy has resulted in increased interest in phage application at the field level (Ly-Chatain, 2014; Wernicki et al., 2017; Sevilla-Navarro et al., 2018; Vikram et al., 2021). Previous studies have reported positive results regarding the delivery of microencapsulated *Salmonella* phages in a simulated gastrointestinal model and in one-day-old chicks (Lorenzo-Rebenaque et al., 2021; Malik, 2021). The present research is the first study that addresses the spatial and temporal effects of microencapsulated and non-encapsulated bacteriophages delivery at different ages of application during the chicken rearing period. These data suggest that microencapsulated bacteriophages formulated using the polymer Eudragit® L100 has the potential to be used throughout the six-week chicken rearing period.

Results obtained in this study demonstrate the efficient protective effect of L100 delivery through the GIT. The FP administered in drinking water were substantially inactivated in the PV-gizzard conditions. The first week of rearing represents a critical moment for the chicks, as the immune system of the animals is still immature, facilitating the rapid colonization and multiplication of *Salmonella,* thereby affecting the entire production cycle (Marin and Lainez, 2009; Hashemzadeh et al., 2010; Koutsoumanis et al., 2019; Groves et al., 2021). Protection in young animals in which the immune system and GIT microbiota are not fully mature could ensure a *Salmonella*-free flock at the field level (Kempf et al., 2020).

Results of our study during the first week of life are in agreement with previous reports in which no antimicrobial effect was observed after application of oral therapy (Alali et al., 2013; Arsi et al., 2015; Nabil et al., 2018). Nabil et al. (2018) reported the need for several phages doses to obtain *Salmonella* reduction, however, the lack of phage effectiveness during the first week of rearing could be due to the low pH and short retention times in the chick's intestinal tract during the first 7 days of its life, that prevents the phages from reaching the *Salmonella* colonization site (Pan and Yu, 2013).

The application of bacteriophages from the second week onwards showed that regardless of the delivery method (FP and L100), some of the administered phage dose was able to reach the gut and cecum. The effect of the encapsulation allowed L100 to overcome the

adverse environmental conditions of the PV-gizzard. A small amount of the FP could pass through the GIT perhaps protected by the buffering effect of feed (Ma et al., 2008, 2016; Stanford et al., 2010; Colom et al., 2015).

The highest bacteriophages concentrations were mainly obtained in the crop regardless of the delivery method applied. These results are in line with those previously reported, where a difference of up to 3 Log10 was found between the crop and the gut-cecum concentrations (Ma et al., 2016). The prolonged retention of high doses of phages in the crop may provide protection to any new orally ingested pathogen, such as *Salmonella* (Ma et al., 2016; Colom et al., 2017). The crop is considered, together with the cecum to be the major site of *Salmonella* colonization in the chicken (Tellez et al., 2001). The long phage residence times in the crop would allow phage-host interaction with potential phage amplification (Ma et al., 2016). Moreover, it was shown that for both FP and L100 delivery methods high concentration of phages were present in the caecum. A hypothesis that could explain the high concentration at the end of the GIT may be the ability of phages predation on non-target species (Ganeshan and Hosseinidoust, 2019). The previous host range characterization of the phage FGS011 demonstrated its capability to lyse *Citrobacter* (Lorenzo-Rebenaque et al., 2021), known to be associated with poultry microbiota and *Salmonella* epidemiology (Jahantigh, 2013; Borda-Molina et al., 2018). This lysis against *Citrobacter* enhances the possibility of phages co-evolution in the gut that may lead to increases in bacteriophage concentration (Ganeshan and Hosseinidoust, 2019). Thus, the possibility to control *Salmonella* in the intestinal tract of chickens before slaughter by the application of phages may prevent carcass contamination during the slaughtering process and reduce the risk of *Salmonella* transmission *via* contaminated chicken meat to consumers (Amalaradjou, 2019).

Excretion of the phage in animal faeces, along with the presence of the phage in feed and/or water may result in the presence of the phage in the house environment, facilitating re-infection of animals with the phage, and the protection of animals from future bacterial challenges (Ma et al., 2016; Sevilla-Navarro et al., 2018; Dąbrowska, 2019).

The results of this study highlight the importance of phages survival dynamics following their administration through the GIT. The route of administration was chosen for ease of administration and delivery of the phage, as non-encapsulated phages in drinking water and encapsulated phages in feed. Bacteriophages have been administered *via* feed and

drinking water previously. This is a feasible low cost delivery method for large-scale application in poultry farms (Lim et al., 2012; Adhikari et al., 2017; Vaz et al., 2020). Lim et al. (2012) and Vaz et al. (2020) applied the bacteriophages in the feed and water, respectively to reduce *Salmonella* colonization in broilers. The administration route (feed or water) as well as the delivery method (encapsulated and non-encapsulated) was shown to affect the survival of the phages in the PV-Gizzard and could impact on the efficacy of phage therapy. Other GIT environment factors, such as intestinal volume, local pH variation, viscosity and presence of commensal microbiota, could affect the phages concentration at the target site, resulting in differences in BP-host interactions (Vaz et al., 2020). Combination of phages with dietary supplementation with probiotics have previously been reported (Toro et al., 2005; Kim et al., 2014). A synergistic effect was shown against *Salmonella* infections in broilers (Toro et al., 2005) and the potential to improve the performance in piglets was noted (Kim et al., 2014). This possibility of combination of phages with other antibiotic alternatives employed in poultry production could be of significant future interest to achieve a higher degree of effectiveness against the bacteria.

3.1.2.6 Conclusions

The conclusions of this study highlight that bacteriophage encapsulation with the polymer Eudragit® L100, especially when administered at the beginning and at the end of the cycle, could ensure targeted delivery of high titres of phages to the caecum affording encapsulated phages protection from the harsh environmental conditions found in the PV-Gizzard. Moreover, the fact that more encapsulated phages were found in the crop and caecum, known sites of high *Salmonella* colonization, makes encapsulation of phages a promising tool to control the bacteria at the field level. On the other hand, the easy dissemination of the phages through faeces may also facilitate the control of the bacterium in the farm environment. However, further *Salmonella* challenge studies are necessary to evaluated the beneficial effects of encapsulation of phages using L100 formulation to control the bacteria in the field during the rearing period.

3.1.2.6 References

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3.1.3 Examining the effects of *Salmonella* phage on the caecal microbiome and metabolome features in *Salmonella*-free broilers

An adapted version of this chapter is under review:

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3.1.3.1 Abstract

Bacteriophages selectively infect and kill their target bacterial host, being a promising approach to controlling zoonotic bacteria in poultry production. To ensure confidence in its use, fundamental questions of safety and toxicity monitoring of phage therapy should be raised. Due to its high specificity, a minimal impact on the gut ecology is expected; however, more in-depth research into key parameters that influence the success of phage interventions has been needed to reach a consensus on the impact of bacteriophage therapy in the gut. In this context, this study aimed to investigate the interaction of phages with animals; more specifically, we compared the caecum microbiome and metabolome after a *Salmonella* phage challenge in *Salmonella*-free broilers, evaluating the role of the phage administration route. To this end, we employed 45 caecum content samples from a previous study where *Salmonella* phages were administered *via* drinking water or feed for 24 hours from 4, 5 and 6-weeks-old broilers. High-throughput 16S rRNA gene sequencing showed a high level of similarity (beta diversity) but revealed a significant change in alpha diversity between broilers with *Salmonella*-phage administered in the drinking water and control. Our results showed that the phages affected only a few genera of the microbiota's structure, regardless of the administration route. Among these, we found a significant increase in *Streptococcus* and *Sellimonas* in the drinking water and *Lactobacillus*, *Anaeroplasma* and *Clostridia_vadinBB60_group* in the feed. Nevertheless, the LC-HRMS-based metabolomics analyses revealed that despite few genera were significantly affected, a substantial number of metabolites, especially in the phage administered in the drinking water were significantly altered (64 and 14 in the drinking water and feed groups, respectively). Overall, our study shows that preventive therapy with bacteriophages minimally alters the caecal microbiota but significantly impacts their metabolites, regardless of the route of administration.

3.1.3.2 Introduction

Bacteriophage therapy is a promising approach to controlling zoonotic bacteria, replacing antibiotics to treat or prevent bacterial diseases in poultry production (Wernicki et al., 2017d; Żbikowska et al., 2020; Zhao et al., 2022; Clavijo et al., 2022). Specifically, lytic bacteriophages (phages) are 'natural predators' that selectively infect and kill their target bacterial host (Mu et al., 2021; Zhao et al., 2022). Compared to antibiotics, phages have high specificity that usually attacks only their targeted bacterial hosts, indicating minimal disruption to the niche microbiota (Cieplak et al., 2018; Gindin et al., 2019). Nevertheless, phages targeting *Salmonella* can potentially lyse phylogenetically related genera, such as *Escherichia coli* or *Citrobacter spp* (Lorenzo-Rebenaque et al., 2021; Zhao et al., 2022). Different challenge experiments in poultry have revealed the efficacy of bacteriophage therapy application to control enteric pathogens (Carvalho et al., 2010; Nabil et al., 2018; Sevilla-Navarro et al., 2018; Clavijo et al., 2019; Richards et al., 2019; Huang et al., 2022). Till now, most phage-based products have been targeted against the main foodborne pathogens, such as *Campylobacter jejuni*, *Salmonella spp*, *Escherichia coli*, *Listeria monocytogenes*, *Staphylococcus aureus*, and *Clostridium perfringens* (Żbikowska et al., 2020). However, to ensure confidence in the use of phages, fundamental questions of safety and toxicity monitoring of phage therapy should be raised (Drilling et al., 2017; Krut and Bekeredjian-Ding, 2018; Dufour et al., 2019; Liu et al., 2021). Likewise, further research into crucial parameters that influence the success of phage interventions is needed to reach a consensus on the impact of bacteriophage therapy in the gut (Javaudin et al., 2021). In poultry, the data on the effects of phage treatment on dysbiotic events in gut microbiota are scarce (Zhao et al., 2022; Clavijo et al., 2022). Thus, before making any decisions on the use of phage as a therapy, our knowledge of phage-host interactions must be increased (Sutton and Hill, 2019). Bacteriophages have the potential to interact with the immune system directly (Ivanenkov and Menon, 2000; Barr et al., 2013, 2015; Nguyen et al., 2017), and given their larger size relative to other biological therapeutic agents, makes phages a more complex therapeutic agent than any biotherapeutics that have preceded them (Sutton and Hill, 2019). Even though ample research on bacteriophage therapy applications has provided many positive conclusions, there are still some unknowns regarding their role in gastrointestinal ecological homeostasis (Loc-Carrillo and Abedon, 2011).

The symbiotic interactions between host and gastrointestinal tract microbiota are fundamental to poultry health, as they have a positive impact on the immune system and broiler productivity (Brisbin et al., 2008). It is well known that gastrointestinal microbiota contributes to the reduction and prevention of enteric pathogen colonisation by competitive exclusion and the synthesis of bacteriostatic and bactericidal compounds in broilers (Clavijo and Flórez, 2018). Conversely, an unbalanced microbiota can induce several gut disorders, such as inflammation and leaky gut (Teague et al., 2017; Jacquier et al., 2019). Accordingly, a logical first step in exploring the safety of phage therapy would be to rule out that dysbiotic changes in the gut microbial community occur. In this way, a recent report demonstrated that *Salmonella* phages induce changes in the intestinal microbiota of *Salmonella*-free chicks at early life stages (Zhao et al., 2022). In addition, in mammals it has been observed that exposure to a commercial bacteriophage preparation results in dysbiosis with increased inflammation and intestinal permeability (Tetz et al., 2017).

From this perspective, this study aimed to investigate the interaction of phages with the animal. More specifically, we compared the caecal microbiome and metabolome after a *Salmonella* phage challenge in *Salmonella*-free broilers, evaluating the role of the phage administration route.

3.1.3.3 Material and Methods

3.1.3.3.1 Caecal content origin

The Directorate-General approved this study for Agriculture, Fisheries and Livestock from the Valencian Community (2021/VSC/PEA/0003). A total of 45 caecal content samples were obtained in a previous study on the use of phages in poultry, carried out at the Centre for Animal Research and Technology (CITA, IVIA, Segorbe, Spain) (Lorenzo-Rebenaque et al., 2022). The animals involved in this study were *Salmonella*-free Ross male chicks (one-day-old), that purchased from a commercial hatchery and housed in the growing room under commercial rearing conditions on an experimental farm. Briefly, the house was supplied with wood shavings as bedding material, programmable electrical lighting, automated electric heating and forced ventilation. The environmental temperature was gradually reduced from 32 °C on arrival day to 19 °C at 39 days post-hatch (Montoro-Dasi et al., 2020). All animals had free access to food and

water. Two different age commercial diets were offered to the animals, a pelleted starter diet from arrival until 21 days post-hatch (*Camperbroiler iniciación, Alimentación Animal Nanta, Spain*) and a pelleted grower diet from 21 days post-hatch to the slaughter day (*Pollos crecimiento G, Alimentación Animal Nanta, Spain*).

Weekly from week 4 to week 6 of the rearing period (fully competent immune system birds age), 15 birds were moved to another room (experimental room) and randomly divided into three groups, with 5 birds in each group (phages in drinking water -water group, phages in feed -feed group and no phages -control group) (**Figure 21**) (Lorenzo-Rebenaque et al., 2022). The phage used in this study is described in detail in Sevilla-Navarro *et al.* (2020) and Lorenzo-Rebenaque *et al.* (2021). The water group received a 10^8 PFU/mL phage concentration *via* drinking water, the feed group received a 10^8 PFU/g phage concentration *via* feed (encapsulated), and the control group did not receive a phage. Caecal samples were obtained 1 day after delivery of the phage. All animals of each group were slaughtered and the caecum was removed. Individual caecal content was divided into two flash-frozen aliquots in liquid nitrogen for subsequent microbiome and metabolome analyses.

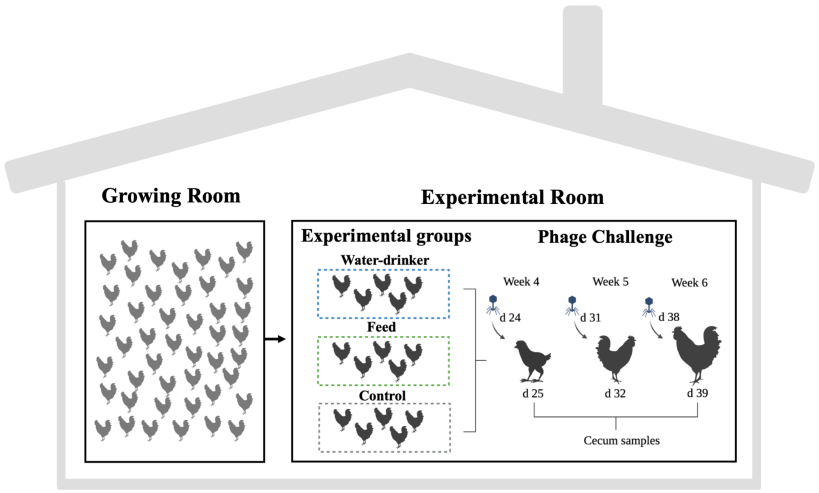

Figure 21. Experimental design of phage challenge. The water group received a 10^8 PFU/mL phage concentration *via* drinking water. The feed group received a 10^8 PFU/g phage concentration *via* feed (encapsulated). The control group did not receive a phage. Created with BioRender.com

3.1.3.3.2 Microbiota analysis

3.1.3.3.2.1 DNA extraction, 16S rRNA gene amplification and MiSeq sequencing

First, caecal content was removed and homogenised. Then, the DNA was extracted from 250 mg of each sample according to the manufacturer's instructions (QIAamp Power Fecal Pro DNA kit, Werfen, Barcelona, Spain). DNA concentration and purity were measured using a Nanodrop ND-1000 spectrophotometer (Thermo Scientific, Wilmington, USA), and verified using a Qubit fluorometer (Life Technologies, Paisley, UK). The DNA was frozen at −20 ºC for shipment at the *Instituto de Investigación Sanitaria y Biomédica de Alicante - ISABIAL* (Alicante, Spain), following the manufacturer's instructions. Once there, 16S rRNA gene amplification and MiSeq sequencing was performed. To this end, from 12.5 ng of DNA (evaluated in Qubit) of each sample, the library was prepared following the instructions of the 16S rRNA Metagenomic Sequencing Library Preparation (Illumina) protocol (Illumina, 2022). Primer sequences cover the V3–V4 regions of the 16S rRNA gene. The following primers also include the Illumina adapters: 16S Amplicon PCR Forward Primer = 5′ TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCCTACGGGNGGCWGCAG; and 16S Amplicon PCR Reverse Primer = 5′ GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGACTACHVGGGTATCTA ATCC. NGS Libraries were analysed using the Agilent 4200 TapeStation System to ensure their integrity. The sequencing run was performed in a MiSeq (Illumina) system in 2 × 300 bp format. The quality of the raw unprocessed reads was evaluated using the FastQC software (Babraham Bioinformatics, 2022).

3.1.3.3.2.2 Bioinformatic analysis

To perform the bioinformatic analysis, demultiplexed paired FASTQ sequences were imported into the QIIME2 v2021.4 (Bolyen et al., 2019). The DADA2 pipeline incorporated into QIIME2 was used for the denoising, filtering and chimera removal of the sequences and assigned reads into Amplicon Sequence Variants (**ASVs**). Then, taxonomic annotation was obtained using the SILVA v138 database (Quast et al., 2013; Campos et al., 2022), and sequences not assigned to any taxa or classified as eukaryote, archaea or only bacteria were filtered out. Sequencing statistical analyses were done using QIIME2 v2021.4.

3.1.3.3.3 Metabolomics analysis

3.1.3.3.3.1 Sample preparations

The sample preparations were performed according previous published method with slight modifications (Coppola et al., 2019). Briefly, caecal contents were lyophilised and homogenised. Then, 10 mg of the sample was mixed with 0.75 mL of cold 75 % (v/v) methanol and 0.1 % (v/v) formic acid, spiked with 10µg/ml formononetin as internal standard, the mix was shaken for 40' at 20 Hz using a Mixer Mill 300 (Qiagen) and centrifuged at 20,000 xg for 15 min at 4 °C. The suspension (600 µL) was transferred to a new 2-mL conical tube. For the LC-ESI-MS analysis, samples were transferred to HPLC tubes and an aliquot of 3 µL was injected. Finally, the supernatant was collected, filtered with HPLC filter tubes (0.22 µm pore size, WhatmannTM) and 3 µL were subjected to LC-ESI-HRMS analysis using an LTQ-Orbitrap Discovery mass spectrometry system (Thermo Fisher Scientific) as previously described (Garcia-Dominguez et al., 2020).

3.1.3.3.3.2 LC-ESI-HRMS analysis

Untargeted LC-ESI-HRMS analyses of the caecal semipolar metabolome were performed as reported above (Garcia-Dominguez et al., 2020) in the *Agenzia nazionale per le nuove tecnologie, l'energia e lo sviluppo economico sostenibile* (ENEA, Roma, Italy). Compound Discoverer software (Thermofisher Scientific) was used to identify the differentially accumulated peaks, by chromatogram alignment and peak alignment/picking/filtration, and public database (e.g., ChemSpider, KEGG, Metabolika) querying based on accurate masses (m/z). After chromatogram alignment and retrieval of all the detected frames (e.g., ions), the data generated were normalised with respect to the internal standard. For metabolite identification, a manual curation using the Metlin database was performed (https://metlin.scripps.edu/). Tentative identifications were validated comparing chromatographic and spectral properties with authentic standards (when available) and reference spectra, in house database, literature data, and based on the m/z accurate masses, as reported in the Pubchem database (http://pubchem.ncbi.nlm.nih.gov/) for monoisotopic mass identification, subsequently confirmed by MS/MS fragmentation.

3.1.3.3.4 Statistical analysis

Statistical analysis of microbiome and metabolome composition was performed following the same methodology. No outlier samples were identified using a principal component analysis with the dataset without zeros, so all samples remained in the datasets. Genera and metabolites with more than 20% zeros within each treatment were removed (Bijlsma et al., 2006). The remaining zeros were replaced by one for microbiome data and by half of the minimum value detected for each metabolite. A total of 124 genera from 44 samples and 718 metabolites from 37 samples remained in the datasets. Datasets were transformed using the additive log-ratio (**ALR**) transformation following:

$$\boldsymbol{ALR(j|ref) = log\left(\frac{x_j}{x_{ref}}\right) = \log(x_j) - \log(x_{ref})} \quad (1)$$

where j is the total number of variables in the dataset, x_j is the values for the genera or metabolite j, and (x_{ref}) is the reference variable used to transform the data. The reference variable for metabolome data was a standard chemical (formononetin) injected in the platform run at a fixed concentration. For microbiome data, $x_{ref}$was the one with the lowest coefficient of variation (x_{ref}; *Anaerofustis*). The lack of isometry was checked using a Procrustes correlation analysis (Greenacre et al., 2021). ALRs were auto scaled with mean of 0 and standard deviation of 1.

A partial least square-discriminant analysis (**PLS-DA**) was used to identify the genera and metabolites that allow classification or discrimination among the treatments. PLS-DA models were computed with the mixOmics packages in R (Rohart et al., 2017), using the treatments as the categorical vector y, and the ALR dataset for genera or metabolites as the matrix X. The balance error rate (**BER**) for the Mahalanobis distance, computed by a 4-fold cross-validation repeated 100 times was used to select the optimal number of components of the model in each iteration process. In each iteration, variables with a variable importance prediction (**VIP**) lower than 1 were removed from the X matrix, as they are not informative for the classification among the treatments (Galindo-Prieto et al., 2014). After the variable selection, a new PLS-DA model was computed. Variable selection and the PLS-DA model computation were done until the lowest BER was achieved, meaning that the best classification and prediction performance was achieved for the model. The prediction performance of the final PLS-DA model was checked with

the construction of a confusion matrix and a permuted-confusion matrix using a 4-fold cross-validation repeated 10,000 times. The former allows us to determine the ability of the model to predict each treatment according to the variables selected by the PLS-DA. The latter determines if the performance achieved is due to a spurious selection of variables throughout the PLS-DA iterations. The prediction performance was considered spurious when the percentage of true positives for each treatment was far from their random probabilities (33 % for three categories and 50 % for two categories).

Bayesian statistics were used complementary to the PLS-DA to measure the relevance of the differences in abundance of genera and metabolites between the trearment (drinking water and feed groups) and the control group. Hence, a model with a single effect of 'treatment' and flat priors was fitted. The marginal posterior distribution of the unknows was done with MCMC (Gibbs sampling) using four chains with a length of 50,000 iterations, a lag of 10, and a burn-in of 1,000 interations. The posterior mean of the differences in genera or metabolites abundances was estimated as the mean of the marginal posterior distribution of differences between the control and each of the treatments. These differences wereestimates were reported as units of standard deviations (SD) of each genera or metabolite. The differences in the mean abundance of the genera and metabolites between the control and the treatments were considered relevant when these differences were higher than 0.5 units of SD, and the probability of the differences (Blasco, 2017) being higher (if the difference is positive) or lower (if negative) than 0 (P0) was higher than 0.9.

The alpha- and beta diversity were computed using the ALR at species level to measure the differences in microbiome composition among groups. The alpha diversity was measured by Shannon's (H') and inverse Simpson indexes to analyse the species diversity and evenness. Differences in the distribution of alpha diversity among groups were considered when the *p-value* of a Mann-Whitney U test was lower than 0.05. Beta diversity was measured by the Bray-Curtis dissimilarity matrix and a nonmetric multidimensional scaling (NMDS) was carried out to retrieve the loadings of the first two dimensions. Differences in microbial genera composition were tested by the permutational multivariate analysis of variance (PERMANOVA; $p\text{-}value < 0.05$) on the loadings of the two first MDS dimensions.

3.1.3.4 Results

3.1.3.4.1 Changes in caecal microbiota

The caecal microbiota was characterised in 44 samples from the three groups (15 of the water group, 14 of the feed group and 15 of the control group) taken after 24 hours of phage application at weeks 4, 5 and 6 of the chickens' rearing period. The total of sequencing reads was 7,044,611 (average 156546.9 reads/sample), with an average read length of 404.5±14.79 pb. A total of 4,192,062 sequences and 2,778 ASVs were generated. A total of 4,144,140 sequences were left for ASVs table generation and database alignment. After filtering, a total of 2,735 ASVs were left for taxonomic assignment. The datasets generated and analysed are available at NCBI's BioProject PRJNA876127.

A PLS-DA with ALR transformed variables were used to evaluate the effect of the administration route (drinking water and feed groups) in the caecal microbial abundance in *Salmonella*-free broilers. The analysis identified 11 relevant variables (genera) in the final model: 2 for water *vs* control group (final PLS-DA model classification performance: water=59.83 % and control=71.99 %, **Figure 22A**), and 7 for feed *vs* group (final PLS-DA model classification performance: feed=74.13 % and control=77.91 %, **Figure 22B**). Overall, the results show that a few genera (2 and 7) were the most potential to discriminate the effect of the phage administration.

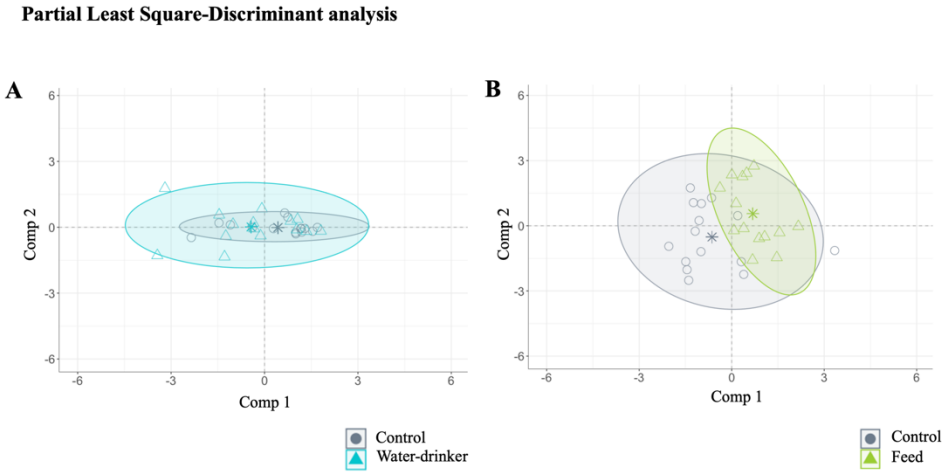

Figure 22. Caecal microbiota features characterised for *Salmonella*-free broilers treated with a *Salmonella* phage. Caecal microbiota composition dissimilarity through the representation of the first (Comp 1) and second components (Comp 2) of the final partial least square-discriminant analysis (PLS-DA) models (A) from the water *vs* control groups, and (B) from the feed *vs* control

groups. The water group (blue) received a 10^8 PFU/mL phage concentration *via* drinking water. The feed group (green) received a 10^8 PFU/g phage concentration *via* feed (encapsulated). The control (grey) group did not receive a phage.

The Shannon diversity index, which is more sensitive to species richness (Johnson and Burnet, 2016), showed that the microbiota diversity of the water group was significantly different from that of the control and feed groups (Kruskal–Wallis test, water *vs* control: *p-value* = 0.02, and feed *vs* control: *p-value* = 0.78, **Figure 23A**). For the inverse Simpson index, which is more sensitive to species evenness (Johnson and Burnet, 2016), no significant differences were observed between groups (Kruskal–Wallis test, water *vs* control: *p-value* = 0.07, feed *vs* control: *p-value* = 0.81, **Figure 23B**). Moreover, in pairwise PERMANOVA comparisons between groups using Bray-Curtis, there were no significant differences between groups in the microbiome composition (*p-value* =0.559; **Figure 23C**). These results showed that despite the few genera identified by PLS-DA, they are enough to show differences in alpha diversity in the water group. On the other hand, for the feed and control group, in general both populations displayed a similar microbiome composition, except for the 7 relevant genera identified by the PLS-DA.

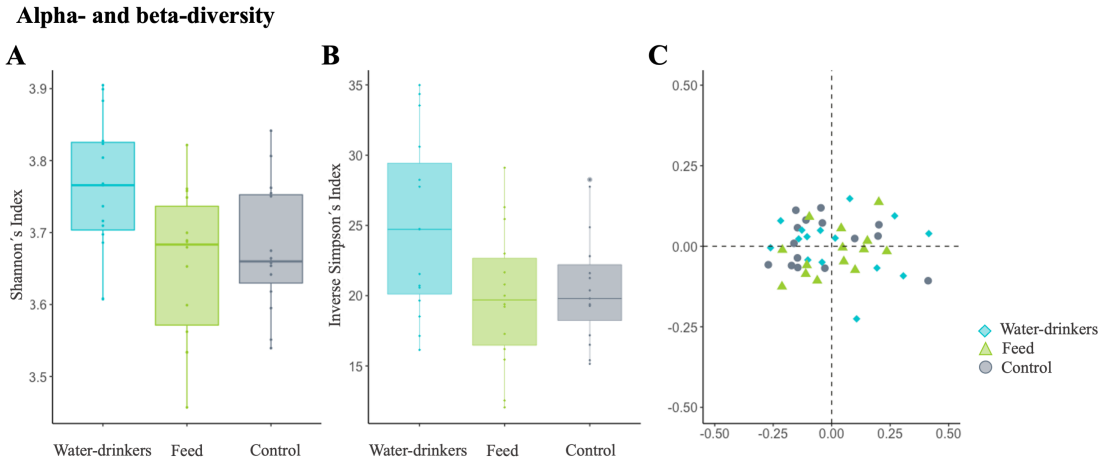

Figure 23. Examining the effects of *Salmonella* phage on the caecal microbiota in *Salmonella*-free broilers. Caecal microbiota composition dissimilarity through the representation of the and alpha- and beta diversity scores from water, feed and control groups. The alpha- and beta diversity scores were calculated with the additive log-ratio of each species abundance according to a reference genera (Anaerofustis). Alpha diversity was computed using (A) Shannon's H index and (B) Inverse Simpson index. Beta diversity was computed by calculating (C) the Bray-Curtis dissimilarity matrix. Differences among populations were established with a *p-value* ≤ 0.05. The water group (blue) received a 10^8 PFU/mL phage concentration *via* drinking water. The feed group (green) received a

10^8 PFU/g phage concentration *via* feed (encapsulated). The control (grey) group did not receive a phage.

A Bayesian statistical analysis was performed to better understand the effect of *Salmonella* phage on the caecal microbiota from the initial relevant genera identified by PLS-DA. The Bayesian results showed that few of the variables included in the PLS-DA model were key variables for discriminating between groups, with relevant differences in mean abundance (**Supplementary Table 1**).

As seen in **Table 3**, in the water group, 2 of the 124 genera detected were different from those of the control group: *Streptococcus* and *Sellimonas*. Both genera, indeed, were more abundant in the water group, and were from the *Firmicutes* phyla. As seen in **Table 3,** in the feed group, 3 of the 124 genera detected were different from those of the control group: *Lactobacillus, Anaeroplasma* and *Clostridia_vadinBB60_group*. All genera were more abundant in the feed group and were from the *Firmicutes* phyla.

Table 3. Caecal microbiota features characterised for *Salmonella*-free broilers treated with a *Salmonella* phage. Key genera identified by partial least square-discriminant analysis (PLS-DA) for discriminating between groups with relevant differences in mean abundance based on Bayesian statistical analysis in water- and feed-phage treated chickens compared with the control group, computed as control *vs* water and control *vs* feed. The water group received a 10^8 PFU/mL phage concentration *via* drinking water. The feed group received a 10^8 PFU/g phage concentration *via* feed (encapsulated). The control group did not receive a phage.

Experimental groups	Family	Genera	HPD95	P0	D
Control *vs* Water	*Streptococcaceae*	*Streptococcus*	[-1.34,0.14]	94.97	-0.62
	Lachnospiraceae	*Sellimonas*	[-1.34,0.12]	94.73	-0.60
Control *vs* Feed	*Lactobacillaceae*	*Lactobacillus*	[-1.32,0.18]	93.39	-0.57
	Acholeplasmataceae	*Anaeroplasma*	[-1.62,-0.17]	99.11	-0.89
	Clostridia_vadinBB60_group	*Clostridia_vadinBB60_group*	[-1.45,0.03]	96.87	-0.70

HPD95%= The highest posterior density region at 95% of probability. P0= Probability of the difference (Dcontrol-water or Dcontrol-feed) being greater than 0 when Dcontrol-water or Dcontrol-feed > 0 or lower than 0 when Dcontrol-water or Dcontrol-feed < 0. D = Mean of the difference control *vs* water or control *vs* feed (median of the marginal posterior distribution of the difference between the control group and the water group or feed group). Statistical differences were assumed if | Dcontrol-water | or | Dcontrol-feed | surpass R value and its P0>0.90.

3.1.3.4.2 Changes in caecal metabolome

The caecal metabolome included 37 samples, namely 12 individuals in the water group, 13 individuals in the feed group and 12 individuals in the control group, obtained after 24 hours of phage application at weeks 4, 5 and 6 of the chickens' growth phase. First of all, an untargeted LC–HRMS-based metabolomics pipeline was used to analyse the

metabolic regulation in phage-treated chickens (water and feed-treated). In this way, a total of 717 peaks were retained. This data is available at the NIH Common Fund's National Metabolomics Data Repository (**NMDR**) website, the Metabolomics Workbench, https://www.metabolomicsworkbench.org where it has been assigned DataTrack ID 3454.

A PLS-DA with ALR transformed variables were used to evaluate the effect of the administration route (drinking water and feed groups) in the caecal metabolome variations of *Salmonella*-free adult broilers. Overall, the analysis identified 70 relevant variables (metabolites) in the final model: 64 for water compared with the control group (final PLS-DA model classification performance: water=86.90 % and control=84.15 %, **Figure 24A**), and 14 for feed compared with the control group (final PLS-DA model classification performance: feed=97.24 % and control=93.92 %, **Figure 24B**). Notably, only 8 metabolites were common to both administration routes. The results showed, thus, that after phage administration, regardless of the administration route, some metabolites (64 and 14 from 717 metabolites identified for water and feed groups, respectively) were the most potential to discriminate the effect of the phage administration.

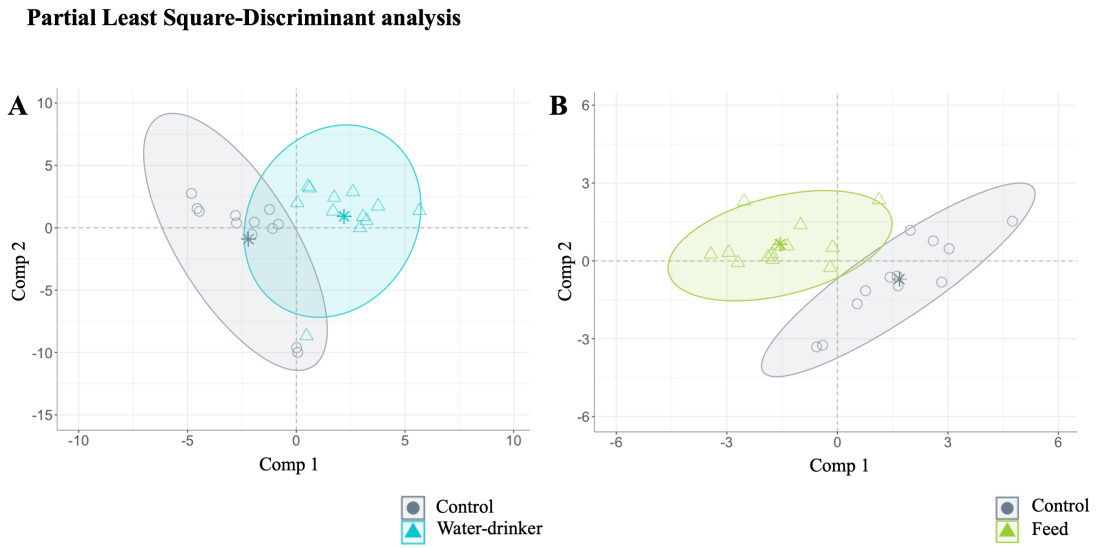

Figure 24. The caecal metabolome features characterised for *Salmonella*-free broilers treated with a *Salmonella* phage. Caecal metabolome composition dissimilarity through the representation of the first (Comp 1) and second components (Comp 2) of the final partial least square-discriminant analysis (PLS-DA) models (A) from the water *vs* control groups, and (B) from the feed *vs* control groups. The water group (blue) received a 10^8 PFU/mL phage concentration *via* drinking water. The feed group (green) received a 10^8 PFU/g phage concentration *via* feed (encapsulated). The control (grey) group did not receive a phage.

We further verified the relevant metabolites identified by PLS-DA and Bayesian statistical analysis, with showed that 27 variables from the initial 64 identified for water compared with the control group and 14 from the initial 14 identified for feed compared with the control group by PLS-DA analysis (**Supplementary Table 2**) had a posterior mean of the differences of at least 0.5 of the SD of the variable, in which the probability of differences being higher or lower than 0 (P0) was higher than 0.90.

For the water group, 16 of the 27 significant metabolites were down-regulated and 11 were up-regulated compared to the control group. Of these, 14 could be tentatively identified. The structures of the identified metabolites included organic acids and derivates (6), organic oxygen compounds (3), phenylpropanoids and polyketides (2), and Benzenoids (2), and organoheterocyclic compounds (1) (**Table 4**). For the feed group, 5 of the 14 significant metabolites were down-regulated and 9 were up-regulated compared to the control group. Of these, 6 could be tentatively identified. The structures of the identified metabolites included lipids and lipid-like molecules (2), organic oxygen compounds (2), organic acids and derivates (1), and phenylpropanoids and polyketides (1) (**Table 4**).

Table 4. Caecal metabolome features characterised for *Salmonella*-free broilers treated with a *Salmonella* phage. Key metabolites identified by partial least square-discriminant analysis (PLS-DA) for discriminating between groups with relevant differences in mean abundance based on Bayesian statistical analysis in water- and feed-phage treated chickens compared with the control group, computed as control *vs* water and control *vs* feed. The water group received a 10^8 PFU/mL phage concentration *via* drinking water. The feed group received a 10^8 PFU/g phage concentration *via* feed (encapsulated). The control group did not receive a phage.

Experimental groups	Super class	Class	Subclass	Name	HPD95	P0	D
Control *vs* Water	Organic oxygen compounds	Organooxygen compounds	Carbohydrates and carbohydrate conjugates	Dimboa glucoside	[-1.57,0.02]	96.95	-0.76
			Carbonyl compounds	Stearyl monoglyceridyl citrate	[-0.12,1.52]	95.24	0.70
				2-Hydroxy-4-methoxyacetophenone 5-sulphate	[-0.16,1.47]	93.64	0.63
	Organic acids and derivates	Peptidomimetics	Hybrid peptides	L-beta-aspartyl-L-alanine	[-0.06,1.59]	96.22	0.73
		Carboxylic acids and derivates	Amino acids, peptides and analogues	Tolmetin glucuronide	[-0.08,1.55]	96.13	0.73
				Hydroxyphenylacetylglycine	[-0.2,1.45]	93.54	0.63
				Nicotinamide Adenine Dinucleotide Phosphate	[-1.36,0.31]	90.70	-0.55
			Carboxylic acid derivates	N-Acetylcadaverine	[-0.04,1.55]	97.30	0.78
	Lipids and lipid-like molecules	Prenol lipids	Monoterpenoids	Monomenthyl succinate	[-0.2,1.47]	93.64	0.64
		Fatty Acyls	Eicosanoid	PGA3	[-1.55,0.1]	95.40	-0.70
	Benzenoids	Phenols	Phenol ethers	Dictagymnin	[-0.12,1.55]	95.71	0.72
		Benzene and substituted derivates	Benzene and substituted derivates	4-Methyl-1-phenyl-2-pentanone	[-0.13,1.53]	95.85	0.73
	Phenylpropanoids and polyketides	Cinnamic acids and derivatives	Hydroxycinnamic acids and derivatives	(R)-2-Feruloyl-1-(4-Hydroxyphenyl)-1,2-ethanediol	[-1.68,-0.15]	98.93	-0.92
		Kavalactones	Kavalactones	5,6-Dihydro-11-methoxyyangonin	[-1.42,0.26]	90.94	-0.56
	Non-identified metabolite 150				[-1.48,0.19]	93.99	-0.66
	Non-identified metabolite 152				[-1.44,0.22]	93.34	-0.63
	Non-identified metabolite 203				[-1.68,-0.09]	98.67	-0.89
	Non-identified metabolite 213				[-1.57,0.02]	97.29	-0.77
	Non-identified metabolite 233				[-1.57,0]	97.59	-0.79
	Non-identified metabolite 273				[-1.94,-0.57]	99.96	-1.25
	Non-identified metabolite 326				[-0.03,1.62]	97.07	0.79
	Non-identified metabolite 400				[0.21,1.78]	99.35	1.00
	Non-identified metabolite 566				[-0.3,1.37]	91.27	0.57
	Non-identified metabolite 568				[-0.08,1.56]	95.68	0.71
	Non-identified metabolite 572				[-0.09,1.56]	96.34	0.76
	Non-identified metabolite 573				[-0.3,1.35]	90.15	0.54
	Non-identified metabolite 678				[-0.04,1.6]	97.16	0.80
Control vs Feed	Organic oxygen compounds	Organooxygen compounds	Carbohydrates and carbohydrate conjugates	Dimboa glucoside	[-1.47,0.1]	96.08	-0.70
			Carbonyl compounds	4-(2-Aminophenyl)-2,4-dioxobutanoic acid	[-1.44,0.16]	93.74	-0.63
	Organic acids and derivates	Carboxylic acids and derivates	Amino acids, peptides and analogues	L-Agaritine	[0.17,1.71]	99.23	0.96
		Steroids and steroid derivates	Sulphated steroids	Androsterone sulphate	[-1.54,0.04]	96.57	-0.74

Lipids and lipid-like molecules	Prenol lipids	Quinone and hydroquinone lipid	7C-aglycone	[-1.48,0.12]	95.20	-0.68
Phenylpropanoids and polyketides	Cinnamic acids and derivatives	Hydroxycinnamic acids and derivatives	(R)-2-Feruloyl-1-(4-Hydroxyphenyl)-1,2-ethanediol	[-1.65,-0.12]	98.86	-0.90
Non-identified metabolite 154				[0.59,1.9]	99.97	1.24
Non-identified metabolite 203				[-1.5,0.05]	96.43	-0.71
Non-identified metabolite 213				[-1.61,-0.06]	98.63	-0.88
Non-identified metabolite 233				[-1.64,-0.11]	98.85	-0.89
Non-identified metabolite 273				[-2.04,-0.7]	99.98	-1.36
Non-identified metabolite 400				[-0.12,1.41]	94.89	0.63
Non-identified metabolite 420				[-0.04,1.54]	96.65	0.75
Non-identified metabolite 557				[-0.16,1.46]	94.79	0.66

HPD95%= The highest posterior density region at 95% of probability. P0= Probability of the difference (Dcontrol-water or Dcontrol-feed) being greater than 0 when Dcontrol-water or Dcontrol-feed > 0 or lower than 0 when Dcontrol-water or Dcontrol-feed < 0. D = Mean of the control *vs* water or control *vs* feed difference (median of the marginal posterior distribution of the difference between the control group and the water group or feed group). Statistical differences were assumed if | Dcontrol-water | or | Dcontrol-feed | surpass R value and its P0>0.90.

3.1.3.5 Discussion

Bacteriophages are considered as a potent biocontrol agent to control zoonotic bacteria replacing antibiotics in poultry production (Wernicki et al., 2017; Zbikowska et al., 2020; D'Angelantonio et al., 2021; Clavijo et al., 2022; Zhao et al., 2022). In fact, the US food safety and inspection service allows the use of *Salmonella*-specific phage against bacterial contamination in live poultry before processing (Huff et al., 2002; Zhang et al., 2019). Nevertheless, although several challenge experiments in poultry have revealed the efficacy of phage therapy to control enteric pathogens (Carvalho et al., 2010; Nabil et al., 2018; Sevilla-Navarro et al., 2018; Clavijo et al., 2019; Richards et al., 2019; Huang et al., 2022), few studies have evaluated the role of bacteriophage exposure in animals and the possible effects on gut physiology (Tetz and Tetz, 2016; Tetz et al., 2017; Hsu et al., 2019; Zhao et al., 2022). Gut microbiota has been recognised as a hidden 'metabolic organ', with a great impact on host biological functions with long-term physiological effect (Robinson et al., 2022).

In this respective, the results of this study showed that the application of phages did not modulate the caecal microbiota beta diversity in target bacteria-free chickens. This fact is expected based on the nature of the bacteriophages, as a virus that has one-to-one correspondence with specific bacteria (Loc-Carrillo and Abedon, 2011). Notably, when phage was administered in the drinking water, microbial alpha diversity was altered. Overall, we detected an increase in the richness and diversity of caecal microbiota, indicating that the total number of bacterial species increased after phage treatment compared to the baseline pre-treatment data. Previous authors also reported an increase in the Shannon index after the *Salmonella*-phage application on animals free of the target bacteria (Tetz et al., 2017; Zhao et al., 2022). Zhao *et al.* (2022) showed that its application during the establishing and development of the intestinal microbiota in the first stages of the chicken production cycle leads to the greatest effect of the phage. Nevertheless, our results displayed that these changes could also take place in the later stages. The differences between the two groups may be because encapsulation and delivery methods delay the phage effect (Colom et al., 2017), and microbiota has been considered temporally stable with a dynamic equilibrium, with alterations that have complex and difficult to predict responses and consequences (Tetz et al., 2017).

Nevertheless, we found a minimal modification in the relative abundance of some microorganisms, regardless of the phage administration route. This result was consistent with previous studies (Tetz et al., 2017; Hsu et al., 2019; Zhao et al., 2022). Phage predation on non-targeted species has been reported previously. Different theories seek to shed light on these phenomena, such as the molecular changes, as single amino acid substitutions and unusual homologous intragenomic recombination that could promote the viral host jump and the diversification of the phage-host spectrum (de Sordi et al., 2017). Notably, the abundance of *Streptococcus* and *Sellimonas* was higher when phages were applied *via* drinking water. *Streptococcus* is a common microorganism found in the gastrointestinal tract of poultry (Yadav and Jha, 2019). However, higher abundance of this genus may not be of interest, as it could cause diseases in broilers and has been negatively correlated with body weight (Thibodeau et al., 2015; Lundberg et al., 2021). Conversely, a higher abundance of *Sellimonas* has been reported to be involved in recovered intestinal homeostasis after dysbiosis events (Muñoz et al., 2020). The abundance rates of *Lactobacillus*, *Anaeroplasma* and *Clostridia_vadinBB60_group* were higher when phages were included in the feed. The *Lactobacillus* genus is part of the group of commensal bacteria that function on vitamin production and antibacterial properties (Wang et al., 2014; Rodrigues et al., 2020). A higher relative abundance of *Anaeroplasma* was also reported in broilers after essential oil administration (Chen et al., 2020), and it has been reported to be positively corrected with the digestibility in other livestock production animals (Zhong et al., 2021). Finally, we also found higher representativeness of *Clostridia_vadinBB60_group*, which is considered one of the most dominant microorganisms in the caecum, with an essential role in carbohydrate fermentation and short-chain fatty acid production (Memon et al., 2022). However, we observed that these few altered genera significantly impact the caecum metabolome, with the most significant effect when phage was administered in the drinking water, and particularly affecting lipid metabolism and organic oxygen compounds notably. Overall, this result is consistent with previous studies on altered metabolites after gastrointestinal therapy, such as lipids and lipid-like molecules, organic acids and organoheterocyclic compounds (Li et al., 2018; Chen et al., 2020; Tang et al., 2021; Wu et al., 2021). Indeed, phage predation could knock down associated metabolic products (Hsu et al., 2019). In this sense, Han *et al.* (2022) showed the metabolic changes occurring in *Klebsiella pneumoniae* following phage application, by reporting alterations in the metabolism of amino acids and nucleotides, which are essential for phage genome replication and

completion of its infection cycle (Han et al., 2022). In our study, the differences observed between the water and the feed group may be due to the timing of the infective cycle of the phage due to phage arrival time and bacterial stress (Han et al., 2022).

The relationship of gut microbiota distortion with the individual's metabolic state was reported by previous authors, who showed how alterations of the gut microbiota in mice by phage administration affected the host gut metabolic phenotype (Hsu et al., 2019). For example, the microbiota is responsible for transforming complex carbohydrates from the feed into products such as lactate, pyruvate or succinate and short-chain fatty acids; or degrading proteins, leading to the production of amino acids, branched-chain fatty acids, amines and harmful phenolic compounds, among others (Aldars-García et al., 2021). Moreover, the gastrointestinal microbiota could modify host-derived metabolites (such as bile acids or cholesterol) or synthesise de novo metabolites (Aldars-García et al., 2021). In this sense, certain bacteria of the phyla *Bacteroidetes* and *Firmicutes* have been related to using amino acid to form short-chain fatty acids (Kumar et al., 2019). Thus, the changes observed in genera from these phyla could be related to these altered metabolites. Considering that gut metabolites could not only impact the balance of intestinal microecology but could also regulate anatomically distant biological systems from the gut *via* the bloodstream (Lu et al., 2021; Tomasova et al., 2021), it will be important to shed light and better investigate on all the changes that are taking place.

3.1.3.6 Conclusions

Our study shows that preventive therapy with bacteriophages minimally alters the intestinal microbiota but significantly impacts their metabolites, regardless of the route of administration. Further studies are needed to understand the potential interplay between differentially abundant bacterial species and significantly altered metabolites to clarify phage treatment implications.

3.1.3.7 Supplementary material

Supplementary Table 1. Caecal microbiota features characterised for *Salmonella*-free broilers treated with a *Salmonella* phage. Bayesian statistical analysis of the relevant genera identified by partial least square-discriminant analysis (PLS-DA) in water- and feed-phage treated chickens compared with the control group, computed as control *vs* water and control *vs* feed. The water group received a 10^8 PFU/mL phage concentration

via drinking water. The feed group received a 10^8 PFU/g phage concentration *via* feed (encapsulated). The control group did not receive a phage.

Supplementary Table 2. Caecal microbiota features characterised for *Salmonella*-free broilers treated with a *Salmonella* phage. Bayesian statistical analysis of the relevant metabolite identified by partial least square-discriminant analysis (PLS-DA) in water- and feed-phage treated chickens compared with the control group, computed as control *vs* water and control *vs* feed. The water group received a 10^8 PFU/mL phage concentration *via* drinking water. The feed group received a 10^8 PFU/g phage concentration *via* feed (encapsulated). The control group did not receive a phage.

Supplementary Table 1. Caecal microbiota features characterised for *Salmonella*-free broilers treated with a *Salmonella* phage. Bayesian statistical analysis of the relevant genera identified by by partial least square-discriminant analysis (PLS-DA) in water- and feed-phage treated chickens compared with the control group, computed as control *vs* water and control *vs* feed. The water group received a 108 PFU/mL phage concentration *via* drinking water. The feed group received a 108 PFU/g phage concentration *via* feed (encapsulated). The control group did not receive a phage.

Experimental groups	Family	Genera	HPD95	P0	D
Control *vs* Water	*Streptococcaceae*	*Streptococcus*	[-1.34,0.14]	94.97	-0.62
	Lachnospiraceae	*Sellimonas*	[-1.34,0.12]	94.73	-0.60
Control *vs* Feed	*Ruminococcaceae*	*Faecalibacterium*	[-0.52,1.01]	72.41	0.23
		Ruminococcus	[-0.55,0.99]	71.52	0.22
		Incertae_Sedis	[-0.95,0.58]	67.74	-017
		Ruminococcus_torques_group	[-1,0.51]	74.63	-0.25
		Ruminococcus_gauvreauii_group	[-1.04,0.48]	75.57	-0.26
	Lactobacillaceae	*Lactobacillus*	[-1.32,0.18]	93.39	-0.57
	Butyricicoccaceae	*Butyricicoccus*	[-0.67,0.88]	58.95	0.09
	Acholeplasmataceae	*Anaeroplasma*	[-1.62,-0.17]	99.11	-0.89
	Clostridia_vadinBB60_group	*Clostridia_vadinBB60_group*	[-1.45,0.03]	96.87	-0.70

HPD95%= The highest posterior density region at 95% of probability. P0= Probability of the difference (Dcontrol-water or Dcontrol-feed) being greater than 0 when Dcontrol-water or Dcontrol-feed > 0 or lower than 0 when Dcontrol-water or Dcontrol-feed < 0. D = Mean of the difference control *vs* water or control *vs* feed (median of the marginal posterior distribution of the difference between the control group and the water group or feed group). Statistical differences were assumed if | Dcontrol-water | or | Dcontrol-feed | surpass R value and its P0>0.90.

Supplementary Table 2. Caecal microbiota features characterised for *Salmonella*-free broilers treated with a *Salmonella* phage. Bayesian statistical analysis of the relevant metabolite identified by partial least square-discriminant analysis (PLS-DA) in water- and feed-phage treated chickens compared with the control group, computed as control *vs* water and control *vs* feed. The water group received a 108 PFU/mL phage concentration *via* drinking water. The feed group received a 108 PFU/g phage concentration *via* feed (encapsulated). The control group did not receive a phage.

Experimental groups	Super class	Class	Subclass	Name	Formula	ION	HPD95	P0	D
Control *vs* Water	Organic oxygen compounds	Organooxygen compounds	Carbohydrates and carbohydrate conjugates	7-Hydroxy-2-Methyl-4-oxo-4H-1-benzopyran-5-carboxylic acid 7-glucoside	C17H18O10	[M+F]-	[-1.22,0.48]	81.61	-0.38
				Dimboa glucoside	C15H19NO10	[M+F]-	[-1.57,0.02]	96.95	-0.76
				D-Glucono-1,5-lactone 6-phosphate	C15H14O4	[M-H]-	[-0.89,0.83]	55.43	-0.06
				Ribose-1-arsenate	C5H11AsO8	[M-H]-	[-1.15,0.55]	76.07	-0.30
				Chavicol O-beta-glucopyranoside	C15H20O6	[M-H]-	[-0.95,0.72]	60.60	-0.11
			Carbonyl compounds	Stearyl monoglyceridyl citrate	C28H54O12	[M+Na-2H]-	[-0.12,1.52]	95.24	0.70
				2-Hydroxy-4-methoxyacetophenone 5-sulfate	C9H10O7S	[M-H]-	[-0.16,1.47]	93.64	0.63
	Organic acids and derivates	Hydroxy acids and derivatives	Beta hydroxy acids and derivatives	Diethyl L-malate	C8H14O5	[M+H]+	[-0.45,1.24]	82.13	0.39
		Peptidomimetics	Hybrid peptides	L-beta-aspartyl-L-alanine	C7H12N2O5	[M+2Na-H]+	[-0.06,1.59]	96.22	0.73
				Cysteinyl-Hydroxyproline	C8H14N2O4S	[M+K]+	[-0.8,0.91]	55.54	0.07
		Carboxylic acids and derivates	Amino acids, peptides and analogues	Tolmentin glucuronide	C21H23NO9	[M+H-2H2O]+	[-0.08,1.55]	96.13	0.73
				Hydroxyphenylacetylglycine	C10H11NO4	[M-H2O-H]-	[-0.2,1.45]	93.54	0.63
				Nicotinamide Adenine Dinucleotide Phosphate	C21H28N7O17P3	[M+2H+Na]3+	[-1.36,0.31]	90.70	-0.55
				Pentosidine	C17H26N6O4	[M+CH3COO]-	[-0.52,1.16]	77.44	0.32
				DL-o-Tyrosine	C9H11NO3	[M+H]+	[-0.63,1.08]	70.11	0.23
				DL-Methionine sulfoxide	C5H11NO3S	[M+FA-H]-	[-0.53,1.14]	74.96	0.28
				Tauropine	C5H11NO5S	[M-H]-	[-0.44,1.24]	84.61	0.42
				D-Glutamate	C5H9NO4	[M+H]+	[-0.99,0.71]	63.47	-0.15

Lipids and lipid-like molecules	Prenol lipids	Carboxylic acid derivates	2-Aminoheptanoate	C7H15NO2	[M+ACN+H]+	[-0.38,1.3]	85.70	0.45
			N-Acetylcadaverine	C7H16N2O	[M+H]+	[-0.04,1.55]	97.30	0.78
			N-(3,4-Dichlorophenyl)-malonamate	C9H7Cl2NO3	[M+NH4]+	[-1.16,0.54]	77.44	-0.32
		Oxo carboxylic acid	4-Oxododecanedioic acid	C12H20O5	[M-H]-	[-1.37,0.33]	88.97	-0.52
		Quinone and hydroquinone lipid	7C-aglycone	C18H18O4	[M-H]-	[-1.36,0.3]	89.39	-0.51
		Monoterpenoids	Monomenthyl succinate	C14H24O4	[M+FA-H]-	[-0.2,1.47]	93.64	0.64
	Fatty Acyls	Eicosanoid	PGA3	C20H28O4	[M-H]-	[-1.55,0.1]	95.40	-0.70
		Fatty acids and conjugates	2-Hydroxyoctadecanoic acid	C18H36O3	[M-H]-	[-0.78,0.94]	58.20	0.09
Lignans, neolignans and related compounds	Furanoid lignans	Tetrahydrofuran lignans	Enterolactone	C18H18O4	[M+FA-H]-	[-1.2,0.48]	81.33	-0.37
Benzenoids	Phenols	Methoxyphenols	Vanylglycol	C18H36O4Si3	[M-H]-	[-0.77,0.94]	57.77	0.09
		Phenol ethers	Dictagymnin	C14H18O	[M+H-H2O]+	[-0.13,1.53]	95.85	0.73
	Benzene and substituited derivates	Benzene and substituited derivates	4-Methyl-1-phenyl-2-pentanone	C12H16O	[M+H-H2O]+	[-0.12,1.55]	95.71	0.72
Phenylpropanoids and polyketides	Isoflavonoids	Isoflavans	3'-Hydroxyequol	C15H14O4	[M-H]-	[-0.5,1.2]	79.78	0.36
		O-methylated isoflavonoids	Isosativan	C17H18O4	[M-H]-	[-1.17,0.5]	78.46	-0.33
	Cinnamic acids and derivatives	Hydroxycinnamic acids and derivatives	(R)-2-Feruloyl-1-(4-Hydroxyphenyl)-1,2-ethanediol	C18H18O6	[M-H]-	[-1.68,-0.15]	98.93	-0.92
			Avenanthramide A2	C18H19NO7	[M-H]-	[-1.18,0.49]	80.39	-0.35
	Phenylpropanoids and polyketides	Phenylpropanoids acids	3-(3,5-Diiodo-4-hydroxyphenyl)lactate	C9H8I2O4	[M+FA-H]-	[-1.2,0.51]	80.03	-0.35
	Kavalactones	Kavalactones	5,6-Dihydro-11-methoxyyangonin	C16H18O5	[M-H2O-H]-	[-1.42,0.26]	90.94	-0.56
Non-identified metabolite 150						[-1.48,0.19]	93.99	-0.66
Non-identified metabolite 152						[-1.44,0.22]	93.34	-0.63
Non-identified metabolite 203						[-1.68,-0.09]	98.67	-0.89
Non-identified metabolite 213						[-1.57,0.02]	97.29	-0.77

Non-identified metabolite 232	[-0.81,0.94]	58.55	0.09
Non-identified metabolite 233	[-1.57,0]	97.59	-0.79
Non-identified metabolite 244	[-0.59,1.15]	75.66	0.30
Non-identified metabolite 273	[-1.94,-0.57]	99.96	-1.25
Non-identified metabolite 277	[-1.04,0.64]	67.04	-0.19
Non-identified metabolite 326	[-0.03,1.62]	97.07	0.79
Non-identified metabolite 400	[0.21,1.78]	99.35	1.00
Non-identified metabolite 514	[-0.55,1.16]	77.81	0.33
Non-identified metabolite 566	[-0.3,1.37]	91.27	0.57
Non-identified metabolite 568	[-0.08,1.56]	95.68	0.71
Non-identified metabolite 571	[-0.35,1.33]	88.98	0.51
Non-identified metabolite 572	[-0.09,1.56]	96.34	0.76
Non-identified metabolite 573	[-0.3,1.35]	90.15	0.54
Non-identified metabolite 595	[-0.74,0.96]	58.66	0.10
Non-identified metabolite 637	[-1.31,0.38]	85.93	-0.46
Non-identified metabolite 644	[-1.23,0.46]	82.57	-0.39
Non-identified metabolite 647	[-1.3,0.34]	87.08	-0.47
Non-identified metabolite 648	[-1.28,0.39]	86.12	-0.46
Non-identified metabolite 669	[-0.86,0.88]	51.02	0.02
Non-identified metabolite 678	[-0.04,1.6]	97.16	0.80
Non-identified metabolite 683	[-0.56,1.16]	75.82	0.30
Non-identified metabolite 698	[-0.62,1.08]	70.59	0.23
Non-identified metabolite 705	[-1.03,0.67]	69.12	-0.21
Non-identified metabolite 712	[-1.19,0.49]	79.47	-0.34

Control *vs* Feed	Organic oxygen compounds	Organooxygen compounds	Carbohydrates and carbohydrate conjugates	Dimboa glucoside	C15H19NO10	[M+F]-	[-1.47,0.1]	96.08	-0.70
			Carbonyl compounds	4-(2-Aminophenyl)-2,4-dioxobutanoic acid	C10H9NO4	[M+H]+	[-1.44,0.16]	93.74	-0.63
	Organic acids and derivates	Carboxylic acids and derivates	Amino acids, peptides and analogues	L-Agaritine	C12H17N3O4	[M+K-2H]-	[0.17,1.71]	99.23	0.96
	Lipids and lipid-like molecules	Steroids and steroid derivates	Sulfated steroids	Androsterone sulfate	C19H30O5S	[M+CH3COO]-	[-1.54,0.04]	96.57	-0.74
		Prenol lipids	Quinone and hydroquinone lipid	7C-aglycone	C18H18O4	[M-H]-	[-1.48,0.12]	95.20	-0.68
	Phenylpropanoids and polykeides	Cinnamic acids and derivatives	Hydroxycinnamic acids and derivatives	(R)-2-Feruloyl-1-(4-Hydroxyphenyl)-1,2-ethanediol	C18H18O6	[M-H]-	[-1.65,-0.12]	98.86	-0.90
	Non-identified metabolite 154						[0.59,1.9]	99.97	1.24
	Non-identified metabolite 203						[-1.5,0.05]	96.43	-0.71
	Non-identified metabolite 213						[-1.61,-0.06]	98.63	-0.88
	Non-identified metabolite 233						[-1.64,-0.11]	98.85	-0.89
	Non-identified metabolite 273						[-2.04,-0.7]	99.98	-1.36
	Non-identified metabolite 400						[-0.12,1.41]	94.89	0.63
	Non-identified metabolite 420						[-0.04,1.54]	96.65	0.75
	Non-identified metabolite 557						[-0.16,1.46]	94.79	0.66

HPD95%= The highest posterior density region at 95% of probability. P0= Probability of the difference (Dcontrol-water or Dcontrol-feed) being greater than 0 when Dcontrol-water or Dcontrol-feed > 0 or lower than 0 when Dcontrol-water or Dcontrol-feed < 0. D = Mean of the difference control *vs* water or control *vs* feed (median of the marginal posterior distribution of the difference between the control group and the water group or feed group). Statistical differences were assumed if | Dcontrol-water | or | Dcontrol-feed | surpass R value and its P0>0.90.

3.1.3.8 References

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Part II

3.2 Bacteriophage dynamics in *Salmonella*-infected broilers and its influence on microbiota and metabolome

3.2.1 Microencapsulated Bacteriophages Incorporated in Feed for *Salmonella* Control in Broilers

An adapted version of this chapter has been published with the reference:

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3.2.1.1 Abstract

Bacteriophage inclusion as a feed additive could offer the prospect of its *en masse* application and reduce the intestinal carriage of *Salmonella* by broiler chickens. The objective of this study was to evaluate the application of microencapsulated phages as a strategy to control *Salmonella* and assess the impact of their use during the broiler rearing period. One-hundred one-day-old chicks were randomly divided in two identical poultry houses according to the two experimental groups (control *vs* Φ-treated group) simulating field production conditions. Half of the animals in each experimental group, were challenged with 10^5 CFU/bird of *Salmonella* Enteritidis *per os*. In the Φ-treated group, microencapsulated *Salmonella*-phage encapsulated in Eudragit®L100 (a pH-responsive formulation) were incorporated in the starter diet feed. To assess *Salmonella* colonization, excretion and diffusion, cecum samples, cloacal swabs and boot swabs were taken weekly. *Salmonella* detection was based on ISO 6579-1:2017 (Annex D). *Salmonella* colonization was significantly reduced in most of the rearing period, meanwhile the excretion was significantly reduced on the 2^{nd}, 4^{th} and 5^{th} week of rearing. Moreover, *Salmonella* contamination of the farm environment was eliminated at the end of the cycle. This study provides important insights into the potential use of phages as a preventative and biocontrol strategy against *Salmonella* infection from farm-to-table.

3.2.1.2 Introduction

Salmonella spp. cause foodborne illness worldwide representing the second most commonly reported zoonotic pathogen in the EU (EFSA and ECDC, 2021). *S*. Enteritidis is the most common serovar causing food borne outbreaks, representing a substantial problem for public health (EFSA and ECDC, 2021; Li et al., 2021). Furthermore, quantitative risk studies have predicted that up to 25 % of *Salmonella* human outbreaks, illnesses and hospitalization arise from poultry sources (Chai et al., 2017).

Young poultry are particularly susceptible to *Salmonella* colonization as it could survive the relatively mild acidic pH of the GIT in young birds and colonize the poultry gut; excretion thereafter in faeces results in contamination of the environment and infection of other birds (Cosby et al., 2015). Moreover, the horizontal transmission of the bacteria from seeder chicks to contact chicks through the litter represents a potential route of transmission (Cosby et al., 2015; El-Shall et al., 2020). Poultry meat contaminated with digesta during the slaughter process is a key in the risk to public health (Alali and Hofacre, 2016). Thus, on-farm interventions should be improved to control the spread of the infection among the chickens (Ruvalcaba-Gómez et al., 2022).

In the EU, the NSCP have been implemented in accordance with EU legislation targeting *S.* Enteritidis and *S*. Typhimurium (EC, 2007). In addition, to improve the established on-farm biosecurity measures, innovative preharvest strategies such as bacteriophages (phages) could be beneficial to minimize *Salmonella* infection in poultry production (Alali and Hofacre, 2016; Ruvalcaba-Gómez et al., 2022). Phages are viruses that specifically infect bacterial cells. Phages are high specific, self-replicating, self-limiting, well tolerated, and accessible from multiple sources (Yin et al., 2021; Ruvalcaba-Gómez et al., 2022). The use of *Salmonella* specific phages to reduce the intestinal carriage of *Salmonella* by broiler chickens showed promising results in controlled trials (Borie et al., 2008; Ahmadi et al., 2016; Nabil et al., 2018) and in poultry house applications (Sevilla-Navarro et al., 2018; Clavijo et al., 2019). Its inclusion as a feed additive offers the prospect of its *en masse* application and a possible solution to control the pathogenic microbial populations (Kim et al., 2013; Upadhaya et al., 2021; Lorenzo-Rebenaque et al., 2022). The current problem is the loss of its antibacterial activity due to its susceptibility to environmental stresses such as the gastric acid, digestive enzymes and bile salts encountered *in vivo* during the treatment of chicken intestinal diseases (Colom

et al., 2017; Yin et al., 2021). Moreover, their stability during food storage could compromise and limit its use in animal production at field level (Yin et al., 2021). These problems could be successfully addressed through microencapsulation. A variety of phage encapsulation materials are available including carbohydrates, lipids, proteins as well as synthetic and biopolymers that could protect the phages from environmental and processing stresses (Yin et al., 2021). Protection of phages against the harsh GIT environment whilst enabling burst or and slow continuous release strategies may improve phage bioavailability and therapy outcomes (Yin et al., 2021).

In this context, the objective of this study was to evaluate the application of microencapsulated phages delivered in animal feed during the six week broiler production cycle as a strategy to control *Salmonella* in the animals and in the environment.

3.2.1.3 Material and methods

In this trial, the handling of experimental animals was approved by the Ethical Review Panel of the Directorate-General for Agriculture, Fisheries and Livestock from the Valencian Community, by the code 2021/VSC/PEA/0003, according to Spanish Royal Decree 53/2013 (Spain, 2013).

3.2.1.3.1 Bacterial Strain

The *S.* Enteritidis strain was selected from a database of *Salmonella* strains (identification code 1712791) isolated from the *Salmonella* National Control Program (CAECAV, in its Spanish acronym Centro de Calidad Avícola y Alimentación Animal de la Comunidad Valenciana, Castellón, Spain).

3.2.1.3.2 Bacteriophage encapsulation

Salmonella phage FGS011 used in this study was isolated by Sevilla-Navarro et al. (2020) and characterized by Lorenzo-Rebenaque et al., (2021). The phage FGS011 was micro-encapsulated with the polymers Eudragit® L100 (L100). Encapsulation was performed according to Malik (2021) and Lorenzo-Rebenaque et al. (2021).

Commercially available Eudragit® polymer L100 has been specifically designed for enteric delivery applications with the aim of protecting therapeutics from gastric acidity and allowing controlled release of therapeutics using a pH-dependent trigger mechanism.

L100 is a copolymer of methacrylic acid and methyl methacrylate with different amounts of carboxylic acid residues providing differences in pH dissolution characteristics, the ratio of free carboxyl groups to ester groups is 1:1. To dissolve Eudragit® L100, the pH of the water was changed to alkaline (pH 12) *via* addition of 4 M NaOH (Fisher Scientific, Hampshire, UK) to allow polymer dissolution, followed by pH adjustment to pH 7 using 0.1 M HCl prior to addition of trehalose powder (Fisher Scientific, Hampshire, UK), to finally added the phages to the solution. Typically, 10% (v/v) high-titre phage (~10^{10} PFU/mL) was added to the solution, yielding phage titres of ~10^{9} PFU/mL in the final formulation. The phage-containing solutions were spray-dried using a commercially available LabPlant spray-dryer SD-06 (LabPlant, UK Limited), which is a co-current dryer with a pneumatic atomiser and a cylindrical drying chamber of dimensions 215 mm outer diameter and 420 mm height. The diameter of the atomization nozzle used throughout the work was 0.5 mm with the measured feed liquid flow rate at 280 mL·h−1 and a drying gas air flow rate of ~20 L·s−1. The air inlet temperatures were set at 100 ºC resulting in corresponding air outlet temperatures of 60 ± 2 ºC respectively.

3.2.1.3.3 Experiment design

The experimental timeline is summarized in **Figure 25**. One hundred day-old-chicks (Ross®, Aviagen, USA) were placed into 2 groups of 50 animals each. Group 1 (control group) was challenged with *Salmonella* and Group 2 (Φ-treated group) was challenged with *Salmonella* and received feed supplemented with microencapsulated FGS011. Based on the group, chicks were randomly placed into two identical poultry houses of an experimental poultry farm at the Center for Research and Animal Technology (CITA-IVIA, in its Spanish acronym Centro de Investigación y Tecnología Animal, Segorbe, Spain) in a final stocking density of 35 kg/m2. The animals were handled according to common practice in poultry production (Ross, 2018). The houses were supplied with wood shavings as bedding material, programmable electrical lights, automated electric heating and forced ventilation. The environmental temperature was gradually reduced from 32 °C on arrival day to 19 °C at 42 days post hatch.

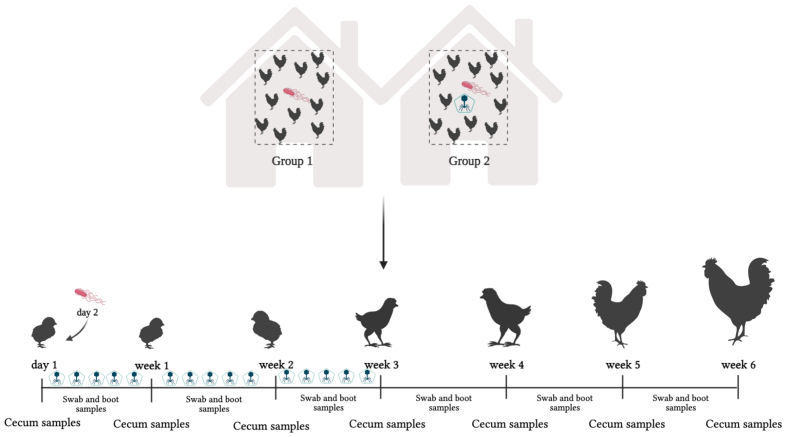

Figure 25. Experimental design throughout the entire production cycle. Group 1 (control group) was challenged with *Salmonella* and Group 2 (Φ-treated group) was challenged with *Salmonella* and received feed supplemented with microencapsulated FGS011. Created with BioRender.com

From hatching day until 21 days of age, chicks were fed with a pelleted starter diet (*Camperbroiler iniciación, Alimentación Animal Nanta, Valencia, Spain*), and from 21 days of age to the slaughter day (42 days of age) the poultry were fed a pelleted grower diet (*A-32 broiler, Alimentación Animal Nanta, Valencia, Spain*). Feed has been pelleted at a 50-65 ºC. Nutritional and product analysis were assessed before the arrival of animals. For each house, body weight was recorded weekly; mortality and the presence of diarrhea were recorded daily.

3.2.1.3.3.1 *Salmonella* infection

Salmonella status of the chicken houses was tested before the arrival of the animals in accordance with ISO/TS 6579-1:2017. In addition, *Salmonella* status of the animals was tested at the day of arrival. For this purpose, samples of meconium (n=25) and delivery box liners (n=4) were collected. Then, to carry out the analysis of samples according to the ISO/TS 6579-1:2017, samples were pre-enriched in 1:10 vol/vol in BPW (Scharlau®, Barcelona, Spain), and were incubated at 37±1°C for 18±2 hours. Then, pre-enriched samples were transferred to MRSV (Difco®, Valencia, Spain), and were incubated at 41.5±1°C for 48 h. Suspicious plates were transferred onto two different agar plates, XLD agar (Liofilchem®, Valencia, Spain), and a selective chromogenic medium (ASAP; bioMerieux®, Marcy l'Étoile, France), and were incubated at 37±1°C for 24±3 hours. A biochemical test (API-20®, bioMerieux, Madrid, Spain) was performed to confirm

Salmonella. Finally, *Salmonella* strains isolated were serotyped using the Kauffman-White scheme (Grimont and Weill, 2007) and stored at -80 ºC for further analysis.

At 24 hours after placing, 50% of chickens of both houses were inoculated *per os* with 100 µL of S. Enteritidis at 10^6 CFU/mL (Amerah et al., 2012; Laptev et al., 2019). The experimental infection was done with 10^5 CFU/bird. To confirm the CFU used to inoculate the seeder chicks, ten-fold dilutions were streaked onto XLD plates and triplicate measurements were taken. The plates were incubated at 37.5°C for 24 h. Once incubated, the colonies were counted and CFU values determined.

3.2.1.3.3.2 Bacteriophage application

Experimental treatment was offered until 21 days of age. To this end, the encapsulated phages in L100 were added to the started diet at a concentration of 1 g/kg. To achieve a homogeneous mixture, an electric paddle mixer was used. After homogenization, samples were taken from various points of the mixed feed to verify that the mixture had been homogeneous. In addition, to assess the phage stability, feed samples were stored, and phage concentration was measured weekly. Finally, the control group were fed in an identical manner except without the addition of phage to the feed. Moreover, the average dose ingested per chicken was calculated by measuring phage numbers in feed samples and the feed intake per chicken.

To this end, triplicate 1 g samples were diluted in 9 mL of Sorensons buffer at pH 7 and mixed thoroughly. After waiting 10 min to achieve decapsulation of the bacteriophage, samples were centrifuged at 8,000 x g and 4 °C for 10 min and filtered through a 0.45 µm filter. Serial dilutions of the samples were prepared in LB. Then, 10 µL of each dilution with 200 µL of the bacterial host suspension was mixed with 5 mL of LB 0.6 % top agar layer and placed over a 1.6 % LB agar bottom layer. To perform the bacterial host suspension, a log-phase culture of the bacterial suspensions in LB, at an optical density (OD) 600 nm of 0.2 (~10^8 CFU/mL) was used. All dilutions were prepared in triplicate, and the plaques were counted after 24 hours of incubation at 37 ºC.

3.2.1.3.3.3 *Salmonella* colonization

To assess *Salmonella* colonization, cecum samples from 4 chickens were collected at weekly intervals throughout the growing period (7,14, 21, 28, and 35 days of age) (Pal et

al., 2021). Finally, at day 42, cecum samples from the remaining animals were also collected. Once in the laboratory, *Salmonella* detection was performed as described previously following ISO/TS 6579-1:2017.

3.2.1.3.3.4 *Salmonella* excretion

In order to study *Salmonella* shedding and dissemination, 30 cloacal swabs and 2 boot swabs were collected per group twice a week throughout the growing period (120 cloacal swabs/per week and 8 boot swabs/per week, respectively) (Borsoi et al., 2011). Once in the laboratory, *Salmonella* detection was assessed following ISO/TS 6579-1:2017.

3.2.1.3.3.5 Organ Weight

To assess the effect of the phage supplementation on organs, the liver and the immune organs (bursa of Fabricius and spleen) were removed and weighed at weekly intervals throughout the growing period (7,14, 21, 28, 35 and 42 days of age). Organ weights were expressed as a relative percentage to the whole body weight (Upadhaya et al., 2021).

3.2.1.3.3.6 Enumeration of bacteriophages

To monitor the number of phages released in the cecum, phage counts were determined from the content of the caecal samples. Briefly, 1 g of caecal content from each chicken was removed, weighed, and diluted 1:10 in LB. The mix was homogenized thoroughly. Moreover, 3 litter samples per group were taken weekly for bacteriophage count, and diluted 1:10 in LB. All samples were centrifuged (8,000 x g and 4 °C for 10 min) and filtered (0.45 μm). Then, serial dilutions were prepared on LB. Finally, 10 μL of each dilution with 200 μL of the bacterial host suspension was mixed with 5 mL of LB 0.6 % top agar layer, and placed over a 1.6 % LB agar bottom layer. All dilutions were prepared per triplicate, and the plaques were evaluated after 24 hours of incubation at 37 ºC.

3.2.1.3.4 Statistical analysis

A GLM, which assumed a binomial distribution for *Salmonella* excretion, and colonization, was fitted to the data to determine the effect of the treatment (control *vs* Φ-treated group) during the rearing period (weeks 1 to 6). As a fixed effect was included the experimental group (control *vs* Φ-treated group). Differences due to phage treatment in weight, and in the relative weight of liver and immune organs (bursa of Fabricius and

spleen) release during the rearing period (weeks 1 to 6) was evaluated using a GLM. Phage concentrations (Log10 CFU/g) in the feed, in the caeca and in the litter were analyzed using the GLM to assess the dynamics of the phage during the rearing period (weeks 1 to 6). Statistical differences were based on a *p-value* level of < 0.05. Statistical analyses were performed using SPSS 27.0 software package (SPSS Inc., Chicago, IL, USA).

3.2.1.4 Results

3.2.1.4.1 Production parameters

Salmonella phages had no detrimental effects on the production performance parameters, without significant differences in the weight of the animals throughout the productive cycle (*p-value* > 0.05). At the end of the cycle, the weight of the chickens was 1997.9±50.4 g for the control group and 1922.7±38.8 g for the Φ-treated group (*p-value* > 0.05). Concerning clinical signs, 28% of the chickens in the control group and 10% of the chickens in the Φ-treated group presented diarrhea, as liquid faeces that sticks to the cloaca area, only between days two and four after *S.* Enteritidis administration. No differences were observed in the mortality rate between the two groups.

3.2.1.4.2 *Salmonella* colonization

At the beginning of the trial, negative *Salmonella* status of the chicken houses and the day-old-chickens was confirmed. The concentration of *S.* Enteritidis used for the animal infection was 3×10^5 CFU/chick. Moreover, all the *Salmonella* strains isolated during this study were serotyped as *S.* Enteritidis. *S.* Enteritidis prevalence in the cecum at different weeks of rearing from broilers is presented in **Table 5**. There were significant differences among groups with respect to *Salmonella* prevalence in caeca samples at different ages during the growing period (*p-value* < 0.05, **Table 5**).

Table 5. Percentage of *S.* Enteritidis positive in the cecum of the chickens for the control and the phage groups throughout the rearing period (% ± standard error).

	Week of rearing					
	1	**2**	**3**	**4**	**5**	**6**
Control	100*	75.0±21.7	100*	25.0±21.7	100*	54.0±9.2*
Φ -Treated	50.0±25.0	75.0±21.7	50.0±25.0	0	25.0±21.7	21.0±7.5

Φ-treated: experimental group of chickens that received feed supplemented with microencapsulated FGS011* Star indicates statistically significant difference between groups within weeks (*p-value* < 0.05).

3.2.1.4.3 *Salmonella* excretion

The results of the *Salmonella* excretion analysis are represented in **Figure 26**. Statistically significant differences were found between groups (control *vs* Φ-treated) in *Salmonella* excretion in weeks 2, 4 and 5.

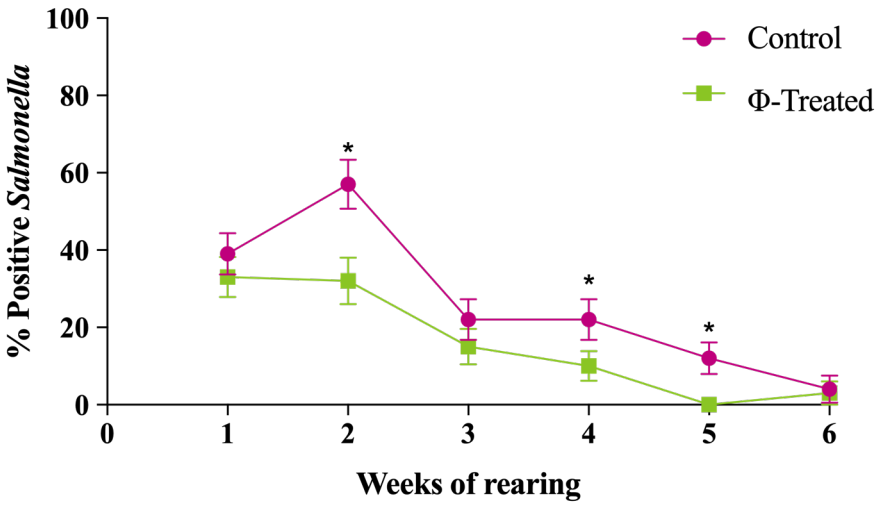

Figure 26. Percentage of *Salmonella*-positive cloacal swabs in the control and Φ-treated groups during the rearing period (weeks). * Star indicates statistically significant difference between groups within weeks (*p-value* < 0.05).

All the boot swabs samples analyzed from the control group were positive during the rearing period. For the phage treated group, boot swabs samples were positive until the fourth week, 50 % (2/4) were negative in the fifth week, and all boot swabs samples were negative for *S.* Enteritidis in the sixth week of rearing.

3.2.1.4.4 Organ Weight

The relative percentage of the weight values obtained in liver and immune organs (bursa of Fabricius and spleen) are presented in **Table 6**. As shown in Table 2, control group presented significantly higher values in the liver at week 1 (4.71±0.19 *vs* 3.22±0.60, for control *vs* Φ-treated, *p-value* < 0.05). In the case of the bursa of Fabricius, significantly higher values were obtained for the Φ-treated group at the end of the rearing period (0.04±0.01 *vs* 0.13±0.03, for control *vs* Φ-treated, *p-value* < 0.05). For the spleen, no differences were obtained among both groups during the cycle (*p-value* > 0.05).

Table 6. Relative percentage at different weeks of rearing of the weight values of the liver and immune organs (bursa of Fabricius and spleen) (weight (%) ± standard error).

		Week of rearing					
		1	2	3	4	5	6
Liver	Control	4.71±0.19*	3.16±0.15	2.61±0.26	2.30±0.11	2.33±0.22	2.12±0.11
	Φ -Treated	3.22±0.60	2.86±0.15	2.46±0.12	2.13±0.13	2.28±0.15	2.11±0.05
Bursa of Fabricious	Control	0.20±0.01	0.23±0.02	0.24±0.04	0.17±0.03	0.13±0.03	0.04±0.01
	Φ -Treated	0.19±0.01	0.26±0.03	0.23±0.03	0.23±0.02	0.21±0.04	0.13±0.03*
Spleen	Control	0.10±0.04	0.09±0.01	0.08±0.01	0.1±0.01	0.12±0.02	0.11±0.02
	Φ -Treated	0.07±0.03	0.08±0.01	0.06±0.01	0.09±0.01	0.11±0.01	0.10±0.02

*** Star indicates statistically significant difference between groups within weeks (*p-value* < 0.05).**

3.2.1.4.5 Enumeration of bacteriophages

The theoretical average dose ingested was 7.9 Log10 PFU/chick. The phage stability in the feed showed no statistically relevant differences in the phage count during storage for 6 weeks (*p-value* <0.05).

The phage counts in caecal contents were determined for each chicken (4 chickens/ group/ week). All broilers (100 %) in Φ-treated group were positive for phages in their caecal contents in weeks 1, 3 and 4. In week 2, 75 % of broilers tested positive for phage isolation and no phage was detected in the caeca in weeks 5 and 6. The phage counts in the caeca are presented in **Table 7.** Moreover, the mean phage titer in litter were showed in **Table 7**.

Table 7. Phage counts (PFU/g) in the caeca and in the litter in Φ-treated group during the rearing period. Data are expressed as mean±standard error of mean.

	Week of rearing					
	1	2	3	4	5	6
Caeca	3.1±1.0 ^{b}	3.1±1.0 ^{b}	4.5±0.2 ^{a}	3.7±0.2 ^{b}	0 ^{c}	0 ^{c}
Litter	4.9±0.5 ^{a}	5.0±0.5 ^{a}	5.5±0.2 ^{a}	4.9±0.1 ^{b}	3.4±0.8 ^{b}	0 ^{c}

^{a,b,c} Different superscripts within each row indicates significant differences between means at *p-value* < 0.05.

3.2.1.5 Discussion

Phage therapy is considered a promising tool to control *Salmonella* in poultry (Ruvalcaba-Gómez et al., 2022), however, finding the most effective application delivery remains a challenge. Previous studies have shown that L100 was able to deliver phages to the target site of *Salmonella* colonization, the caeca, in a simulated gastrointestinal

model and at different ages during the broiler rearing period without bacterial challenge (Lorenzo-Rebenaque et al., 2021, 2022). In the present study, the application of L100 encapsulated phages reduced the *Salmonella* caecal carriage and its excretion at key moments during the rearing period, such as the second, and fifth weeks of rearing (Marin and Lainez, 2009), and removed the bacteria from the environment in the last weeks of the rearing period (Sevilla-Navarro et al., 2018). Thus, the application of L100 as a feed additive in the starter diet had significant implications for the *Salmonella* epidemiology in the broiler rearing cycle, without altering production parameters.

Salmonella is considered one of the major infection agents responsible for foodborne human outbreaks caused mainly by contamination of poultry-derived products (Kosznik-Kwaśnicka et al., 2020). In the EU, the detection of specific serovars of *Salmonella*, such as Enteritidis and Typhimurium, in poultry could lead to the elimination of the whole poultry flock (Kosznik-Kwaśnicka et al., 2020). Consequently, poultry sector demands more practical, foolproof, and cost-effective solutions able to control the *Salmonella* excretion and, therefore, to reduce the infective pressure within the flock (Ruvalcaba-Gómez et al., 2022). It has been shown that the administration of phages in the chickens could inhibit bacterial colonization thereby improving food safety (Alali and Hofacre, 2016; Clavijo et al., 2019). Nevertheless, Bardina et al (2012) highlighted the need for continuous treatment of the animals to achieve a significant reduction in colonised bacteria, especially in young chicks, after phage application. Moreover, the method of administration needs to be practical from a commercial point-of-view for its *en masse* application (Thanki et al., 2021). Thus, implementation of phage therapy using a starter diet could overcome these limitations. Since the viability of orally-administered phages may be rapidly reduced due to the harsh conditions of the gastrointestinal tract (Lorenzo-Rebenaque et al., 2021), the application of microencapsulated phages in the feed may protect phages against environmental stresses resulting in improved delivery of high titres of active phage at the target site (caecum) (Lorenzo-Rebenaque et al., 2022). Our results showed that dietary supplementation with 0.1 (% wt) of L100 in the feed was able to control the *S*. Enteritidis in the batch at the end of the rearing period, a key moment to avoid the entry of *Salmonella* in the slaughterhouse (Machado Junior et al., 2020).

Salmonella excretion prevalence in the control group was significantly higher than in the Φ-treated group on the second and fifth weeks of rearing, two moments that have been described in the literature as important for increases in *Salmonella* excretion (Marin and

Lainez, 2009). At the 2 week period of rearing, the highest *Salmonella* excretion occurs coinciding with changes in the immune system and in the gut microbiota (Marin and Lainez, 2009; Ballou et al., 2016). At 5 weeks of rearing, an increase in *Salmonella* detection in commercial farms has been reported, related with the process of flock thinning (Marin and Lainez, 2009). It has been described that the stress of this practice could induce a gut microbiota disturbance, resulting in higher rates of bacterial shedding (Marin and Lainez, 2009). However, in our study, this was not carried out. Thus, a gradual decrease was observed in the control group until the end of the rearing period; this has previously been noted in published studies (El-Shall et al., 2020). Moreover, when chickens are more susceptible, phage therapy may provide an effective biocontrol mitigation strategy. Despite the fact that *Salmonella* was significantly reduced in the chickens in different moments of the cycle, it must be noted that total elimination of the bacteria was not reached using phages (Adhikari et al., 2017; Nabil et al., 2018). In this respect, previous authors have described its synergistic effect with other alternatives (such as probiotics, prebiotics, symbiotic, etc.), and their use in combination may be important for total *Salmonella* control in the animal, and therefore in the reduction of human outbreaks (Kim et al., 2013; Ruvalcaba-Gómez et al., 2022).

In addition, phage microencapsulation is important to ensure the stable and controlled delivery of treatment in *Salmonella*-infected chickens (Colom et al., 2017). The microencapsulation of the phage in L100 allowed ease of application of the phage and phage stability was maintained during storage. Previous authors highlighted this fact, due to the importance of the impact that storage conditions have on phage stability (Thanki et al., 2021). Moreover, when phage administration was stopped, the phage remained present in caeca and litter until the 4^{th} and the 5^{th} weeks of rearing, respectively. This coincided with bacteria reduction in the cecum of the chickens and its elimination in the litter. Previous authors have suggested that a threshold density of bacteria may be needed for phage amplification or maintenance of high titres of phage in the gut, however, the absence of bacteria in the GIT would lead to phage removal (Colom et al., 2017). Thus, at the end of the animal rearing period, it may be possible to achieve chickens and a farm environment free of *Salmonella*.

Measurement of immune organ weight is a possible method for evaluating the immune status in broilers (Abd El Tawab et al., 2015). There was a significant difference in the mean of the relative weight of bursa of Fabricius at the end of the cycle, with high values

in the Φ-treated group. The obtained results were in line with previous authors who revealed that *Salmonella* challenge could depress the immune organ growth, and the application of antibiotic alternatives, such as symbiotics, could produce significant increases in weight (Abd El Tawab et al., 2015). In poultry, high bursa weight could be related to high immune activity due to it being a major lymphoid organ, with implications for immune cell phenotypes, proliferation, and antibody production (Abd El Tawab et al., 2015). However, this study did not carry out immunological testing of the chickens, which may be helpful to complement the information reported here. On the other hand, the spleen and liver were considered the main filtration organs and major sites of phage accumulation (Dąbrowska, 2019; Van Belleghem et al., 2019). The inclusion of L100 did not increase the relative weight of liver and spleen to the body weight compared with the control group. These results agree with those reported by Li et al. (2020), they did not report significant changes in the liver after phage application, highlighting that phage residues were not harmful to the animal. Wang et al. (2013) by contrast did not find differences in the spleen relative weight but did note that the liver weight increased after phage treatment. These findings suggest the need for further studies to study the effect of phage therapy on chicken host immunity and metabolome after the use of phages for therapeutic purposes (Hsu et al., 2019).

3.2.1.6 Conclusion

In conclusion, the results from this study demonstrate that *S.* Enteritidis flock contamination may be markedly curtailed through L100 encapsulated phage application as a feed additive in the starter diet during rearing. A reduction in *Salmonella* colonization and excretion was noted with complete elimination of bacteria recorded from the environment at the end of the rearing period. However, higher phage doses, improved delivery protocols and/or combination with other strategies may be necessary to achieve total elimination of *salmonella* from the animals. This study provides important insights into the use of phages as a preventative and biocontrol strategy against *Salmonella* infection from farm-to-table.

3.2.1.7 References

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3.2.2 Bacteriophage therapy against *Salmonella* in broilers modulates the caecal microbiota and metabolome with no biological significance in broilers

3.2.2.1 Abstract

Bacteriophage therapy has been postulated as one of the most promising tools to control zoonotic bacteria, such as *Salmonella*, during broiler production period. Compared to antibiotics, phages have high specificity that usually attacks only their targeted bacterial hosts, indicating minimal disruption to the niche microbiota. Nevertheless, little data exist about the effects of the clearance of pathogenic bacteria on the gut environment after phage therapy in poultry. Therefore, the present study was conducted to investigate the influence of *Salmonella* phage on host physiology through cecal microbiota and metabolome modulation using high-throughput 16S rRNA gene sequencing and an untargeted metabolomics approach. To this end, we employed 24 caecum content samples and 24 blood serum samples from 4, 5 and 6-weeks-old broilers from a previous study where *Salmonella* phages were administered *via* feed in *Salmonella*-infected broilers. Broilers were weighed individually weekly from seven days to the end of the rearing period. Phage did not affect the alpha diversity and beta diversity. Specifically, we found changes in the relative abundances of 14 genera using the PLS-DA and Bayes approaches. However, these subtle changes at the genus level were accompanied by substantial changes in cecal metabolites (63 up-regulated and 37 down-regulated). Nevertheless, the magnitude of the changes in blood serum in the phage-treated group appears not to cause a significant physiological response. As such, these results were confirmed by the observation that the phages administration did not influence the broilers's performance from early to later growth stages. In summary, the results of the current study showed that the application of *Salmonella* phages under production conditions modulates the cecal microbiome and metabolome profiles in broilers without impacting host physiology in terms of growth performance.

3.2.2.2. Introduction

Salmonella is one of the most frequently isolated foodborne pathogens worldwide. Currently, this bacterium has been related with the 3 % of the bacterial foodborne disease, accounting for 80 million infections and 155,000 deaths globally (Tzani et al., 2021; El-Saadony et al., 2022). In Europe, despite the efforts to control this pathogen in poultry production, poultry products continue to be the primary source of the infection (EFSA and ECDC, 2021). Indeed, broilers could acquire the bacteria and not exhibit any clinical illness, being a silent source of infection (El-Saadony et al., 2022). Carrier animals are a silent source of infection not only to other broilers in co-housing facilities but also to the processing facilities, with the human health hazards that entails (El-Saadony et al., 2022; Marin et al., 2022). Despite the biosecurity practices implemented on farms, its control remains a major challenge worldwide, and new alternatives are still necessary (Abd El-Hack et al., 2022; El-Saadony et al., 2022; Ayalew et al., 2022). In this context, bacteriophage therapy has been postulated as one of the most promising tools to control zoonotic bacteria in broilers (Wernicki et al., 2017; Żbikowska et al., 2020; Ruvalcaba-Gómez et al., 2022; Zhao et al., 2022; Clavijo et al., 2022). Indeed, several commercial phages against *Salmonella* in the poultry industry are available (Bafasal®, Biotector®S, SalmoFreshTM, SalmoPro®, SalmonelexTM (PhageGuard), PhageGuard STM, BacWashTM and SalmoFREE®).

Bacteriophages (or phages) are viruses that selectively infect and replicate in their target bacterial host. Compared to antibiotics, phages have high specificity that usually attacks only their targeted bacterial hosts, indicating minimal disruption to the niche microbiota (Cieplak et al., 2018; Gindin et al., 2019). Contrary to that, the indiscriminately broad-spectrum of antibiotics has high risk of exert an “imbalance” in the gut commensal microbial community (dysbiosis) (Baümler and Sperandio, 2016). In this context, little data exist about the effects the clearance of a pathogenic bacteria on the gut environment after phage therapy in poultry (Kosznik-Kwaśnicka et al., 2022; Clavijo et al., 2022). The gut microbiome is a complex ecosystem that comprises an extremely large number of different indigenous bacteria, archaea, bacteriophages, eukaryotes, viruses, and fungi, which acts as a key intermediate between environmental inputs and host metabolism (Tang et al., 2019; Chen et al., 2019). Cumulative evidence showed that the microbiome plays a crucial role in important metabolic functions, with a great influence on host biological functions, health states, disease progression and performance (Tang et al.,

2019; Chen et al., 2019). Indeed, the host and gut microbiota influence each other through a metabolic axis *via* small molecule metabolites and co-metabolites (Chen et al., 2019). The study of circulating metabolites through metabolomics allows the understanding of the mechanisms of biological and biochemical processes in complex systems that could impact in the well-being and production in livestock (Xiao et al., 2012; Ye et al., 2021). Moreover, gut microbiota unquestionably plays a critical role in the successful colonization and infection development caused by enteric pathogens, such as *Salmonella* (Grzymajlo, 2022). Indeed, *Salmonella* strongly interacts with the chicken gut microbiome, altering the microbiota composition and richness (Grzymajlo, 2022; Clavijo et al., 2022). Therefore, the integrative analysis of cecal microbiota and metabolite profiles of the ceca and serum can help to understand the changes in the host's physiological condition under a *Salmonella* infection and the impact of the phage therapy (Clavijo et al., 2022). Therefore, the present study investigated the influence of *Salmonella* phage on host physiology through modulation of the microbiota and the cecal metabolome in late-stage broiler rearing.

3.2.2.3 Material and Methods

3.2.2.3.1 Caecal content origin

Samples derived from a previous study related with the application of phages to control *Salmonella* during the broiler rearing period (Lorenzo-Rebenaque et al., 2022), carried out in the Centre for Animal Research and Technology (CITA, IVIA, Segorbe, Spain). Briefly, a total of 100 Ross one-day-old male broilers *Salmonella*-free were randomly divided into two treatment groups (phage-treated [Φ-treated] and non-phage [control]). Twenty-four hours after arriving, 50 % of the chicks from each experimental group were challenged with *Salmonella* Enteritidis (10^5 CFU/bird). For the first 21 days, the birds from Φ-treated group were fed with 0.1 % encapsulated *Salmonella*-phage (10^8 PFU/g). The birds in the control group were fed without phage during all rearing period.

Throughout the 42-day experiment, all birds were reared on floor pens with free access to food and water, and 23 hours photoperiod. In the first week, the room temperature was 32 °C and was decreased to 19 ºC at 42-day-old (Montoro-Dasi et al., 2020). The following feeding schedule was used; started feed on day 1 to 21 (*Camperbroiler iniciación, Alimentación Animal Nanta, Spain*), and finisher feed on day 21 to 42 (*Pollos*

crecimiento G, Alimentación Animal Nanta, Spain). The Directorate-General approved this study for Agriculture, Fisheries and Livestock from the Valencian Community (2021/VSC/PEA/0003).

At 4, 5 and 6 week of age, 4 broilers from each group were euthanized for sampling (**Figure 27**). Approximately 2 mL of blood was centrifuged at 4,000 × g for 15 min, and the serum was preserved at –80 °C for metabolome analysis. The cecal content was collected and aliquoted into two parts and immediate snap freezing with liquid nitrogen and kept frozen at −80 °C for DNA and metabolomes extraction. For the analysis of the microbiota and metabolome, samples corresponding to weeks 4 to 6 were chosen, according to the microbiota maturation and stabilization (Clavijo et al., 2022).

3.2.2.3.2 Growth Performance

Broilers were weighed individually weekly from seven days of age to determine the body weight.

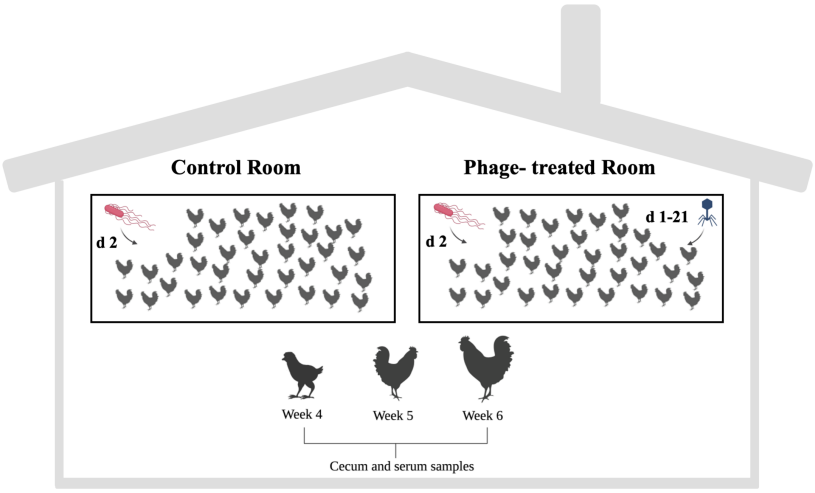

Figure 27. Experimental design of the study. Half of the animals in both groups were challenge with *Salmonella* Enteritidis at 2 the second day of the rearing period. Φ-treated group received 0.1 % encapsulated *Salmonella*-phage (10^8 PFU/g) with the feed (days 1 to 21 of the rearing period). The control group did not receive a phage. Created with BioRender.com

3.2.2.3.3 Microbiota analysis

3.2.2.3.3.1 DNA extraction, 16S rRNA gene amplification and MiSeq sequencing

The DNA was extracted from 250 mg of each homogenized cecal content following the manufacturer's instructions (QIAamp Power Fecal Pro DNA kit, Werfen, Barcelona, Spain). The DNA quality were determined using Nanodrop Nanodrop ND-1000 spectrophotometer (Thermo Scientific, Wilmington, USA), and DNA quantity were determined using Qubit fluorometer (Life Technologies, Paisley, UK). The DNA was frozen at −20ºC for shipment to the *Instituto de Investigación Sanitaria y Biomédica de Alicante* - *ISABIAL* (Alicante, Spain). The 16S rRNA gene amplicon libraries, were prepared using the 16S Metagenomic Sequencing Library Preparation, Preparing 16S Ribosomal RNA Gene Amplicons for the Illumina MiSeq System (Illumina®) protocol. Primer sequences cover the V3–V4 regions of the 16S rRNA gene. The following primers also include the Illumina adapters: 16S Amplicon PCR Forward Primer = 5′ TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCCTACGGGNGGCWGCAG; and 16S Amplicon PCR Reverse Primer = 5′ GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGACTACHVGGGTATCTA ATCC. The MiSeq (Illumina) system in 2 × 300 bp format sequencing was performed to sequence the Illumina libraries. To evaluate the quality of the raw unprocessed reads the FastQC software was used (Babraham Bioinformatics, 2022).

3.2.2.3.3.2 Bioinformatic analysis

Raw sequencing data were processed by QIIME2 v2021.4. The DADA2 pipeline incorporated into QIIME2 was used to the denoising, filtering, and chimera removal of the sequences and reads were clustered in Amplicon Sequence Variants (ASVs). Each ASV was taxonomical assigned using the SILVA v138 database (Quast et al., 2013; Campos et al., 2022). Reads not assigned to any taxa or classified as Eukaryote or Archaea, were removed from the analysis. Sequencing statistical analyses were performed using QIIME2 v2021.4.

3.2.2.3.4 Metabolomics analysis

3.2.2.3.4.1 Sample Preparations

The cecal metabolites were extracted from 10 mg of each homogenized cecal content following a published method with a little modification (Coppola et al., 2019). Briefly, samples were dissolved in cold aqueous methanol (75 µL, 75 %), and formic acid (0.1 %), spiked with 10 µg/mL formononetin as internal standard. Then, the mix was shaked for 40' at 20 Hz using a Mixer Mill 300 (Qiagen). After centrifugation at 20,000 xg for 15 min at 4 °C, 600 µL of the supernatant were gained, and transferred to a new 2-mL conical tube. The supernatants were transferred to HPLC filter tubes (0.22 µm pore size, WhatmannTM) and an aliquot of 3 µL of each sample was injected for the analysis. To LC-ESI-HRMS analysis LTQ-Orbitrap Discovery was used as mass spectrometry system (Thermo Fisher Scientific) as previously described (Garcia-Dominguez et al., 2020).

The serum metabolites were extracted from 100 µL of each serum samples following a published method with a little modification (Yu et al., 2021). Briefly, samples were dissolved in cold aqueous methanol (200 µL, 75 %), and acetronitrile (200 µL, 75 %), spiked with 10 µg/ml formononetin as internal standard. After centrifugation at 20,000 xg for 15 min at 4 °C, 200 µL of the supernate were gained, and dried under low-temperature vacuum (Thermo Scientific, USA). The samples were redissolved resuspended with 100 µL of methanol (10 %) and transferred to HPLC tubes and an aliquot of 3 µL was injected for the analysis.

3.2.2.3.4.2 LC-ESI-HRMS analysis

Untargeted LC-ESI-HRMS analyses of the caecum and serum samples was conducted as reported before (Garcia-Dominguez et al., 2020) in the *Agenzia nazionale per le nuove tecnologie, l'energia e lo sviluppo economico sostenibile* (ENEA, Roma, Italy). The data were further processed with Compound Discoverer software (Thermofisher Scientific). After detection of the features (the m/z and rt for each peak), and chromatogram alignment, the data generated were normalized with respect to internal standard. After chromatogram alignment and retrieval of all the detected frames (e.g., ions), the data generated were normalised with respect to the internal standard. For metabolite identification, a manual curation using the Metlin database was performed (https://metlin.scripps.edu/). Tentative identifications were validated comparing chromatographic and spectral properties with authentic standards (when available) and reference spectra, in house database, literature data, and based on the m/z accurate

masses, as reported in the Pubchem database (http://pubchem.ncbi.nlm.nih.gov/) for monoisotopic mass identification, subsequently confirmed by MS/MS fragmentation.

3.2.2.3.5 Statistical analysis

3.2.2.3.5.1 Body weight statistical analysis

A Generalised Linear Model (GLM) was used to evaluate the differences due to phage treatment on the body weight. As a fixed effect was included the experimental group (Φ-treated *vs* control group). Statistical differences were based on a *p-value* level of < 0.05. Statistical analyses were performed using SPSS 27.0 software package (SPSS Inc., Chicago, IL, USA).

3.2.2.3.5.2 Cecal microbiome and cecal and serum metabolome statistical analysis

Statistical analysis of cecal microbiome and cecal and serum metabolome composition was performed following the same methodology. No outlier samples were identified using a principal component analysis with the dataset without zeros, so all samples remaining in the datasets. Genera and metabolites with almost 20 % zeros within each treatment were removed (Bijlsma et al., 2006). The remaining zeros were replaced by one for microbiome data and by half of the minimum value detected for each metabolite. A total of 110 genera, 1,112 cecal metabolites and 612 serum metabolites from 24 samples each, remaining in the datasets. Datasets were transformed using the additive log-ratio (ALR) transformation following:

$$ALR(j \mid ref)=\log(x\_j/x\_ref)=\log_{f0}(x\_j)-\log_{f0}(x\_ref) \quad (1)$$

where j is the total number of variables in the dataset, x_j is the values for the genera or metabolite j, and x_ref is the reference variable used to transform the data. The reference variable for metabolome data was a standard chemical (formonetin) injected in the platform run at a fixed concentration. For microbiome data, X_ref was the one with the lowest coefficient of variation (X_ref; *Family_XIII_AD3011_group*). The lack of isometry was checked using Procrustes correlation (Greenacre et al., 2021). ALRs were auto-scaled with mean of 0 and standard deviation of 1.

A partial least square-discriminant analysis (PLS-DA) were used to identify the genera and metabolites that allow to classify or discriminate among the treatments. PLS-DA models were computed with the mixOmics packages in R (Rohart et al., 2017), using the treatments as the categorical vector y, and the ALR dataset for genera or metabolites as the matrix X. The balance error rate (BER) for the Mahalanobis distance, computed by a 4-fold cross-validation repeated 100 times was used to select the optimal number of components of the model in each iteration process. In each iteration, variables with a variable importance prediction (VIP) lower than 1 were removed from the X matrix because are not informative for the classification among the treatments (Galindo-Prieto et al., 2014). After variable selection a new PLS-DA model was computed. Variable selection and PLS-DA model computation were done until the lowest BER was achieved. The prediction performance of the final PLS-DA model was checked with the construction of a confusion matrix and a permuted-confusion matrix using a 4-fold cross-validation repeated 10,000 times. The former allows to determine the ability of the model to predict each treatment according to the variables selected by the PLS-DA. The latter determines if the performance achieved is due to a spurious selection of variables throughout the PLS-DA iterations. The prediction performance was considered spurious when the percentage of true positives for each treatment was far from their random probabilities (33 % for three categories and 50 % for two categories).

Bayesian statistics were used complementary to the PLS-DA to measure the relevance of the differences in the genera and metabolites abundance between the control and the treatments. A model with a single effect of “treatment” and flat priors was fitted. The estimation of the marginal posterior distribution of the unknows were done with MCMC using four chains of 50,000 iterations, with a burn-in of 1,000 and a lag of 10. The mean of the marginal posterior distribution of the differences between the control and each one of the two types of phage administration were used to estimate the posterior mean of the differences in genera or metabolites between the control and the treatments. These estimates were reported as unit of standard deviations (SD) of each variable. The differences in the mean abundance of the genera and metabolites between the control and the treatments were considered relevant when these differences were higher than 0.5 units of SD, and the probability of the differences (Blasco, 2017) being higher (if the difference is positive) or lower (if negative) than 0 (P0) was higher than 0.9.

The alpha- and beta-diversity were computed using the ALR at the species level to measure the differences in microbiome composition among groups. The alpha-diversity was measured by Shannon's (H') and inverse Simpson indexes to analyze the species diversity and evenness. Differences in the distribution of alpha-diversity among groups were considered when the *p-value* of a Mann-Whitney U test was lower than 0.05. Beta-diversity was measured by the Bray-Curtis dissimilarity matrix and a nonmetric multidimensional scaling (NMDS) was carried out to retrieve the loadings of the first two dimensions. Differences in microbial genera composition were tested by the permutational multivariate analysis of variance (PERMANOVA; *p-value* < 0.05) on the loadings of the two first MDS dimensions.

3.2.2.4 Results

3.2.2.4.1 Effects of phage on bodyweight

There were no significant differences in the bodyweight of birds among treatments from week 1 to 7 (*p-value* <0.05) (**Figure 28**).

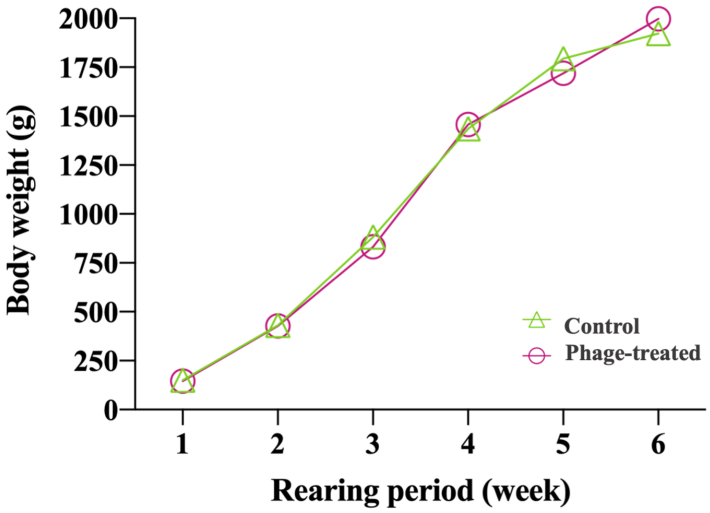

Figure 28. Weekly body weight of broilers (g/animal) of the Φ-treated (green) and control (pink) groups. Φ-treated group (green) received 0.1% *Salmonella*-phage (10^8 PFU/g). The control (pink) group did not receive a phage

3.2.2.4.2 Effects of phage on cecal microbiota

High-throughput sequencing obtained 3,258,381 sequencing reads (average 161,928.5 reads/sample), with an average read length of 403.8±13.18 pb. After denoising, removing

chimeras, and filtering low quality sequences, a total of 2,201,366 sequencies and 1,049 ASVs were generated. After filtering a total of 681 ASVs were left for taxonomic assignment. The 16S rRNA gene amplicon sequencing results are available at NCBI's (BioProject PRJNA880003).

A PLS-DA with ALR transformed variables were used to elucidate the phage administration influence on cecal microbial variations in *Salmonella*-infected broilers. The analysis identified 17 relevant variables (genera) in the final model (final PLS-DA model classification performance: Φ-treated =98.76% and control=99.85%, **Figure 29**). The results show that a several genera (17) were relevant for the classification among the groups.

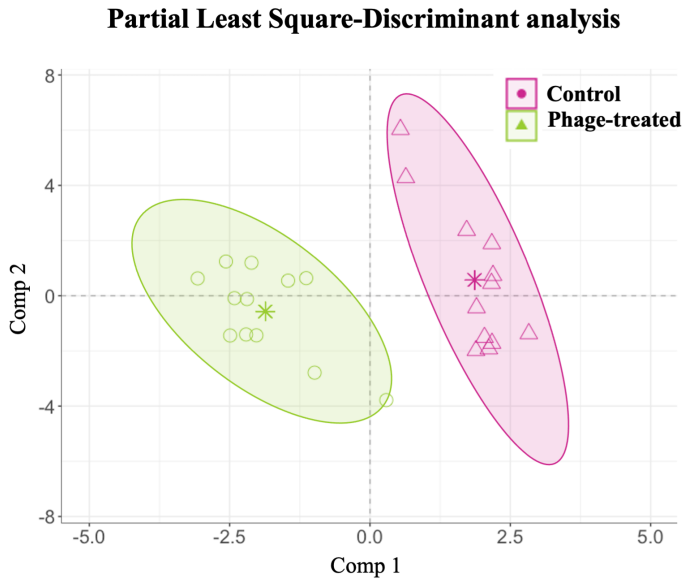

Figure 29. Examining the effects of *Salmonella* phage on the cecal microbiota in *Salmonella*-infected broilers. Cecal microbiota composition dissimilarity through the representation of the first (Comp 1) and second components (Comp 2) of the final partial least square-discriminant analysis (PLS-DA) models from the Φ-treated (green) and control (pink) groups. Φ-treated group (green) received 0.1 % *Salmonella*-phage (10^8 PFU/g) *via* feed. The control group (pink) did not receive a phage.

The Shannon´s diversity index, that is more sensitive to species richness (Johnson and Burnet, 2016), and the inverse Simpson index, that is more sensitive to species evenness (Johnson and Burnet, 2016), showed that no significant differences were observed between Φ-treated and control groups in the alpha diversity (Kruskal–Wallis test, Shannon´s diversity index: *p-value* = 0.2, inverse Simpson index: *p-value* = 0.22; **Figure 30A and 30B**). Moreover, in pairwise permanova comparisons between groups using Bray Curtis, there were no significant differences between groups in the microbiota composition (*p-value* =0.09; **Figure**

30C). These results showed that despite several genera identified by PLS-DA, in general both populations have a similar microbiota composition.

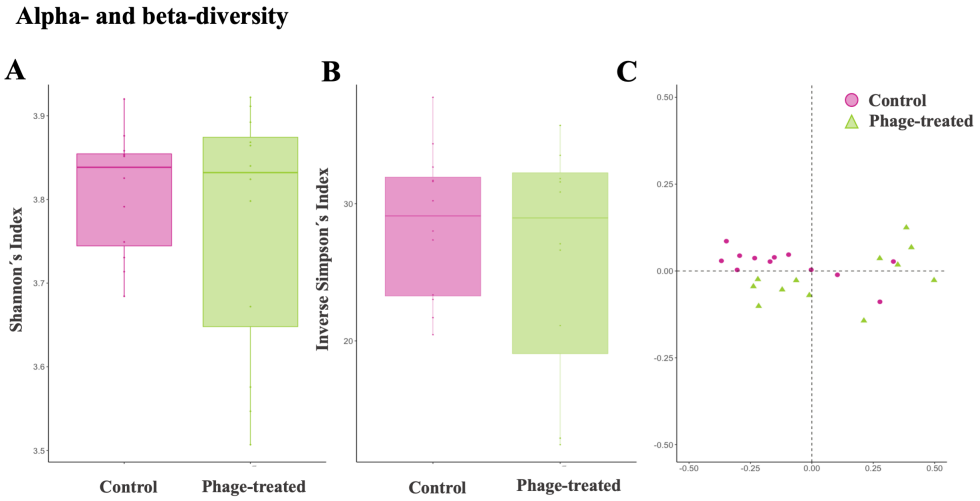

Figure 30. Examining the effects of *Salmonella* phage on the cecal microbiota in *Salmonella*-infected broilers. Cecal microbiota composition dissimilarity through the representation of the and alpha- and beta-diversity scores from control and Φ-treated groups. The alpha- and beta-diversity scores were calculated with the additive log-ratio of each species abundance according to a reference genera (*Family_XIII_AD3011_group*). Alpha-diversity was computed using (A) Shannon's H index and (B) Inverse Simpson index. Beta-diversity was computed by calculating (C) the Bray Curtis dissimilarity matrix. Differences among populations were established with a *p-value* lower than 0.05. Φ-treated group (green) received 0.1 % *Salmonella*-phage (10^8 PFU/g) *via* feed. The control group (pink) did not receive a phage.

To better understand the effect of phage application on the *Salmonella*-infected cecal microbiota a Bayesian statistical analysis was performed from the initial relevant genera identified by PLS-DA. The Bayesian results showed that several of the variables included in the PLS-DA model are key variables for discriminating between groups, with relevant differences in mean abundance (**Supplementary Table 3**).

As see in Table 1, 14 of the 110 genera detected were different between Φ-treated and control group. Among them, *Streptococcus*, *Paludicola*, *Romboutsia*, *Hydrogenoanaerobacterium*, *UCG005*, *Weissella*, *Frisingicoccus*, *Marvinbryantia*, *Turicibacter* and *Family_XIII_UCG001* from *Firmicutes* phylum and *Bacteroides* from *Bacteroidota* phylum were more abundant in Φ-treated group. While *Faecalibacterium*, *Monoglobus*, *Erysipelatoclostridium* from *Firmicutes* phylum were less abundant in the Φ-treated group.

Table 8. Examining the effects of *Salmonella* phage on the cecal microbiota in *Salmonella*-infected broilers. Key genera identified by partial least square-discriminant analysis (PLS-DA) for discriminating between groups with relevant differences in mean abundance based on Bayesian statistical analysis in Φ-treated broilers compared with the control group, computed as Φ-treated *vs* control. Φ-treated group received a 0.1 % *Salmonella*-phage (10^8 PFU/g) *via* feed. The control group did not receive a phage.

Phylum	Family	Genus	$HPD95_{phage-control}$	$P0_{phage-control}$	$D_{phage-control}$
Firmicutes	*Streptococcaceae*	*Streptococcus*	[-0.03,1.59]	96.71	0.76
	Staphylococcaceae	*Faecalibacterium*	[-1.51,0.15]	94.89	-0.68
	Ruminococcaceae	*Paludicola*	[-0.2,1.47]	92.69	0.61
	Peptostreptococcaceae	*Romboutsia*	[0.11,1.68]	98.62	0.90
	Oscillospirales	*Hydrogenoanaerobacterium*	[0.04,1.65]	97.99	0.84
	Oscillospiraceae	*UCG005*	[0.66,1.97]	99.98	1.31
	Monoglobaceae	*Monoglobus*	[-1.49,0.16]	94.64	-0.67
	Leuconostocaceae	*Weissella*	[-0.18,1.5]	92.95	0.62
	Lachnospiraceae	*Frisingicoccus*	[0.63,1.95]	99.97	1.29
		Marvinbryantia	[0.55,1.94]	99.89	1.22
	Erysipelotrichaceae	*Turicibacter*	[0.77,2.01]	99.99	1.40
	Erysipelatoclostridiaceae	*Erysipelatoclostridium*	[-1.65,-0.09]	98.64	-0.89
	Anaerovoracaceae	*Family_XIII_UCG001*	[-0.25,1.43]	92.26	0.60
Bacteroidota	*Bacteroidaceae*	*Bacteroides*	[0.7,1.98]	99.99	1.33

HPD95phage-control = The highest posterior density region at 95 % of probability. P0= Probability of the difference (Dphage-control) being greater than 0 when Dphage-control > 0 or lower than 0 when Dphage-control < 0. Dphage-control = Mean of the difference - Φ-treated -control (median of the marginal posterior distribution of the difference between the control group Φ-treated group). Statistical differences were assumed if | Dphage-control | surpass R value and its P0>0.90.

3.2.2.4.3 Effects of phage on caecal metabolome

An un-targeted LC–MS-based metabolomics platform was used to analyze the metabolic regulation in Φ-treated *Salmonella*-infected broilers. A total of 1,112 metabolites were retained. This data is available at the NIH Common Fund's National Metabolomics Data Repository (NMDR) website, the Metabolomics Workbench, https://www.metabolomicsworkbench.org where it has been assigned Study ID ST002311. The data can be accessed directly via it's Project DOI: http://dx.doi.org/10.21228/M8598K.

A PLS-DA with ALR transformed variables were used to elucidate the phage administration influence on cecal metabolome variations in *Salmonella*-infected broilers. The analysis identified 118 relevant variables (metabolites) in the final model for cecal samples (final PLS-DA model classification performance: control=100.00 % and Φ-treated =99.92 %, **Figure 31**). The results show that after phage administration, several cecal metabolites (118) were relevant for classifying the groups.

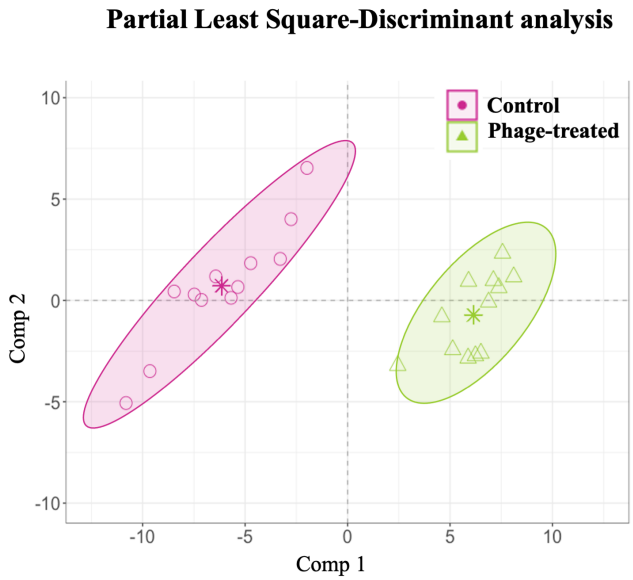

Figure 31. Examining the effects of *Salmonella* phage on the cecal metabolome in *Salmonella*-infected broilers. Cecal metabolome composition dissimilarity through the representation of the first (Comp 1) and second components (Comp 2) of the final partial least square-discriminant analysis (PLS-DA) models from the Φ-treated (green) and control (pink) groups. Φ-treated group (green) received 0.1% *Salmonella*-phage (10^8 PFU/g) *via* feed. The control group (pink) did not receive a phage.

We further verified the relevant metabolites identified by PLS-DA by Bayesian statistical analysis. The Bayesian statistical analysis showed that 100 variables from the initial 118 identified in samples by PLS-DA analysis (**Supplementary Table 4**) had a posterior mean of the differences of at least 0.5 of the SD of the variable in which the probability of differences being higher or lower than 0 (P0) was higher than 0.90.

In cecum samples, from the 100 significant metabolites 63 were up-regulated and 37 were down-regulated compared to the control group. From all of them, 21 could be tentative identified. The structures of the identified metabolites included lipids and lipid-like molecules (12), organic acids and derivates (2), organic oxygen compounds (2), phenylpropanoids and polykeides (2), organoheterocyclic compounds (1), benzenoids (1), and organic nitrogen compounds (1)(**Table 9**).

Table 9. Examining the effects of *Salmonella* phage on the caecal metabolome in *Salmonella*-infected broilers. Key metabolites identified by partial least square-discriminant analysis (PLS-DA) for discriminating between groups with relevant differences in mean abundance based on Bayesian statistical analysis in Φ-treated broilers compared with the control group, computed as Φ-treated *vs* control. Φ-treated group received a 0.1 % *Salmonella*-phage (10^8 PFU/g) *via* feed. The control group did not receive a phage.

Superclass	Class	Subclass	Metabolite	HPD95 $_{phage-control}$	P0 $_{phage-control}$	D $_{phage-control}$
Benzenoids	Benzene and substituited derivates	Phenyl methylcarbamates	2-(Ethylsulfonylmethyl)phenyl methylcarbamate	[0.3,1.8]	99.47	1.03
Lipids and lipid-like molecules	Fatty acyls	Eicosanoids	9-deoxy-9-methylene-PGE2	[-1.43,0.27]	92.19	-0.60
		Fatty alcohols	Persenone A	[0.54,1.93]	99.88	1.21
		Stigmasterols and C24-ethyl derivatives	5alpha,8alpha-epidioxy-stigmasta-6,9(11),22E-trien-3beta-ol	[-1.73,-0.18]	98.92	-0.93
	Steroids and steroid derivates	Cholesterol and derivates	9,11alpha-epoxy-6alpha-acetoxy-cholest-7-en-3beta,5alpha,19-triol	[0.37,1.82]	99.80	1.11
		Steroid ester	Estra-1,3,5(10)-triene-3,6beta,17beta-triol triacetate	[0.53,1.91]	99.93	1.22
		Sulfate steroids	Pregnanolone sulfate	[-1.82,-0.35]	99.70	-1.08
		Bile acids, alcohols and derivates	Perulactone	[1.05,2.11]	100.00	1.57
		stigmastanes and derivates	7-Oxostigmasterol	[0.82,2.03]	100.00	1.42
	Sphingolipids	Phosphosphingolipids	SM(d18:1/0:0)	[-1.82,-0.35]	99.69	-1.08
	Prenol lipids	Isoprenoids	(+)-3beta-Hydroxy-ursan-28-oic acid	[-1.71,-0.14]	98.85	-0.92
	Glycerophospholipids	Glycerophosphoethanolamines	PE(14:0/0:0)	[0.37,1.81]	99.80	1.09
			PC(18:2(2E,4E)/0:0)	[-1.62,-0.03]	97.83	-0.83
Organic acids and derivates	Carboxylic acids and derivates	Amino acids, peptides, and analogues	Yersiniabactin	[1.73,2.12]	100.00	1.91
		Monocarboxylic acid	1-(3,4-Dihydroxyphenyl)-1-decene-3,5-dione	[-1.11,0.6]	72.98	-0.26
		Carbocylic acid derivates	(S,E)-Lyratol propanoate	[0.68,1.98]	99.98	1.33
Organic nitrogen compounds	Organonitrogen compounds	Organic nitroso compounds	3-[(3-Methylbutyl)nitrosoamino]-2-butanone	[0.82,2.03]	99.99	1.43
Organic oxygen compounds	Organooxygen compounds	Carbohidrates and carbohydrate conjugates	D-Glucosamine 1-phosphate	[0.59,1.94]	99.97	1.28
		Carbohydrates and carbohydrate conjugates	Glucosyl (E)-2,6-Dimethyl-2,5-heptadienoate	[-1.88,-0.46]	99.90	-1.18
Organoheterocyclic compounds	Tetrapyrroles and derivates	Bilirubins	Mesobilirubinogen	[0.18,1.71]	99.22	0.95

Phenylpropanoids and polykeides	Stilbenes	Stilbenes	Batatasin III	[-1.84,-0.37]	99.73	-1.09
	Flavonoids	Falavans	Kaempferol 7,4'-dimethyl ether 3-(6"-(E)-p-coumarylglucoside)	[1.27,2.15]	100.00	1.71
Non-Indentified metabolite 66				[0.24,1.77]	99.31	0.97
Non-Indentified metabolite 101				[0.24,1.77]	99.35	0.99
Non-Indentified metabolite 102				[0.09,1.65]	98.78	0.89
Non-Indentified metabolite 116				[0.03,1.63]	97.95	0.85
Non-Indentified metabolite 117				[0.03,1.65]	97.59	0.82
Non-Indentified metabolite 122				[-0.01,1.6]	97.28	0.80
Non-Indentified metabolite 132				[-1.89,-0.51]	99.95	-1.20
Non-Indentified metabolite 195				[0.33,1.8]	99.62	1.06
Non-Indentified metabolite 196				[0.48,1.9]	99.87	1.17
Non-Indentified metabolite 199				[0.26,1.76]	99.39	0.99
Non-Indentified metabolite 210				[-1.9,-0.53]	99.95	-1.24
Non-Indentified metabolite 214				[-1.97,-0.64]	99.94	-1.28
Non-Indentified metabolite 216				[-2,-0.74]	99.98	-1.37
Non-Indentified metabolite 217				[0.25,1.77]	99.40	1.02
Non-Indentified metabolite 266				[-2.14,-1.27]	100.00	-1.70
Non-Indentified metabolite 267				[-2.13,-1.4]	100.00	-1.78
Non-Indentified metabolite 384				[-2,-0.74]	99.99	-1.36
Non-Indentified metabolite 386				[0.48,1.87]	99.90	1.17
Non-Indentified metabolite 394				[-1.75,-0.24]	99.37	-1.00
Non-Indentified metabolite 398				[1.63,2.13]	100.00	1.88
Non-Indentified metabolite 399				[1.66,2.13]	100.00	1.89
Non-Indentified metabolite 400				[1.76,2.09]	100.00	1.93
Non-Indentified metabolite 402				[1.74,2.1]	100.00	1.92
Non-Indentified metabolite 433				[0.64,1.96]	99.99	1.31
Non-Indentified metabolite 434				[0.6,1.96]	99.94	1.26
Non-Indentified metabolite 436				[0.62,1.95]	99.96	1.28
Non-Indentified metabolite 449				[1.52,2.14]	100.00	1.83
Non-Indentified metabolite 462				[-1.76,-0.21]	99.26	-0.98
Non-Indentified metabolite 463				[-1.78,-0.29]	99.53	-1.02
Non-Indentified metabolite 465				[0.99,2.1]	100.00	1.54
Non-Indentified metabolite 466				[-2.06,-0.89]	100.00	-1.46
Non-Indentified metabolite 501				[-2.12,-1.16]	100.00	-1.64
Non-Indentified metabolite 506				[-1.98,-0.69]	99.98	-1.33
Non-Indentified metabolite 509				[-1.85,-0.41]	99.87	-1.14
Non-Indentified metabolite 510				[-1.89,-0.46]	99.83	-1.15
Non-Indentified metabolite 530				[-1.89,-0.51]	99.94	-1.22
Non-Indentified metabolite 532				[0.3,1.78]	99.55	1.05
Non-Indentified metabolite 533				[0.64,1.96]	99.97	1.30
Non-Indentified metabolite 536				[-2.13,-1.21]	100.00	-1.68

Non-Indentified metabolite 571	[0.12,1.68]	98.98	0.92
Non-Indentified metabolite 586	[0.37,1.81]	99.83	1.10
Non-Indentified metabolite 615	[0.31,1.8]	99.58	1.06
Non-Indentified metabolite 616	[0.28,1.78]	99.48	1.01
Non-Indentified metabolite 617	[0.28,1.79]	99.51	1.04
Non-Indentified metabolite 633	[-1.88,-0.46]	99.88	-1.17
Non-Indentified metabolite 660	[-1.6,-0.02]	97.86	-0.81
Non-Indentified metabolite 661	[0.02,1.62]	97.70	0.82
Non-Indentified metabolite 664	[-1.54,0.12]	95.54	-0.72
Non-Indentified metabolite 665	[-1.56,0.03]	97.16	-0.77
Non-Indentified metabolite 666	[-1.43,0.27]	92.68	-0.61
Non-Indentified metabolite 680	[0.37,1.84]	99.72	1.08
Non-Indentified metabolite 692	[-1.6,0.01]	97.31	-0.79
Non-Indentified metabolite 695	[-1.61,-0.03]	98.05	-0.84
Non-Indentified metabolite 724	[0.56,1.95]	99.92	1.22
Non-Indentified metabolite 748	[0.19,1.71]	99.17	0.95
Non-Indentified metabolite 813	[0.66,1.96]	99.97	1.31
Non-Indentified metabolite 822	[0.54,1.9]	99.92	1.22
Non-Indentified metabolite 823	[0.5,1.9]	99.85	1.20
Non-Indentified metabolite 856	[1,2.09]	100.00	1.54
Non-Indentified metabolite 859	[-2,-0.79]	99.99	-1.41
Non-Indentified metabolite 860	[-1.93,-0.59]	99.96	-1.28
Non-Indentified metabolite 866	[0.54,1.91]	99.94	1.24
Non-Indentified metabolite 872	[0.44,1.85]	99.86	1.17
Non-Indentified metabolite 889	[0.24,1.77]	99.17	0.98
Non-Indentified metabolite 893	[-2.02,-0.81]	99.99	-1.43
Non-Indentified metabolite 897	[0.51,1.89]	99.93	1.23
Non-Indentified metabolite 901	[0.29,1.79]	99.59	1.04
Non-Indentified metabolite 903	[0.64,1.95]	99.97	1.31
Non-Indentified metabolite 904	[0.66,1.97]	99.98	1.32
Non-Indentified metabolite 941	[0.68,1.99]	99.97	1.31
Non-Indentified metabolite 964	[0.61,1.94]	99.98	1.26
Non-Indentified metabolite 970	[0.79,2.01]	99.99	1.41
Non-Indentified metabolite 977	[-1.87,-0.45]	99.86	-1.18
Non-Indentified metabolite 989	[0.33,1.8]	99.62	1.06
Non-Indentified metabolite 1003	[1.2,2.11]	100.00	1.67
Non-Indentified metabolite 1020	[1.04,2.08]	100.00	1.56
Non-Indentified metabolite 1025	[0.8,2.04]	99.99	1.41
Non-Indentified metabolite 1037	[0.32,1.8]	99.58	1.06
Non-Indentified metabolite 1080	[-1.69,-0.16]	98.82	-0.91

HPD95phage-control = The highest posterior density region at 95 % of probability. P0= Probability of the difference (Dphage-control) being greater than 0 when Dphage-control > 0 or lower than 0 when Dphage-control < 0. Dphage-control = Mean of the difference - Φ-treated -control (median of the marginal posterior distribution of the difference between the control group Φ-treated group). Statistical differences were assumed if | Dphage-control | surpass R value and its P0>0.90.

Regarding the 63 metabolites that were probably *up-regulated* in Φ-treated group based on Bayesian statistical significance, the structure of the identified metabolites was showed in **Figure 32A**, and corresponds mainly to steroids and steroid derivates (lipids and lipid like molecules), and carboxylic acids (organic acids and derivates). The 37 metabolites that were probably *down-regulated* in Φ-treated group based on Bayesian statistical significance, the structure of the identified metabolites was showed in **Figure 32B**, and corresponds to lipids and lipid like molecules, organoxigen compounds, and phenylpropanoids and polykeides.

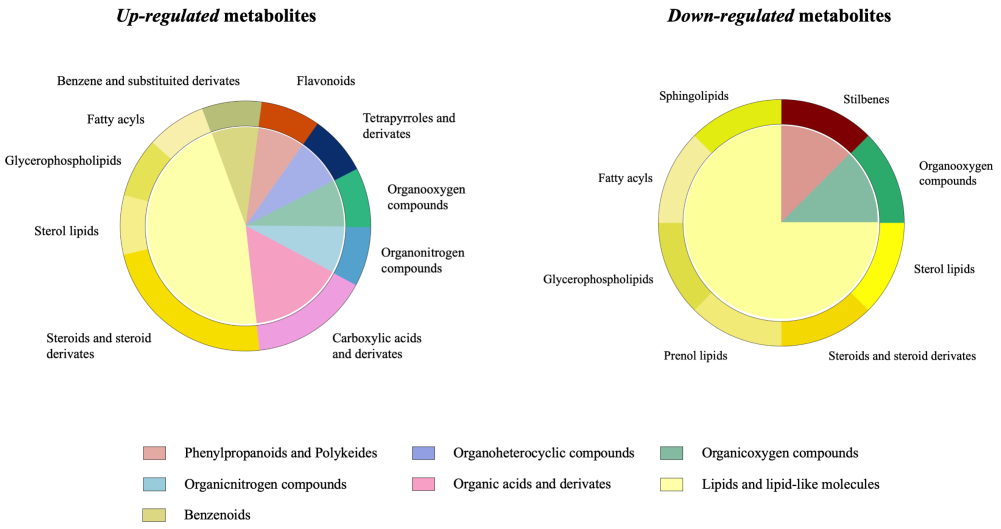

Figure 32. Examining the effects of *Salmonella* phage on the cecal metabolome in *Salmonella*-infected broilers. Class (inside of the cycle) and subclass (outside of the cycle) of significant (A) up-regulated and (B) down-regulated metabolites in Φ-treated identified by partial least square-discriminant analysis (PLS-DA) for discriminating between groups with relevant differences in mean abundance based on Bayesian statistical analysis in Φ-treated broilers compared with the control group, computed as Φ-treated *vs* control. Φ-treated group received 0.1 % *Salmonella*-phage (10^8 PFU/g) *via* feed. The control group did not receive a phage.

3.2.2.4.4 Effects of phage on serum metabolome

A total of 612 metabolites were retained. This data is available at the NIH Common Fund's National Metabolomics Data Repository (NMDR) website, the Metabolomics Workbench, https://www.metabolomicsworkbench.org where it has been assigned Study ID ST002312. The data can be accessed directly via it's Project DOI: http://dx.doi.org/10.21228/M8598K.

A PLS-DA with ALR transformed variables were used to elucidate the phage administration influence on serum metabolome variations in *Salmonella*-infected broilers. The analysis identified 45 relevant variables (metabolites) in the final model (final PLS-DA model classification performance: control=99.66 % and Φ-treated =99.26 %, **Figure 33**). The results showed that after phage administration, several cecal metabolites (45) were relevant for classifying the groups. We further verified the relevant metabolites identified by PLS-DA by Bayesian statistical analysis. The Bayesian statistical analysis showed that 16 variables from the initial 45 identified by PLS-DA analysis (**Supplementary Table 3**) had a posterior mean of the differences of at least 0.5 of the SD of the variable in which the probability of differences being higher or lower than 0 (P0) was higher than 0.90.

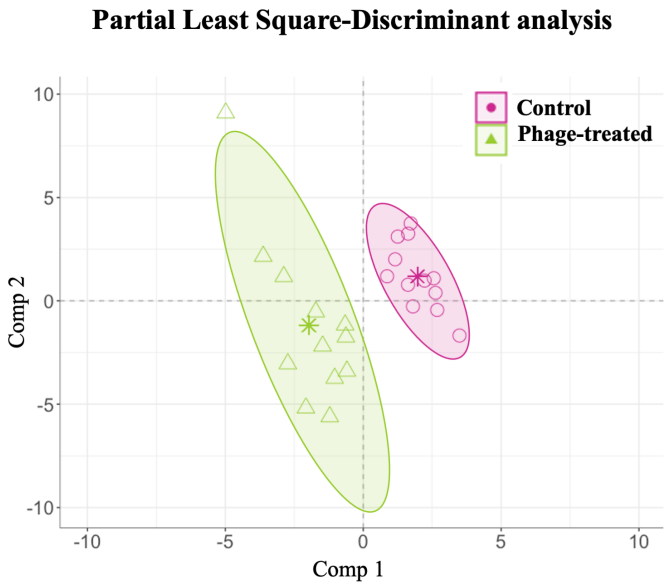

Figure 33. Examining the effects of *Salmonella* phage on the serum metabolome in *Salmonella*-infected broilers. Serum metabolome composition dissimilarity through the representation of the first (Comp 1) and second components (Comp 2) of the final partial least square-discriminant analysis (PLS-DA) models from the Φ-treated (green) and control (pink) groups. Φ-treated group (green) received 0.1 % *Salmonella*-phage (10^8 PFU/g). The control group (pink) did not receive a phage.

For the 16 significant metabolites 4 were up-regulated and 12 were down-regulated compared to the control group. Of them, 8 could be tentative identified. The structures of the identified metabolites included organoheterocyclic compounds (3), lipid and lipid-like molecules (2), organic acids and derivates (1), organic oxygen compounds (1), and phenylpropanoids and polykeides (1)(**Table 10**).

Table 10. Examining the effects of *Salmonella* phage on the serum metabolome in *Salmonella*-infected broilers. Key metabolites identified by partial least square-discriminant analysis (PLS-DA) for discriminating between groups with relevant differences in mean abundance based on Bayesian statistical analysis in Φ-treated broilers compared with the control group, computed as Φ-treated *vs* control. Φ-treated group received 0.1 % *Salmonella*-phage (10^8 PFU/g) *via* feed. The control group did not receive a phage.

Super Class	Class	Sub Class	Name	HPD95phage-control	P0phage-	Dphage-control
Organoheterocyclic compounds	Indoles and derivates	Tryptamines and derivates	5-Methoxytryptophan	[-1.8,-0.3]	99.63	-1.05
	Pteridines and derivates	Pterins and derivates	6-Lactoyltetrahydropterin	[-1,63,-0,03]	97.69	-0.82
	Heteroarene	Polycyclic heteroarene	Indolylmethylthiohydroximate	[-1,88,-0,48]	99.92	-1.20
Organic Oxygen compounds	Organooxygen compounds	Carbohydrates and carbohydrate conjugates	D-Mannitol	[-1.7,-0.14]	98.80	-0.92
Organic acids and derivates	Carboxylic acids and derivates	Amino acids.Peptides. and analogues	Prolyl-Tyrosine	[-0.61,1.13]	69.97	0.22
			L-Ornithuric acid	[0,35,1,8]	99.69	1.08
Lipids and lipid-like molecules	Steroids and steroid derivates	Bile acids. alcohols and derivates	Murocholic acid	[-1.41,0.27]	91.60	-0.58
	Glycerophospholipids	Glycerophosphoethanolamines	PE(17:0/0:0)	[-0,26,1,42]	92.76	0.61
Phenylpropanoids and Polykeides	Isoflavonoids	O-methylated isoflavonoids	Homoferreirin	[-1.43,0.27]	91.27	-0.57
Non-identified metabolite 50				[-1,7,-0,16]	98.85	-0.93
Non-identified metabolite 322				[-1,81,-0,32]	99.63	-1.06
Non-identified metabolite 323				[-1,87,-0,42]	99.81	-1.12
Non-identified metabolite 346				[-1,78,-0,29]	99.62	-1.06
Non-identified metabolite 395				[-1,81,-0,34]	99.65	-1.05
Non-identified metabolite 458				[-1,81,-0,32]	99.59	-1.05
Non-identified metabolite 461				[0,27,1,76]	99.51	1.02
Non-identified metabolite 490				[-0,3,1,39]	90.14	0.54

HPD95phage-control = The highest posterior density region at 95 % of probability. P0= Probability of the difference (Dphage-control) being greater than 0 when Dphage-control > 0 or lower than 0 when Dphage-control < 0. Dphage-control = Mean of the difference - Φ-treated -control (median of the marginal posterior distribution of the difference between the control group Φ-treated group). Statistical differences were assumed if | Dphage-control | surpass R value and its P0>0.90.

For the 4 metabolites that were probably *up-regulated* in Φ-treated group based on Bayesian statistical significance, the structure of the identified metabolites was showed in **Figure 34A**, and corresponds to glycerophospholipids such as glycerophosphoethanolamines (lipids and lipid like molecules) and to carboxylic acids and derivates such as amino acids, peptides, and analogues (organic acids and derivates compounds). For the 12 metabolites that were probably *down-regulated* in Φ-treated group based on Bayesian statistical significance, the structure of the identified metabolites was showed in **Figure 34B**, and corresponds mainly to mainly to pteridines and derivates, indoles and derivates such as tryptamines and derivates heteroarene such as polycyclic heteroarene (organoheterocyclic compounds).

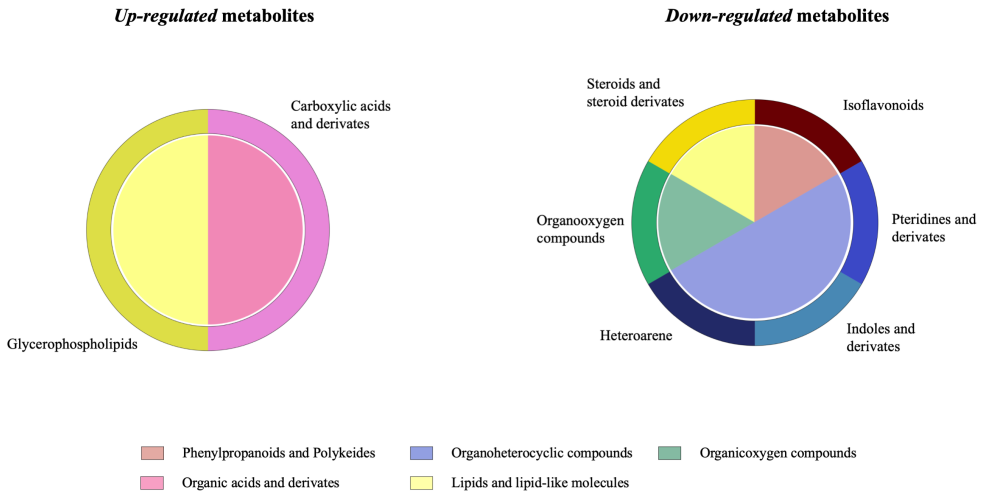

Figure 34. Examining the effects of *Salmonella* phage on the serum metabolome in *Salmonella*-infected broilers. Class (inside of the cycle) and subclass (outside of the cycle) of significant (A) up-regulated and (B) down-regulated metabolites in Φ-treated identified by partial least square-discriminant analysis (PLS-DA) for discriminating between groups with relevant differences in mean abundance based on Bayesian statistical analysis in Φ-treated broilers compared with the control group, computed as phage-treated *vs* control. Φ-treated group received 0.1 % *Salmonella*-phage (10^8 PFU/g) *via* feed. The control group did not receive a phage.

3.2.2.5 Discussion

The therapeutic potential of bacteriophages to support *Salmonella* control in poultry flocks has been demonstrated over the last years (Hong et al., 2013; Ahmadi et al., 2016; Adhikari et al., 2017; Nabil et al., 2018; Vaz et al., 2020; Sorour et al., 2020). Nevertheless, it is increasingly established that phages influence host physiology through

microbiota modulation by depleting bacterial species important for homeostasis (Sausset et al., 2020). However, studies to detect changes in the gut microbiome in infected (bacterial) and treated (phage) or uninfected and treated animals have yielded contradictory results on the non-targeted bacteria (Galtier et al., 2016; Tetz et al., 2017; Dissanayake et al., 2019; Hsu et al., 2019; Clavijo et al., 2022). Our study provided evidence that *Salmonella* phage modulates the cecal microbiota and metabolome, but with no effect on the body weight and with a minimal influence on the blood serum metabolome, suggesting that phage treatment may indeed have no biological significance in broilers.

Although mammals phage therapy seems to be safe and well-tolerated, a complete understanding of phage-host interactions is lacking (Liu et al., 2021). The main theoretical advantages of phages over antibiotics are that they do not affect the gut microbial community (Clavijo et al., 2022). Nevertheless, several studies have demonstrated that phages induce changes in the microbiome, although these do not appear to be of biological significance (Sarker et al., 2012; McCallin et al., 2013; Galtier et al., 2016; Sarker et al., 2016; Sarker et al., 2017; Clavijo et al., 2022). In this study, the phage group, had altered the microbiome and metabolome profiles compared to the non-treated group. Still, the phage group did not affect the number of species found (alpha diversity) or the number of unique species (beta diversity) in agreement with previous studies (Zhao et al., 2022). Specifically, we found changes in the relative abundances of a few genera using the PLS-DA, and Bayes approaches. However, our findings suggest that subtle changes at the genus level are accompanied by substantial changes in cecal metabolites. These changes in metabolic profile based on gut microbiota agree with previous research comparing non-treated and treated *Salmonella*-infected broilers with different antimicrobials (Rubinelli et al., 2017). Cecal alterations presented a context-specific singularity that entails changes in the interaction between microbes (probably with altered abundances) and hosts epithelial/immune cells, leading to alterations in the shedding of microbial-associated molecular patterns in the gut, and in the availability of metabolites produced by gut microbiota (Lee et al., 2022). Still, we do not know whether these metabolic changes reflect a direct involvement of phages in the central microbiome or are the result of altering the relative abundance of the identified genera. Further studies are needed to elucidate this issue.

We found that most of the altered genera in the Φ-treated group present an increase in bacterial abundance. For instance, the abundance of *Bacteroides* was significantly enhanced. In previous studies, this beneficial bacterial genus has been found in *Salmonella*-infected chickens treated with probiotics (Khan and Chousalkar, 2020; Aljahdali et al., 2020). This genus has been related to acetic acid production and its influence on lipid metabolism (Jiang et al., 2021). Lipids regulate biological processes such as immunity and inflammation (Li et al., 2022). In addition, *Bacteroides* has also been associated with the metabolism of bile acids, proteins, fats and carbohydrates (Jiang et al., 2021). These observations could be consistent with the alteration of cecal metabolites observed in this study. Note that the level of bile acids in the gut can affect microbial community abundance (Li et al., 2022). Moreover, bile acids have been related to regulating hepatic metabolic pathways (Li et al., 2022), which have a protective effect against sepsis *via* different mechanisms such as bacterial clearance and adaptation to inflammation (Strnad et al., 2017; Li et al., 2022). After treatment, the *Romboutsia* genus has been identified in *Salmonella*-infected laying hens (Khan and Chousalkar, 2020). This genus has been described as part of the commensal bacteria involved in carbohydrate utilisation, simple amino acid fermentation and anaerobic respiration (Memon et al., 2022). The *Weissella* genus from the *Leuconostocaceae* family and the *Turicibacter* genus from the *Erysipelotrichaceae* family has been previously described in the chicken and mammalian gut (Khan and Chousalkar, 2020; Gilroy et al., 2021). We also identified an increase in the abundance of the *Weissella* genus after *Salmonella* treatment, similar to previous studies in layers (Khan and Chousalkar, 2020). The *Weissella* genus has been described as a *Salmonella* antimicrobial (Tenea and Lara, 2019). *Weissella* is a heterofermentative lactic acid bacteria that are part of the autochthonous microbiota that helps in host health status maintenance and gut homeostasis (Cupi and Elvig-Jørgensen, 2019; Memon et al., 2022). Meanwhile, *Turicibacter* has been related to subclinical infections in the mammalian gut and colitis but has also been considered a healthy genus (O'Cuív et al., 2011; Zhou et al., 2021). As for the genus *Hydrogenoanaerobacterium*, from the *Oscillospirales* family, a sugar-fermenting and hydrogen-producer, it positively correlated with body weight (Baniel et al., 2021; Zhang, 2021). Low levels of *Oscillospirales* in patients have been described as associated with dysbiosis (Chen et al., 2020). Finally, higher levels in the phage group were also presented for *Family_XIII_UCG001* from the *Anaerovoracaceae* family. This family function in the gut was unknown yet; however, it belongs to the class of *Clostridia*,

which is involved in the fermentation of plant polysaccharides (Reyer et al., 2021). It is worth mentioning that highly relative abundant of *Lachnospiraceae* (*Frisingicoccus* and *Marvinbryantia* genus) and *Ruminococcaceae* (*Paludicola* genus) families have been observed in cecal microbiota composition in *Salmonella* infected chickens (Mon et al., 2015). Moreover, members of these families are considered butyric acid and short-chain fatty acids producers through the carbohydrate fermentation that presents a potential protective role in *Salmonella*-colonization resistance in the gut (Mon et al., 2015). Highlight that fatty acids have been related to reducing *Salmonella* virulence through the restriction of host invasion, the maintenance of the gut barrier integrity and intestinal immunity activation (Li, 2018; Lee et al., 2022*).* Admittedly, most of the significantly different fatty acids identified in our study were up-regulated in the phage group.

On the other hand, a decrease in *Ruminococcaceae* has been associated with an increase in *Salmonella* colonisation susceptibility (Cazals et al., 2022). Genera *Faecalibacterium*, *Monoglobus* and *Erysipelatoclostridium* were decreased in the phage group. *Faecalibacterium* and *Monoglobus* are considered commensals in the chicken caecum and whose role has been linked to pectin degradation (Wang et al., 2016; Kim et al., 2019; Bindari and Gerber, 2022). Moreover, *Faecalibacterium* was identified as a butyrate-producing genus with anti-inflammatory properties by regulating inflammatory gene expressions and apoptosis in host cells (Wang et al., 2016; Kumar et al., 2019; Bindari and Gerber, 2022). Regarding the genus *Erysipelatoclostridium*, it has been suggested that it interacts positively to displace *Salmonella* in the poultry gut microbiota (Khan and Chousalkar, 2020).

The underlying question deriving from our results is whether perturbations of the cecal microbiome and metabolome confer phenotypic alterations. Although it is known that gut microbiota plays an essential role in health, and this has been receiving increasing attention in recent years, the specific role of bacteria is currently unknown partly due to the complex bacteria interactions (Sausset et al., 2020). Admittedly, gut microbiota and its metabolic activities have essential effects on chickens´ health status and performance (Calik and Ergün, 2015; Borda-Molina et al., 2018; Clavijo and Flórez, 2018; Yang et al., 2020). Likewise, it has been reported that phages can modulate bacterial communities, but also phages influence the gut ecosystem by interacting directly with the immune cells, thereby modulating host immune activity (Carroll-Portillo and Lin, 2019; Sinha and Maurice, 2019; Van Belleghem et al., 2019). Moreover, the phage can cross the epithelial

barrier through a process known as transcytosis (Nguyen et al., 2017) and interact directly with the immune cells. Since blood serum profiles reflect changes in the host's metabolism rather than those in the gut microbial activity (Aldars-García et al., 2021), our comparison between the blood serum metabolome of Φ-treated and no-treated groups showed a high similarity in the metabolic profile. For example, the phage group observed that glycerophospholipids levels were high. These metabolites have been considered antimicrobial and immunomodulatory in broilers (Li et al., 2022), which would be expected after the phage treatment. In addition, lower levels of tryptamines and derivates (5-Methoxytryptophan) were noted in the phage group. Note that high levels of tryptamine derivatives have been described in inflammatory gut diseases (Vila et al., 2022). Overall, the magnitude of the changes in blood serum in the Φ-treated group appears not to cause a significant physiological response. As such, these results are confirmed by the observation that the phages administration did not influence the chick's performance from early to later growth stages (with or without bacterial target challenge), which is consistent with several previous studies (Adhikari et al., 2017; Wang et al., 2017; Noor et al., 2020; Sarrami et al., 2022). The translational value of these findings to other production systems, like laying hens or other species, could potentially be a source of bias due to the short period of rearing in broilers (6 weeks-old). Therefore, further long-term studies are required to assess its sustained effects and investigate the role of phages in the immune response.

3.2.2.6 Conclusion

In summary, the results of the current study showed that the application of *Salmonella* phages under production conditions modulates the cecal microbiome and metabolome profiles in broilers. However, the response in blood serum metabolites and growth performance suggests that the phage modulation seems have no biological significance. Further studies are required to assess whether such a shift implies that *Salmonella* phages shift the microbiota composition, which promotes the change in metabolic profile, or whether the phages are actively involved in metabolite changes

3.1.3.7 Supplementary material

Supplementary Table 3. Examining the effects of *Salmonella* phage on the cecal microbiota in *Salmonella*-infected broilers. Bayesian statistical analysis of the relevant genera identified by partial least square-discriminant analysis (PLS-DA) in Φ-treated

chickens compared with the control group, computed as Φ-treated *vs* control. Φ-treated group received 0.1% *Salmonella*-phage (10^8 PFU/g) *via* feed. The control group did not receive a phage.

Supplementary Table 4. Examining the effects of *Salmonella* phage on the cecal metabolome in *Salmonella*-infected broilers. Bayesian statistical analysis of the relevant genera identified by partial least square-discriminant analysis (PLS-DA) in Φ-treated chickens compared with the control group, computed as Φ-treated *vs* control. Φ-treated group received 0.1% *Salmonella*-phage (10^8 PFU/g) *via* feed. The control group did not receive a phage.

Supplementary Table 5. Examining the effects of *Salmonella* phage on the serum metabolome in *Salmonella*-infected broilers. Bayesian statistical analysis of the relevant genera identified by partial least square-discriminant analysis (PLS-DA) in Φ-treated chickens compared with the control group, computed as Φ-treated *vs* control. Φ-treated group received 0.1% *Salmonella*-phage (10^8 PFU/g) *via* feed. The control group did not receive a phage.

Supplementary Table 3. Examining the effects of *Salmonella* phage on the cecal microbiota in *Salmonella*-infected broilers. Bayesian statistical analysis of the relevant genera identified by partial least square-discriminant analysis (PLS-DA) in Φ-treated chickens compared with the control group, computed as Φ-treated *vs* control. Φ-treated group received 0.1% *Salmonella*-phage (10^8 PFU/g) *via* feed. The control group did not receive a phage.

Phylum	Family	Genus	HPD95 $_{\Phi\text{-control}}$	P0 $_{\Phi\text{-control}}$	D $_{\Phi\text{-control}}$
	Streptococcaceae	*Streptococcus*	[-0.03,1.59]	96.71	0.76
	Staphylococcaceae	*Faecalibacterium*	[-1.51,0.15]	94.89	-0.68
		Jeotgalicoccus	[-0.71,1.05]	64.42	0.16
	Ruminococcaceae	*Paludicola*	[-0.2,1.47]	92.69	0.61
	Peptostreptococcaceae	*Romboutsia*	[0.11,1.68]	98.62	0.90
	Oscillospirales	*Hydrogenoanaerobacterium*	[0.04,1.65]	97.99	0.84
Firmicutes	*Oscillospiraceae*	*UCG005*	[0.66,1.97]	99.98	1.31
		Flavonifractor	[-1.37,0.33]	88.70	-0.51
	Monoglobaceae	*Monoglobus*	[-1.49,0.16]	94.64	-0.67
	Leuconostocaceae	*Weissella*	[-0.18,1.5]	92.95	0.62
	Lachnospiraceae	*Frisingicoccus*	[0.63,1.95]	99.97	1.29
		Marvinbryantia	[0.55,1.94]	99.89	1.22
	Erysipelotrichaceae	*Turicibacter*	[0.77,2.01]	99.99	1.40
	Erysipelatoclostridiaceae	*Erysipelatoclostridium*	[-1.65,-0.09]	98.64	-0.89
	Clostridia_vadinBB60_group	*Clostridia_vadinBB60_group*	[-1.25,0.49]	81.10	-0.37
	Anaerovoracaceae	*Family_XIII_UCG001*	[-0.25,1.43]	92.26	0.60
Bacteroidota	*Bacteroidaceae*	*Bacteroides*	[0.7,1.98]	99.99	1.33

HPD95Φ-control = The highest posterior density region at 95% of probability. P0= Probability of the difference (DΦ-control) being greater than 0 when DΦ-control > 0 or lower than 0 when DΦ-control < 0. DΦ-control = Mean of the difference - Φ-treated-control (median of the marginal posterior distribution of the difference between the control group Φ-treated group). Statistical differences were assumed if | Dcontrol-water | or | Dcontrol-feed | surpass R value and its P0>0.90.

Supplementary Table 4. Examining the effects of *Salmonella* phage on the cecal metabolome in *Salmonella*-infected broilers. Bayesian statistical analysis of the relevant genera identified by partial least square-discriminant analysis (PLS-DA) in Φ-treated chickens compared with the control group, computed as Φ-treated *vs* control. Φ-treated group received 0.1% *Salmonella*-phage (10^8 PFU/g) *via* feed. The control group did not receive a phage.

Superclass	Class	Subclass	Metabolite	Formula	Ion	HPD95 $_{\Phi\text{-control}}$	P0 $_{\Phi\text{-control}}$	D $_{\Phi\text{-control}}$
Benzenoids	Benzene and substituited derivates	Phenyl methylcarbamates	2-(Ethylsulfonylmethyl)phenyl methylcarbamate	C11H15NO4S	[M-H]-	[0.3,1.8]	99.47	1.03
		Phenylpropanes	1-Methoxy-1-(2,4,5-trimethoxyphenyl)-2-propanol	C13H20O5	[M-H2O-H]-	[-1.36,0.34]	88.36	-0.50
Lipids and lipid-like molecules	Fatty acyls	Eicosanoids	9-deoxy-9-methylene-PGE2	C21H34O4	[M-H]-	[-1.43,0.27]	92.19	-0.60
			15-keto-Prostaglandin E2	C20H30O5	[M-H]-	[-0.87,0.91]	53.02	0.03
		Fatty alcohols	Persenone A	C23H38O4	[M-H]-	[0.54,1.93]	99.88	1.21
	Sterol lipids	Stigmasterols and C24-ethyl derivatives	Norselic acid B	C29H44O4	[M+FA-H]-	[-0.92,0.86]	56.98	-0.08
			5alpha,8alpha-epidioxy-stigmasta-6,9(11),22E-trien-3beta-ol	C29H44O6	[M+H]+	[-1.73,-0.18]	98.92	-0.93
		Cholesterol and derivates	9,11alpha-epoxy-6alpha-acetoxy-cholest-7-en-3beta,5alpha,19-triol	C29H46O6	[M+H-2H2O]+	[0.37,1.82]	99.80	1.11
		Steroid ester	Estra-1,3,5(10)-triene-3,6beta,17beta-triol triacetate	C24H30O6	[M+H]+	[0.53,1.91]	99.93	1.22
	Steroids and steroid derivates	Sulfate steroids	Pregnanolone sulfate	C21H34O5S	[M+FA-H]-	[-1.82,-0.35]	99.70	-1.08
		Bile acids, alcohols and derivates	3-Oxo-5beta-chola-8(14),11-dien-24-oic Acid	C24H34O3	[M+H]+	[-0.33,1.37]	89.11	0.52
			Perulactone	C30H46O7	[M-H]-	[1.05,2.11]	100.00	1.57
			(25S)-3-oxo-cholest-1,4-dien-26-oic acid	C28H42O3	[M+FA-H]-	[-1.32,0.39]	86.52	-0.47
		Ergosterols and derivates	Momordenol	C29H46O2	[M+ACN+H]+	[-1.24,0.49]	82.77	-0.40
		stigmastanes and derivates	7-Oxostigmasterol	C29H46O2	[M+H]+	[0.82,2.03]	100.00	1.42
	Sphingolipids	Phosphosphingolipids	SM(d18:1/0:0)	C23H49N2O5P	[M+Cl]-	[-1.82,-0.35]	99.69	-1.08
		Ceramides	3-Sulfodeoxycholic acid	C24H40O7S	[M-H]-	[-0.37,1.34]	87.46	0.49
	Prenol lipids	Isoprenoids	(+)-3beta-Hydroxy-ursan-28-oic acid	C30H50O3	[M+ACN+H]+	[-1.71,-0.14]	98.85	-0.92
	Glycerophospholipids	Glycerophosphoethanolamines	PE(14:0/0:0)	C19H40NO7P	[M-H]-	[0.37,1.81]	99.80	1.09
		Glycerophosphocholines	LysoPC(18:2(9Z,12Z)/0:0)	C26H50NO7P	[M+H]+	[-0.33,1.36]	88.93	0.52

			PC(18:2(2E,4E)/0:0)	C26H50NO7P	[M+FA-H]-	[-1.62,-0.03]	97.83	-0.83
Organic acids and derivates	Carboxylic acids and derivates	Amino acids, peptides, and analogues	Yersiniabactin	C21H27N3O4S3	[M+ACN+H]+	[1.73,2.12]	100.00	1.91
		Monocarboxylic acid	1-(3,4-Dihydroxyphenyl)-1-decene-3,5-dione	C16H20O4	[M+ACN+H]+	[-1.11,0.6]	72.98	-0.26
		Carbocylic acid derivates	(S,E)-Lyratol propanoate	C13H20O2	[M+FA-H]-	[0.68,1.98]	99.98	1.33
Organic nitrogen compounds	Organonitrogen compounds	Organic nitroso compounds	3-[(3-Methylbutyl)nitrosoamino]-2-butanone	C9H18N2O2	[M+H]+	[0.82,2.03]	99.99	1.43
		Amines	Stearoylethanolamide	C20H41NO2	[M+FA-H]-	[-0.71,1.05]	67.89	0.20
Organic oxygen compounds	organooxygen compounds	Carbohidrates and carbohydrate conjugates	D-Glucosamine 1-phosphate	C6H14NO8P	[M-H]-	[0.59,1.94]	99.97	1.28
		Carbohydrates and carbohydrate conjugates	Glucosyl (E)-2,6-Dimethyl-2,5-heptadienoate	C16H18N3O5	[M+Na-2H]-	[-1.88,-0.46]	99.90	-1.18
Organoheterocyclic compounds	Tetrapyrroles and derivates	Bilirubins	Mesobilirubinogen	C33H44N4O6	[M+H]+	[0.18,1.71]	99.22	0.95
Phenylpropanoids and polykeides	Stilbenes	Stilbenes	Batatasin III	C16H18O3	[M+CH3COO]-	[-1.84,-0.37]	99.73	-1.09
	Flavonoids	Falavans	Kaempferol 7,4'-dimethyl ether 3-(6"-(E)-p-coumarylglucoside)	C32H30O13	[M-H]-	[1.27,2.15]	100.00	1.71
Non-Indentified metabolite 66						[0.24,1.77]	99.31	0.97
Non-Indentified metabolite 101						[0.24,1.77]	99.35	0.99
Non-Indentified metabolite 102						[0.09,1.65]	98.78	0.89
Non-Indentified metabolite 116						[0.03,1.63]	97.95	0.85
Non-Indentified metabolite 117						[0.03,1.65]	97.59	0.82
Non-Indentified metabolite 122						[-0.01,1.6]	97.28	0.80
Non-Indentified metabolite 132						[-1.89,-0.51]	99.95	-1.20
Non-Indentified metabolite 195						[0.33,1.8]	99.62	1.06
Non-Indentified metabolite 196						[0.48,1.9]	99.87	1.17
Non-Indentified metabolite 199						[0.26,1.76]	99.39	0.99
Non-Indentified metabolite 210						[-1.9,-0.53]	99.95	-1.24
Non-Indentified metabolite 214						[-1.97,-0.64]	99.94	-1.28
Non-Indentified metabolite 216						[-2,-0.74]	99.98	-1.37
Non-Indentified metabolite 217						[0.25,1.77]	99.40	1.02

Non-Indentified metabolite 266	[-2.14,-1.27]	100.00	-1.70
Non-Indentified metabolite 267	[-2.13,-1.4]	100.00	-1.78
Non-Indentified metabolite 384	[-2,-0.74]	99.99	-1.36
Non-Indentified metabolite 386	[0.48,1.87]	99.90	1.17
Non-Indentified metabolite 394	[-1.75,-0.24]	99.37	-1.00
Non-Indentified metabolite 398	[1.63,2.13]	100.00	1.88
Non-Indentified metabolite 399	[1.66,2.13]	100.00	1.89
Non-Indentified metabolite 400	[1.76,2.09]	100.00	1.93
Non-Indentified metabolite 402	[1.74,2.1]	100.00	1.92
Non-Indentified metabolite 433	[0.64,1.96]	99.99	1.31
Non-Indentified metabolite 434	[0.6,1.96]	99.94	1.26
Non-Indentified metabolite 436	[0.62,1.95]	99.96	1.28
Non-Indentified metabolite 449	[1.52,2.14]	100.00	1.83
Non-Indentified metabolite 462	[-1.76,-0.21]	99.26	-0.98
Non-Indentified metabolite 463	[-1.78,-0.29]	99.53	-1.02
Non-Indentified metabolite 465	[0.99,2.1]	100.00	1.54
Non-Indentified metabolite 466	[-2.06,-0.89]	100.00	-1.46
Non-Indentified metabolite 501	[-2.12,-1.16]	100.00	-1.64
Non-Indentified metabolite 506	[-1.98,-0.69]	99.98	-1.33
Non-Indentified metabolite 509	[-1.85,-0.41]	99.87	-1.14
Non-Indentified metabolite 510	[-1.89,-0.46]	99.83	-1.15
Non-Indentified metabolite 530	[-1.89,-0.51]	99.94	-1.22
Non-Indentified metabolite 532	[0.3,1.78]	99.55	1.05
Non-Indentified metabolite 533	[0.64,1.96]	99.97	1.30
Non-Indentified metabolite 536	[-2.13,-1.21]	100.00	-1.68
Non-Indentified metabolite 571	[0.12,1.68]	98.98	0.92

Non-Indentified metabolite 586	[0.37,1.81]	99.83	1.10
Non-Indentified metabolite 615	[0.31,1.8]	99.58	1.06
Non-Indentified metabolite 616	[0.28,1.78]	99.48	1.01
Non-Indentified metabolite 617	[0.28,1.79]	99.51	1.04
Non-Indentified metabolite 633	[-1.88,-0.46]	99.88	-1.17
Non-Indentified metabolite 660	[-1.6,-0.02]	97.86	-0.81
Non-Indentified metabolite 661	[0.02,1.62]	97.70	0.82
Non-Indentified metabolite 664	[-1.54,0.12]	95.54	-0.72
Non-Indentified metabolite 665	[-1.56,0.03]	97.16	-0.77
Non-Indentified metabolite 666	[-1.43,0.27]	92.68	-0.61
Non-Indentified metabolite 667	[-0.72,1.03]	63.55	0.15
Non-Indentified metabolite 669	[-0.94,0.8]	55.14	-0.05
Non-Indentified metabolite 680	[0.37,1.84]	99.72	1.08
Non-Indentified metabolite 692	[-1.6,0.01]	97.31	-0.79
Non-Indentified metabolite 695	[-1.61,-0.03]	98.05	-0.84
Non-Indentified metabolite 724	[0.56,1.95]	99.92	1.22
Non-Indentified metabolite 748	[0.19,1.71]	99.17	0.95
Non-Indentified metabolite 813	[0.66,1.96]	99.97	1.31
Non-Indentified metabolite 822	[0.54,1.9]	99.92	1.22
Non-Indentified metabolite 823	[0.5,1.9]	99.85	1.20
Non-Indentified metabolite 856	[1,2.09]	100.00	1.54
Non-Indentified metabolite 859	[-2,-0.79]	99.99	-1.41
Non-Indentified metabolite 860	[-1.93,-0.59]	99.96	-1.28
Non-Indentified metabolite 866	[0.54,1.91]	99.94	1.24
Non-Indentified metabolite 872	[0.44,1.85]	99.86	1.17
Non-Indentified metabolite 889	[0.24,1.77]	99.17	0.98

Non-Indentified metabolite 893	[-2.02,-0.81]	99.99	-1.43
Non-Indentified metabolite 897	[0.51,1.89]	99.93	1.23
Non-Indentified metabolite 901	[0.29,1.79]	99.59	1.04
Non-Indentified metabolite 903	[0.64,1.95]	99.97	1.31
Non-Indentified metabolite 904	[0.66,1.97]	99.98	1.32
Non-Indentified metabolite 941	[0.68,1.99]	99.97	1.31
Non-Indentified metabolite 964	[0.61,1.94]	99.98	1.26
Non-Indentified metabolite 970	[0.79,2.01]	99.99	1.41
Non-Indentified metabolite 977	[-1.87,-0.45]	99.86	-1.18
Non-Indentified metabolite 989	[0.33,1.8]	99.62	1.06
Non-Indentified metabolite 1003	[1.2,2.11]	100.00	1.67
Non-Indentified metabolite 1010	[-0.95,0.8]	54.83	-0.05
Non-Indentified metabolite 1016	[-0.83,0.92]	57.78	0.08
Non-Indentified metabolite 1019	[-1.33,0.38]	87.88	-0.50
Non-Indentified metabolite 1020	[1.04,2.08]	100.00	1.56
Non-Indentified metabolite 1025	[0.8,2.04]	99.99	1.41
Non-Indentified metabolite 1026	[-1.21,0.53]	77.04	-0.32
Non-Indentified metabolite 1027	[-0.52,1.17]	77.04	0.31
Non-Indentified metabolite 1029	[-0.41,1.28]	86.44	0.46
Non-Indentified metabolite 1037	[0.32,1.8]	99.58	1.06
Non-Indentified metabolite 1080	[-1.69,-0.16]	98.82	-0.91

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Super Class	Class	Sub Class	Name	Formula	Ion	HPD95$_{control-\Phi}$	P0 $_{control-\Phi}$	D$_{control-\Phi}$
Organoheterocyclic compounds	Indoles and derivates	Tryptamines and derivates	5-Methoxytryptophan	C12H14N2O3	[M+H]+1	[-1.8,-0.3]	99.63	-1.05
	Pteridines and derivates	Pterins and derivates	6-Lactoyltetrahydropterin	C9H18C9H13N5O3O8	[M+CH3OH+H]+	[-1,63,-0,03]	97.69	-0.82
	Heteroarene	Polycyclic heteroarene	Indolylmethylthiohydroximate	C10H10N2OS	[M+CH3COO]-	[-1,88,-0,48]	99.92	-1.20
Organic Oxygen compounds	Organooxygen compounds	Carbohydrates and carbohydrate conjugates	D-Mannitol	C6H14O6	[M+H]+1	[-1.7,-0.14]	98.80	-0.92
Organic acids and derivates	Organic sulfuric acids and derivates	Arylsulfates	Dihydroferulic acid 4-sulfate	C10H12O7S	[M-H]-	[-0.58,1.17]	74.45	0.28
	Carboxylic acids and derivates	Amino acids.Peptides. and analogues	Prolyl-Tyrosine	C14H18N2O4	[M+H]+1	[-0.61,1.13]	69.97	0.22
			Alpha-N-Phenylacetyl-L-glutamine-like	C13H16N2O4	[M-H2O-H]-	[-1,17,0,56]	74.40	-0.28
			L-Ornithuric acid	C19 H20 N2 O4	[M+H]+1	[0,35,1,8]	99.69	1.08
Lipids and lipid-like molecules	Steroids and steroid derivates	Bile acids. alcohols and derivates	Murocholic acid	C24H40O4	[M+FA-H]-	[-1.41,0.27]	91.60	-0.58
		Steroid ester	11alpha,17beta-Dihydroxyandrost-4-en-3-one diacetate	C23H32O5	[M+ACN+Na]+	[-1,02,0,74]	62.60	-0.13
	Glycerophospholipids	Glycerophosphoethanolamines	PS(20:3(8Z,11Z,14Z)/0:0)	C26H46NO9P	[M+Na-2H]-	[-1,07,0,67]	66.72	-0.18
			PS(19:0/0:0)	C25H50NO9P	[M-H]-1	[-1,06,0,69]	66.29	-0.19
			PS(18:1(9Z)/0:0)	C24H46NO9P	[M+Na-2H]-	[-0,58,1,15]	73.63	0.27
			PE(6:0/6:0)	C17H34NO8P	[M+H]+1	[-0,4,1,3]	83.83	0.42
			PE(22:6(4Z,7Z,10Z,13Z,16Z,19Z)/0:0)	C27H44NO7P	[M+H]+1	[-0,79,0,97]	54.99	0.06
			PE(20:4(5Z,8Z,11Z,14Z)/0:0)	C25H44NO7P	[M-H]-1	[-0,94,0,82]	55.63	-0.06
			PE(20:4(5Z,8Z,11Z,14Z)/0:0)	C25H44NO7P	[M-H]-1	[-1,19,0,56]	76.35	-0.30
			PE(18:2(9Z,12Z)/0:0)	C23H44NO7P	[M-H]-1	[-0,54,1,2]	77.41	0.32
			PE(17:0/0:0)	C22H46NO7P	[M+H]+1	[-0,26,1,42]	92.76	0.61
			LysoPC(20:5(5Z,8Z,11Z,14Z,17Z))	C28H48NO7P	[M+H]+1	[-0,42,1,28]	84.59	0.43
			LysoPC(18:2)	C26H50NO7P	[M+H]+1	[-0,83,0,91]	53.17	0.03
Phenylpropanoids and Polykeides	Isoflavonoids	O-methylated isoflavonoids	Homoferreirin	C17H16O6	[M-H]-1	[-1.43,0.27]	91.27	-0.57
Non-identified metabolite 50						[-1,7,-0,16]	98.85	-0.93
Non-identified metabolite 154						[-0,76,0,98]	58.28	0.09
Non-identified metabolite 170						[-0,55,1,2]	79.09	0.34
Non-identified metabolite 189						[-1,02,0,72]	63.62	-0.14
Non-identified metabolite 198						[-0,33,1,4]	87.55	0.48
Non-identified metabolite 214						[-1,16,0,59]	76.81	-0.31
Non-identified metabolite 215						[-1,06,0,68]	67.97	-0.20
Non-identified metabolite 216						[-1,04,0,72]	63.43	-0.14
Non-identified metabolite 217						[-1,19,0,54]	78.15	-0.33

Non-identified metabolite 218	[-0,8,0,96]	56.33	0.06
Non-identified metabolite 219	[-1,07,0,67]	67.77	-0.20
Non-identified metabolite 322	[-1,81,-0,32]	99.63	-1.06
Non-identified metabolite 323	[-1,87,-0,42]	99.81	-1.12
Non-identified metabolite 346	[-1,78,-0,29]	99.62	-1.06
Non-identified metabolite 364	[-1,02,0,74]	63.02	-0.14
Non-identified metabolite 374	[-1,12,0,63]	71.18	-0.24
Non-identified metabolite 395	[-1,81,-0,34]	99.65	-1.05
Non-identified metabolite 424	[-1,24,0,49]	82.16	-0.39
Non-identified metabolite 458	[-1,81,-0,32]	99.59	-1.05
Non-identified metabolite 461	[0,27,1,76]	99.51	1.02
Non-identified metabolite 490	[-0,3,1,39]	90.14	0.54
Non-identified metabolite 609	[-0,79,0,96]	57.56	0.08

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3.2.2.8 References

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Chapter IV. General Discussion

Poultry production is one of the most important agriculture-based industry due to its high degree of specialisation and the production of safe, nutritive, appealing, and healthy poultry foods (OECD/FAO, 2022). The consumers increasingly demand must be accompanied of higher quality standards to prevent the spread of pathogens that could pose a risk not only to animals, but also to humans (Borda-Molina et al., 2018, Espinosa et al., 2020). In this context, poultry sector with governs, private companies, universities, and research centers have made substantial investments to control important zoonotic microorganism such as *Salmonella*. Despite their efforts, currently *Salmonella* continuous to be one of the most frequently isolated foodborne pathogens worldwide, and the second zoonotic foodborne disease in the EU; being poultry products considered the main source of the infection (EFSA and ECDC, 2021). To improve the control of this bacterium, phages has been considered an innovative preharvest strategy in poultry production (Alali and Hofacre, 2016; Ruvalcaba-Gómez et al., 2022). Research has focused on the use of phages to reduce zoonotic bacteria pre-harvest (Carvalho et al., 2010; Loc-Carrillo and Abedon, 2011; Nabil et al., 2018; Ruvalcaba-Gómez et al., 2022), post-harvest (Duc et al., 2018; Esmael et al., 2021), and/or the surface in livestock facilities and food industries (Woolston et al., 2013). However, it is important to highlight that the administration method needs to be practical and suitable from a commercial point-of-view, and also it must take into account its impact on the gut ecology homeostasis (Thanki et al., 2021a; Clavijo et al., 2022). In this sense, gut microbiota plays a key role in vital metabolic functions, with a great impact on host biological functions, health states, disease progression and performance (Tang et al., 2019; Chen et al., 2019). In light of the above, before making any decisions regarding the use of phage as a therapy, our knowledge of phage-host interactions must be increased (Sutton and Hill, 2019). Thus, this doctoral thesis has been focused on evaluating the effect of phage application in free-target bacteria broilers and in *Salmonella*-infected broilers, both in the dynamics of phage, and *Salmonella* control, as well as its effect on the intestinal ecology homeostasis.

Oral phage therapy has been considered an applicable tool for *Salmonella* control in poultry production (Ahmadi et al., 2016; Adhikari et al., 2017; Nabil et al., 2018), especially for the *en masse* treatment application, overcoming a major limiting factor for large scale poultry (Reynaud et al., 1992). However, inherent challenges during the GIT transit, could compromise its efficacy or lead to divergent results, due to the lack of phage stability to gastric acidity, digestive enzymes and bile salts, or the relatively short

residence times in the intestinal tract (Sabouri et al., 2016; Ma et al., 2016; Malik et al., 2017a; Abdelsattar et al., 2019). In this sense, encapsulation has provided a protective delivery technique for the release of the phages with minimal loss at the caeca, the predilected *Salmonella* colonization site, incorporating approaches for burst release and/or sustained release (Ahmadi et al., 2016; Dąbrowska, 2018; Vinner et al., 2019). So, the aim of the first part was to study the phage gastrointestinal dynamics in *Salmonella*-free broilers and its influence on microbiota and metabolome (**3.1**).

For this purpose, firstly, to assess the phage dynamics within the GIT of *Salmonella*-free broilers, an *in vitro* model that simulates the GIT conditions of the broilers was used (**3.1.1**). To improve the efficacy of the orally administered phages, the phages were encapsulated (L100 and S100). Encapsulation not only could provide protection from gastric acidity but also, could incorporate approaches for burst release and/or sustained release on the pathogen colonization site (Ly-Chatain, 2014; Hussain et al., 2015; Rastogi et al., 2016). The main results obtained showed that compared to non-encapsulated phages, microencapsulation could protect phages from GIT conditions (PV-gizzard pH) and that they could be released until the caeca under simulated conditions *in vitro*. Then, when phages were assessed *in vivo* in day-old chicks (**3.1.1**), when animals are particularly susceptible to *Salmonella* infection and consequently could compromise the entire production cycle. The results demonstrated that encapsulated and non-encapsulated phages were subject to the complex external factors that influence the treatment success. In this sense, the encapsulated and non-encapsulated phage could survive through the GIT and were excreted in the faeces in one-day-old chicks. Regarding the survival of phages formulated in different encapsulated forms administered to one-day-old chicks, slightly higher concentrations of phage encapsulated in L100 were found to be delivered to the intestine compared with the S100 formulation. The apparent dissimilar results between groups were most likely since the encapsulations required extra and different times to release and distribute the phage, among the GIT digesta while being carried at the rate of feed passage (Ma et al., 2016). Since the GIT conditions change throughout the life of the chicken, the phage dynamics in the GIT throughout the rearing period of broilers have been studied to assess the best intervention moment to prevent the spread of *Salmonella* in chicken farms.

However, phage application during the entire rearing period may not be interesting from an economical point of view, so it is interesting to know when it is the best moment to

apply phages maximizing its efficiency. Then, the dynamics of the phage delivery along the GIT depending on the moment of administration during the rearing period and its effect on the microbiota and metabolome was evaluated (**3.1.2**). Results obtained showed that L100 delivery protected the phage through the GIT in each week of the chicken rearing period, especially if they are administered at the beginning of the production cycle (week 1 of rearing). The first week of rearing is a critical and decisive moment for the chicks, as the immune system of the animals is still immature and their gut microbiota is naïve, chicks are more susceptible to *Salmonella* colonization and multiplication, thereby compromising the entire production cycle (Marin and Lainez, 2009; Hashemzadeh et al., 2010; Koutsoumanis et al., 2019; Groves et al., 2021). Thus, to ensure a *Salmonella*-free flock at the field level a pivotal fact is the protection of young animals (Kempf et al., 2020). Moreover, it was shown that for both FP and L100 delivery methods, high concentrations of phages was present in the caecum. A hypothesis that could explain the high concentration at the end of the GIT may be the ability of phages to increase its concentration after interacting with the microbiota (non-target species) (Ganeshan and Hosseinidoust, 2019).

In addition to the possible interaction of the phages with the rest of the microbiota, to ensure confidence in the use of phages, is needed to reach a consensus on the impact of bacteriophage therapy in the gut environment (Javaudin et al., 2021) (**3.1.3**). In this sense, gut microbiota plays a symbiotic role as a "metabolic organ" ensuring several metabolic functions essential to the host with long-term physiological effects (Robinson et al., 2022). Thus, the knowledge of phage-host microbiota interactions must be increased to make decisions in the development of phage as a therapy (Sutton and Hill, 2019). Based on the nature of the phages, as a virus that has a one-to-one correspondence with specific bacteria (Loc-Carrillo and Abedon, 2011), slight changes have been observed in the caecal microbiota after the oral application of non-encapsulated and encapsulated *Salmonella* phages in *Salmonella*-free animals. Different theories seek to shed light on these phenomena, such as the molecular changes, as single amino acid substitutions and unusual homologous intragenomic recombination that could promote the viral host jump and the diversification of the phage-host spectrum (de Sordi et al., 2017). After FP and L100 administration, genera involved in recovered intestinal homeostasis after dysbiosis events (Muñoz et al., 2020), vitamin production and antibacterial properties (Wang et al., 2014; Rodrigues et al., 2020), digestibility (Zhong et al., 2021) carbohydrate fermentation

and short-chain fatty acid production (Memon et al., 2022) showed alterations. Despite few genera were altered, significantly alterations were observed on the metabolome, with the most significant effect in FP, and particularly affecting lipid metabolism and organic oxygen compounds notably. Phage predation could knock down associated metabolic products (Hsu et al., 2019). In this sense, previous authors showed that after phage application, the metabolism of amino acids and nucleotides were altered, (Han et al., 2022). In our study, the differences observed between FP and L100 may be due to the timing of the infective cycle of the phage due to phage arrival time and bacterial stress (Han et al., 2022). Taking into account that gut metabolites could not only impact the balance of intestinal microecology but could also regulate anatomically distant biological systems from the gut *via* the bloodstream (Lu et al., 2021; Tomasova et al., 2021), it will be important to shed light and better investigate on all the changes that are taking place.

In view of these results, further *Salmonella* challenge studies have been necessary to evaluate the control effects of the administration *via* feed of encapsulated phages in the field during the rearing period. So, the aim of the second part was to assess the bacteriophage dynamics in *Salmonella*-infected broilers and its influence on microbiota and metabolome (**3.2**).

For this purpose, firstly, L100 was administered with the starter diet on the 21 first days of the rearing period (**3.2.1**). The implementation of phage therapy using a starter diet could overcome different challenges such as the *en mass* application in broiler large productions or the highest susceptibility of young chickens to the *Salmonella* infection and colonization (Barrow et al., 2004; Marin and Lainez, 2009; Foley et al., 2013; Berry and Wells, 2016; Thanki et al., 2021). Bacteriophage supplementation decreased the *S.* Enteritidis carriage in the caeca and decreased the excretion in the second, fourth and fifth weeks of rearing, key moments of the rearing period (Marin and Lainez, 2009), and removed the bacteria from the environment at the end of the rearing (Sevilla-Navarro et al., 2018). Nevertheless, phage application didn't reach total bacteria elimination (Adhikari et al., 2017; Nabil et al., 2018). In this respect, the synergistic effects of combining phages with other alternatives (such as probiotics, prebiotics, symbiotic, etc.) could be a promising approach to the total bacteria elimination (Kim et al., 2013; Ruvalcaba-Gómez et al., 2022). Moreover, the evaluation of the immune status of these animals thought the measurement of the immune organs (bursa of Fabricius, spleen, and

liver), showed that the phage application was not harmful to the animal, and even improve the weight of the bursa of Fabricius in the treated animals. Beyond that phages is a potential 360-degree *Salmonella* control strategy in poultry, as we have already mentioned, the microbiota plays an essential role in the evolution of pathogen infections, being in constant dynamic equilibrium. Therefore, its modulation when therapy is carried out may not only affect production parameters, but also the achievement of animals free of pathogenic bacteria. Therefore, the study of caecal ecology can shed light on the impact that phage therapy against *Salmonella* will have (**3.2.2**).

The chickens microbiota after *Salmonella* elimination will not undergo modifications in alpha or beta diversity, unlike when an antibiotic is administered (Kairmi et al., 2022). In fact, the wide range of action of antibiotics often leads to dysbiosis after treatment (Elokil et al., 2020). Moreover, when microbiota alterations of treated broilers have been detected after phage treatment, the changes have been transitory, unlike the changes produced by antibiotics (Kosznik-Kwaśnicka et al., 2022). There are certain bacteria that will be altered after phage therapy. This is not surprising, as the elimination of the bacteria will lead to cascading effects on the other bacteria that will produce a change in the intestinal paradigm (Hsu et al., 2019; Kosznik-Kwaśnicka et al., 2022). In addition to the changes observed in the microbiota, there are also changes in the metabolome of these animals, as changes in the regulation of metabolites are observed. In addition, host metabolomics may enable global metabolite alterations in response to the therapies mediated by caecal secretion (Elokil et al., 2020). However, the caecal changes are not reflected at the systemic level, as the serum metabolome and production parameters of these animals were not altered. However, given the fact of the short production period of broilers, we cannot expect that slight alterations will produce major changes in their physiology, nevertheless, further studies on long-term effects may be observed in long-life production systems such as laying hens or breeders have to be done to study phage treatment effects.

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Chapter V. Conclusions

1. Significant differences were observed between phage delivery results of *in vitro* studies compared with *in vivo* results. In one-day-old chicks there were no statistically significant differences between phage delivered along the GIT for the encapsulated and non-encapsulated phage (the gut being the exception, but differences were small here too). Encapsulation of the phage using the polymers Eudragit® L100 and Eudragit® S100 resulted in delivery of phage in day-old chicks with no adverse reactions observed in the animals. Further studies are needed to better understand the dynamics of the encapsulated phage released during transit through the GIT of the chickens during the entire production cycle.

2. Bacteriophage encapsulation with the polymer Eudragit® L100, especially when administered at the beginning and at the end of the cycle, could ensure targeted delivery of high titres of phages to the caecum affording encapsulated phages protection from the harsh environmental conditions found in the PV-Gizzard. Moreover, the fact that more encapsulated phages were found in the crop and caecum, known sites of high *Salmonella* colonization, makes encapsulation of phages a promising tool to control the bacteria at the field level. On the other hand, the easy dissemination of the phages through faeces may also facilitate the control of the bacterium in the farm environment. However, further *Salmonella* challenge studies are necessary to evaluated the beneficial effects of encapsulation of phages using L100 formulation to control the bacteria in the field during the rearing period.

3. Preventive therapy with bacteriophages minimally alters the intestinal microbiota but significantly impacts their metabolites, regardless of the route of administration. Further studies are needed to understand the potential interplay between differentially abundant bacterial species and significantly altered metabolites to clarify phage treatment implications.

4. *S*. Enteritidis flock contamination may be markedly curtailed through L100 encapsulated phage application as a feed additive in the starter diet during rearing. A reduction in *Salmonella* colonization and excretion was noted with complete elimination of bacteria recorded from the environment at the end of the rearing period. However, higher phage doses, improved delivery protocols and/or combination with other strategies may be necessary to achieve total elimination of *Salmonella* from the animals. This study provides important insights into the use of phages as a preventative and biocontrol strategy against *Salmonella* infection from farm-to-fork.

5. The application of *Salmonella* phages under production conditions modulates the cecal microbiome and metabolome profiles in broilers. However, the response in blood serum metabolites and growth performance suggests that the phage modulation seems have no biological significance. Further studies are required to assess whether such a shift implies that *Salmonella* phages shift the microbiota composition, which promotes the change in metabolic profile, or whether the phages are actively involved in metabolite changes.