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Selection and Assessment of Bacteriophages against *Salmonella* in the Poultry Sector

PhD Thesis by
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Selection and Assessment of Bacteriophages against *Salmonella* in the Poultry Sector

A thesis submitted to the Polytechnic University of Valencia in partial fulfilment of the requirements for the degree of Doctor of Philosophy

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ABSTRACT/RESUMEN/RESUM

ABSTRACT

Millions of human salmonellosis cases are reported worldwide every year, and the disease results in thousands of deaths. In the United States, *Salmonella* causes around 1.2 million, whereby 23,000 hospitalisations and 450 deaths every year. In 2018, a total of 94,203 confirmed cases in humans were reported in Europe of which 8,730 were in Spain. The main source of infection are poultry products as eggs and chicken meat. The main serovars related to these outbreaks are *Salmonella* Enteritidis, *Salmonella* Typhimurium and *Salmonella* Typhimurium monophasic variant. However, in recent years, the prevalence of other serotypes such as *Salmonella* Infantis, which are currently the most prevalent serovars in broiler chickens, has been on the rise.

National *Salmonella* Control Programmes together with biosecurity measures, cleaning and disinfection protocols and good hygiene practices have succeeded in reducing the prevalence of the bacteria at the field level. However, new cases of salmonellosis in humans continue to emerge every year and the presence of the bacterium continues to be detected in poultry farms. Therefore, new alternatives continue to be sought in the fight against the bacteria, such as the use of additives in feed, good management at field level or the use of bacteriophages.

Bacteriophages or phages are ubiquitous viruses in the environment and are widely distributed in nature. They are microorganisms that specifically attack bacteria, altering them until they are destroyed. These characteristics make phages a very promising tool for the elimination of *Salmonella* in poultry farms as a complementary tool for cleaning and disinfection, and as another control tool to be included in good hygiene practices at field level.

Thus, in this doctoral thesis we have focused our studies on determining the phage diversity in poultry farms and studying the application of phage therapy for the control of *Salmonella* as a complementary measure to cleaning and disinfection.

To this end, three experiments were carried out during the period from January 2017 to December 2019, where phages were isolated from different samples taken from poultry farms and their epidemiology was studied. Moreover, phages isolated were purified,

multiplied and used against *Salmonella* Enteritidis and *Salmonella* Infantis to assess their efficacy as sanitisers in poultry farms.

The objective of the **first experiment** was to assess *Salmonella*-phage prevalence in commercial poultry farms in terms of the production livestock type: layers or broilers. The most prevalent *Salmonella* serovars isolated in poultry production were used for phage isolation. *Salmonella*-specific phages were isolated from 141 faeces samples taken from layers (n = 108) and broilers (n = 33) from different farms located in the Valencia Region during June and July 2019. Analysis of the samples revealed that 100% presented *Salmonella* phages, the most prevalent being the *Salmonella* phage serovar *Salmonella* Enteritidis (93%), followed by *Salmonella* Virchow (59%), *Salmonella* Typhimurium (55%), *Salmonella* Infantis (52%) and *Salmonella* Ohio (51%). These results indicate that poultry farms could represent an important source of *Salmonella* phages. Moreover, we have shown a close relationship between *Salmonella* phage prevalence and *Salmonella* serovar prevalence in poultry farms, suggesting that phages co-exist within their serovar. Furthermore, the fact of isolating a higher number of phages against *Salmonella* Enteritidis makes the vaccine a double control measure, as it immunises the animals and increases the prevalence of phages against public health isolates. Finally, the more bacteria we find in the environment, the more serovar-specific phages may be present. In this context, we could isolate the wild *Salmonella* strains, increase the concentration of the environmental wild *Salmonella* phages, and apply them in poultry *Salmonella*-positive farms to control *Salmonella* contamination at field level.

The purpose of the **second experiment** was to assess the effect of phages against *Salmonella* Infantis and *Salmonella* Enteritidis on farm surfaces and evaluate bacteriophage application procedure as sanitiser against *Salmonella* in field conditions. Thus, the most prevalent serovars in poultry production were selected (*Salmonella* Infantis and *Salmonella* Enteritidis) to contaminate farm facilities. Then, two specific phages isolated from poultry faeces were applied against them. Results showed *Salmonella* Infantis and *Salmonella* Enteritidis decreased by 4.55 $\log_{10}$ CFU/mL and 3.85 $\log_{10}$ CFU/mL, respectively; the maximum reduction in *Salmonella* was on the 5^{th} day, after 10^8 PFU/mL and 10^3 PFU/mL bacteriophage application (7.00 $\log_{10}$ CFU/mL and 4.10 $\log_{10}$ CFU/mL, respectively). These results highlight phages as a promising tool

together with cleansing and disinfection. However, more studies are needed to demonstrate their efficacy as sanitisers in poultry farms.

Finally, the aim of the **third experiment** was to assess the application of autophages for the control of *Salmonella* Enteritidis in a naturally contaminated laying hen farm. The study was carried out in an experimental house whose health status revealed the presence of *Salmonella* Enteritidis. The autophage was isolated and the phenotype from environmental samples taken from the same shed where it was subsequently applied to evaluate its effect as a *Salmonella* control tool. It was sprayed onto the animals and on the surfaces of the facilities at two times separated by 24 hours. A total of 48 samples (40 faeces samples and 8 cloth samples) taken in 4 different sampling sessions were analysed. Swab cloth samples taken before the autophage application showed the presence of *Salmonella*, but after the first application of the autophage, all swab samples were negative for *Salmonella*. Concerning faeces samples, statistically significant differences were obtained between the results obtained before (2.34 $\log_{10}$ CFU/g) and after (1.07 $\log_{10}$ CFU/g) application of the autophage. This study shows that autophages could be used not only as a measure to reduce the excretion of *Salmonella* by infected animals, but also as a complementary measure in the cleaning and disinfection of the facilities. In addition, the fact that autophages eliminated the *Salmonella* from the environment, even with the animals inside the facility, could avoid horizontal transmission of *Salmonella* among infected and non-infected animals.

In conclusion, phages are bacteria-specific viruses that can be used not only as an antimicrobial to treat animal infection, but also as a complementary tool in the cleaning and disinfection process of poultry farms. The most relevant results obtained from this doctoral thesis are that the poultry sector has a promising, economic and ecological tool able to significantly reduce the prevalence of *Salmonella* when current measures cannot eliminate it from poultry farms. However, further studies are needed to demonstrate the efficacy of phages in combination with other current control measures, such as additives or cleaning and disinfection protocols.

Salmonella es una de las principales causas de toxiinfecciones alimentarias en el mundo. En Estados Unidos, causa cerca de 1,2 millones de casos anuales, de los cuales 23.000 son hospitalizados y 450 fallecen. En Europa, los últimos datos recogidos por la Autoridad Europea de Seguridad Alimentaria publicaron un total de 94.203 casos, de los cuales 8.730 fueron en España. La principal fuente de la infección son los productos de origen animal, principalmente los huevos y la carne de pollo, siendo *Salmonella* Enteritidis, *Salmonella* Typhimurium y *Salmonella* Typhimurium monofásica los principales serotipos relacionados con las toxiinfecciones humanas. Sin embargo, en los últimos años está aumentando la prevalencia de otros serotipos de gran relevancia para la salud pública, como *Salmonella* Infantis, llegando a ser el más prevalente en pollo de engorde.

Los Programas Nacionales de Control de *Salmonella* junto con las medidas de bioseguridad, protocolos de limpieza y desinfección, así como buenas prácticas de higiene, han logrado reducir la prevalencia de la bacteria a nivel de campo. Sin embargo, cada año siguen surgiendo nuevos casos de salmonelosis en la especie humana, y se sigue detectando la presencia de la bacteria en las explotaciones avícolas. Por ello, se continúan buscando nuevas alternativas en la lucha contra la bacteria, como pueden ser el empleo de aditivos en el pienso, un buen manejo a nivel de campo o el uso de bacteriófagos.

Los bacteriófagos son virus ubicuos en el ambiente y se encuentran ampliamente distribuidos en la naturaleza. Son microorganismos que atacan específicamente a bacterias, alterándolas hasta destruirlas. Estas características hacen de los fagos una herramienta muy prometedora para la eliminación de *Salmonella* en explotaciones avícolas como medida complementaria a la limpieza y desinfección, y como una herramienta más de control para incluir en las buenas prácticas de higiene a nivel de campo.

En este contexto, en esta tesis doctoral hemos centrado nuestros estudios en conocer la diversidad fágica en las explotaciones avícolas, y estudiar la aplicación de la terapia fágica para el control de *Salmonella* como medida complementaria a la limpieza y desinfección.

Para ello, se realizaron tres experimentos durante el periodo de enero de 2017 y diciembre de 2019, en los que se aislaron bacteriófagos de diferentes muestras procedentes de granjas avícolas y se estudió su epidemiología para después multiplicarlos y enfrentarlos a dos de los principales serotipos, *Salmonella* Enteritidis y *Salmonella* Infantis.

El objetivo del **primer experimento** fue evaluar la prevalencia de bacteriófagos frente a *Salmonella* en granjas comerciales de gallinas ponedoras y broilers. Para ello, se aislaron bacteriófagos específicos de *Salmonella* a partir de 141 muestras de heces de granjas de gallinas ponedoras (n = 108) y broilers (n = 33) localizadas en la Comunidad Valenciana. Para el aislamiento de los fagos, se emplearon los serotipos más relevantes aislados en avicultura (*Salmonella* Enteritidis, *Salmonella* Typhimurium, *Salmonella* Typhimurium monofásica, *Salmonella* Infantis, *Salmonella* Hadar, *Salmonella* Virchow, *Salmonella* Senftenberg, *Salmonella* Ohio y *Salmonella* Kentucky). El análisis de las muestras reveló que el 100% presentaban fagos frente a *Salmonella*, siendo los más prevalentes los bacteriófagos frente a *Salmonella* Enteritidis (93%), seguidos de *Salmonella* Virchow (59%), *Salmonella* Typhimurium (55%), *Salmonella* Infantis (52%) y *Salmonella* Ohio (51%). Estos resultados indican que las granjas avícolas podrían representar una importante fuente de fagos de *Salmonella*. Además, se ha observado una estrecha relación entre la prevalencia de bacteriófagos frente a *Salmonella* y la prevalencia de serotipos de *Salmonella* en las granjas avícolas, lo que sugiere que los fagos coexisten con su serotipo. Además, el hecho de aislar un mayor número de bacteriófagos frente a *Salmonella* Enteritidis sugiere que la vacuna sea una doble medida de control, ya que inmuniza a los animales y aumenta la prevalencia de fagos contra los aislados de importancia en salud pública. Por último, a mayor concentración bacteriana en el medioambiente puede aumentar la probabilidad de aislar bacteriófagos frente a ella. En este contexto, se podrían aislar los bacteriófagos presentes en las granjas contaminadas con *Salmonella*, aumentar su concentración y aplicarlos frente a las mismas granjas avícolas positivas a *Salmonella.*

El objetivo del **segundo experimento** fue evaluar el efecto de los bacteriófagos contra *Salmonella* Infantis y *Salmonella* Enteritidis en las superficies de las granjas, y evaluar la aplicación del procedimiento de los bacteriófagos como desinfectante contra *Salmonella* en las condiciones de campo. En este estudio se seleccionaron dos serotipos de gran relevancia en la producción avícola (*Salmonella* Infantis y *Salmonella* Enteritidis) para contaminar las superficies del suelo. Posteriormente, se aplicaron contra ellos dos

bacteriófagos aislados de las mismas granjas avícolas. El estudio se realizó dentro de una granja experimental para imitar las condiciones de producción reales. La concentración de *Salmonella* Infantis y *Salmonella* Enteritidis en las superficies de la nave disminuyó en 4,55 $\log_{10}$ UFC/mL y 3,85 $\log_{10}$ UFC/mL, respectivamente, después de dos aplicaciones consecutivas de bacteriófagos. La mayor reducción de *Salmonella* Infantis y *Salmonella* Enteritidis se obtuvo el 5º día después de la primera aplicación (7,00 $\log_{10}$ CFU/mL y 4,10 $\log_{10}$ CFU/mL, respectivamente). Estos resultados ponen de manifiesto que los bacteriófagos podrían ser una herramienta prometedora para utilizar en combinación con los procedimientos de limpieza y desinfección. Sin embargo, es necesario realizar más estudios para demostrar la eficacia de los bacteriófagos como desinfectantes en las explotaciones comerciales.

Por último, el objetivo del **tercer experimento** fue evaluar la aplicación experimental de los autofagos para el control de *Salmonella* Enteritidis en una granja de gallinas ponedoras contaminada de forma natural. El estudio se llevó a cabo en una nave experimental cuyo estatus sanitario reveló presencia de *Salmonella* Enteritidis. Se aisló y fenotipó el autofago a partir de muestras ambientales tomadas de la misma nave donde posteriormente fue aplicado para evaluar su efecto como herramienta de control de *Salmonella*. Se aplicó vía pulverización sobre los animales y sobre las superficies de la nave en dos tiempos separados por 24 horas. Se analizaron un total de 48 muestras (40 muestras de heces y 8 muestras de paños) tomadas en 4 sesiones de muestreo diferentes. Las muestras de paños tomadas previa aplicación del autofago dieron resultado de presencia de *Salmonella*, sin embargo, tras la primera aplicación del autofago, todas de las muestras de paños dieron ausencia a *Salmonella*. Por lo que respecta a los recuentos obtenidos de las muestras de heces tras la aplicación del bacteriófago sobre los animales, se obtuvieron diferencias estadísticamente significativas entre los resultados obtenidos antes (2,34 $\log_{10}$ UFC/g) y después (1,07 $\log_{10}$ UFC/g) de la aplicación del autofago. Este estudio pone en evidencia que los autofagos se podrían emplear, no solo como una medida para reducir la excreción de *Salmonella* por parte de los animales infectados, sino como medida complementaria en la limpieza y desinfección de las instalaciones con una gran eficacia. Además, el hecho de que el autofago eliminara la *Salmonella* del medio ambiente, aún con los animales en el interior de la nave, podría evitar la re-contaminación horizontal entre animales infectados y no infectados.

En conclusión, los bacteriófagos son virus específicos de bacterias que pueden utilizarse no solo como antimicrobianos para tratar la infección de animales, sino también como una herramienta complementaria del proceso de limpieza y desinfección de las granjas avícolas. Los resultados más relevantes que se han obtenido de esta tesis doctoral es que el sector avícola dispone de una herramienta prometedora, económica y ecológica capaz de reducir significativamente la prevalencia de *Salmonella* cuando las medidas actuales no son capaces de eliminarla de las granjas avícolas. Sin embargo, se necesitan más estudios para demostrar la eficacia de los fagos en combinación con otras medidas de control actuales, como los aditivos o los protocolos de limpieza y desinfección.

Salmonella és una de les principals causes de toxiinfeccions alimentàries en el món. En Estats Units, causa prop de 1,2 milions de casos anuals, dels quals 23.000 són hospitalitzats i 450 moren. A Europa, les últimes dades arreplegats per l'Autoritat Europea de Seguretat Alimentària van reportar 94.203 casos, dels quals 8.730 es van produir a Espanya. La principal font d'infecció són els productes d'origen animal, principalment els ous i la carn de pollastre. Els principals serotips relacionats amb estes toxiinfeccions són *Salmonella* Enteritidis, *Salmonella* Typhimurium i *Salmonella* Typhimurium monofàsica, no obstant, en els últims anys està augmentant la prevalença d'altres serotips com a *Salmonella* Infantis, arribant a ser el més prevalent en la producció càrnica de pollastre.

Els Programes Nacionals de Control de *Salmonella* junt amb les mesures de bioseguretat, protocols de neteja i desinfecció, així com bones pràctiques d'higiene, han aconseguit reduir la prevalença a nivell de camp. No obstant, cada any continuen sorgint nous casos de salmonel·losi, i es continua detectant la presència de la bacteria en algunes explotacions avícoles. Per això, es continua buscant noves alternatives en la lluita contra la bacteria, com pot ser l'ocupació d'additius en el pinso, un bon maneig a nivell de camp o l'ús de bacteriòfags.

Els bacteriòfags són virus ubics en l'ambient i es troben àmpliament distribuïts en la naturalesa. Són microorganismes que ataquen específicament a les bacteries, alterant-les fins a destruir-les. Estes característiques fan dels bacteriòfags una ferramenta molt prometedora per a l'eliminació de *Salmonella* en explotacions avícoles com a mesura complementària a la neteja i desinfecció i com una ferramenta més de control per a incloure en les bones pràctiques d'higiene.

En este context, en esta tesi doctoral hem centrat els nostres estudis a conèixer la diversitat fàgica en les explotacions avícoles i estudiar l'aplicació de la teràpia fágica per al control de *Salmonella* com a mesura complementària a la neteja i desinfecció. Per a això, es van realitzar tres experiments durant el període de gener de 2017 i desembre de 2019, en els que es van aïllar bacteriòfags de diferents mostres procedents de granges avícoles i es va

estudiar la seua epidemiologia per a després multiplicar-los i enfrontar-los a dos dels principals serotips, *Salmonella* Enteritidis i *Salmonella* Infantis.

L'objectiu del **primer experiment** va ser avaluar la prevalença de bacteriòfags enfront de *Salmonella* en granges comercials de gallines ponedores i pollastres. Per a això, es van aïllar bacteriòfags específics de *Salmonella* a partir de 141 mostres d'excrements de granges de gallines ponedores (n = 108) i pollastres (n = 33) localitzades a la Comunitat Valenciana. Per a l'aïllament dels bacteriòfags, es van emprar els serotips més rellevants aïllats en avicultura (*Salmonella* Enteritidis, *Salmonella* Typhimurium, *Salmonella* Typhimurium monofàsica, *Salmonella* Infantis, *Salmonella* Hadar, *Salmonella* Virchow, *Salmonella* Senftenberg, *Salmonella* Ohio i *Salmonella* Kentucky). L'anàlisi de les mostres va revelar que el 100% presentava fagos enfront de *Salmonella*, sent els més prevalents els fagos enfront de *Salmonella* Enteritidis (93%, seguits de *Salmonella* Virchow (59%), *Salmonella* Typhimurium (55%), *Salmonella* Infantis (52%) i *Salmonella* Ohio (51%). Estos resultats indiquen que les granges avícoles podrien representar una important font enfront de bacteriòfags de *Salmonella*. A més, s'ha observat una estreta relació entre la prevalença de bacteriòfags enfront de *Salmonella* i la prevalença de serotips de *Salmonella* en les granges avícoles, la qual cosa suggereix que els bacteriòfags coexisteixen amb el seu serotip. A més, el fet d'aïllar un nombre més gran de bacteriòfags enfront de *Salmonella* Enteritidis suggereix que la vacuna siga una doble mesura de control, ja que immunitza els animals i augmenta la prevalença de bacteriòfags contra els aïllats d'importància en salut pública. Finalment, a major concentració bacteriana en el medi ambient pot augmentar la probabilitat d'aïllar bacteriòfags enfront d'ella. En este context, es podrien aïllar els bacteriòfags presents en les granges contaminades amb *Salmonella*, augmentar la seua concentració i aplicar-los enfront de les mateixes granges avícoles positives a *Salmonella*.

L'objectiu del **segon experiment** va ser avaluar l'efecte dels bacteriòfags contra *Salmonella* Infantis i *Salmonella* Enteritidis en les superfícies de les granges, i avaluar l'aplicació del procediment dels bacteriòfags com a desinfectant contra *Salmonella* en les condicions de camp. En este estudi es van seleccionar dos serotips de gran rellevància en la producció avícola (*Salmonella* Infantis i *Salmonella* Enteritidis) per a contaminar les superfícies del sòl i es van aplicar contra ells dos bacteriòfags aïllats de les granges avícoles. L'estudi es va realitzar dins d'una granja experimental per a imitar les condicions

de producció reals. La concentració de *Salmonella* Infantis i *Salmonella* Enteritidis va disminuir 4,55 $\log_{10}$ UFC/mL i 3,85 $\log_{10}$ UFC/mL, respectivament, després de dos aplicacions consecutives de bacteriòfags. La major reducció de *Salmonella* Infantis i *Salmonella* Enteritidis es va obtindre el 5º dia després de la primera aplicació (7,00 $\log_{10}$ CFU/mL i 4,10 $\log_{10}$ CFU/mL, respectivament). Estos resultats manifesten que els bacteriòfags són una ferramenta prometedora per a utilitzar en combinació amb els procediments de neteja i desinfecció. No obstant, es necessiten més estudis per a demostrar l'eficàcia dels bacteriòfags com a desinfectants en les explotacions comercials.

Finalment, l'objectiu del **tercer experiment** va ser avaluar l'aplicació experimental dels autòfags per al control de *Salmonella* Enteritidis en una granja de gallines ponedores. L'estudi es va dur a terme en una nau experimental l'estatus sanitari de la qual va revelar presència de *Salmonella* Enteritidis. Es va aïllar i fenotip l'autòfag a partir de mostres ambientals preses de la mateixa nau on posteriorment va ser aplicat per a avaluar el seu efecte com a ferramenta de control de *Salmonella*. Es va aplicar via polvorització sobre els animals i sobre les superfícies de la nau en dos temps separats per 24 hores. Es van analitzar un total de 48 mostres (40 mostres d'excrements i 8 mostres de draps) preses en 4 sessions de mostratge diferents. Les mostres de draps preses amb l'aplicació prèvia de l'autòfag van donar resultat de presència de *Salmonella*, no obstant , després de la primera aplicació de l'autòfag, el 100% de les mostres de draps van donar absència a *Salmonella*. Pel que fa a les mostres d'excrements, es van obtindre diferències estadísticament significatives entre els resultats obtinguts abans (2,34 $\log_{10}$ UFC/g) i després (1,07 $\log_{10}$ UFC/g) de l'aplicació de l'autòfag. Este estudi posa en evidència que els autofagos es podrien emprar com a mesura complementària en la neteja i desinfecció de les instal·lacions amb una gran eficàcia i per a reduir els recomptes de *Salmonella* en animals infectats. A més, el fet de que l'autòfag eliminarà la *Salmonella* del medi ambient, encara amb els animals en l'interior de la nau, podria evitar la recontaminació horitzontal entre animals infectats i no infectats.

En conclusió, els bacteriòfags són virus específics de bactèries que poden utilitzar-se no sols com antimicrobians per a tractar la infecció d'animals, sinó també com una ferramenta complementària del procés de neteja i desinfecció de les granges avícoles. Els resultats més rellevants que s'han obtingut d'esta tesi doctoral és que el sector avícola disposa d'una ferramenta prometedora, econòmica i ecològica capaç de reduir

significativament la prevalença de *Salmonella* quan les mesures actuals no són capaços d'eliminar-la de les granges avícoles. No obstant, es necessiten més estudis per a demostrar l'eficàcia dels bacteriòfags en combinació amb altres mesures de control actuals, com els additius o els protocols de neteja i desinfecció.

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%	Percentage
AMP	Ampicillin
AMR	Antimicrobial Resistance
ANOVA	Analysis of Variance
AZM	Azithromycin
BOE	*Boletín Oficial del Estado* (Official State Gazette)
BPW	Buffered Peptone Water
C	Chloramphenicol
$CaCl_2$	Calcium Chloride
CAZ	Ceftazidime
CFU	Colony Forming Units
CIP	Ciprofloxacin
COL	Colistin
CTX	Cefotaxime
DNA	Deoxyribonucleic Acid
EC	European Commission
ECDC	European Centre for Disease Prevention and Control
EFSA	European Food Safety Authority
EU	European Union
g	Grams
GLM	General Linear Model
GN	Gentamicin
h	Hours
ICTV	International Virus Taxonomy Committee

ISO	International Organisation for Standardisation
LB	Luria-Bertani
M	Molar
MDR	Multidrug Resistance
MERO	Meropenem
$MgSO_4$	Magnesium Sulphate
min	Minutes
mL	Millilitres
MPN	Most Probable Number
MS	Member States
MSRV	Modified Semi-Solid Rappaport Vassiliadis
n	Number of samples
NAL	Acid Nalidixic
NCP	National Control Programmes
ºC	Celsius Degrees
OD	Optical Density
PCNV	Provisional Committee for Viral Nomenclature
PFU	Plaque Forming Units
RET	Ready to Eat Food
RNA	Ribonucleic Acid
RPM	Revolutions per minute
sec	Seconds
SMX	Sulphamethoxazole
spp.	Species
T	Times

TET	Tetracycline
TGC	Tigecycline
TM	Trimethoprim
XLD	Xylose-Lysine-Deoxycholate
µL	Microlitres
µm	Micrometres

CHAPTER I. GENERAL INTRODUCTION

1. General introduction

1. General aspects of bacteriophages

1.1.1 Historical context of bacteriophages

Bacteriophages or phages have been the subject of many studies throughout the world since they were discovered at the beginning of the 20th Century, firstly by Frederick William Twort (1915), and secondly by Felix d'Hérelle (1917), both acknowledged as the fathers of phage biology (Ackermann, 2011; Casey *et al*., 2018) (Figure 1). Before F.W. Twort and Felix d'Hérelle discovered phages, a British bacteriologist, Ernest Hanbury Hankin, reported that something in water from rivers in India had unexpected antibacterial properties against cholera. Moreover, although water had been passed through a fine porcelain filter, it kept this distinctive feature (Hankin, 1896). Later, F.W. Twort, observed "glassy and transparent" spots, which turned out to be zones of dead bacteria. Moreover, these zones were transmissible and specific to the type of bacteria that were contaminating his plates. He proposed three hypotheses for this phenomenon; (i) a manifestation of the bacteria life cycle; (ii) an enzyme produced by the bacteria; or (iii) an ultra-microscope virus. However, after some years, F.W. Twort abandoned his research on phages (Keen, 2015).

It was just two years later when Felix d'Hérelle published similar observations to Twort during World War I. The unsanitary conditions in the trenches on the battle-fronts caused infections such as dysentery, affecting numerous soldiers. Thus, Felix d'Hérelle studied the bacterial cause of these infections and identified *Shigella* as the etiological agent of this rampaging infectious disease. Then, he co-incubated soldiers' faecal filtrates with the bacteria isolated on petri dishes, resulting in the death of the bacteria. In contrast to Twort, he was quite convinced that the phenomenon he observed was a virus able to infect bacteria (Norkin, 2013). During the 1920s and 1930s d'Hérelle developed phage-based treatments against a range of human infections including *Shigella dysenteriae, Salmonella* Typhi, *Escherichia coli*, *Pasteurella multocida* and *Vibrio cholerae* (Casey *et al*., 2018). Moreover, he was first to designate these microorganisms "bacteriophages", a name that came from bacteria and the Greek root *phagein* (from Greek "to eat"), which means "bacteria eaters" (Ackermann, 2011; Keen, 2015).

Figure 1. Fathers of phage biology: Frederick William Twort (left) and Felix d'Hérelle (right).

1.1.2 Characteristics of bacteriophages

Phages are the most abundant microorganism on Earth and have played an important role in molecular biology and biotechnology development (Jurczak-Kurek *et al.*, 2016; Maciejewska *et al.*, 2018). They remain stable in thermal conditions from 30ºC to 60ºC and pH ranges from 3 to 13 (Akhwale *et al.*, 2019). They are described as a natural predator of bacteria, ubiquitous, and estimated to be present at over 10^{31} phage particles on the planet (Keen, 2015; Furfaro *et al.*, 2018). Phages have been isolated from every environment where bacteria exist, and it is claimed that at least one type of phage specifically infects each bacteria strain (Comeaut *et al.*, 2008; Keen, 2015). Phages occur everywhere in the biosphere and have colonised even such habitats as volcanic hot springs (Ackermann, 2011). However, their main habitats are the ocean and topsoil, and the main reservoirs are lysogenic bacteria (Ackermann, 2011).

Phages, unlike other viruses, are virus that infect only prokaryotic cells (Clokie *et al.*, 2011). They have either DNA (Deoxyribonucleic Acid) or RNA (Ribonucleic Acid) as their genetic material, in either circular or linear configuration, as a single- or a double-stranded molecule. Phage morphology can be diverse, but commonly consists of a **head** containing the nucleic acid, and a **tail** that is used by the phage to attach itself to the

susceptible bacteria and inject its nucleic acid into the target bacteria to initiate the infection (Freeman, 2005) (Figure 2).

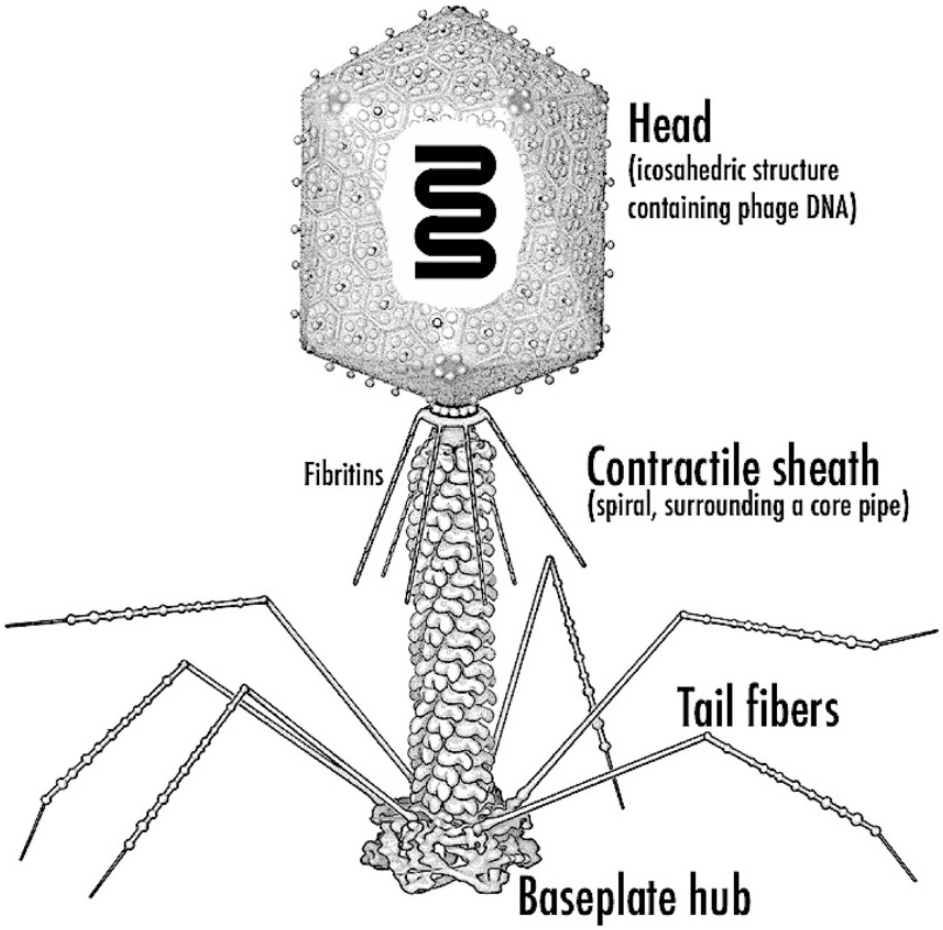

Figure 2. Phage structure
(Harada *et al.*, 2018).

All known phages can be divided into two main groups according to the type of infection. One group is characterised by a **lytic or virulent infection** and the other is represented by a **lysogenic or temperate infection** (Olszak *et al.*, 2017). Their biological cycle involves the attachment and invasion of the bacterium. Nevertheless, before infection, phage structures have to match strain-specific variants of bacterial receptors (Principi *et al.*, 2019).

Phages could present different kinds of replication cycles. For the lytic cycle, the phage injects its genetic material into the host bacterium and the phage genome synthesises early proteins that break down the host DNA, allowing the phage to take control of the cellular

machinery. Heads and sheaths are assembled separately, then a new genetic material is packed into the head and new progeny phage particles are constructed. During this process, the host cells gradually become weakened by phage enzymes and eventually burst, releasing on average 10-200 new phage progeny into the surrounding environment (Steward, 2018). On the other hand, lysogenic cycle is characterised by integration of the phage DNA into the host cell genome or as a plasmid. Incorporated phage DNA will be replicated along with the host bacteria genome and new bacteria will inherit the viral DNA. Such transition of viral DNA could take place through several bacterial generations without major metabolic consequences for the bacterium. Thus, lysogenic phages are not suitable for phage therapy (Steward, 2018) (Figure 3).

It is important to highlight that, regarding environmental conditions (temperature, pH, etc.) and the type of bacterial cell, there are several different pathways of phage infection, including **chronic**, **pseudolysogeny** and **abortive infection**. It is important to emphasise that not all phage cycles end with bacterial death, and replication of phage particles (Wernicki *et al.*, 2017).

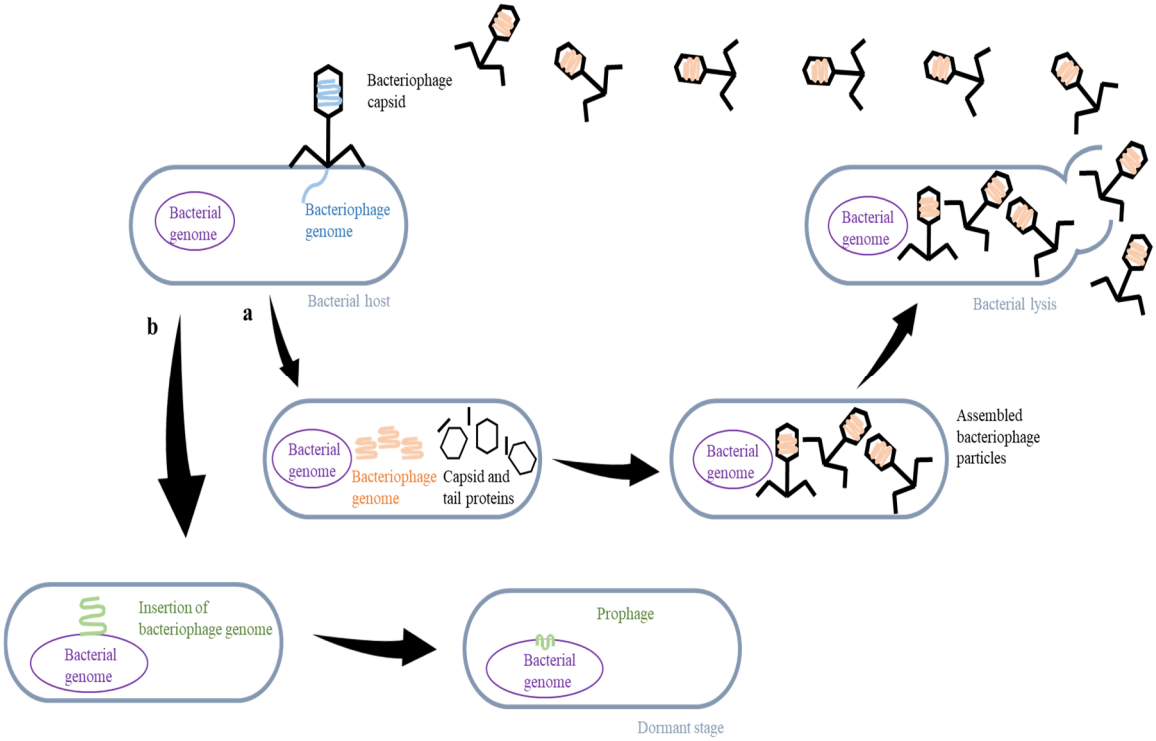

Figure 3. Scheme of lytic (a) and lysogenic cycle (b) (Adapted from: Harada *et al.*, 2018).

1.1.3 Bacteriophage nomenclature

The main objective of classification is generalisation and simplification, and thus virus research and understanding.

Phages are the most abundant biological, primitive and simple structures on the planet. Their number is estimated at 10^{30} to 10^{32} in the environment, playing an important role in the microbiological balance of the environment. More than 6,000 phages are known and every year 100 new ones are described, making them the largest group of known viruses (Ackermann, 2011) (Figure 4).

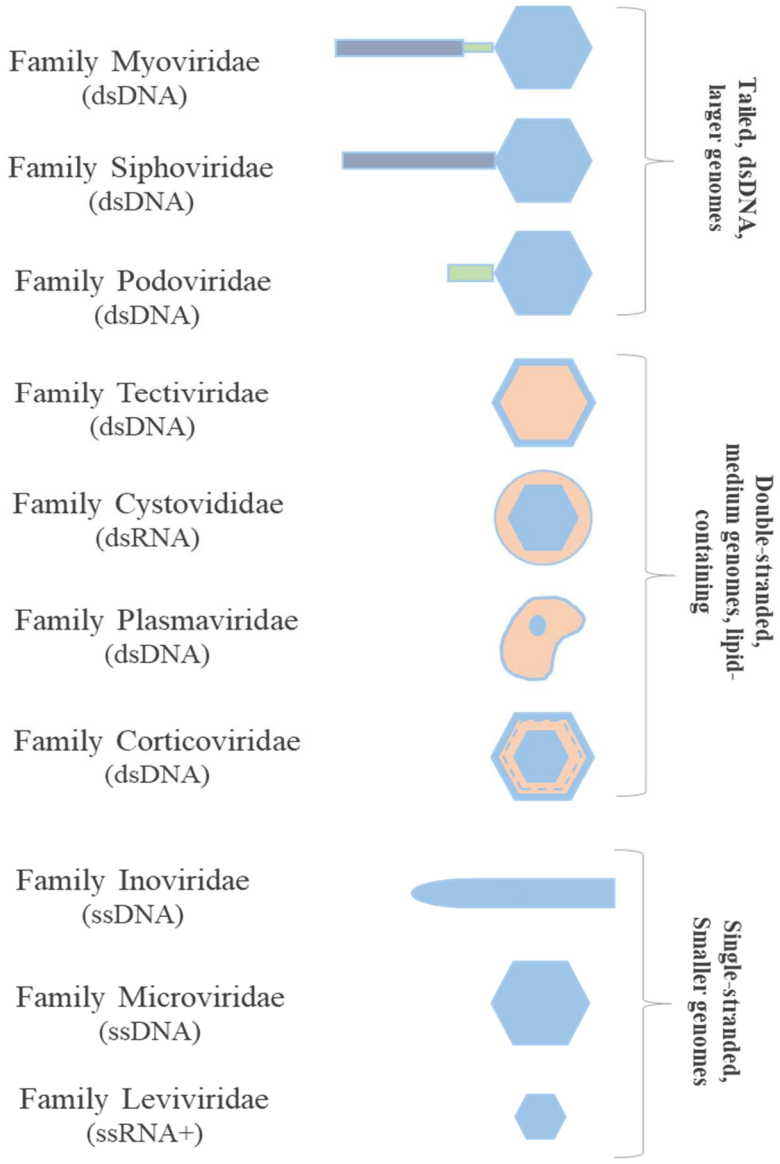

Figure 4. General scheme of phages family. Morphological and genomic classification (Adapted from: Hyman and Abedon, 2012).

The predecessor of the phage classification was Sir Macfarlane Burnet (1936), an Australian microbiologist, who showed that phages differed in size and resistance against different chemical agents. Nevertheless, in 1939, Ruska proposed that phage classification should be consistent with the morphology observed by the electron microscope due to the wide morphological diversity. Moreover, in 1962, Lwoff, Horne and Tournier began the phage classification based on viral properties and their nucleic acid. To this end, they created the Provisional Committee for Viral Nomenclature (PCNV), which was renamed in 1971 as International Virus Taxonomy Committee (ICTV); this committee is considered the starting point for phage classification. ICTV classifies prokaryotic viruses according to their host range, physical characteristics (structure, capsid size and shape), type of genomic material (single or double-stranded DNA or RNA), genome size and resistance to organic solvents (Sharma *et al*., 2017). Viral particles could be virus with tail, polyhedral, filamentous or pleomorphic (Ackerman, 2011).

Currently, 873 species, 204 genera and 14 subfamilies are recognised. Around 96% of the known phages belong to 3 families: *Myoviridae*, *Podoviridae* and *Siphoviridae*, all belonging to the order of the *Caudovirales*, which means virus with tail (Figure 5). The *Myoviridae* and *Siphoviridae* families possess long tails, contractile and not contractile, respectively. *Podoviridae* family phages have a short non-contractile tail (Nobrega *et al*., 2018). Long-tailed phage tails end on a basal plate to which the receptor binding proteins bind, as well as the tail fibres and spicules. In contrast, members of the short non-contractile tailed phages lack this base plate, so that both receptor binding proteins and fibres and spicules bind directly to the tail (Nobrega *et al*., 2018). Moreover, two new families have been suggested for inclusion in the *Caudovirales* order: *Ackermannviridae* and *Herelleviridae* (ICTV, 2019).

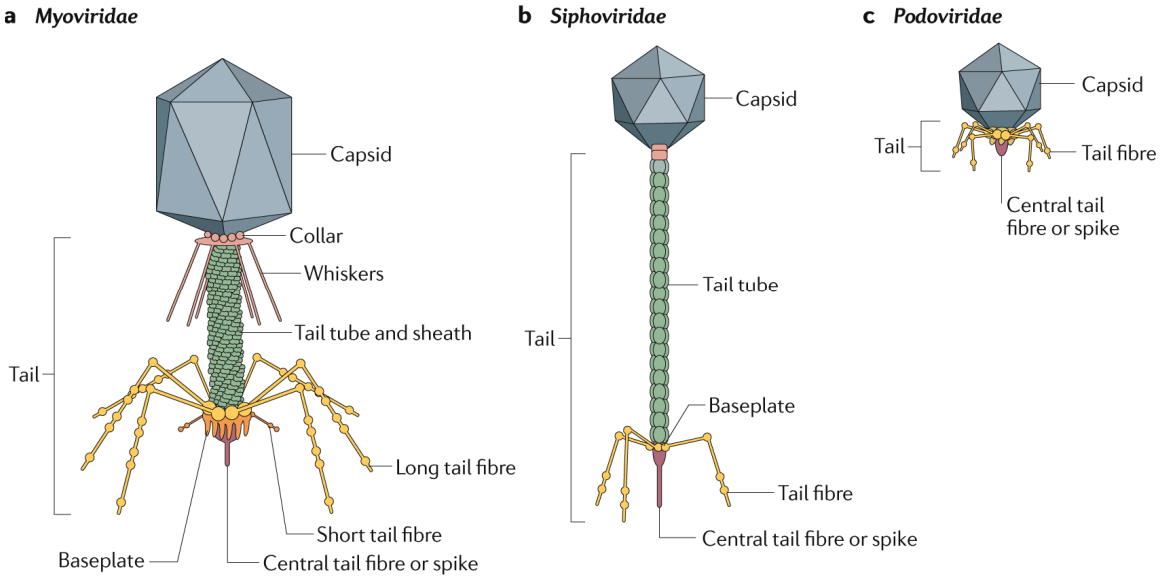

Figure 5. Phages with tail morphology (Nobrega *et al.*, 2018).

1.1.4 Isolation, identification and characterisation

1.1.4.1 Bacteriophage isolation

The isolation of phages is often presented as a fairly simple technique, and every environment where the pathogenic host is present could represent a potential source of phages (Hyman, 2019).

There is no gold standard technique for phage isolation, and since Felix d'Hérelle described phages several modifications have been implemented. Phage isolation will vary depending mainly on the host bacteria, and the type of sample (Hyman, 2019). However, phage isolation is based on a mixing step of the phage-containing sample with host bacteria, followed by a removal of bacterial debris, firstly by centrifugation and then by filtration (Hyman, 2019). For phage isolation, the next steps could be as follows:

1. ***Sample processing***: The bacteria has to be mixed with an environmental sample (faeces, water, litter, etc.) and the mixture then has to be incubated overnight (pre-enrichment procedure). Temperature and time of incubation depend on the target bacteria characteristics. After incubation, the bacteria must be removed from the culture by centrifugation and then filtration (0.22 µm pore size filters are commonly used). Once the bacteria have been removed, the filtrated liquid may contain the phage (Hyman, 2019). Moreover, a phage isolation procedure without

a pre-enrichment step has been described. For this purpose, environmental samples have to be cultured with the target bacteria directly on a Luria-Bertani (LB) agar plate. After incubation, the plate is checked for a clearing spot or plaque presence. However, this procedure requires a high concentration of phage in the original sample (Bhunchoth *et al.*, 2016; Gencay *et al.*, 2017).

1. ***Detection of bacteriophages***: There are many ways to detect phages regarding the type of target bacteria. One simple and common approach is "**the spot test**". In this test, a LB agar plate is inoculated with the target bacteria and then small drops of phage filtrate are placed on the plate surface. After incubation, a zone of lysis indicates the presence of a specific phage (Oliveira *et al.*, 2017; Hyman, 2018). Another method is "**the plaque test**". In this method, phage filtrate obtained in the previous step is mixed with the target bacteria and LB soft agar. Then, the mixture is placed on LB agar and incubated overnight according to bacteria characteristics. After incubation, the plate is checked for the appearance of lysis plaques (Pallavali *et al.*, 2017; Hyman, 2019). The last method is "**the culture lysis test**"; the phage filtrate is added to a 4h target bacteria pre-enrichment in LB broth. The mixture is then incubated under bacteria characteristics. The diminished or increased turbidity is monitored to assess bacterial metabolic activity (Sullivan *et al.,* 2003) (Figure 6).

The spot test | The plaque test | The culture lysis test

Figure 6. Different techniques for phage detection.

2. ***Plaque purification***: After the presence of phages has been assessed, the isolation of pure plaques is performed through multiple rounds of plaque purification (Hyman, 2018). Phages isolated from a sample are considered undefined, as it

may contain a mixture of phages. To isolate a single phage, individual plaques are collected by scraping the top agar and added to 200 µL of PBS. Serial dilutions are performed and 100 µL of each dilution, 100 µL of the target bacterial suspension and 5 mL soft LB agar are placed as a layer on the top of an LB agar (Oliveira *et al.,* 2009). This step is repeated three times until each picked plaque represents a clone derived from a single phage (Pallavali *et al*., 2017).

1.1.4.2 Bacteriophage identification and characterisation

Identification and characterisation of phages is based on lytic activity, morphological characterisation and genotypic characterisation.

1. ***Lytic activity***: For phage therapy lytic phages are preferred over lysogenic phages. For most phages, clear plaques are considered as an indicator of lytic phage activity (Jurczak-Kurek *et al*., 2016; Hyman, 2019). However, whole sequence genome should be performed for phages to be screened for the presence of toxin genes or genes associated to lysogenic cycle life.
2. ***Morphology by electron microscopy***: Morphology is considered essential for classifying novel phages and phages with different morphologies. This technique is especially important to develop new cocktails with phages from different families (Aprea *et al.,* 2015).
3. ***Genome sequence***: The sequence of a phage can confirm the presence or lack of toxin genes and the ability to form a lysogen. This is currently the preferred method for screening the multiple properties of phages (Hyman, 2019).

1.2 Bacteriophage therapy

1.2.1 Phage therapy approaches

Phages are described as harmless to humans, animals and plants, and for this reason they are proposed as a safe alternative to conventional antibiotics (González-Menéndez *et al.*, 2018). The use of phages in Eastern Europe and the former Soviet Union has been widespread since they were discovered (Furfaro *et al.*, 2018). Nevertheless, the potential of phage therapy is currently being studied according to rigorous standards, such as the selection and characterisation of the phage, selection of the subjects (animals or humans) and selection of the bacteria (Kutter *et al.*, 2010; Villarroel *et al.*, 2017; Furfaro *et al.*, 2018).

One approach of phage therapy is as biocontrol along the food chain, as a promising disinfectant to reduce the risk of contamination produced by biofilms and as sanitiser at field level and as a complementary tool for cleaning and disinfection (Kutter *et al.*, 2010). In 2006, the United States FDA (Food and Drug Administration) approved a preparation of phages to be used directly in food against *Lysteria Monocytogenes* (Listex^{TM}). In the following years, the FDA also approved Salmofresh^{TM} and PhageGuard^{TM}, which were granted GRAS (Generally Recognised as Safe) status (Moye *et al.*, 2018). The success of phage therapy relies on their high specificity against the target bacterium, their self-amplification and auto-limiting nature and their evolving capacity against antimicrobial resistance bacteria (Wernicki *et al.*, 2017).

Another approach of the therapeutic use of phage is as a formulation of cocktails, which contain two or more phage types, to treat different diseases (Wittebole *et al.*, 2014). The main advantage of this type of formulation is its broader spectrum of activity in comparison to individual phage isolates. They can target different bacteria, achieving a higher effectiveness (Chan and Abedon, 2012). However, when resistance against phages appears, an alternative is the use of autophages, which means a phage isolated from the environment where the target bacterium is present.

From a therapeutic viewpoint, phage therapy provides many benefits over chemotherapy, as they are effective against antibiotic-resistant bacteria and no side effects have been described during phage treatment (Wei *et al.*, 2019) (Figure 7).

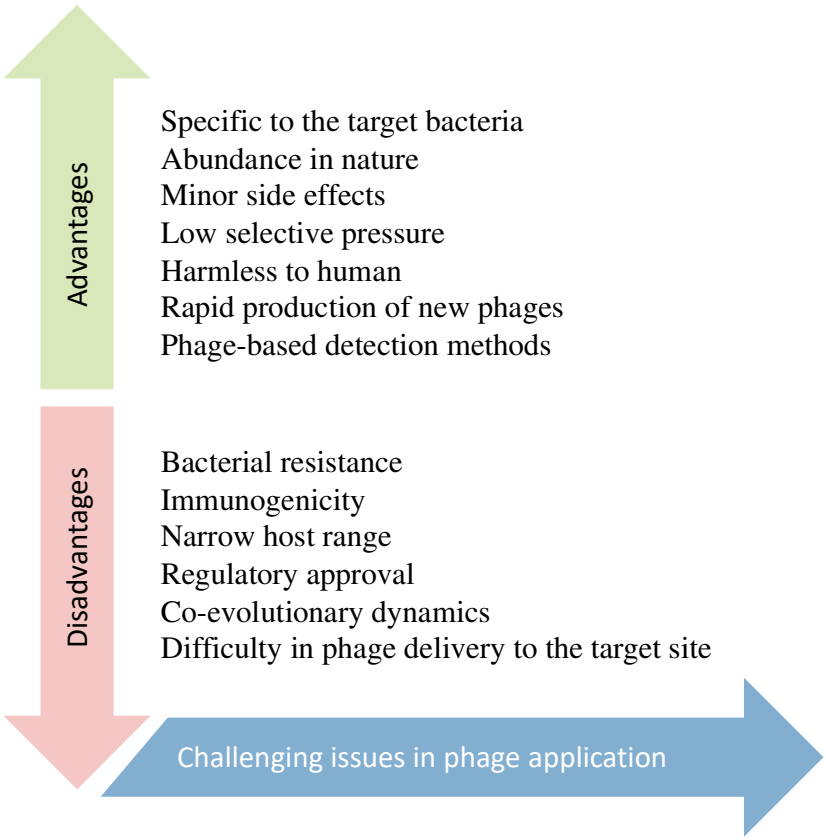

Figure 7. Advantages and disadvantages of using bacteriophages for the treatment of *Salmonella*
(Adapted from: Xie *et al.*, 2019).

1.2.2 Bacteriophages encapsulation

The way phages are applied depends on their specific use. They can be delivered by oral administration or by direct spraying onto food or industrial surfaces (Bueno *et al.*, 2012; Moye *et al.*, 2018). However, in many cases phages can encounter some difficulties in killing the bacteria (temperature conditions, environmental pH, UV light, etc.). For this reason, a manageable possibility to overcome this problem is the encapsulation of phages (González-Menéndez *et al.*, 2018).

Phage encapsulation may increase the circulation time of a phage for treating systemic or intracellular infections, or as prophylactic treatment (Malik *et al.*, 2017). Micro or nano-encapsulation is a method that allows the packaging of different material in vesicles that can release their contents at controlled rates (González-Menéndez *et al.*, 2018). Different methods described for phage encapsulation include freeze drying (lyophilisation), spray drying, in this emulsions, polymeric nanoparticles and liposomes (Malik *et al.*, 2017).

1.2.3 Bacteriophage therapy resistance

The main weaknesses of phage therapy are the production of neutralising antibodies, the emergence of phage-resistant bacterial strains and the efficacy of phages only when administered shortly after bacterial infection (Capparelli *et al*., 2010; Soni *et al*., 2010; Kysela *et al*., 2007).

Studies based on the immune effects of clinical application of lytic phages are minimal (Krut and Bekeredjian-Ding, 2018). Biswas *et al.* (2002) reported that the immunogenicity of phages could develop the formation of phage-neutralising antibodies that can hamper the therapeutic success. However, it seems that immunogenicity of phages does not represent a relevant safety risk for patients. Indeed, many studies refer to phages selected for diagnostic immunisation or tumour therapy (Krut and Bekeredjian-Ding, 2018). On the other hand, the emergence of bacterial resistance against phages is also possible. The bacteria can develop several mechanisms to prevent viral infections, such as hiding the phage's access to the receptor through the production of extracellular matrix, change or loss of receptor, secretion of substances (such as enzymes) to avoid phage adhesion to the bacterial membrane and activation of measures for blocking DNA injection into the cells and thereafter the inhibition of phage replication and release (Seed *et al*., 2015; Oeschlin *et al*., 2018; Principi *et al*., 2019). However, as phage and bacterial hosts coevolve, a new phage could be re-isolated from the environment. This is an advantage of phage discovery compared to the extensive time required to find new antibiotics. In this regard, phage resistance would not be as problematic as drug resistance (Haq *et al.,* 2012).

1.3 Bacteriophage therapy against *Salmonella*

1.3.1 *Salmonella* epidemiology in humans

Millions of human salmonellosis cases are reported worldwide every year, and the disease results in thousands of deaths. In the United States, *Salmonella* causes around 1.2 million cases, leading to 23,000 hospitalisations and 450 deaths every year (WHO, 2019). In 2018, a total of 94,203 confirmed cases in humans were reported in Europe by the European Surveillance System by 28 EU (European Union) MS (Member States), and 91,867 were confirmed. The salmonellosis trend in humans has been established over the

last five years (2014 to 2018). Most of the outbreaks were caused by *Salmonella* Enteritidis (*S.* Enteritidis), showing an increase of 36.3% compared to 2017 (EFSA and ECDC, 2019).

The main way *Salmonella* infections occur is by consumption of contaminated food of animal origin, such as poultry products (eggs and meat). Moreover, another source of cross contamination due to a lack of hygiene measures has been described (WHO, 2019; EFSA and ECDC, 2019). Salmonellosis caused by *S.* Enteritidis has been related to broiler and layer production (57.4% and 37.4%). Likewise, *Salmonella* Typhimurium (*S.* Typhimurium) has been associated with broilers (27.3%), followed by pigs (13.9%), layers (13.1%) and cattle (2.3%) production. Monophasic *Salmonella* Typhimurium (mST) has been associated with broilers and pigs (43.4% and 39.6%, respectively), and *Salmonella* Infantis (*S.* Infantis) was markedly related to broiler production (93%). Finally, *Salmonella* Derby (*S.* Derby) was associated with pigs and turkeys (61.8% and 30.3%, respectively) (EFSA and ECDC, 2019) (Figure 8).

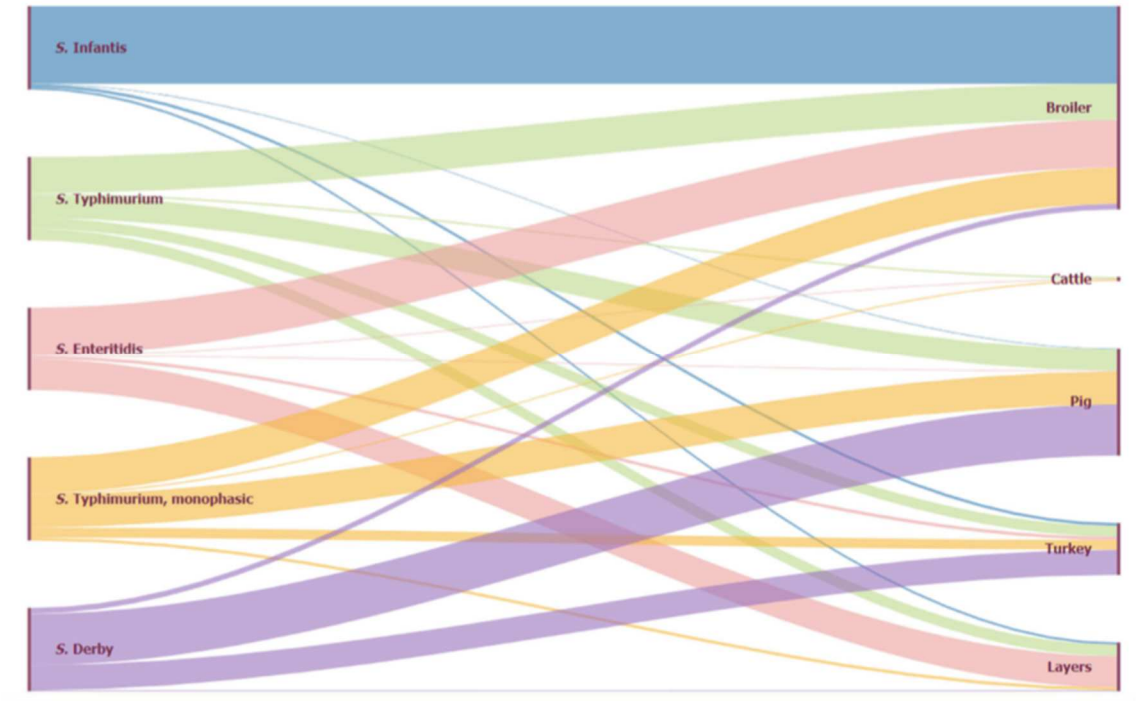

Figure 8. Sankey diagram of the distribution of the human EU top-five *Salmonella* serovars, across different food and animal sources (broiler, cattle, pig, turkey and layers) (EFSA and ECDC, 2019).

1.3.1.1 Clinical aspects

Salmonella is one of the main pathogens involved in human gastroenteritis worldwide (EFSA and ECDC, 2019). The most common presentation of non-typhoidal *Salmonella* infection is an acute gastroenteritis. Onset of the intestinal salmonellosis is usually characterised by acute fever, abdominal pain, diarrhoea, nausea and sometimes vomiting. The onset of disease symptoms occurs 6-72 h after bacteria ingestion, and the illness lasts 2-7 days. Symptoms are relatively mild and self-limiting. However, in some patients, such as children and the elderly, the associated dehydration can become severe and life-threatening (severe bacteraemia, meningitis and other forms of extraintestinal infections) (Capparrelli *et al*., 2010; WHO, 2019).

1.3.1.2 Salmonellosis treatment in humans

Treatment in severe cases is mainly electrolyte replacement and rehydration. Antimicrobial therapy is not recommended for mild or moderate cases in healthy individuals. They are only administered in severe and extraintestinal disease, mainly in immunocompromised patients (children and old people), and the choice of the antibiotics would depend on the susceptibility pattern of the strain and the clinical condition. Options include ampicillin, trimethoprim-sulphamethoxazole, fluoroquinolones and third generation cephalosporins (ceftriaxone). Recent studies have indicated that a short-course therapy (3–5 days) for patients with severe gastroenteritis would lead to a faster clinical recovery. However, a side effect of antibiotic use is the emergence and dissemination of resistant bacteria, not only in pathogenic bacteria but also in the endogenous flora (Chen *et al*., 2013).

Recent data published by the EFSA (European Food Safety Authority) reported *Salmonella* resistance against sulphonamides/sulphamethoxazole (32.8%), tetracyclines (30.2%) and ampicillin (27.5%) (EFSA and ECDC, 2019). Moreover, resistance to ciprofloxacin was reported in 13.0% of the isolates which was a slight increase compared with 2016. Resistance to cefotaxime or ceftazidime was observed in 1.9% and 1.1% of the isolates which was similar to the levels in 2016. These antimicrobials represent the most important antimicrobial classes (fluoroquinolones and third generation cephalosporins) used for treatment of salmonellosis, and they have been classified by World Human Organisation (WHO) as the highest priority (WHO, 2017).

1.3.2 Phage therapy in humans

Phages as bactericidal agents have been employed for 90 years as a means of treating bacterial infections in humans as well as other species, a process known as phage therapy (Abedon *et al*., 2011). They form part of the physiological mammalian microbiota, and lytic phages could regulate the composition of the microbiome and support its diversity and resilience. In human intestine, DNA phages could infect *Firmicutes*, *Bacteroidetes*, *Proteobacteria* and *Actinobacteria*, whereas RNA phages are claimed to be ingested with food and are present only transiently (Mirzaei *et al*., 2017; Zhang *et al*., 2006). Furthermore, it is described that phages could adhere to the mucosal surfaces of diverse animals, reducing microbial colonisation and pathology (Barr *et al*., 2015).

Mikeladze *et al*. (1936) described a phage treatment of an acute colitis caused by *Shigella* or *Salmonella*. An ampoule of 5 mL was administered orally every 2 h to the patients; the results showed a decrease in fever and an improvement of feeble and rapid pulse, intestinal pain and tenesmus. Other researchers have studied phage therapy in humans against *Salmonella* as a prophylactic measure rather than a therapeutic measure (Abedon *et al*., 2011).

Another approach of *Salmonella* phage therapy in humans is to control zoonotic *Salmonella* in ready-to-eat food (RET). In this context, several authors have described the success of phage therapy. Huang *et al*. (2018) described a decrease of 0.52 $\log_{10}$ *Salmonella* counts in a sausage and 0.49 $\log_{10}$ in lettuce after phage application. Whichard *et al*. (2003) described a reduction of 1.8-2.1 logs after phage application to chicken frankfurters. In the same line, Kang *et al*. (2013) and Hungaro *et al*. (2013) decreased *Salmonella* counts from chicken skin by 3 $\log_{10}$ and 0.5-2 $\log_{10}$, respectively, after application of a single phage. For this reason, even though salmonellosis phage therapy in human is not so common, the use of phage therapy to combat major foodborne pathogens could be an interesting approach to reduce salmonellosis in humans.

1.3.2.1 *Salmonella* epidemiology in poultry production

According to Regulation (EC) No 2160/2003 and its following amendments, MS have to set up *Salmonella* National Control Programmes (NCP) aimed at reducing the prevalence of *Salmonella* serovars, which are considered relevant for public health in poultry flocks.

Currently, prevalence targets have been defined for breeding flocks of *Gallus* (laying hens, broilers and breeding and fattening turkeys) and correspond to the maximum annual percentage of flocks remaining positive for relevant serovars: *S.* Enteritidis and *S.* Typhimurium, including its monophasic variants, and for breeding flocks *S.* Infantis, *Salmonella* Virchow (*S.* Virchow) and *Salmonella* Hadar (*S.* Hadar) are considered to be relevant as well. These NCPs are based on Regulation (EC) No 200/2010 and the prevalence target 1% or less and 2% or less was set for all commercial-scale adult breeding and broiler flocks and laying flocks, respectively.

The latest data recorded in 2019 showed that 2.04% of breeding flocks tested were positive for *Salmonella* spp., and 0.54% were positive for any of the five target serovars (*S.* Enteritidis, *S.* Typhimurium, mST, *S.* Virchow, *S.* Infantis and *S.* Hadar) (EFSA and ECDC, 2019). For laying hens, *Salmonella* was found in 4% of the flocks, although *Salmonella* prevalence of the two target serovars (*S.* Enteritidis and *S.* Typhimurium) was 1.1%. The most commonly reported serovar was *S.* Enteritidis. Regarding broiler production, *Salmonella* was found in 3.5% of the flocks; nevertheless, the prevalence of the target serovars was 0.20%. The prevalence was higher for *S.* Typhimurium than for *S.* Enteritidis. However, among outside target serovars, the most common serovar reported was *S.* Infantis (EFSA and ECDC, 2019).

Considering the production chain for meat and meat products, the highest percentage of positive samples were found for fresh broiler (7.15%). With respect to eggs and egg products, 0.37% and 3.52% of tested egg units and egg products, respectively, were *Salmonella*-positive (data collected from 13 and 9 MS, respectively) (EFSA and ECDC, 2019).

1.3.2.2 Main sources of *Salmonella* contamination in the poultry sector

Many epidemiological studies have demonstrated the wide variety of routes by which *Salmonella* can be disseminated within integrated poultry companies (Davies and Breslin, 2003; Namata *et al.*, 2008). Several factors are related to *Salmonella* colonisation in poultry, including the age and genetic susceptibility of the birds, stress, level of pathogen exposure (infectious dose), competition with gut microbiota, and the infecting *Salmonella* serovar (Foley *et al.*, 2011). Moreover, other infection routes to take into account for *Salmonella* contamination of the farms are feed, water, day-old chicks

infected, farmer management, farm pests (rodents in laying farms and mice in broilers) (Cox *et al.*, 2000; Kemp *et al.*, 2005; Carrique-Mas *et al.*, 2008; Marin *et al.*, 2011; Heyndrickx *et al.*, 2012) (Figure 9).

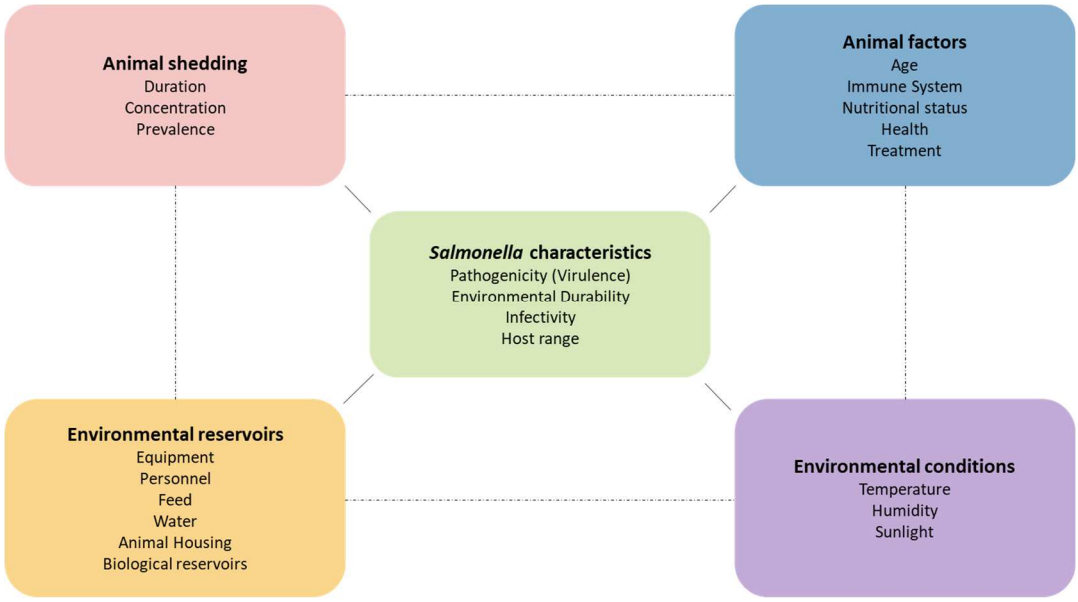

Figure 9. Factors affecting *Salmonella* prevalence (Adapted from: The Poultry Site, 2011).

There are two ways of *Salmonella* transmission, vertical and horizontal.

4. ***Vertical transmission***: Eggs are contaminated either from the ovary tissue or on their passage through the cloaca (Gama *et al.*, 2003). *S.* Enteritidis is able to contaminate the reproductive tissues of hens (ovaries and oviducts), where egg contents can be infected before the shell is formed (Keller *et al.*, 1995). Moreover, it has been shown that serovars more capable of vertical transmission have higher levels of virulence for humans or the ability to spread in poultry populations (EFSA and ECDC, 2019).

1. ***Horizontal transmission***: This could be via the digestive or respiratory tract (Cox *et al.*, 1996). *Salmonella* excretion in their caecal droppings will result in the contamination of other birds and the house environment (Cox *et al.*, 1996). Moreover, the bacteria could infect the chicken via the respiratory tract, reach the lungs and penetrate the mucosa until the internal organs without defensive barriers (Cox *et al.*, 1996).

Prevention of *Salmonella* contamination in poultry products requires detailed knowledge of the most important sources associated with its presence in the production system (Slader *et al*., 2002; Marin *et al*., 2011). Good management practices are important to control *Salmonella* prevalence in poultry farms. These good management practices are described below.

1.3.2.3 Main control measures for *Salmonella* in the poultry sector

Since 2007, NCPs for *Salmonella* have been implemented to control the bacteria in poultry farms, and thus reduce its prevalence. Biosecurity and hygiene measures, together with prophylactic methods, regular monitoring and cleaning and disinfection protocols on the farms have been proposed to decrease *Salmonella* prevalence, according to legislation level (ECDC and EFSA, 2017).

1. ***Biosecurity measures and hygiene measures***: *Salmonella* prevention could be achieved by adopting Good Agricultural Practices and Hazard Analysis Critical Control Point (Khan *et al*., 2018). To avoid cross contamination between flocks, overshoes, disinfection dips for boots, boot changes between different poultry houses and washing hands before and after visits could be applied as hygiene measures. Moreover, protocols for rodents, flies and wild birds should be implemented to control entry of the bacterium to farms (Meunier *et al*., 2015).
2. ***Prophylactic protocols***: Vaccination is an efficient strategy for the reduction of *Salmonella* infection in poultry. According to Regulation 2016/2003, vaccination in layer production is mandatory, but is optional in breeders in Spain (except in the Valencia Region, where it is also compulsory) (EC, 2003; PAZ, 2018). The target serovars are those with importance in public health; *S.* Enteritidis and *S.* Typhimurium (the latter only in the Valencia Region), and sometimes there is a cross-immunisation against mST variant. As vaccination is applied in poultry, the public health risk has decreased (Li *et al*., 2019).
3. ***Salmonella surveillance or monitoring***: Surveillance should be carried out according to Regulation (EC) 2160/2003 to check the *Salmonella* status of poultry flocks and, in the case of positive flocks, to take the measures that will reduce the prevalence and risk of transmission of *Salmonella* to humans (WHO, 2019).

4. ***Cleaning and disinfection***: Cleaning and disinfection is considered an essential on-farm step to control *Salmonella* (Fosse *et al*., 1994; Carrique-Mas *et al*., 2009; Andres *et al*., 2015). Before the introduction of a new flock, all the farm facilities and equipment should be cleaned and disinfected and bacteriological monitoring should be performed after the procedure to assess the absence of the bacterium (Martelli *et al*., 2017).

1.3.2.4 Phage therapy against *Salmonella* in the poultry sector

Several studies have demonstrated phage therapy success in the poultry sector for *Salmonella* control, as phage therapy has been shown to reduce side effects compared to traditional antibiotic treatments, due to its specificity (Nabil *et al*., 2018).

At field level, Fiorentin *et al*. (2005) reduced *S*. Enteritidis colonisation by 3.5 $\log_{10}$ CFU in the digestive tract with a single oral dose of phages. Moreover, the prophylactic use of phages has proven to be 100% effective against *S*. Enteritidis, with elimination from chicken tonsils after the application of an oral gavage of phage suspension (Ahmadi *et al*., 2016). In the same line, Lim *et al*. (2012) reduced the morbidity and severity of *Salmonella* infection after the application of phages as a feed additive.

Different approaches have been used to assess the success of phage therapy in controlling *Salmonella* in foodstuffs. They have studied using biofilms and bacterial growth biocontrol on carcasses and equipment surfaces to reduce bacterial loads. Moreover, phages have been applied on food as a natural preservative to treat chicken carcasses against *Salmonella* (Goode *et al*., 2003; Carvalho *et al*., 2012). Their results showed non-recoverable *Salmonella* after phage application, resulting in elimination of the pathogen. In the same way, Higgins *et al*. (2005) reduced natural *Salmonella* contamination from broiler and turkey carcasses rinses by 100% and 60%, respectively.

1.4 Study cornerstone

Poultry is an important production sector all over the world. In 2018, close to 123 million tonnes of meat and 87 million tonnes of eggs were produced and it is estimated that production is likely to increase in the following years (FAO, 2019). In Europe, Spain is

the 3rd producer of eggs and poultry meat, producing close to 1.5 million and 1 million tons of eggs and meat, respectively.

Salmonella has long been recognised as an important zoonotic pathogen of economically significant losses in the poultry sector. There are numerous sources of human salmonellosis, but eggs and poultry meat are reported to be the most common source (EFSA and ECDC, 2019). In this sense, introduction of NCP has resulted in an important reduction in the prevalence of poultry *Salmonella* serovars throughout Europe. However, total elimination of the bacterium from poultry flocks is still difficult to achieve and new cases of salmonellosis emerge every year. For this reason, innovative techniques should be implemented to control *Salmonella* at farm level (Wang *et al.,* 2010).

The progressive increase in the number of multidrug resistant bacteria and the complete ban on the use of antibiotics in livestock feed in the EU have led to the growth of alternatives to antibiotic research, such as the use of phages to combat bacterial infections in humans and animals (Wernicki *et al*., 2017).

In this context, the following chapters were intended to assess the use of phages to control *Salmonella* at field level, and to give the poultry sector an alternative measure or complementary tool against the bacteria.

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CHAPTER II. OBJECTIVES

2. Objectives

The content of this doctoral thesis aims to provide new knowledge of alternative measures that will help to control *Salmonella* in the poultry sector. Thus, the main purpose of this study is to assess the epidemiology of phages in the poultry sector and evaluate the use of phages as a promising tool against *Salmonella* in the field. To achieve this goal, the following objectives have been proposed:

1. Determine *Salmonella*-phage prevalence in poultry commercial farms, regarding the production livestock: layers and broilers.

2. Assess the effect of bacteriophages against *S*. Infantis and *S*. Enteritidis on farm surfaces and evaluate phage procedure application as sanitiser against *Salmonella* in field conditions.

3. Evaluate the application of autophages to reduce *S*. Enteritidis in environmental and faecal samples on a layer farm.

CHAPTER III. EXPERIMENTAL CHAPTERS

3. Experimental chapters

3.1 Serovar bacteriophage diversity according to *Salmonella* serovar in layer and broiler poultry farms

S. Sevilla-Navarro, P. Catalá-Gregori, C. Marin. 2020. Serovar bacteriophage diversity according to *Salmonella* serovar in layer and broiler in poultry farms. Poultry Science. *Under review*.

3.1.1 Abstract

The emergence of several *Salmonella* serovars resistant to multiple antibiotics in poultry-derived products underscores a significant food safety hazard. The exploration of novel non-antibiotic interventions in the field, such as the use of phages, is therefore necessary to avoid the presence of antibiotic-resistant isolates. Phages are a group of viruses widely distributed in nature whose life cycle is strictly associated with the prokaryotic cell. Researchers have demonstrated the success of phage therapy in poultry products, reducing *Salmonella* counts after phage administration. However, the impact that phage concentration in the environment may have against certain *Salmonella* serovars with relevance in food safety is not well understood. Therefore, the aim of this study was to assess *Salmonella* phage prevalence in commercial poultry farms in terms of the production livestock type: layers and broilers. The most prevalent *Salmonella* serovars isolated in poultry production were used for phage isolation. *Salmonella*-specific phages were isolated from 141 faeces samples taken from layers (n = 108) and broilers (n = 33) from different farms located in the Valencia Region during June and July 2019. Analysis of the samples revealed that 100% presented *Salmonella* phages, the most prevalent being the *Salmonella* phage serovar *S.* Enteritidis (93%), followed by *S.* Virchow (59%), *S.* Typhimurium (55%), *S.* Infantis (52%) and *S.* Ohio (51%). These results indicate that poultry farms could represent an important source of *Salmonella* phages. Moreover, we have shown a close relationship between *Salmonella* phage prevalence and *Salmonella* serovar prevalence in poultry farms, suggesting that phages co-exist within their serovar. Furthermore, the fact of isolating a higher number of phages against *S.* Enteritidis makes the vaccine a double control measure, as it immunises the animals and increases the prevalence of phages against public health isolates. Finally, the more bacteria we find in the environment, the more serovar-specific phages may be present. In this context, we could isolate the wild *Salmonella* strains, increase the concentration of the environmental wild *Salmonella* phages, and apply them in poultry *Salmonella*-positive farms to control *Salmonella* contamination at field level.

3.1.2 Introduction

Salmonella spp. remains one of the main bacteria involved in foodborne outbreaks and is a worldwide major public health hazard (WHO, 2019). It is estimated that non-typhoidal *Salmonella* worldwide cause around 94 million cases of illness and 155,000 deaths (Ao *et al.*, 2015). The latest data published by the EFSA reported 91,857 human cases, 43.2% of which hospitalised (EFSA and ECDC, 2019). The main serovars involved in human outbreaks were *S.* Enteritidis, *S.* Typhimurium, and mST variant (EFSA and ECDC, 2019). However, new serovars such as *S.* Infantis or *S.* Kentucky are emerging (EFSA and ECDC, 2019). The emergence of several *Salmonella* serovars resistant to multiple antibiotics in poultry-derived products underscores a significant food safety hazard (Nair *et al.*, 2018). For this reason, the exploration of novel non-antibiotic interventions in the field is necessary to avoid the presence of antibiotic-resistant strains (Nair *et al.*, 2018). In this context, owing to increasing reports of antimicrobial resistance (AMR) in *Salmonella*, the importance of controlling this pathogen by finding alternatives to the use of antibiotics to reduce the bacteria from poultry farms might be studied.

Phages are a group of viruses widely distributed in nature, whose life cycle is strictly associated with the prokaryotic cell (Wernicki *et al.*, 2017; Moye *et al.*, 2018). The use of host-specific phages has been promoted as a cost-effective and adaptable approach to control zoonotic bacteria (Atterbury *et al.*, 2007; Borie *et al.*, 2008; Ahmadi *et al.*, 2016; Sevilla-Navarro *et al.*, 2018). Moreover, phages seem to be a good alternative due to their minimal environmental impact, self-perpetuating, self-limiting and specificity (Wang *et al.*, 2010). Researchers have demonstrated the success of phage therapy in poultry products, reducing *Salmonella* counts from broiler carcasses after phage administration. Higgins *et al.* (2005) reduced *Salmonella* counts in 100% of broiler carcasses where phages were inoculated. Moreover, Kang *et al.* (2013) decreased *Salmonella* counts on chicken skin by up to 3 logs after the application of a single phage. Other research showed *Salmonella* decreasing counts by 1 log on fresh eggshells after application of the phage (Moye *et al.*, 2018).

However, the impact that phage concentration in the environment may have against certain *Salmonella* serovars with relevance in food safety is not well understood. Thus, an improved understanding of *Salmonella* phage diversity will provide a better insight

into the roles of phages in *Salmonella* ecology and diversity, and a biocontrol approach for diagnostic applications (Wongsuntornpoj *et al*., 2014; Crabb *et al*., 2018). Therefore, the aim of this study was to assess *Salmonella* phage prevalence in poultry commercial farms regarding the production livestock type: layers and broilers.

3.1.3 Material and Methods

3.1.3.1 *Salmonella* strains selected for phage isolation

Salmonella isolates used for phage isolation were selected from a database of *Salmonella* strains isolated in 2019 from the NCP (*Centro de Calidad Avícola y Alimentación Animal de la Comunidad Valenciana*-CECAV). One strain was randomly selected from each of most prevalent serovars isolated in poultry production in Europe: *S*. Enteritidis, *S*. Typhimurium, mST, *S*. Kentucky, *S*. Hadar, *Salmonella* Senftemberg (*S*. Senftemberg), *Salmonella* Ohio (*S*. Ohio), *S*. Infantis and *S*. Virchow (EFSA and ECDC, 2019). The selected strains were thawed and revived on nutrient agar (Oxoid Ltd., England, UK) and incubated at 37.5 ± 2°C for 18 ± 4 h. For characterisation of the strains, the antimicrobial sensibility pattern was performed. To this end, *Salmonella* Sensititre Plates (Gram Negative MIC Plate) were used to assess antimicrobial susceptibility of isolated strains. A 10 µL aliquot of the inoculum was aseptically transferred to 10 mL Sensititre cation-adjusted Mueller-Hinton Broth, and plaques were inoculated according to manufacturer instructions. Plates were read at 18 h to 24 h manually by visualisation of a growth button on the bottom of the microtitre well by using a light box. Reading the results was performed according to the manufacturer's instructions. The antibiotics selected were those set forth in Decision 2013/653 (European Union, 2013), including: two quinolones: Ciprofloxacin (CIP) and Nalidixic Acid (NAL); two B-lactams: Meropenem (MERO) and Ampicillin (AMP), one phenicol: Chloramphenicol (C); one pyrimidine: Trimethoprim (TM); one tetracycline: Tetracycline (TET); one macrolide: Azithromycin (AZM); one glycylcycline: Tigecycline (TGC); two cephalosporin: Ceftazidime (CAZ) and Cefotaxime (CTX); one polymyxin: Colistin (COL); one potentiated sulphonamide: Sulphamethoxazole (SMX), and one aminoglycoside: Gentamicin (GN). Multidrug resistance (MDR) was defined as acquired resistance to at least one agent in three or more antimicrobial classes (EFSA and ECDC, 2016).

3.1.3.2 *Salmonella*-bacteriophage isolation

Salmonella-specific phages were isolated from 141 faeces samples taken from layers (n = 108) and broilers (n = 33) from different farms included in the NCP and located in Valencia Region during June and July 2019 (Figure 10).

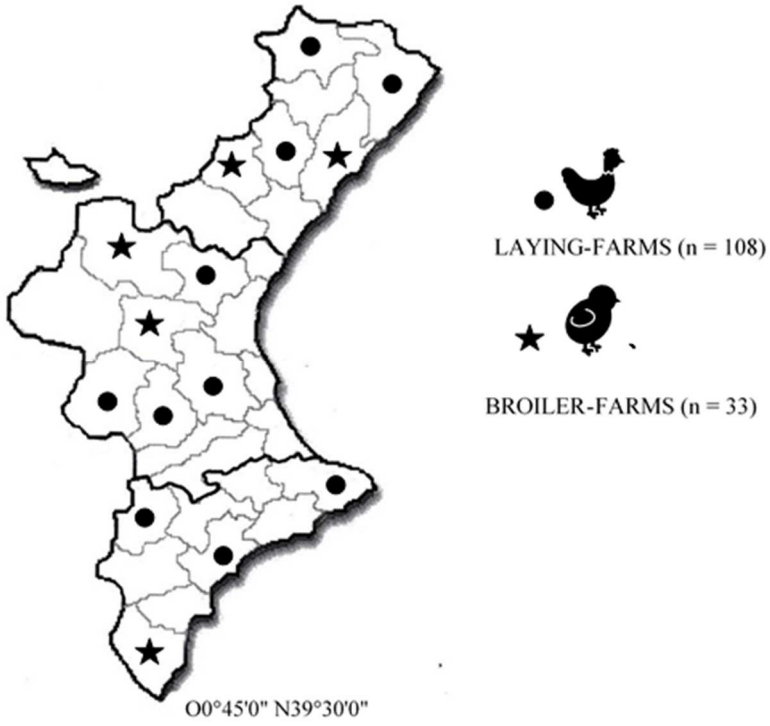

Figure 10. Geographical distribution of poultry farms included in the study.

Broiler age was from 35 days onwards and samples from layers were taken during the laying period (20 weeks onwards). *Salmonella* negative samples following ISO 6579-1:2017 were selected for this study.

The phages were isolated by an enrichment procedure. Faeces samples were placed in sterile pots and 10 g of each sample was diluted in 90 mL of LB (Luria-Bertani, VWR Chemicals, Barcelona, Spain) and incubated along with each selected *Salmonella* serovar overnight at 37ºC. After incubation, 2 mL of this enrichment culture was centrifuged 16,000 x g for 5 min. The supernatant was then filtered through a 0.22 µm membrane. Phages were isolated and purified in spot test by double agar method. Briefly, bacterial suspensions of each serovar were adjusted to an optical density at 600 nm (OD = 600) of

0.2 (~10^8CFU/ml) in LB and incubated at 37°C for 4 h. Then, 200 µL of cultures were added to 5 mL of LB agar (LB with 0.6% agar) and tempered to 45°C and poured onto previously prepared and dried LB basal agar (with 1.6% agar). Then, 10 µL of each filtrate was spotted onto the surfaces of *Salmonella* lawns and incubated overnight at 37ºC. After the incubation, morphologically different plaques were selected and resuspended in 1 mL of PBS. Ten-fold serial dilutions of the phage suspension were plated by the double agar layer method and phages that produced clear plaques were selected. This procedure was repeated 3 times to obtain a single type of phage (Cortés *et al*., 2015).

3.1.3.3 Statistical analysis

We tested whether occurrence of phages against *Salmonella* was related to the livestock production type. To do so, we fitted a generalised linear model (GLM) where occurrence of *Salmonella* phage was the response variable and the sample type (faeces from different broiler and layer farms) and serovar were the factors. For this analysis, the error was designated as having a binomial distribution and the probit link function was used. Binomial data for each sample were assigned a 1 if *Salmonella* phage was isolated or a 0 if not. A *P-value* < 0.05 was considered to indicate a statistically significant difference. Analyses were carried out using a commercially available software program (SPSS 21.0 software package; SPSS Inc., Chicago, IL, 2002).

3.1.4 Results

3.1.4.1 Antimicrobial *Salmonella* susceptibility

From the *Salmonella* strains isolated, 57% were resistant to at least one of the fourteen antibiotics tested, and 50% were MDR to 3 or more of the groups of antibiotics tested. The highest percentages of AMR were found to be TET (50%) followed by SMX (36%) and AMP (36%), CIP (29%) and NAL (29%), C (14%) and TM (14%). Resistance to MERO, AZM, TGC, CAZ, COL, GN and CTX was not observed.

3.1.4.2 Bacteriophage prevalence in poultry farms

A total of 141 faecal samples from broilers and layers were obtained from farms located in different geographic areas of Valencia Region. Analysis of the samples revealed that 100% presented *Salmonella* phages.

In addition, from 1,269 phage isolation tests (each sample x each serovar), statistically significant differences were found according to livestock type ($P<0.05$). In laying hens, 42% (n = 408) of samples contained *Salmonella* phages. Regarding samples from broiler farms, 53% (n = 156) presented at least 1 phage capable of infecting *Salmonella* spp.

3.1.4.3 Prevalence of *Salmonella* bacteriophages per serovar and livestock production type

From the 141 samples, statistically significant differences were shown among serovars. The most prevalent *Salmonella* phage serovar was *S*. Enteritidis (93%) followed by *S*. Virchow (59%), *S*. Typhimurium (55%), *S*. Infantis (52%) and *S*. Ohio (51%) (Table 1).

Table 1. Percentage of phages isolated from faeces regarding *Salmonella* serotype.

Strain	n	%	SEM
SE	131	93^{g}	0.022
ST	78	55^{f}	0.042
mST	36	26^{cd}	0.037
SK	12	9^{a}	0.023
SH	29	21^{b}	0.034
SS	50	35^{de}	0.040
SO	72	51^{f}	0.042
SI	73	52^{f}	0.042
SV	83	59^{f}	0.041

^{a,b,c,d,e,f,g}: percentage with different superscripts means statistically significant difference within column; SEM: standard error of the mean; n: number of samples positive for the presence of a phage against *Salmonella*; SE: *S*. Enteritidis; ST: *S*. Typhimurium; mST: *S*. Typhimurium monophasic variant, SK: *S*. Kentucky; SH: *S*. Hadar; SS: *S*. Senftenberg; SO: *S*. Ohio; SI: *S*. Infantis; SV: *S*. Virchow.

Regarding broiler production, statistically significant differences were shown among serovars ($P<0.05$). The highest percentage of *Salmonella* phages were isolated against *S*. Vichow (97%) and *S*. Enteritidis (91%), followed by *S*. Ohio (76%) and *S*. Typhimurium (64%). However, none of the samples presented phages able to generate lysis plaques on the *S*. Kentucky strain (0%). With respect to layer production, statistically significant differences were shown among serovars ($P<0.05$). The highest percentage of *Salmonella* phages were isolated against and *S*. Enteritidis (94%) followed by *S*. Typhimurium (53%) and *S*. Infantis (52%), *S*. Virchow (47%) and *S*. Ohio (44%) (Table 2).

Table 2. Percentage of phages isolated per serovar within livestock production type.

	Livestock production					
	Broilers			Layers		
Strain	**n**	**(%)**	**SE**	**n**	**(%)**	**SEM**
SE	30	91^{ef}	0.050	101	94^{E}	0.024
ST	21	$64d^{e}$	0.084	57	53^{D}	0.048
mST	2	6^{b}	0.042	34	31^{B}	0.045
SK	0	0^{a}	0.000	12	11^{A}	0.030
SH	17	52^{cd}	0.087	12	11^{A}	0.030
SS	12	36^{c}	0.084	38	35^{BC}	0.046
SO	25	76^{e}	0.075	47	44^{CD}	0.048
SI	17	52^{cd}	0.087	56	52^{D}	0.048
SV	32	97^{f}	0.030	51	47^{D}	0.048

^{a,b,c,d,e,f}: percentage with different superscripts means statistically significant difference within column; ^{A,B,C,D,E}: percentage with different superscripts means statistically significant difference within column; SE: Standard error of the mean; n: number of samples positive for the presence of a phage against *Salmonella*; SE: *S.* Enteritidis; ST: *S.* Typhimurium; mST: *S.* Typhimurium monophasic variant, SK: *S.* Kentucky; SH: *S.* Hadar; SS: *S.* Senftenberg; SO: *S.* Ohio; SI: *S.* Infantis; SV: *S.* Virchow.

Moreover, statistically significant differences were shown between each livestock and serovar. From broiler farms, a higher prevalence of *Salmonella* phages was observed against *S.* Virchow, *S.* Ohio and *S.* Hadar. Conversely, with regard to mST and *S.* Kentucky, the higher phage prevalence was obtained from samples from laying hens ($P<0.05$). On the other hand, no statistically significant differences ($P>0.05$) were found between broiler and layer phage isolation against *S.* Enteritidis, *S.* Typhimurium, *S.* Infantis and *S.* Senftenberg strains (Figure 11).

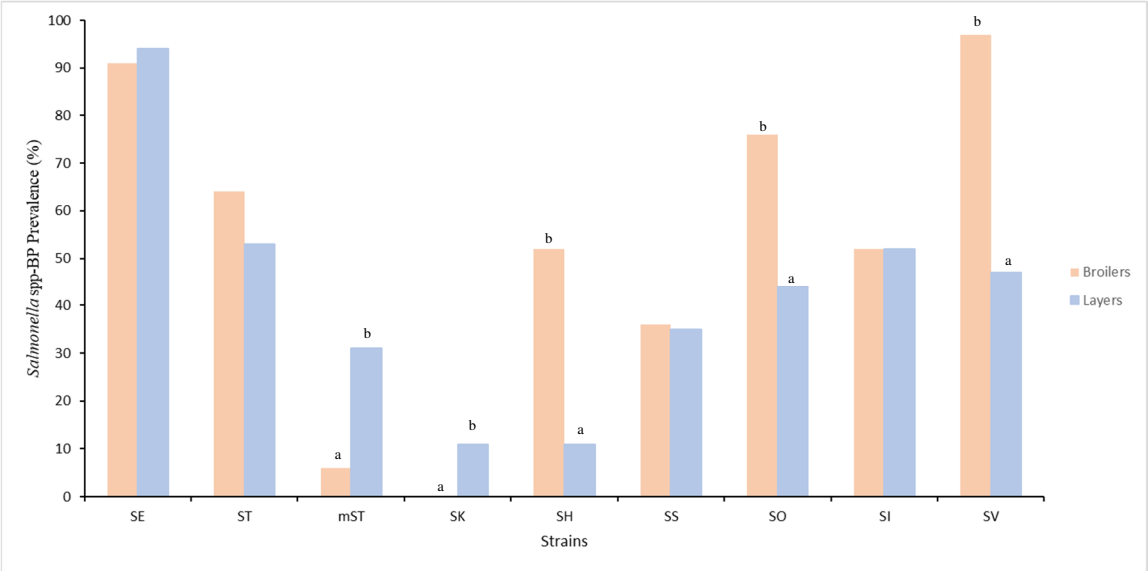

Figure 11. Prevalence of *Salmonella*-phage isolation regarding serovar and livestock production.

[a,b] superscript indicated significant differences of *Salmonella*-phage prevalence according to livestock production type. SE: *S.* Enteritidis; ST: *S.* Typhimurium; mST: *S.* Typhimurium monophasic variant, SK: *S.* Kentucky; SH: *S.* Hadar; SS: *S.* Senftenberg; SO: *S.* Ohio; SI: *S.* Infantis; SV: *S.* Virchow; BP: bacteriophage.

3.1.5 Discussion

In this study, the diversity of *Salmonella* phages in poultry farms regarding livestock production (broilers or layers) and *Salmonella* serovar was analysed. Although *Salmonella* spp. was not present in any of the samples examined, phages for several serovars of public health importance were present in 100% of the samples. Thus, the presence of infecting phages would suggest that the bacteria strain was present at some point in the recent past (Wang *et al.,* 2009; Petsong *et al.,* 2019). Our results indicate that animal farm environment, especially on poultry farms, could represent an important source of *Salmonella* phages (Bao *et al.*, 2011; Hungaro *et al.,* 2013).

A wide *Salmonella* phage diversity was present in the broiler and layers farms analysed, being more varied in broilers. Regarding *Salmonella* serovar-phages isolated, the most prevalent was *S.* Enteritidis (93%), followed by *S.* Virchow (59%), *S.* Infantis (52%), *S.* Typhimurium (55%) and *S.* Ohio (51%). Regardless of poultry production type, the phages isolated were those from most important serovars involved in human salmonellosis (EFSA and ECDC, 2007; Petsong *et al.,* 2019). Different hypotheses could explain this fact; one could be that *S.* Enteritidis, *S.* Typhimurium, *S.* Infantis, and *S.* Virchow were most prevalent serovars in Spanish poultry farms years ago (EFSA and

ECDC, 2007). In recent years, control measures applied in the field have removed the wild bacteria from most of the farms, but their specific phages have survived and remained in the house environment (Wang *et al*., 2009). Another hypothesis could be the strict vaccination programmes implemented in poultry production. Vaccination against *S*. Enteritidis is mandatory in all layers (EC 1177/2006). Moreover, the vaccination programme is stricter in the Valencia Region where, since 2008, it is mandatory to vaccinate not only against *S*. Enteritidis, but also against *S*. Typhimurium (PAZ 2008; PAZ 2009). The latest data recovered from official controls in the Valencia Region showed that 100% of *S*. Enteritidis strains isolated from rearing layers were *S*. Enteritidis vaccine strains (unpublished data). Vaccination spreads the vaccine strain throughout the house environment, resulting in cross contamination between animals (EFSA and ECDC, 2004; Greenwood, 2014; Lee, 2015). In addition, vaccination in breeders can result in vaccine strain vertical transmission to their progeny (Dórea *et al*., 2010).

S. Virchow, *S*. Infantis and *S*. Ohio phages were the main serovars isolated from *Salmonella* control programme in the Valencia Region (unpublished data). These results suggest that the presence of phages infecting *Salmonella* in farms roughly parallels the serovar prevalence (field or vaccine strain). On the other hand, *S*. Virchow is a serovar with public health significance, as the latest data published by the EFSA showed that it was one of the 10 serovars responsible for sporadic cases in humans (EFSA and ECDC, 2019).

Regarding *Salmonella* phage production type, *S*. Virchow, *S*. Ohio and *S*. Hadar were more prevalent in broilers than in layers. These results are in line with data recovered from the *Salmonella* control in the Valencia Region, as no *S*. Virchow nor *S*. Hadar were isolated from laying farms (unpublished data). Marin and Lainez (2009) demonstrated that the main serovars isolated from broiler farms in the Valencia Region were *S*. Virchow, *S*. Ohio and *S*. Hadar. Moreover, the Valencia Region *Salmonella* control programme showed, in 2019, that the *S*. Ohio serovar was more prevalent in layers than in broilers (16.84% and 1.05%, respectively). This data is consistent with Adesiyun *et al*. (2005) and Marin *et al*. (2009) who demonstrated that *S*. Ohio was one of the main serovars isolated from layer samples.

Regarding mST variant and *S.* Kentucky phages, they were more prevalent in layer production. One reason that could explain this fact is that oral administration of *S.* Typhimurium vaccine strain could provide cross-immunisation against mST variant (Kilroy *et al.*, 2015). Although *S.* Kentucky serovar is associated with broiler production (EFSA and ECDC, 2019), several authors have isolated *S.* Kentucky from environmental samples in laying farms (Sivaramalingam *et al.*, 2013; USDA and NASS, 2014; Amand *et al.*, 2017).

This study aims to understand the abundance and diversity of *Salmonella* phage in poultry farms environments. We have shown a close relationship between *Salmonella* phage prevalence and *Salmonella* serovar prevalence in poultry farms, suggesting that phages co-exist within its serovar (Petsong *et al.*, 2019). Furthermore, the fact of isolating a higher number of phages against *S.* Enteritidis or *S.* Typhimurium makes the vaccine a double control measure; on the one hand it immunises the animals and on the other it increases the prevalence of phages against public health strains, helping their control on farms.

Finally, the more bacteria we find in the environment, the more serovar-specific phages may be present. In this context, we could isolate the wild *Salmonella* strains, increase the concentration of environmental wild *Salmonella* phages, and apply them in poultry *Salmonella*-positive farms to control *Salmonella* contamination at field level (Sevilla-Navarro *et al.*, 2018; Sevilla-Navarro *et al.*, 2020).

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3.2 *Salmonella* Infantis and *Salmonella* Enteritidis specific bacteriophages isolated from poultry faeces as a complementary tool for cleaning and disinfection against *Salmonella*

S. Sevilla-Navarro, P. Catalá-Gregori, C. García, V. Cortés, C. Marin. 2020. *Salmonella* Infantis and *Salmonella* Enteritidis specific bacteriophages isolated from poultry faeces as a complementary tool for cleaning and disinfection against *Salmonella.* Comparative Immunology, Microbiology and Infectious Diseases. 68: 101405.

3.2.1 Abstract

Salmonellosis represents an important public health concern. Several authors point out the inefficiency of the cleaning and disinfection protocols used to remove the bacteria from the field. For this reason, innovative techniques, such as phages, could be implemented to control the bacteria. The main objectives of this study were to assess the effect of phages against *S.* Infantis and *S.* Enteritidis on farm surfaces and evaluate bacteriophage procedure application as sanitiser against *Salmonella* in field conditions. So, the most prevalent serovars in poultry production were selected (*S.* Infantis and *S.* Enteritidis) to contaminate farm facilities. Then, two specific phages isolated from poultry faeces were applied against them. Results showed that *S.* Infantis and *S.* Enteritidis decreased by 4.55 $\log_{10}$ CFU/mL and 3.85 $\log_{10}$ CFU/mL, respectively; the maximum reduction in *Salmonella* was on the 5^{th} day, after 10^8 PFU/mL and 10^3 PFU/mL bacteriophage application. These outcomes highlight phages as a promising tool together with cleaning and disinfection.

3.2.2 Introduction

Salmonella is widely recognised as one of the most important zoonotic pathogens with economic impact in animal and humans. There are roughly 550 million gastrointestinal cases worldwide, and *Salmonella* is one of the main pathogens in these disease outbreaks (WHO, 2018). In the United States, this is a significant public health concern, and *Salmonella* causes around 1.2 million cases and 450 deaths every year (CDC, 2019). In Europe, salmonellosis was responsible for 91,662 cases in humans, of which 9,426 were reported in Spain the same year (EFSA, 2019a). The main sources of infection are poultry products, particularly meat and eggs (EFSA, 2019b). Main serovars involved in these food outbreaks are *S.* Enteritidis, *S.* Typhimurium and mST variant. However, last year *S.* Infantis prevalence increased considerably, being the most prevalent serovar in broilers (EFSA, 2019b).

These zoonotic bacteria represent an important public health concern and controlling the disease has become a vital challenge in most countries (EFSA, 2019a; FAO/WHO, 2009). Thus, *Salmonella* NCP, in accordance with Regulation (EC) Nº 2160/2003, together with biosecurity measures, cleaning and disinfection protocols and prophylactic measures, have resulted in a decreased prevalence at field level (Vandeplas *et al.,* 2010). However, despite all these measures, new cases of salmonellosis emerge every year and survival of the bacteria is still being demonstrated in some poultry farms (EFSA, 2019a). Several authors have pointed out the inefficiency of the hygiene programmes and cleaning and disinfection protocols (Marin *et al*., 2009) not only because of incorrect practice, but also due to the bacteria's resistance to disinfectants (Corcoran *et al*., 2014). For this reason, innovative techniques applied at farm level, such as the use of phages, must be implemented to complement the cleaning and disinfection protocols (Wernicki *et al*., 2017; Sevilla-Navarro *et al.,* 2018).

Phages are ubiquitous agents that infect and replicate in the prokaryotic cells (Kim *et al.,* 2013; Adhikari *et al.,* 2017). These viruses only attack bacteria, altering them until they are destroyed. Their success lies in their high specificity against the target bacterium, their self-amplification and auto-limiting nature and their evolving capacity against antimicrobial resistant bacteria (Wernicki *et al.,* 2017). The effectiveness of phage therapy depends on the individual bacteria, on the given phage concentration, the adaptive mechanism of the bacteria and time of applications (Sevilla-Navarro *et al*., 2018). These

characteristics make phages a very promising tool for the elimination of *Salmonella* in those farms where disinfectants fail to eradicate it (Sevilla-Navarro *et al.,* 2018; Lin *et al*., 2017). Although some phage products are already being commercialised, no precise official guidelines of phage production have been issued, for this reason phage production is still in development and there are some challenges to overcome (Drulis-Kawa *et al*., 2015; García *et al*., 2019). Cocktails of phages, are the most often used against the bacteria, however resistance against the target bacteria can occur and there is the possibility of producing auto-phages, when the cocktails are not active against the field isolates (Sevilla-Navarro *et al.,* 2018; Drulis-Kawa *et al*., 2017; Oeschlin *et al*., 2018).

Few articles describe the use of phages as a sanitiser at field level in poultry farms, although it appears as an emergent measure in the food industry, where it is applied as sanitiser against biofilm bacteria (Oliveira *et al*., 2015; Gutiérrez *et al*., 2016; Hungaro *et al.*, 2013).

Therefore, the main objectives of this study were to assess the effect of phages against *S.* Infantis and *S*. Enteritidis on farm surfaces, and to evaluate phage procedure application as sanitiser against *Salmonella* in field conditions.

3.2.3 Material and Methods

3.2.3.1 *S*. Infantis and *S*. Enteritidis bacteria

The two *Salmonella* isolates (*S.* Infantis and *S*. Enteritidis) employed in this study were isolated CECAV from the *Salmonella* NCP. The isolates were isolated following the ISO 6579-1:2017 and serotyped according to the Kauffman-White-Le Minor technique. They have been stored at -80ºC for further studies.

3.2.3.2 Bacteriophage isolation

3.2.3.2.1 *S.* Infantis bacteriophage isolation

Phage against *S.* Infantis was isolated from faeces collected from different farms. Briefly, 25 g of faeces were homogenised and diluted 1:10 in LB (Luria-Bertani, VWR Chemicals, Barcelona, Spain). Samples were centrifuged at 16,000 x g for 5 min. The supernatant was then filtered through a 0.22 µm membrane. Phage detection was performed by spotting samples on *S.* Infantis lawns as described by Kropinski *et al.* (2007). These plates were incubated overnight at 37ºC. After incubation, a clear zone in the plate resulting from the lysis of host bacteria cells indicates the presence of a specific phage (Hungaro *et al.*, 2013). A single lysis plaque from each positive sample was purified by serial dilutions and plated onto LB agar supplemented with $MgSO_4$ and $CaCl_2$ (Luria-Bertani, VWR Chemicals, Barcelona, Spain). To do so, 200 µL from the host culture and 100 µL of phage containing sample were mixed with 5 mL of 0.6% LB agar and overlaid onto 1.5% LB agar plates, then the mix was incubated overnight at 37ºC. Lysates of single plaques from a single phage were mixed in PBS (Phosphate Buffered Saline, VWR Chemicals, Barcelona, Spain) and centrifuged at 5,000 x g for 5 min. Phage suspensions were recovered and filtered using membranes with a pore size of 0.45 µm and 0.22 µm. Phages were stored at 4ºC. Phage titre was analysed by successive dilutions of the phage suspension performed in PBS. One hundred µL of each dilution together with 100 µL of the respective bacterial host suspension were mixed with 5 mL of LB 0.6% top agar layer and placed over a 1.5% LB agar bottom layer. Plates were incubated overnight at 37ºC. Phage titration was performed three times (Oliveira *et al.*, 2009).

3.2.3.2.2 *S.* Enteritidis bacteriophage isolation

The *S.* Enteritidis phages used in this study were previously obtained and characterised by Sevilla-Navarro *et al.* (2018).

3.2.3.3 Bacteriophage multiplication

Finally, the phage was multiplied until a concentration of 10^{12} PFU/mL, 10^{8} PFU/mL and 10^{3} PFU/mL were reached and stored at 4ºC until use (Adams, 1959). For this purpose, 400 mL of host culture (*S.* Infantis and *S.* Enteritidis, respectively) was grown to OD_{600} = 0.2 at 37ºC. Phage lysate was added to a Multiplicity of Infection (MOI) = 0.1. The sample (bacterium and phage) was incubated under agitation (180 rpm) and every hour up to 8 h, 10 mL of the sample was taken, and several dilutions were prepared into LB. After, 10 µL of each fold dilution were spotted onto double agar layer and incubated overnight at 37ºC. The phage titre was calculated on the basis of counted plaques. Phage titres of 10^{12} PFU/mL, 10^{8} PFU/mL and 10^{3} PFU/mL were selected for the *in situ* trial.

3.2.3.4 Bacteriophage phenotyping

Morphologic plate characteristics were performed to characterise phenotypically whether the phages were lytic or lysogenic according to Jurczak-Kurek *et al.* (2016).

Likewise, the phages were studied in terms of size and morphology by transmission electron microscope as described in previous studies (Sevilla-Navarro *et al.,* 2018). To this end, 10 µL from the phage with a concentration of 10^{8} PFU/mL was fixed in an aqueous solution of paraformaldehyde (2%). A 7.2 V glow was discharged on samples placed on the MESH Cooper grid and incubated in the grids for 15 min. Then, samples were washed in phosphate buffered 0.1 M for 2 min and fixed with glutaraldehyde (1%). Samples were negatively stained with uracil acetate and incubated with methyl cellulose (1%) for 30 sec. Samples were dried until use.

3.2.3.5 *In situ* assay

The floor contamination procedure was performed inside an experimental poultry house at the Animal Research Centre (CITA, Segorbe, Spain) to mimic the real conditions of poultry production. To assess *Salmonella* status of the experimental house before the trial, surface samples were taken in accordance with ISO 6579-1:2017. The material of the phages's application was tested on the cement floor of the house, as house floors have shown a high tendency to resist *Salmonella* disinfection (Davies *et al.*, 2001; Marin *et al.,* 2009).

Two experiments were performed. Phage concentration and times of application were assessed in the first one, and *Salmonella* reduction counts throughout the week after phage application were evaluated in the second.

3.2.3.5.1 Experiment 1 - Definition of bacteriophage concentration and time of application

Each phage (against *S*. Infantis and *S*. Enteritidis) was tested at different concentrations (10^{12}, 10^{8}, 10^{3} PFU/mL) and number of applications (1, 2 or 3) against *S.* Infantis and *S.* Enteritidis, respectively. Each treatment (phage x concentration x application) was evaluated twice. A negative control (only bacteria) was included in the study per concentration, application and session.

For experimental contamination of the house, 80 cm^2 squares were marked on the cement floor. Each square was an experimental unit. First, *Salmonella* (1 mL) was inoculated in each area (*S*. Infantis or *S*. Enteritidis) at a concentration of 10^{8} CFU/mL and spread with a sterile swab. All test areas were allowed to dry under environmental conditions for 3 days (Marin *et al.,* 2009). Before phages were applied, the negative control was swabbed to establish initial *Salmonella* growth according to Commission Regulation (EU) No 2160/2003. Then, each phage was applied on each area at different concentrations and number of applications. To avoid cross contamination between phages and different concentrations, squares were covered with a plastic cover. Finally, each area was swabbed 24h after phages application and *Salmonella* counts were determined according to ISO 6579-2:2017 (Figure 12).

1.

2.

C.

D.

Figure 12. Schedule of experiment 1. A: 80 cm^2 square marked on the cement floor; B: *Salmonella* inoculation; C: Phage application; D: Sample collection for phage effect assessment.

3.2.3.5.2 Experiment 2 - *Salmonella* count monitoring

As cleaning and disinfection procedures are applied with a maximum duration of one week during the downtime in Spanish broiler production (Martínez *et al.,* 2009), the experiment was performed over one week. In accordance with results obtained in experiment 1, the optimum combination of concentration of each phage and number of phages applications were selected for on-farm application. *Salmonella* contamination of the house and the phage application were performed as reported above (Experiment 1). A total of 14 samples per phage were taken and *Salmonella* counts were determined (1 sample x 7 days x 2 sessions) (ISO 6579-2:2012). Moreover, each negative control per experimental unit was assessed as reported above (Experiment 1).

3.2.3.6 Statistical analysis of the *Salmonella* counts

A GLM was used to compare the effect of phage application on *Salmonella* counts, including as fixed effect the number of applications (1, 2 or 3), concentration (10^{12}, 10^{8} and 10^{3} PFU/mL) and number of sessions (n=2). Sessions were not significant and were excluded from the final model (*P*=0.127). The optimal result obtained in *Salmonella* reduction (concentration x application) was used to assess the evolution of *Salmonella* decreasing during the week. A *P-value* less than 0.05 was considered to indicate a statistically significant difference. All statistical analyses were carried out using SPSS 16.0 software.

3.2.4 Results

3.2.4.1 Bacteriophage phenotyping

Phenotypic characterisation showed a *S.* Infantis-phage and a *S.* Enteritidis-phage with a size of 200 nm and an isometric head, which could correspond to the *Myoviridae* family (Figure 13). Moreover, the presence of lytic plaques suggested that both were lytic phages (Jurczak-Kurek *et al.,* 2016).

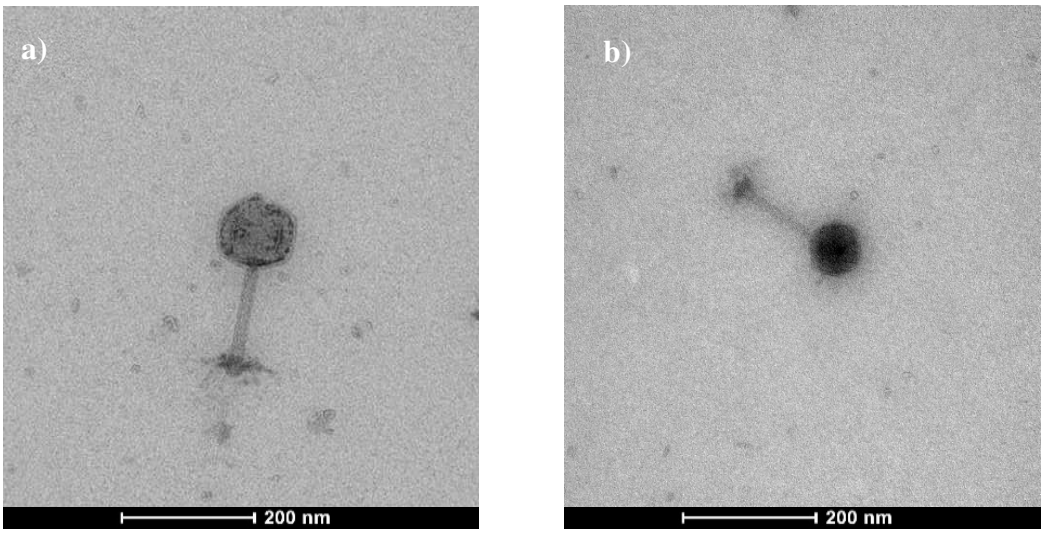

Figure 13. Phenotypic test: observation by transmission electron microscope using negative staining (a) *S.* Infantis-phage; (b) *S.* Enteritidis-phage.

3.2.4.2 Experiment 1 – Definition of bacteriophage concentration and time of application

Regardless of the phage assessed and the concentration applied, statistical differences were found between the number of phage applications and *Salmonella* reduction ($P<0.05$). However, no statistically significant differences were shown between concentration of phages and *Salmonella* reduction.

According to the results obtained after *S.* Infantis-phage application (Table 3), the highest *Salmonella* reduction was obtained after two applications of the phage at a concentration of 10^8 PFU/mL ($P<0.05$). In addition, no statistical differences were observed after the third application. For 10^{12} PFU/mL, the highest *Salmonella* counts reduction was also observed after the second application of the phage, showing no differences after the third application. Finally, at a concentration of 10^3 PFU/mL, no significant differences were found in *Salmonella* reduction despite the number of applications ($P<0.05$).

With respect to the results obtained after *S.* Enteritidis-phage application (Table 4), the optimum reduction in *Salmonella* was obtained after two consecutive applications of the phage at a concentration of 10^3 PFU/mL. In the same line, for 10^{12} PFU/mL and 10^8 PFU/mL *S.* Enteritidis-phage, two applications were necessary to reach the maximum reduction and no statistical differences were found after the third application.

Table 3. *S.* Infantis log_{10} CFU/mL reduction according to phage concentrations and time of application.

	Phage Concentrations (PFU/mL)								
	10^{12}			10^{8}			10^{3}		
	Log_{10} CFU/mL counts	Log_{10} CFU/mL R	SEM	Log_{10} CFU/mL counts	Log_{10} CFU/mL R	SEM	Log_{10} CFU/mL counts	Log_{10} CFU/mL R	SEM
Applications									
C	8.00	0.00^{a}	0.11	8.00	0.00^{a}	0.16	8.00	0.00^{a}	0.21
1	5.02	2.98^{b}	0.18	5.10	2.90^{b}	0.26	4.93	3.07^{b}	0.35
2	4.12	3.88^{c}	0.20	3.45	4.55^{c}	0.28	4.31	3.69^{b}	0.37
3	3.93	4.07^{c}	0.22	3.24	4.76^{c}	0.31	4.03	3.97^{b}	0.41

C: Control group (concentration control group remained constant along the study); $^{a, b, c}$ Means with different superscripts in a column are statistically different ($P<0.05$); SEM: Standard error of the mean; R: reduction.

Table 4. *S.* Enteritidis log_{10} CFU/mL reduction according to phage concentrations and time of application.

	Phage Concentrations (PFU/mL)								
	10^{12}			10^{8}			10^{3}		
	Log_{10} CFU/mL counts	Log_{10} CFU/mL R	SEM	Log_{10} CFU/mL counts	Log_{10} CFU/mL R	SEM	Log_{10} CFU/mL counts	Log_{10} CFU/mL R	SEM
Applications									
C	8.00	0.00^{a}	0.12	8.00	0.00^{a}	0.15	8.00	0.00^{a}	0.14
1	5.64	2.36^{b}	0.20	5.52	2.48^{b}	0.24	5.50	2.50^{b}	0.23
2	4.63	3.37^{c}	0.22	4.83	3.17^{bc}	0.26	4.15	3.85^{c}	0.25
3	4.53	3.47^{c}	0.24	4.61	3.39^{c}	0.29	4.91	3.09^{b}	0.27

C: Control group (concentration control group remained constant along the study); a, b, c Means with different superscripts in a column are statistically different ($P<0.05$); SEM: Standard error of the mean; R: reduction.

3.2.4.3 Experiment 2 - *Salmonella* count monitoring

In accordance with the results obtained in Experiment 1, *Salmonella* counts during a week were analysed after two consecutive applications of phage at different concentrations (10^8 PFU/mL and 10^3 PFU/mL, for *S.* Infantis-phage and *S.* Enteritidis-phage, respectively). After phage application, the highest reduction for both serovars (*S.* Infantis and *S.* Enteritidis) was observed after day 5 of application (7.00 $\log_{10}$ CFU/mL and 4.10 $\log_{10}$ CFU/mL) (P<0.05). However, for *S.* Infantis, no statistically significant differences were shown between the bacteria decrease on days 3 and 5, rising again on days 4, 6 and 7. This occurs similarly with *S.* Enteritidis, where no statistically significant differences were shown between the bacteria reduction on days 5 and 7, showing a rise back up at day 6. Results obtained are summarised in Figure 14.

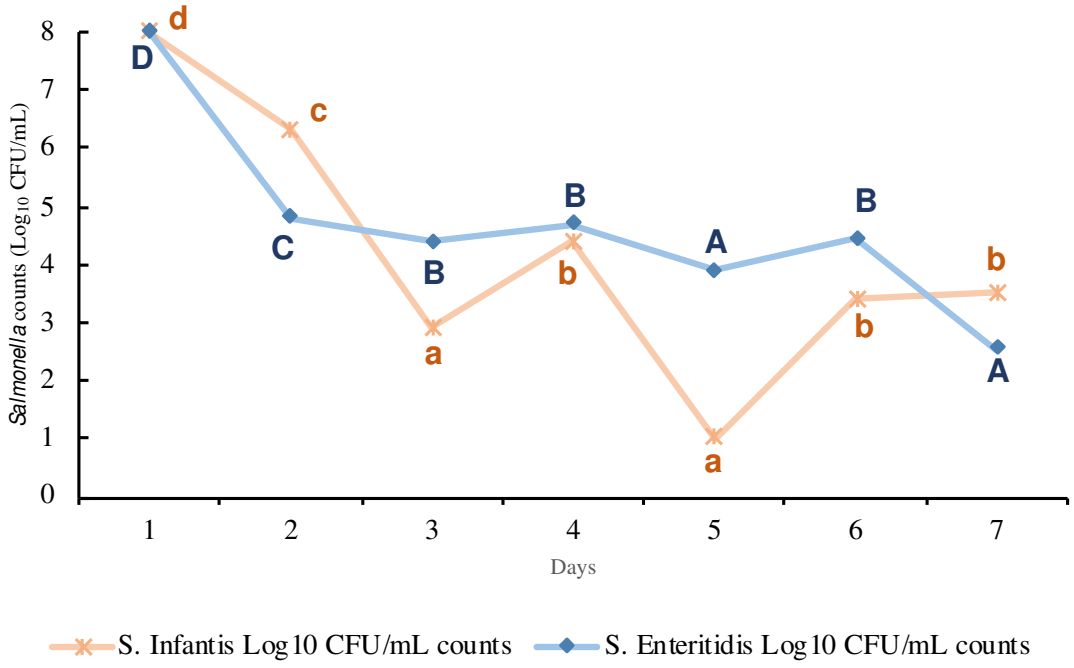

Figure 14. *S.* Infantis and *S.* Enteritidis counts reduction, after two applications of *S.* Infantis-phage and *S.* Enteritidis-phage, respectively.

[a, b, c, d] Means with different superscripts in the same line are statistically different for *S.* Infantis-phage (P<0.05). [A, B, C, D] Means with different superscripts in the same line are statistically different for *S.* Enteritidis-phage (P<0.05).

3.2.5 Discussion

To the best of our knowledge, this is the first study to assess the efficacy of phage as sanitiser against *Salmonella* in poultry farm facilities.

Nowadays, *S.* Infantis and *S.* Enteritidis are the main significant serovars in meat and egg production, respectively (EFSA, 2019a). Due to the impact of these serovars, over the past few years the poultry sector has focused its effort on controlling *Salmonella* in farms. However, the measures are not effective enough, and the bacteria remain in some facilities (Lin *et al.*, 2017). In this context, effective and cost-effective solutions for cleaning and disinfection protocols are seen as a necessary measure for the elimination of *Salmonella* from poultry farms (Carrique-Mas *et al.*, 2009). For this reason, phages have garnered high interest as a potential measure to reduce *Salmonella* contaminations in commercial poultry farms. Phages are useful in a wide range of applications, from health facilities to agriculture and foodstuff industries, to combat bacterial infections (Hungaro *et al.*, 2013; Moye *et al.*, 2018).

The results of our study showed decreases in *S.* Infantis and *S.* Enteritidis of 4.55 $\log_{10}$ CFU/mL and 3.85 $\log_{10}$ CFU/mL, respectively, from the surfaces of farm facilities after consecutive phage application. Similar results were reported by Woolston *et al.* (2013), showing reductions of 4.3 log CFU/surface and 3.0 log CFU/surface after the application of a specific *Salmonella*-phage cocktail. Moreover, after 2 consecutive phage applications, the optimal reduction of *S.* Infantis and *S.* Enteritidis (4.55 $\log_{10}$ CFU/mL and 3.85 $\log_{10}$ CFU/mL, respectively) was reached for 10^8 PFU/ mL and 10^3 PFU/mL, respectively. By comparison, these results are consistent with results obtained by Sevilla-Navarro *et al.* (2018), where the highest *S.* Enteritidis reduction was reached after 2 consecutive phage applications. Furthermore, some authors applied a single phage dose in their studies; however, after the trial they hypothesised that a second application could provide better results (Rombouts *et al.,* 2016). In contrast, Fiorentin *et al.* (2005) had more significant reductions with the use of a single dose of phage in animals than with repeated phage administration, arguing that continuous administration ofphage may lead to resistant *Salmonella.*

No statistically significant differences were found between phage concentration used and *Salmonella* reduction in our study. Different hypotheses could explain this result. Wernicki *et al.* (2017) explained that phages could reach a maximum antimicrobial activity and Carvalho *et al.* (2010) showed that increasing the titre of the phage used could increase the bacterial resistance. Conversely, the bacterium can develop resistance after phage treatment over time (Sharma *et al.,* 2015; Filho *et al.*, 2007). However, the time or the phage dose that could induce this change remain unknown (Pires *et al.*, 2016; Cisek *et al.,* 2017). For this reason, strategies to address the problem of resistance could include the use of cocktails of phages, changes in the phage composition and, therefore, personalising the phage therapy. The different phages present in the cocktail would target different receptors on the bacterial surface, resulting in a lower statistical chance of bacterial co-resistance (Oeschlin, 2018).

With respect to *S.* Enteritidis and *S.* Infantis decrease throughout the week, our results were consistent with those published by Shao and Wang (2008), which reported significant differences in the decrease in *Salmonella* spp. as the week progressed, with day 5 showing the highest reduction in *Salmonella* counts.

Due to antimicrobial and disinfectant resistance, *Salmonella* spp. have become a worldwide concern (Carrique-Mas *et al.,* 2009). Some authors have described the use of additional tools to improve the cleaning and disinfection results and reduce the persistence of pathogens on farm facilities (Maertens *et al.,* 2018). There are some products for the application of phages as disinfectants in food industry facilities, although nothing in the literature describes the use of phages at field level. For this reason, further studies are needed to study the effect of phages on diverse floor surfaces. The development of phage therapy as non-toxic to humans, environmentally friendly and cost-effective holds good prospects for the future as a useful measure for cleaning and disinfection in livestock facilities (Gutiérrez *et al.,* 2016).

However, due to phage therapy's specificity to the host bacteria, phage strategies should not be used alone, but in combination with cleaning and disinfection (Woolston *et al.*, 2013). This way, it could be possible to reduce the infective pressure (3 and 4 logarithms after 2 phage applications) before applying the detergents and disinfectants, achieving an

optimal result of the cleaning and disinfection process. Nevertheless, it is important to highlight that if the bacteria remain in the environment or enter again with a new flock coming into the farm, it will be necessary to apply the phages in combination with an accurate cleaning and disinfection. In this sense, we recommend starting the procedure of cleaning and disinfection by removing any remaining traces of dust and faeces by dry cleaning, followed by a wet cleaning with detergent. Subsequently, on dried facilities, two phage applications will be performed at 24 h intervals. Finally, a double disinfection will be applied, firstly by contact and then by nebulisation.

These promising results showed a new, safe and effective measure to minimise the persistence of pathogens in farm facilities. However, further studies are needed to prove the efficacy of phage in combination with commercial cleaning and disinfection protocols at field level.

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3.3 Autophage as a control measure for *Salmonella* in laying hens

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3.3.1 Abstract

Notwithstanding the *Salmonella* NCP, the latest data published by the EFSA show an increase in *S.* Enteritidis prevalence in laying hen flocks. For this reason, the implementation of innovative techniques such as phage therapy is needed to control *Salmonella* at farm level. Most common phage applications are a cocktail of two or more phages, as it has been reported that cocktails could remove different *Salmonella* serotypes, thus providing cross efficacy. Nevertheless, resistance to the phage cocktail has been reported, resulting in a decrease in their effectiveness. Along these lines, some authors have reported the possibility of using autophage when commercial phage cocktails are not active against field strains. To our best knowledge, no autophage (phage isolated from the same environment where the pathogen is isolated) has been found to control *Salmonella* in laying hens. In this context, the aim of this study was to assess the application of autophage in reducing *S.* Enteritidis in environmental and faecal samples in a layer farm. To this end, the phage was isolated from the same farm where the bacteria was present and was applied onto the facility installations and the animals, at two different times. After phage challenges, swab cloths from facility surfaces and faeces samples were collected at three times, according to the time elapsed after the phage challenge. The results obtained in our study showed that all the surface samples collected from the farm facilities after phage therapy were negative for *Salmonella*. Concerning faeces samples, statistical differences were found in *Salmonella* counts, with the largest decrease (1.78 $\log_{10}$ CFU/g) occurring after the second challenge. Otherwise, depending on the moment of sampling, the results obtained were 2.34 $\log_{10}$ CFU/g, 1.39 $\log_{10}$ CFU/g, 0.56 $\log_{10}$ CFU/g and 0.97 $\log_{10}$ CFU/g, for T0, T1, T3 and T3, respectively. The study highlights the use of autophage therapy not only for *S.* Enteritidis control in animals, but as a sanitiser in cleaning and disinfection. Thus, it could be a measure to avoid the horizontal transmission of *Salmonella* among the animals, as it could remove *Salmonella* from facilities with the presence of the animals.

3.3.2 Introduction

Salmonella spp. is one of the most important zoonotic pathogens with economic impact in animals and humans (EFSA, 2017). The EFSA reported a total of 94,530 cases of human salmonellosis in 2016, with poultry products the main source of infection. In Spain, a total of 9,818 human cases of salmonellosis were reported the same year.

Implementation of the *Salmonella* NCP in accordance with Regulation (EC) No 2160/2003 against *S.* Enteritidis and *S.* Typhimurium, the main serotypes involved in human outbreaks, has resulted in an important reduction in the prevalence of poultry serotypes throughout Europe, especially in Spain (ECDC and EFSA, 2017). In addition to control programmes, *Salmonella* has mainly been controlled through biosecurity and prophylactic measures such as the use of vaccines, prebiotics and probiotics (Colom *et al.*, 2015). However, in laying hens, the prevalence of flocks positive for target serovars, and especially for *S.* Enteritidis, has increased in the last year (EFSA, 2017). For this reason, new and innovative techniques must be implemented to control *Salmonella* at farm level, such as genetic selection, molecular analysis, transcriptomic responses, mutant strains and phages (Anderson *et al.,* 2010; Carvalho *et al.*, 2010; Fife *et al.,* 2010; Filho *et al.,* 2010; Wang *et al.,* 2010).

Phages are viruses that infect and replicate in prokaryotic cells (Kim *et al.*, 2013; Adhikari *et al.*, 2017) and are probably the most widely distributed and diverse entities in the biosphere (Wok *et al.,* 2001; Oliveira 2009). The therapeutic effectiveness of phage therapy depends on their lytic titre, the form and type of application, and the application period (Wernicki *et al.*, 2017). However, despite the call for new antimicrobial drugs, the use of phages in Europe is not allowed, as in other countries as Russia or the United States, where they are used not only at farm level, but also as food additives or in human therapy (Hagens, 2006; Abedon *et al.,* 2011).

Phage therapy seems to be a promising tool for *Salmonella* control at field level (Ahmadi *et al.*, 2016). Different studies have shown significant reduction in *Salmonella* spp. in broilers after the used of phage supplementation (Atterbury *et al.*, 2007, Borie *et al.,* 2008, Ahmadi *et al.*, 2016). In an experimental study in layers, Adhikari *et al.* (2017) reported significant reductions in cecal samples (0.9 $\log_{10}$ CFU/g), internal organs such as spleen, (0.4 $\log_{10}$ CFU/g), liver with gall bladder (0.57 $\log_{10}$ CFU/g) and ovary (0.19 $\log_{10}$ CFU/g)

and in faecal *Salmonella* shedding (0.86 $\log_{10}$ CFU/g) after phage supplementation against *S*. Enteritidis. However, another study showed that phage efficacy should be maximised using a high titre of phage to reduce *Salmonella* at farm level (Wernicki *et al*., 2017).

Most common phage applications are a cocktail of 2 or more phages. It has been reported that cocktails could remove different *Salmonella* serotypes, thus providing crossover efficacy (Wernicki *et al.,* 2017). Nevertheless, resistance to the phage cocktail has been observed, which results in a decrease in their effectiveness (Wernicki *et al.,* 2017). Drulis-Kawa *et al*. (2012) described the possibility of using autophage when the commercial phage cocktail is not active against field strains. This way, lytic autophage could be isolated directly from the environment and prepared for application, being more specific and effective than a commercial phage cocktail. An autophage is a phage isolated from the same environment where the pathogen is isolated. In this context, to our best knowledge, no autophage has been reported to control *Salmonella* in laying hens.

Therefore, the aim of the study was to assess the application of autophage in reducing *S*. Enteritidis in environmental and faecal samples on a layer farm.

3.3.3 Material and Methods

3.3.3.1 Bacterial Strain and Growth Condition

The procedure was based on the official method ISO 6579-1:2017. Faeces samples were homogenised, and 25 g were transferred into 225 mL of BPW (Buffered Peptone Water ISO, VWR Chemicals, Barcelona, Spain). They were then incubated at 37±ºC for 18±2 h. The pre-enriched samples were transferred into MSRV (Modified Semi-Solid Rappaport Vassiliadis agar plate) (MSRV, Difco, Valencia, Spain), which was incubated at 41.5±1ºC for 24-48 h. Suspect plates obtained in MSRV were transferred into XLD (Xylose-Lysine-Deoxycholate) (XLD, Liofilchem, Valencia, Spain) and ASAP (ASAP, bioMerieux, Madrid, Spain), and then incubated at 37±1ºC for 24-48 h. After the incubation period, 5 typical colonies of *Salmonella* were selected and streaked into nutrient agar plates (Scharlab, Barcelona, Spain) 37±1ºC for 24±3 h. *Salmonella* strains isolated were serotyped according to the Kauffman-White-Le Minor technique (Grimont, 2007).

3.3.3.2 Bacteriophage isolation and purification

Phages were isolated from laying hens' faeces collected from 10 different points of the farm. Briefly, 25 g of faeces were homogenised and diluted 1:10 in BPW and a single colony of *S.* Enteritidis previously isolated from the same farm was added to the dilution and incubated overnight at 37ºC. After incubation, 2 mL were transferred into an Eppendorf tube and centrifuged at 16,000 x g for 5 min. The supernatant was then filtered through a 0.22 µm membrane. Phage detection was done by spotting the phage lysate on *S.* Enteritidis lawns, as described by Kropinski *et al.* (2007). These plates were incubated overnight at 37ºC. After incubation, a clear zone in the plate resulting from the lysis of host bacteria cells indicates the presence of phages (Hungaro *et al.,* 2013). A single lysis plaque from each positive sample was purified by serial dilutions and plated to LB agar (Luria-Bertani, VWR Chemicals, Barcelona, Spain). To this end, 200 µL from the host culture and 100 µL of phage-containing sample were mixed with 3 mL of 0.6% LB agar and overlaid onto 1.5% LB agar plates and incubated overnight at 37ºC. Lysates from single plaques were mixed and centrifuged at 5,000 x g during 5 min. Phage suspensions were recovered and filtered with membranes with a pore size of 0.45 µm and 0.22 µm. Phages were stored at 4ºC.

3.3.3.3 Bacteriophage amplification

The amplification of each isolated phage was performed by inoculating 4.5 mL of the purified phage suspensions in 9 mL of a 4-6 h BPW culture in BPW of the respective *Salmonella* hosts. It was incubated overnight at 37ºC, with shaking (120 rpm). The suspension was centrifuged at 9,000 x g for 10 min and filtered through a 0.22 µm membrane. This procedure was repeated by inoculating the resulting phage lysate volume in 100 mL of 4-6 h BPW culture followed by incubation overnight at 120 rpm and 37ºC. The resultant phage suspension was filtered through a 0.22 µm membrane and stored at 4ºC.

Phage titre was analysed according to Adams (1959), with slight modifications. Briefly, successive dilutions of the phage suspension were performed in a saline solution and 100 µL of each dilution together with 100 µL of the respective bacterial host suspension were mixed with 3 mL of LB 0.6% top agar layer and placed over a 1.5% LB agar bottom layer. Plates were incubated overnight at 37ºC. Phage titration was performed in triplicate

(Oliveira, 2009). Finally, autophage was multiplied until a concentration of 10^9 PFU/mL was reached and stored at 4ºC until use.

3.3.3.4 Bacteriophage characterisation

Phenotypic tests, such as observation by transmission electron microscope and morphologic plate characteristics, were performed to characterise whether the autophage was lytic or lysogenic (Li *et al.*, 2016). For this, lawns of indicator strains were prepared as overlays by adding 100 µL of overnight culture to 4 mL of 0.6% LB agar. After mixing, the inoculum was immediately overlaid onto 1.5% LB agar plates and incubated overnight at 37ºC and examined for lysis zones (plaques).

Moreover, the phage was studied in terms of size and morphology by using transmission electron microscope. For this purpose, 10 µL from the phage with a concentration of 10^8 UPF/mL was fixed in an aqueous solution of paraformaldehyde (2%). A 7.2 V glow was discharged on samples placed on the MESH Cooper grid and incubated in the grids for 15 min. Then, samples were washed in phosphate buffered 0.1 M for 2 min and fixed with glutaraldehyde (1%). Samples were negatively stained with uracil acetate and incubated with methyl cellulose (1%) for 30 sec. Samples were dried until use.

3.3.3.5 *In Vivo* Assays in Laying Hens

The effect of the autophage against field *S*. Enteritidis was assessed in a commercial farm were *S*. Enteritidis and autophage were isolated. Twenty thousand-layer hens (60-week-old) were raised in battery cages and reared according to bird age in environmentally controlled temperatures, between 18-20ºC, and air relative humidity (60-70%), within the standards of the line (Lohmann, 2016) (Figure 15). The animals were handled according to the guidelines for experimentation animal care (BOE, 2013).

Figure 15. Laying hens farm.

Before the challenge, to confirm *Salmonella* farm status, 2 swab cloths from the surface of facility installations and 10 faeces samples were randomly taken from the farm (T0). Faeces and swab cloths were collected as reported in the NCP (PNC, 2018). Just after sampling, the autophage was applied onto the animals and the facilities by spray. Phage challenge was performed at 2 times (Figure 16).

Figure 16. Preparation of phage before application onto the facilities.

The first challenge took place on the first day of the trial and the second challenge 24 h later. After challenges, swab cloths from the facilities surfaces and faeces samples were collected at 3 times: 24 h after first phage challenge (T1), 24 h after second challenge (T2) and 7 days after first challenge (T3). To this end, the autophage effect on the facilities

against *Salmonella* was assessed taking sterile swab samples from the surface and the effect on the animal was assessed taking samples from the faeces line (Figure 17).

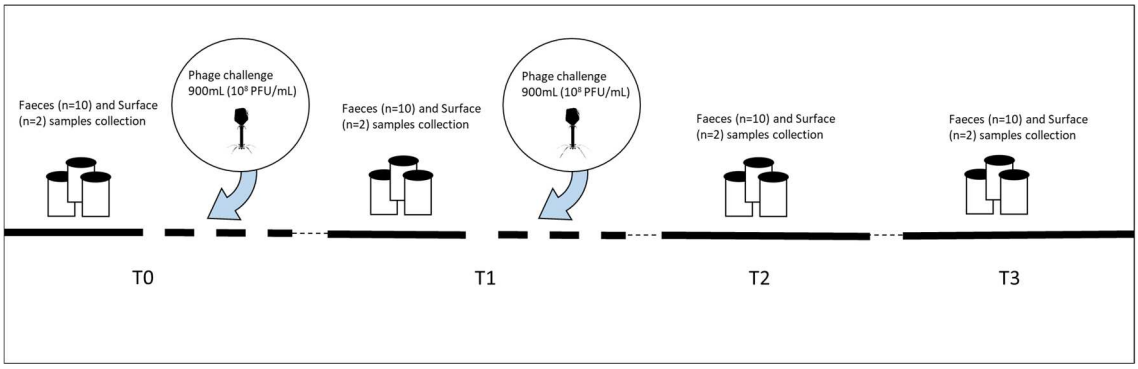

Figure 17. Schematic illustration of the autophage challenge and samples collection within the study.

T0: Sampling before challenge; T1: Sampling 24 h post first challenge; T2: Sampling 24 h post second challenge: T3: Sampling 7 days post first challenge

From facility surfaces, *Salmonella* detected from swab cloths collected were tested according to ISO 6579-1:2017, as described above. Moreover, for *Salmonella* enumeration, the procedure described by Fravalo *et al.* (2003) was performed with slight modifications. Briefly, faeces samples were homogenised, and 25 g were transferred into 225 mL of BPW, then 2.5 mL of the suspension was transferred into an empty tube, and serial 1:5 dilutions were made and incubated at 37ºC for 18±2 h. After incubation, 20 µL of each tube were transferred onto MSRV agar plates and incubated at 41.5ºC for 24-48 h. Suspect plates were plated onto XLD medium and incubated at 37ºC ±1 for 24±3 h. After the incubation period, a characteristic colony was tested by biochemical confirmation and serotyped according to the Kauffman-White-Le Minor technique. Finally, for the estimation of MPN (Most Probable Number), the software described by Jarvis *et al.* (2010) was used and the results were transformed into logarithms (log_{10} CFU/g).

3.3.3.6 Statistical analysis

One-way ANOVA was used to compare the effect of autophage application on *Salmonella* recovery in swab cloth surface samples and on the *Salmonella* counts in faeces samples before and after the autophage challenge, among 4 different times (T0, T1, T2 and T3). A *P*-value of less than 0.05 was considered to indicate a statistically

significant difference. All statistical analyses were carried out using SPSS 16.0 software (SPSS Inc., Chicago, IL).

3.3.4 Results

At each sampling time (T0, T1, T2 and T3), 2 surface swab cloths and 10 faeces samples were collected, with a total of 48 samples.

Surface samples taken before phage infection were positive for *Salmonella* serotype Enteritidis. However, after phage application, all samples collected from the farm facilities were negative for *Salmonella*.

For faeces samples, *Salmonella* counts are shown in Table 5. Mean counts results obtained before (2.34 $\log_{10}$ CFU/g) and after (1.07 $\log_{10}$ CFU/g) autophage application presented statistical differences (P<0.05). Otherwise, depending of the moment of sampling, the results obtained were 2.34 $\log_{10}$ CFU/g, 1.39 $\log_{10}$ CFU/g, 0.56 $\log_{10}$ CFU/g and 0.97 $\log_{10}$ CFU/g, for T0, T1, T2 and T3, respectively.

Table 5. *Salmonella* counts ($\log_{10}$ CFU/g) in laying hens' faeces before and after phage infection.

Sample ID	CFU/g (before challenge)	CFU/g (Day post-challenge)		
	T0	T1 (24h)	T2 (48h)	T3 (7 days)
1	2.9	0.51	1.1	1.6
2	2.9	0	0	0
3	1.8	0.19	0.93	1.8
4	0	0	0	0
5	2.0	0.58	0	0
6	1.3	2.9	0	0
7	2.9	1.1	0.79	2.9
8	2.9	2.9	1.5	1.58
9	2.9	2.9	1.1	1.8
10	2.9	2.9	0.19	0

ID: Identification number of the sample; T0: Sampling before phage challenge; T1: Sampling 24 h post first phage challenge; T2: Sampling 24 h post phage second challenge: T3: Sampling 7 days post phage first challenge.

Statistical differences were found in *Salmonella* counts at the time of sampling (Figure 18). The highest reduction in *Salmonella* shedding (1.78 $\log_{10}$ CFU/g) was observed 48 h after the first autophage challenge.

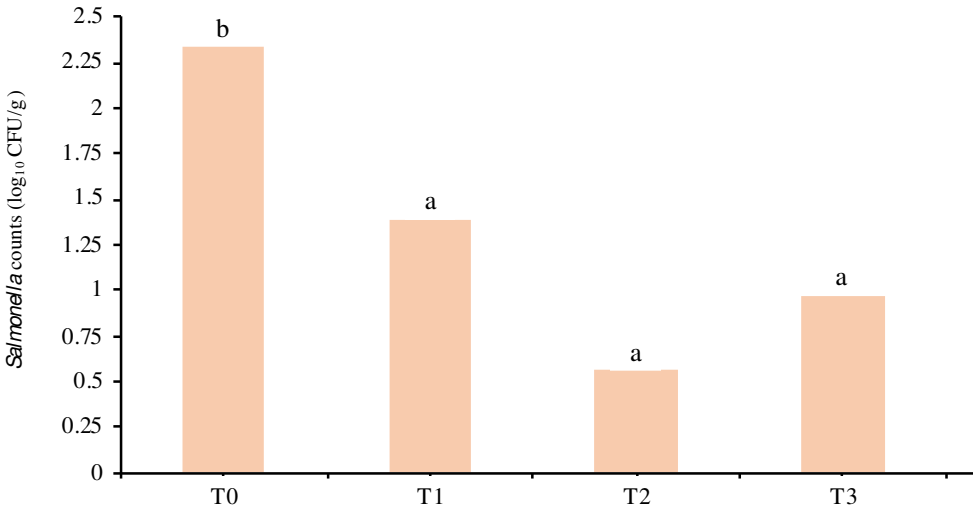

Figure 18. *Salmonella* mean counts (log_{10} CFU/g) in laying hens' faeces before (T0) and after phage challenge (T1 to T3).

T0: Sampling before phage challenge; T1: Sampling 24 h post first phage challenge; T2: Sampling 24 h post second phage challenge: T3: Sampling 7 days post first phage challenge. [a,b] Means with different superscripts are statistically different (P<0.05).

Autophage characterisation showed a phage with a size of 200 nm and isometric head, which could correspond to the *Myoviridae* family (Figure 19a). Moreover, the diameter and clear plaques indicated it was a lytic phage (Figure 19b) (Jurczak-Kurek *et al.*, 2016).

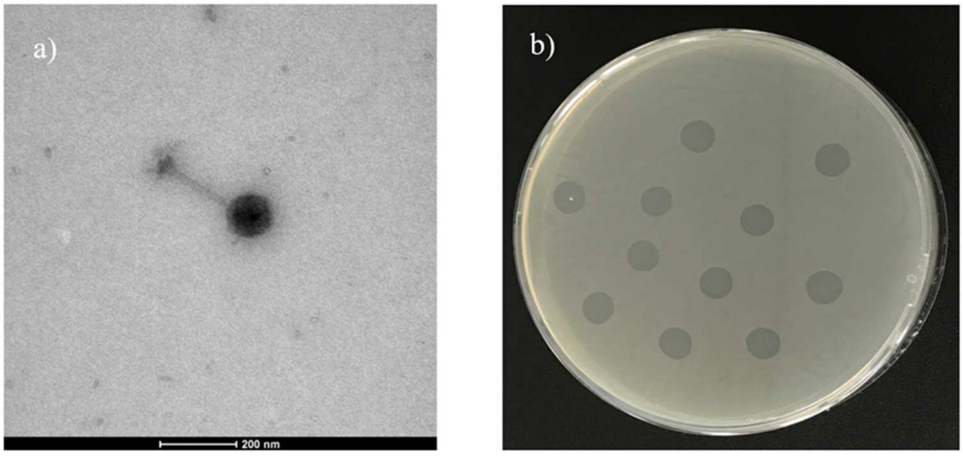

Figure 19. Phenotypic tests: observation by transmission electron microscope and morphologic plate characteristics. a) Electron micrograph of autophage using negative staining; b) Clear lysis zone as result of *S.* Enteritidis lysis due to the presence of autophage.

3.3.5 Discussion

The results showed *S.* Enteritidis removal from farm facility surfaces after autophage application. Moreover, after 2 consecutive autophage applications in layers, there was a reduction in faecal *S.* Enteritidis counts.

The rise of antibiotic-resistant bacterial strains has resulted in an increased interest in alternative measures such as phage therapy (Nilsson, 2014; Ahmadi *et al.,* 2016). However, and despite the need to develop new antibacterial agents, the approval rate of novel alternatives to antibiotics, as phages, remains low or void (Callun *et al*., 2016). This is due to the enormous variation of bacteria-phage combinations, requiring a large number of obligatory clinical trials before being considered as a viable alternative to antibiotics (Callum *et al.,* 2016). For this reason, the need arises to establish a more efficient regulatory pathway to authorise the use of phages. Previous studies on safety, efficacy and quality could possibly assist in the establishing of new regulatory paths.

Virulent phages are abundant worldwide and have been proven to be very effective in *in-vitro* trials (Nilsson, 2014). Clinical trials with a cocktail of phages in poultry production showed promising results, although it has been shown that the treatment is not completely effective (Nilsson, 2014; Wernicki *et al.,* 2017). The effectiveness of phage therapy depends on the individual bacteria, on the given phage and on the adaptive mechanism of the bacteria (Wernicki *et al.,* 2017). For example, *Salmonella* is an intracellular pathogen that diminishes the phage infection and multiplication inside the eukaryote cell (Silva *et al.,* 2012).

There is a huge bibliography showing the efficacy of phages against bacteria in broilers. Fiorentin *et al*. (2005) and Lim *et al.* (2011) reported positive results against *S.* Typhimurium and *S.* Enteritidis infection using a cocktail of more than 2 phages. Ahmadhi *et al*. (2016) reported that a single phage was more effective as a prophylactic measure than as a treatment for *S*. Enteritidis infection. Nevertheless, no references were found that report the efficacy of autophage therapy in laying hens. This study showed significant differences in *Salmonella* counts in faeces 48 h and 7 days after autophage applications. Similar results were obtained by Adhikari *et al.* (2017), who concluded that the phage supplementation reduced faecal *Salmonella* shedding after 6 days post-

challenge. However, in broilers, Filho *et al.* (2007) reported that the use of phage therapy as oral administration in chickens against *S.* Enteritidis inhibits the bacteria only 24-28 h after phage treatment, suggesting that the bacteria could have developed resistance against the phages. Nevertheless, other studies, such as Toro *et al.* (2005) and Fiorentin *et al.* (2005), observed higher reductions. Different hypotheses could explain this fact, such as the age of the animals, the different phage application method and the trial conditions (Huff *et al.,* 2003; Adhikari *et al.*, 2017; Wernicki *et al.*, 2017), or the specificity of the phage against the target bacteria.

The high specificity against target bacteria, the cost-effectiveness ratio, the easy administration and fewer side effects than antibiotics make phage therapy a promising tool to control *Salmonella* or other pathogens in poultry (Drulis-Kawa *et al.*, 2012; Nikkahdi *et al.,* 2017). However, despite the need to determine the etiological factor causing an infection before phage therapy application, this ensure the specificity of the treatment, which only removes the target bacteria. Antibiotic therapies are the other side of the coin, indiscriminately removing pathogens and normal microbiota, in addition to the antimicrobial resistance that the pathogen acquires (Loc-Carrillo and Abedon, 2011; Drulis-Kawa *et al.*, 2012). Moreover, this study highlights the use of autophage not only for *S.* Enteritidis control in animals, but as a sanitiser in cleaning and disinfection. Thus, it could be a measure to avoid the horizontal transmission of *Salmonella* among the animals, as *Salmonella* could be removed from facilities in the presence of the animals.

To conclude, the use of phages could not only be a preventive or prophylactic measure against pathogens with importance in poultry products, but also a complementary tool for cleaning and disinfection. Moreover, the fact that the autophage removed the *Salmonella* from the environment could prevent horizontal recontamination between infected and non-infected animals.

3.3.6 References

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CHAPTER IV. GENERAL DISCUSSION

4. General Discussion

As described at the beginning of this manuscript, three experiments were carried out with the aim of providing knowledge to address some of the challenges facing the poultry sector, such as the resistance of *Salmonella* in the poultry production system despite the strict biosecurity measures set out at field level since 2007.

We have focused our studies on phage therapy strategies that we believe could help poultry producers in their fight against *Salmonella* and would at least help enhance food safety. Moreover, the results obtained might help draft future legislative consideration for phage authorisation as a complementary tool for cleaning and disinfection or as an alternative measure to those currently in place.

Salmonellosis is one of the main infections affecting the commercial poultry market, causing great economic losses in poultry production and posing a public health concern. Among the 1.2 million cases caused by *Salmonella*, contaminated food caused 1 million illness (CDC, 2019). Although there are numerous causes of human salmonellosis, eggs and poultry meat are considered the most common source of human infection (EFSA and ECDC, 2019). For this reason, *Salmonella* in poultry has been subject to an official control programme in the European Union (EU) since 2007. Legislators have been working with the aim of minimising *Salmonella* prevalence in the poultry sector, particularly *S.* Enteritidis and *S.* Typhimurium serovars (Martínez-Avilés, 2019). However, *Salmonella* control is complicated because there are numerous potential sources of contamination by these bacteria in an integrated poultry operation (feed, rodents, wild birds, insects, transport, farm surroundings and the processing plant environment) (Marin *et al.*, 2011). All of this, together with *Salmonella'*s antimicrobial resistance and its transmission through the food chain, has increased the concern of the competent authorities to eliminate the bacteria from primary production.

Phages are known to be highly abundant in multiple environments, and they outnumber their bacterial hosts (Parikka *et al.*, 2017). In this manuscript, we have presented the diversity of *Salmonella* phages in poultry farms in terms of livestock production (broilers and layers). The results showed that 100% of samples collected from poultry farms were positive for the presence of phages. However, no *Salmonella* was isolated from the farm

at that time. This fact could be explained by the bacteria having been present in the flock at some point in the past. The fact of being able to isolate phages where the bacteria has not been found can help to better understand *Salmonella* epidemiology in the field. These results are in accordance with those published by Petsong *et al*. (2019), who recovered a vast number of *Salmonella* phages from different farms, suggesting that farm environments, especially the poultry ones, could represent an important source of abundant *Salmonella* phages (Bao *et al*., 2011; Hungaro *et al.,* 2013).

Regarding *Salmonella* serovar-phages isolated, the most prevalent were *S*. Enteritidis, *S*. Virchow, *S*. Infantis, *S*. Typhimurium and *S*. Ohio. Different hypotheses could explain this. One could be that these serovars were the most prevalent in Spanish poultry farms years ago, and, although they have been eliminated from most poultry facilities, their phages remain in the poultry environment. Another hypothesis could be the strict vaccination programmes implemented in poultry production. It is important to highlight that vaccination spreads the vaccine strain and its phages throughout the house environment, resulting in cross contamination between animals (EFSA and ECDC, 2004). In addition, vaccination in breeders can result in vaccine strain vertical transmission to their progeny (Dórea *et al.,* 2010).

Regarding *Salmonella* phage production type, *S*. Virchow, *S*. Ohio and *S*. Hadar were more prevalent in broiler than in layers. These results are in line with the data recovered from the *Salmonella* control programme in the Valencia Region. Regarding mST and *S*. Kentucky phages, they were more prevalent in layer production. Concerning mST, it could be explained by the administration of the *S*. Typhimurium vaccine strain, which could provide cross contamination against mST. Regarding *S*. Kentucky, several authors have isolated this serovar from environmental samples in poultry farms.

This first study attempted to explain the abundance and diversity of *Salmonella* phages in the environment of poultry farms. A close relationship has been shown between phage prevalence and *Salmonella* serovar prevalence in poultry farms, suggesting that phages co-exist within its serovars. Moreover, the fact of isolating a higher number of phages against *S*. Enteritidis and *S*. Typhimurium makes the vaccine a double control measure; on one hand, the vaccine immunises the animals, and on the other it increases the prevalence of phages against public health strains, helping their control on farms.

Furthermore, the results of this study suggest that it is possible to control the bacteria present in a farm by isolating, amplifying and applying its autophage in the same farm. However, genomic and metagenomic studies should be carried out to begin appreciating the diversity and abundance of phage species on a global scale, especially in poultry farms (Ofir and Sorek 2018).

According to previous results that demonstrated that the role of phages in the environment is to balance the bacterium presence, the use of phages at higher concentrations as a complementary tool for cleaning and disinfection could cause a natural bacteria-phage balance disruption by reducing the presence of the bacteria. After the application of phages against *S.* Infantis and *S.* Enteritidis onto the facilities of an experimental farm, it was shown that *S.* Infantis and *S.* Enteritidis decreased 4.55 $\log_{10}$ CFU/mL and 3.85 $\log_{10}$ CFU/mL, respectively, from the surfaces of the farm after two consecutive phage applications. Moreover, with respect to the reduction throughout the week, significant differences were shown in the *Salmonella* decrease, with the 5th day of the week presenting the highest reduction in *Salmonella* counts.

Phage therapy has been successfully tested in treatment against *Salmonella* with promising results. Phages are considered one of the safest antibacterial treatments, as they are highly specific for their target microorganism and are anything but harmful to humans, animals and plants and they have few side effects (Sulakvelidze, 2011; Abedon, 2015; Pirnay *et al.,* 2015). There are some products for the application of phages as sanitisers, although nothing in the literature describes the use of phages at field level. For this reason, further studies are needed to study the effect of phages on diverse surfaces present in poultry farms.

Additionally, the results have been far more promising when application of the phage was done in a commercial farm naturally contaminated by *Salmonella*. The results obtained showed that 100% of samples were negative for the presence of *Salmonella* in the farm after phage application (Sevilla-Navarro *et al.,* 2018). Furthermore, after two consecutive phage applications, we managed to reduce the *Salmonella* count logarithms (1.27 $\log_{10}$ CFU/mL) from faeces. This could be explained by the fact that *Salmonella* is an intracellular pathogen that has the advantage of surviving inside host cells. It has the capacity to invade intestinal epithelial cells and penetrate intracellularly within

macrophages. These intracellular *Salmonella* characteristics are not easily controlled by phages, as they would be unable to enter the eukaryotic cells (Sulakvelidze *et al.,* 2001; Doss *et al.,* 2017). Similar results were reported by Nabil *et al.* (2018), who described a reduction of *S.* Enteritidis after four consecutive phage applications. Although we did not succeed in eliminating the bacterium from the animals, due to the intracellular character of *Salmonella*, our study demonstrated that the use of phages where animals are infected with the bacterium could reduce the horizontal cross contamination, limiting the spread of infection.

If we reduce the on-farm concentration of the bacteria with biosecurity and prophylactic measures together with the phage therapy, we might be able to eliminate all the *Salmonella* from farms. Phages remain infective under very harsh environmental conditions and tend to continue replicating until the population density of the host bacteria has been significantly reduced. These qualities indicate that phage therapy may require fewer or limited administrations while performing as well or better than conventional treatments (Doss *et al.*, 2017). What is more, the FAO-WHO demonstrated that lowering the on-farm prevalence of the bacteria is an important strategy for reducing the bird excrement entering the processing plant and lowering the risk of contaminated meat products reaching the food chain. For this reason, control of the main risk factors and the implementation of new sanitary measures, such as phages, could be a useful tool to reduce or even eliminate the presence of *Salmonella* on-farm.

In conclusion, phages are natural bacterial killers that can be used not only as antimicrobials for animal infection but also as a complementary tool in the cleaning and disinfection process. This way, phages could be useful for pathogen control and to lower the risk of pathogens infecting the animals and hence the derived food products. The most relevant results obtained from this doctoral thesis is that the poultry sector has a suitable, cost-effective and eco-friendly tool able to reduce *Salmonella* prevalence significantly when the current strategies are not able to eliminate it from the poultry farms. However, further studies are needed to prove the efficacy of phages in combination with other current control measures, such as dietary nutrients or commercial cleaning and disinfection protocols at field level.

4.1 References

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CHAPTER V. CONCLUSIONS

5. Conclusions

1. There is a close relationship between *Salmonella* phage prevalence and *Salmonella* serovar prevalence in poultry farms, suggesting that phages co-exist within their serovar.

2. The fact of isolating a higher number of phages against *S.* Enteritidis and *S.* Typhimurium suggests *Salmonella*-vaccination as a double control measure, as it immunises the animals and increases the prevalence of phages against public health strains.

3. Due to bacteriophage therapy specificity to the host bacteria, bacteriophage strategies should not be used alone, but in combination with cleaning and disinfection strategies.

4. Phage therapy could reduce the *Salmonella* infective pressure before applying the detergents and disinfectants, improving the results of the farms' cleaning and disinfection processes.

5. The use of phages at higher concentrations in farms could cause a natural bacterium-phage balance disruption, thus reducing the *Salmonella* from the farm facilities, providing a new tool to control *Salmonella* at field level.

6. Autophage reduce and remove the *Salmonella* from the animal's faeces and farm facilities, respectively, preventing the horizontal recontamination between infected and non-infected animals.

7. Further studies are needed to prove the efficacy of bacteriophage in combination with commercial cleaning and disinfection protocols at field level.