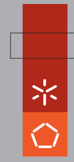

Development of a bacteriophage based product to control colibacillosis in poultry
Ana Cristina Afonso Oliveira
UMinho | 2009

Universidade do Minho
Escola de Engenharia

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Development of a bacteriophage based product to control colibacillosis in poultry

Dissertation for PhD degree in Chemical and Biological Engineering

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August 2009

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Universidade do Minho

Agosto de 2009

The most difficult was to get here.

The easiest was to travel this whole way and just enjoy the journey!

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A ti dedico este meu esforço, este meu empreendimento.

Abstract

Escherichia coli (*E. coli*) is part of the commensal microflora of the chicken intestinal tract, being commonly an opportunistic bacteria that causes disease in immunologic deprived chickens. However, there are extra-intestinal *E. coli* strains, the avian pathogenic *Escherichia coli* (APEC) that are able to cause colibacillosis by itself, due to its invasive ability. The colibacillosis and colisepticemia are responsible for significant economic losses in poultry industries worldwide, mainly due to the low feed conversion rate with consequent weight loss, high cost of treatments during production, poor carcass quality with consequent rejection at slaughter and high mortalities rates. The increasing high patterns of antibiotic resistance acquired by these bacteria, as well as the restriction to the antibiotic usage implemented by the European Union, have encouraged the search of new solutions to control severe infections ensuring good meat quality and minimizing environmental impact.

Bacteriophages (phages), virus infecting exclusively bacteria, have been proposed as valuable alternatives to antibiotics based on their capacity to infect and destroy the bacteria, releasing in few minutes progeny that will infect the surrounding hosts.

The presented work aimed at developing an efficient, safe and competitive phage based product to control colibacillosis in poultry. The work encompassed five different stages: firstly, different bacteriophages active for a wide range of APEC strains were isolated and characterized; secondly, an *in vivo* evaluation of the toxicity of the phage suspensions were performed, thirdly the effect of the route of administration and phage titre on phage dissemination in the chickens' organisms was assessed; fourthly the efficacy of the phages presenting the wider lytic spectrum was evaluated through *in vivo* efficiency trials; finally, large animal trials were performed to validate the efficacy of the phage product.

Phages were isolated from poultry sewage and tested against 148 O-serotyped APEC strains. The results showed that 70.5% of the tested strains were sensitive to a combination of three of the five isolated phages. Taxonomically, two of these three phages, phi F61E and phi F78E look like 16-19, T4-like phages (*Myoviridae*) and the other, phi F258E is a T1-like phage and belongs to the *Syphoviridae* family. All belong to the *Caudovirales* order. Restriction fragment length polymorphism (RFLP) patterns demonstrate that all phages were genetically different. The *in vivo* evaluation of the toxicity of the phage lysate revealed that the phage suspensions did not promote any

decrease in feed and water intake, or body weight lost during the *in vivo* trial and the *post mortem* necropsies did not show any macroscopic lesions in the internal organs. These observations supported that the lysate was not toxic for chickens.

The *in vivo* assessment of the effect of the route of administration and the dosage in the dissemination of the phages in the chicken's organs, revealed that when administered orally and by spray, all the phages reached the respiratory tract, as well as the bloodstream. Intramuscular administration enabled the phages to reach all chickens' internal organs. Results suggested that, besides the intramuscular administration (not feasible to use in flocks), the oral and spray administration can be considered promising administration routes to treat respiratory *E. coli* infections in poultry.

The *in vivo* evaluation of the efficacy of phi F78E to control severe *E. coli* infections revealed that phage performance is dosage dependant and only a high concentration of 10^9 PFU/ml allowed a decrease in 25% and 43% in chickens' mortality and morbidity, respectively. Interestingly, the phage cocktail (of phi F61E, phi F78E and phi F258E) administered in the water drinking and by spray in a single application, and composed by 5×10^7 PFU/ml of each bacteriophage, was able to control the mortality rate in naturally infected chicken flocks, refractive to antibiotherapy. The mortality felt from 2.2% in average, to under 0.5% in no more than three weeks, with no recidivism.

In conclusion, with this work it was possible to obtain an antimicrobial product, comprised by a combination of three different lytic phages, which demonstrated to be safe for chickens and efficient against colibacillosis in the poultry industry.

Sumário

A *Escherichia coli* (*E. coli*) é uma bactéria que integra a flora intestinal das aves, e que frequentemente se comporta como oportunista, causando doença em aves imuno-deprimidas. Contudo, existem estirpes extra-intestinais patogénicas (APEC) que são capazes, pelas suas capacidades invasivas, de causar infecções (colibacilose) que podem degenerar em septicemias. Estas infecções são responsáveis por perdas económicas importantes na indústria avícola, devido a baixas taxas de conversão alimentar com consequentes perdas de peso das aves, elevados custos de tratamento na produção, baixa qualidade da carcaça que leva a rejeições no matadouro e elevadas taxas de mortalidade. O aumento do padrão de resistências a antibióticos adquiridos por estas bactérias, bem como as restrições que a Comunidade Europeia tem vindo a impor no uso destes anti-microbianos, vem relançar a importância do desenvolvimento de alternativas terapêuticas.

Os bacteriófagos (fagos), são vírus que parasitam exclusivamente bactérias e que têm vindo a surgir como alternativa valiosa à terapia tradicional. A sua capacidade de infectar e destruir bactérias hospedeiras, libertando nova geração com potencial para infectar os hospedeiros circundantes são características que corroboram a sua valia.

O presente trabalho visou o desenvolvimento de um produto à base de fagos para o controlo de colibaciloses na indústria avícola, que se mostrasse eficiente e inócuo e que fosse competitivo. O trabalho compreendeu cinco fases diferentes: em primeiro lugar, bacteriófagos distintos que infectavam uma vasta gama de estirpes APEC foram isolados e caracterizados; seguidamente, foi efectuada a avaliação *in vivo* da toxicidade da suspensão de fagos; em terceiro lugar, foi testada a influência da via de administração e da concentração de fagos na sua disseminação no organismo das aves; posteriormente a eficiência dos fagos com o espectro de lise mais amplo foi avaliada, através de experimentação *in vivo*; finalmente foram realizadas experiências em aviários experimentais para validar a eficácia do produto à base de fagos.

Os fagos foram isolados de camas de aviários e testados para 148 APEC serotipadas para o antigénio "O". Destas, 70.5% revelaram-se sensíveis a pelo menos um de três dos fagos. Taxonomicamente, todos os fagos se inserem na ordem dos *Caudovirales*, sendo que dois deles, o phi F61E e o phi F78E são fagos 16-19, do tipo T4 (*Myoviridae*) e o outro, phi F258E é um fago do tipo T1 (*Syphoviridae*). Padrões de RFLP demonstraram que todos os fagos são geneticamente diferentes.

A avaliação *in vivo* da toxicidade do lisado de fagos revelou que a suspensão de fagos não provocou qualquer diminuição na ingestão de alimento e água nem perda de peso, e a avaliação *post mortem* das carcaças não revelou lesões macroscópicas nos órgãos. Estas observações corroboram a inocuidade do produto, para as aves.

As experiências *in vivo* para analisar o efeito da via de administração e da concentração da suspensão, na disseminação dos fagos no organismo das aves, indicou que quando administrados por via oral e por spray, qualquer dos tipos de fago em teste atingiram o tracto respiratório e a corrente sanguínea. A administração intra-muscular, permitiu que os fagos testados fossem recuperados em todos os órgãos analisados. Os resultados sugerem que, para além da administração intramuscular (modo que não é prático para o maneio em bandos), a administração oral e nasal deverão ser veículos eficientes de transporte de fagos para o tratamento de infecções respiratórias por *E. coli* em aves.

A avaliação *in vivo* da eficiência do phi F78E para o controlo de infecções severas de *E. coli*, revelou que o desempenho do fago estava dependente da dose, e que apenas uma concentração elevada, de 10^9 PFU/ml, permitiu um decréscimo na mortalidade e morbilidade das aves, respectivamente de 25% e 43% em média.

Curiosamente, a combinação de fagos (phi F61E, phi F78E e phi F258E), numa concentração de 5×10^7 PFU/ml cada um, administrado na água de bebida e por spray numa única aplicação, foi eficiente no controlo da mortalidade em bandos naturalmente infectados por APEC, e em que os antibióticos não tinham tido sucesso terapêutico. A mortalidade desceu, em média, de 2.2% para valores inferiores a 0.5% em não mais de 3 semanas, sem recidivas. Em conclusão, este trabalho possibilitou o desenvolvimento de um produto constituído por três fagos, que demonstrou ser inócuo e eficiente no controlo de colibacilose na indústria avícola.

Outline of the thesis

This thesis is structured in 7 chapters:

Chapter I presents the background information about the role of *Escherichia coli* (*E. coli*) in the poultry industry, the motivations of finding new alternatives to antibiotics, and the features and research studies that support bacteriophages' therapeutic potential.

In **Chapter II**, the selection and characterization of bacteriophages (phages) to be incorporated in a therapeutic cocktail, aiming at controlling pathogenic *E. coli* strains in poultry is described. The results presented in this chapter comprise the isolation of phages from poultry sewage, the *in vitro* evaluation of phages lytic spectra towards a panel of isolated and O-serotyped APEC strains, the phages morphological characterization by Transmission Electronic Microscopy (TEM), the phages life cycle investigation by the induction of infected hosts with Mytomicin C and the genetic comparison between phages' DNA, performed by restriction fragment length polymorphism (RFLP) patterns.

Chapter III addresses the *in vivo* toxicity evaluation of the cocktail composed by the three selected phages, phi F78E (Myoviridae), phi F258E (Syphoviridae), and phi F61E (Myoviridae).

The results of the influence of the administration route and the phage dosage in the dissemination of the three selected phages in the chickens' organs are presented in **Chapter IV**.

In **Chapter V** are reported the results of confined experiments intending the *in vivo* phage performance evaluation on treating severe respiratory *E. coli* infections in chickens, when administered orally and by spray.

Naturally *E. coli* infected chicken flocks refractive to antibiotherapy, were used in the work described in **Chapter VI**, to perform large scale experiments with the three-phages cocktail, composed by phi F61E, phi F78E and phi F258E.

Chapter VII encloses final conclusions as well as suggestions for future works.

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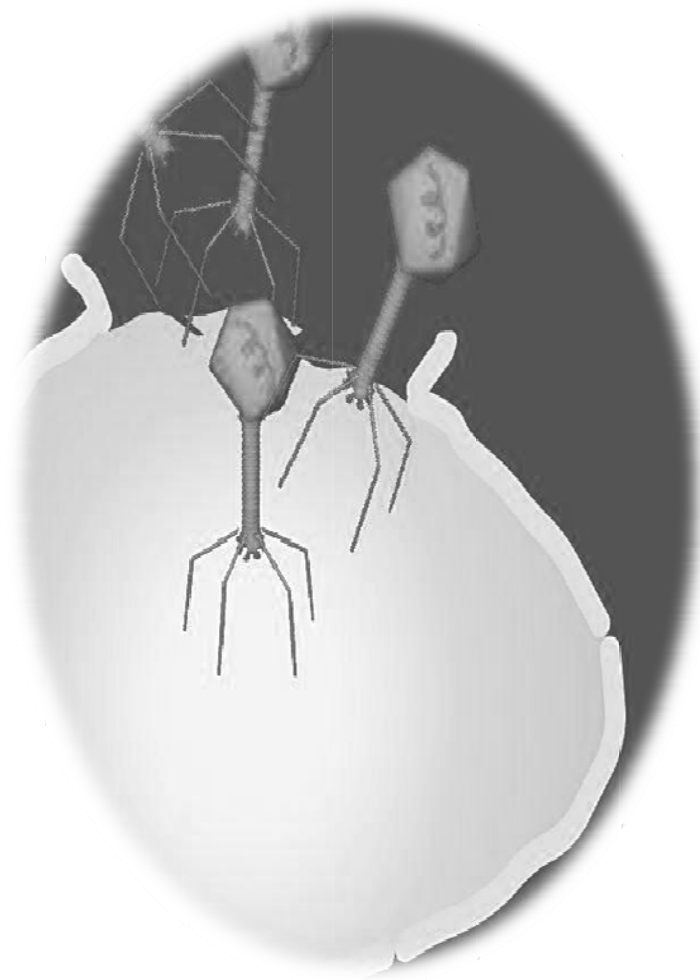
List of symbols and abbreviations

AML	Amoxycillin
AMP	Ampicillin
APEC	Avian Pathogenic *Escherichia coli*
ATCC	American Type Culture Collection
bp	Base Pairs
BW	Body Weight
CFU	Colony Forming Units
CG	Control group
CHG	Challenged group
CHMP	Committee for Medicinal Products for Human Use
CsCl	Cesium Chloride
DGV	Direcção Geral de Veterinária
DNA	Desoxi-ribonucleic Acid
DNase	Desoxi-ribonuclease
DO	Doxycycline
dsDNA	Double-stranded Desoxi-ribonucleic Acid
DSMZ	German Collection of Microorganisms and Cell Cultures
dsRNA	Double-stranded Ribonucleic Acid
EC	European Council
EEC	European Economic Community
ENR	Enrofloxacin
EU	Endotoxin Units
EU	European Union
FDA	Food and Drug Administration
FELASA	Federation of European Laboratory Animal Science Associations
GI	Gastrointestinal
HCl	Hydrochloric Acid
IBV	Infectious Bronchitis Virus
LAL	Limulus Amebocyte Lysate Assay
LB	Luria Bertani
LPS	Lipopolysaccharide
LREC	Laboratorio de Referencia de *Escherichia coli*

$MgSO_4$	Magnesium Sulfate
N/T	Non Typeable
NA	Nalidixic Acid
NaCl	Sodium Chloride
NCBI	National Center for Biotechnology Information
NDV	Newcastle Disease Virus
OA	Oxolinic Acid
PCR	Polymerase Chain Reaction
PFU	Plaque Forming Units
PIP	Pipemidic Acid
PVDF	Polyvinylidene Fluoride
RFLP	Restriction Fragment Length Polymorphism
RNA	Ribonucleic Acid
RNase	Ribonuclease
rpm	Rotation Per Minute
SDS	Sodium Dodecyl Sulfate
SPSS	Statistical Package for the Social Sciences
ssDNA	Single-stranded Desoxi-ribonucleic Acid
ssRNA	Single-stranded Ribonucleic Acid
STX	Sulphamethoxazole/ Trimethropim
TE	Tetracycline
TEM	Transmission Electronic Microscopy
US	United States
USDA	United States Department of Agriculture
UV	Ultra Violet
WHO	World Health Organization
WT	Wild Type

I. INTRODUCTION



Colibacillosis is a severe infection, associated with *Escherichia coli* (*E. coli*), either as part of the commensal microflora of the chicken intestinal tract behaving as opportunistic, or as extra-intestinal - the avian pathogenic *Escherichia coli* (APEC) - that are able to cause colibacillosis by itself due to its invasive ability. The colibacillosis and colisepticemia are responsible for significant economic losses in poultry industries worldwide, due to low feed conversion rate, weight loss, high cost of treatments during production, poor carcass quality with consequent rejection at slaughter and high mortalities rates. The increasing high patterns of antibiotic resistance acquired by these bacteria, as well as the restrictions to the antibiotic usage implemented by the European Union, has encouraged the search of new antimicrobial solutions to control these infections, with the guarantee of good meat quality and the lowest environmental impact.

Bacteriophages (phages) are viruses that obligatory parasites bacteria, and have been proposed as valuable alternatives to antibiotics based on their capacity to infect and destroy the bacteria, releasing progeny to infect the surrounding hosts. This chapter describes the role of *E. coli* in the poultry industry, explains the motivations of finding new alternatives to the antibiotics and presents the bacteriophages as an alternative, describing features and research studies that support its therapeutic potential.

Keywords: bacteriophage; *Escherichia coli*; poultry.

1. COLIBACILLOSIS IN POULTRY INDUSTRY

1.1 *Escherichia coli*

Escherichia coli (*E. coli*) is a Gram-negative bacillus, facultative anaerobic and non-sporulating bacteria that belongs to the family of *Enterobacteriaceae*. Its optimal growth occurs at 37 °C. The cells are about 2-3 micrometres (μm) long and 0.5 μm in diameter. Some strains have multiple "flagells" around the cell to confer motility, and fimbria or adesins that allow its attachment to the intestine walls. These bacteria are normal inhabitants of the intestinal lumen of humans and other warm-blooded animals [21]. As in all Gram-negative bacteria, the outer surface membrane of the cell wall is constituted by complexes of lipopolysaccharides (LPS), macromolecules responsible for several of the bacteria biological properties [126]. These chemical structures, also known as endotoxins, comprise three regions or domains: the lipid A, hydrophobic, is projected into the outer membrane, conferring greater stability and resistance; an intermediate glycosidic part consisting of a linear and hydrophilic region of polysaccharides, and a third region, named O-chain, with repetitive subunits of monosaccharides responsible for much of the immunospecificity of the bacterial cell [17]. The number of the O-chain subunits defines the bacteria O-serotype, a factor conditioning virulence on Gram-negative bacteria [65].

E. coli possess the ability to transfer DNA via bacterial conjugation (through plasmids exchange), by transduction (carried by a bacteriophage), or by transformation (acquired from the environment as "naked" DNA). These processes allow genetic material to spread horizontally through an existing population and might led to transfer genes encoding advantageous proteins, or conversely toxins, from one bacteria to another [13].

1.2 *E. coli* role in poultry industry

Escherichia coli is part of the commensal microflora of the chicken intestinal tract. Particularly in chickens and turkeys' intestines, it can reach concentrations of 10^6 CFU/g of fecal material [7], and are found in several fecal contaminated places, like water, dust, feathers, skin, etc. [50]. Under certain conditions, *E. coli* infections can arise causing colibacillosis. The most important source of transmission seems, thus, to be fecal contamination through the inhalation of the microorganisms into the respiratory

tract [8]. The oxygen exchange zones, in this case lungs and air sacs, are very vulnerable to bacteria incursion and subsequent multiplication. Avian air sacs have no resident cellular defense mechanisms and must rely on an inflammatory influx of heterophils (highly phagocytic granulated leukocytes) as the first line of cellular defense, followed by macrophages [86].

Commonly, *E. coli* is an opportunistic bacteria that causes disease in immunologic deprived chickens. Stressful external agents, as other bacteria or virus infections affecting respiratory system (*Mycoplasma gallisepticum*, infectious bronchitis virus (IBV), Newcastle disease virus (NDV)), or adverse environmental conditions (as temperature, and humidity, high concentrations of ammonia and dust in poultry houses)) frequently contribute to decrease chicken immunologic defenses [24]. However, some *E. coli* strains, named avian pathogenic *Escherichia coli* (APEC), are able to cause colibacillosis by itself, due to its invasive ability (Figure I.1 A). These strains belong to an extra-intestinal pathogenic group, and possess specific virulence traits that are determinant for the host infection and to development of septicemia. Many adhesins promoting the attachment of the bacteria to cell receptors are encoded: type 1 fimbriae have been involved with the initial stages of the upper respiratory colonization, whereas the P fimbriae are involved in colonization of the internal organs [66]. Other virulence factors known to be associated with APEC, include the presence of the K1 antigen, particularly when associated with O1 and O2 serogroups, the ability to secrete aerobactin, the temperature-sensitivity of the hemagglutinin (Tsh), serum resistance, the presence of some pathogen-specific chromosomal regions, and others [23, 24, 28, 86]. Respecting to the O-serotype in poultry, 10 to 15% are pathogenic [56] and belong to specific O-serogroups, as O1, O2, O5, O8, O15 and O78 [24, 133, 25, 52, 77].

The pathogenesis of the infection comprises the colonization of the respiratory tract by the bacteria, the passage through the epithelium and the penetration into the mucosa of the respiratory organs. The survival and multiplication of the bacteria in the blood stream, leads consequently to septicemia that degenerates in multiple organ lesions, typically pericarditis, aerosacculitis, perihepatitis and peritonitis (Figure I.1 B) [49]. *In vivo* experiments showed that, although APEC cells were effectively rescued from blood by macrophages, others were found to be occasionally free in the airways, as the air sac lumen, in interstitial tissues of infected chickens, and also mixed with heterophils, erythrocytes, and fibrin [100].

A. **B.**

Figure I.1 Colisepticemia: A- Chicken with symptoms of colisepticemia. B- Fibrin deposits in carcass.

From the economical point of view, this pathology represents a great problem to poultry industry worldwide, causing important losses due to the low conversion rates causing body weight loss, high mortalities rates, poor carcass quality with consequent rejection at slaughter and high cost of treatments during production [25, 57, 103, 108].

The problems of emerging antibiotic resistant *E. coli* strains, and on the other hand, the recent European Union (EU) rules and legislation restricting the number of active ingredients available for livestock production [29-31], makes urgent to improve the existing therapeutic solutions and above all, to come up with efficient, feasible and non pollutant antimicrobial alternatives.

2. THE ANTIBIOTICS IN LIVESTOCK PRODUCTION

The antimicrobial agents widely used to control bacterial infections in animals and humans are the antibiotics. In the EU, the amount of antibiotic active ingredients available for clinical use increased from five, in 1959, to one hundred and two different molecules in 1997 [20]. Particularly in intensively reared food animals, as in poultry industry, the use of antibiotics, regardless the objective - therapeutic, prophylactic or performance enhancer - induces a high selection pressure for microbial resistance. Mechanisms related with this phenomenon might occur through the differential survival rate conferred to bacteria, in the occasion of one or more spontaneous mutations in the bacterial chromosome or due to acquisition of foreign DNA coding certain mechanisms of resistance. In any case, mutant strains are selected among the sensitive ones (wild

type) with the administration of this drugs, and so are able to multiply and become dominant [66, 67, 100]. These mechanisms result in an unavoidable loss of the efficacy of treatments of the most commonly used active ingredients, in poultry industry.

The birds' pathogenic bacteria serotypes, have a high capacity of dispersion among successive flocks in the same aviary, being thus the most frequently exposed to antibiotics [56, 20, 57, 122, 123].

Despite the indubitably losses in poultry industry, other concerns arise from microbial resistances. In fact, the disrespect for the safety interval between the antibiotic administration and slaughter, might became an important clinical and public health problem [20, 61, 73].

World Health and Life Science organizations are concerned about the deleterious effects that antimicrobial resistant bacteria ingested from animal derived food products may have on human health, like increased duration of illness, treatment failure, and loss of therapeutic options [26, 123]. FDA has also emitted reports commenting this problem and suggesting the application of alternative methods for the control of pathogenic microbes [29, 30].

From the environmental point of view, effluents containing antibiotic residues can create a reservoir of resistance microorganisms on soil and water. Those substances can persist in the environment for long periods of time after treatment, affecting the microbial community as long as they remain intact and at growth inhibitory levels [72, 90].

3. BACTERIOPHAGES

3.1 Structure and life cycle

Bacterial viruses or bacteriophages (phages), are likely to be the most widely distributed and diverse entities in the biosphere. Phages infect exclusively bacteria and are associated with almost all bacterial genera, including cyanobacteria, archaebacteria and mycoplasms. These virus may be grouped on the basis of a few general characteristics including the host range and strategies of infection, the morphology and particle size, the nucleic acid, the molecular weight and the genome sequence, the morphogenesis, the phylogeny, the sensitivity to physical and chemical agents, among others [55].

Bacteriophages are composed of protein or lipoprotein capsids, which are morphologically heterogeneous, ranging from polyhedral (like hexagonal) structures, to

filamentous or to pleomorphic (for example spherical) structures. The capsid, enclose the phage nucleic acid, DNA or RNA, that can be arranged as linear (free extremities) or circular molecules, and in single (ss) or double (ds) strains. Some phages have small genomes (few encoding 12 or fewer genes) and other have large genomes that can reach 480 000 base pairs (bp) [27, 51, 55, 92]. The advent of the electron microscope allowed phage biologists to measure the size of phage structures and to determine the symmetry of the capsid, giving rise to a taxonomy based on morphotypes (Table I.1) [92]. Phages with a polyhedral capsid often carry a more or less complex tail (*Caudovirales* order), to which a base plate, spikes, or tail fibers can be attached. Those are specific connecting structures ensuring the contact of the phage with the receptors of the host cell wall. The tails can be contractile (*Myoviridae* family), long and non-contractile (*Siphoviridae* family) or short and non-contractile (*Podoviridae* family) [27, 51, 92].

Table I.1 Classification of phages according to the International Committee on Taxonomy of Viruses (ICTV) [84]

Order	Family	Morphology	Nucleic acid
Caudovirales	*Myoviridae*[a]	Non-enveloped, contractile tail	Linear dsDNA
	Siphoviridae[a]	Non-enveloped, long non-contractile tail	Linear dsDNA
	Podoviridae[a]	Non-enveloped, short non-contractile tail	Linear dsDNA
	Tectiviridae[b]	Non-enveloped, isometric	Linear dsDNA
	Corticoviridae[b]	Non-enveloped, isometric	Circular dsDNA
	Lipothrixviridae	Enveloped, rod-shaped	Linear dsDNA
	Plasmaviridae	Enveloped, pleomorphic	Circular dsDNA
	Rudiviridae	Non-enveloped, rod-shaped	Linear dsDNA
	Fuselloviridae	Non-enveloped, lemon-shaped	Circular dsDNA
	Inoviridae	Non-enveloped, filamentous	Circular ssDNA
	Microviridae	Non-enveloped, isometric	Circular ssDNA
	Leviviridae	Non-enveloped, isometric	Linear ssRNA
	Cystoviridae[b]	Enveloped, spherical	Segmented dsRNA

[a]Tailed phages
[b]Lipid containing

Phage receptor sites are located on different parts of bacteria, and include structures such as proteins, lypopolyssacharides or sugars, anchored to the cell membrane or as part of the cell wall structure. Some of them are present permanently on the cell while others, as for example the fertility (sex) fimbriae, are produced only by bacteria in the logarithmic growth phase [27,55].

A common characteristic of phages is that, although their genome carries the information required to guarantee their own multiplication, they rely on the energy and on the protein biosyntethic machinery of their bacterial hosts to translate its genome in new virus progeny and complete their lytic cycle. Hence, phages are obligatory parasites. Some develop elaborated mechanisms to reach this goal, being, however, the final result common to all: to reach the intracellular environment becoming able to produce more viruses. Once, by chance, a phage meets an appropriate host, the infection starts by the specific binding to the bacterial receptor (Figure I.2).

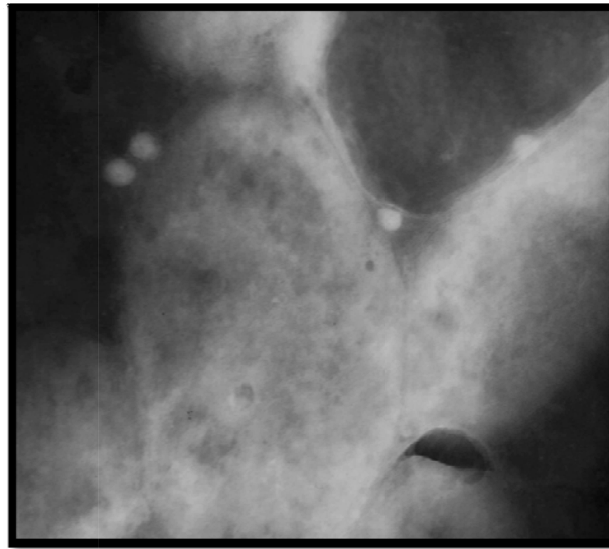


Figure I.2 Phages adsorbed to *Escherichia coli* cell wall. Picture obtained by Transmission Electronic Microscopy (TEM). Magnitude: 38 000 ×.

The type of infection varies, according to the phage life cycle (Figure I.3). Phages might follow two main infection strategies: the lytic cycle, destroying the host bacteria after infection, or the lysogenic cycle, co-existing and replicating with bacteria as a foreign genetic material. In the first case, phages are denominated virulent and in the second one, temperate [71].

For the phage therapy perspective, temperate phages must be discarded. This kind of phages are not able to control a bacterial infection, remaining in the host in a latent form, allowing bacteria to multiply normally until adverse conditions arise, activating the lytic cycle. Furthermore, when a cell becomes lysogenic, bacterial genes might be occasionally carried by phages and expressed in neighbor infected cells. These genes can change the properties of the new bacteria or even induce the expression of hazardous proteins. The phage genome integrated in the host DNA chromosome is

known as a prophage, and remain incorporated either into the bacteria chromosome or existing as an extra chromosomal plasmid [45, 71].

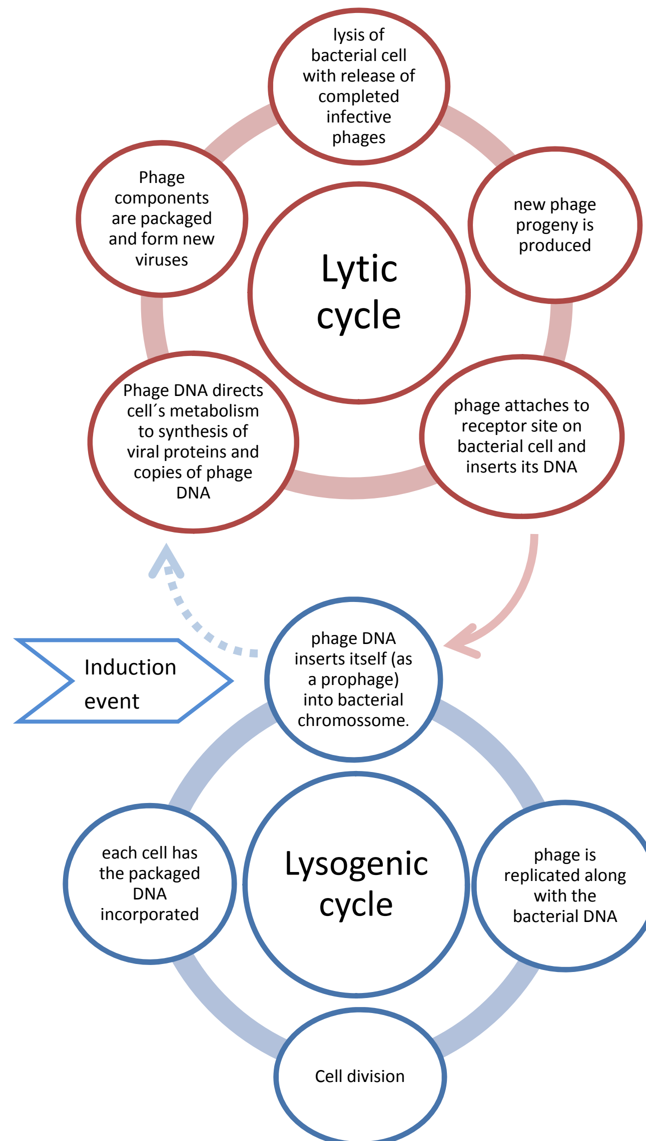


Figure I.3 Phage lytic and lysogenic cycles: main processes (adapted from: http://faculty.irsc.edu/FACULTY/TFischer/micro%20resources.htm).

Conversely, virulent phages are encouraged to be used in therapy, based on their capacity to infect and destroy the bacteria, releasing in few minutes progeny that will infect the surrounding hosts. Briefly, the cells infection mainly comprises the adsorption and the irreversible attachment of the phage to the bacteria. These processes allow phages to get through the bacterial membrane and to inject the nucleic acid through it. The adsorption is mediated by the phage tail fibers from the base plate or by some analogous structure, depending on the phage taxonomy. These structures attach to

specific receptors on the bacteria (LPS, pili, lipoprotein, …). The nucleic acid from capsids is transferred into the bacterial cell by different mechanisms, according to the phage type. In subsequent steps, the viral genome is transcribed using the host metabolic equipment - aminoacids, nucleotides, ribosome, enzymes - beginning its translation on the phage structural components and on genetic material. Particles organize themselves in the intracellular space to be released as infectious viral particles [45, 82]. Some phage strategies are known to promote the host lysis. All the dsDNA encode in its genome a hydrolytic enzyme, named lysine, which degrade the cell wall components, the peptidoglycan or murein. To carry on this feature, this enzyme needs another protein, the holin, also encoded by phage genome sequences [132]. Holins act forming "holes" in the membrane. Those formations allow lysine, stored in the cytoplasm, to reach the peptidoglycan layer and to disaggregate this structure, and the phages produced during the infection are released [107]. In dsDNA type phages, the holin is the factor that controls the lysis moment [127, 132]. A holin inhibitor also encoded by the phage, indicate the finalization of the lysis process. On the other hand, ssDNA and ssRNA phages have single and specific genes for the host lysis. No enzyme capable of degrading the peptidoglycan structure was found in lysates of these phage types, and the genetic analysis suggests no genes encoding those proteins. The lytic activity may occur after phages replication and morphogenesis. Lyses seems to be a secondary activity of structural proteins [124, 132].

3.2 Phage therapy

Many reviews and reports have been published, focusing the problem of bacterial antibiotic resistance, discouraging the use and abuse of antimicrobials in food animal production, and challenging the scientific community to find feasible alternatives of reducing microbial pathogens loads [3, 29, 128].

The evidences of bacteriophage advantages over common therapies have been triggering numerous research works aiming the characterization and evaluation of phages as safe and efficient antimicrobial particles.

One of the phages characteristics supporting its therapeutic use is their exclusivity on prokaryotic infection, being metabolically inert in their extracellular form. Accordingly, phages cannot interact with humans, animals or plants cells, having therefore a highly encouraging safety profile [51]. The idea of phages harmlessness to

human and animals' health is reinforced, taking in account its ubiquity in Nature. Indeed, phages are the most-numerous life form on earth, and phage population in the biosphere is calculated to be around 10^{31} phages, existing 10^{10} phages per liter of surface seawater [78] and 10^7 to 10^9 per g of sediment or topsoil [105, 4, 5, 52, 131]. Thus, it is reasonable to say that phages are regularly consumed in food and usually colonize the intestine [19, 47]. Additionally, another important characteristic sustaining the phages use for therapy is their high specificity for a given host, targeting and recognizing specific receptors in the bacteria. This trait avoids the indiscriminate lysis of the normal microflora, contributing in consequence to preserve the microbial balance. This assumes high importance in therapy since patients may be more protected against secondary infections [85]. Other advantage on using phages for treatments relies on its exponential growth following the host infections. If this event takes place on critical infection sites, it might allow phages to exert a broader therapeutic action to control illness. The phages ability to infect antibiotic resistant bacteria, overcoming resistance problems and the low cost of phage production are other factors supporting its use. Besides all these properties associated with bacteriophages, they sill enclose a great potential to be genetically manipulated in order to improve their efficiency [61].

In the last two decades, phage therapy applied to control infections rising from animal production industries gained special attention. In veterinary Medicine, several studies have already established "the proof of principle" of the phage therapy. For example, researchers of the Institute for Animal Disease Research, in the UK, reported successes on the use of phages in experimental treatments of *E. coli* infections in mice[113] and in infections of diarrhea-causing *E. coli* strains in the alimentary tract of calves, lambs, and piglets [114-116]. Barrow et al. (1998) [9], reported the successful use of phages in preventing septicemia and a meningitis-like infection in chickens, also caused by *E. coli*. Similar studies with encouraging results were reported for mice and guinea pigs infected with *Pseudomonas aeruginosa* and *Acinetobacter* [117-119], *Klebsiella ozaenae, Klebsiella rhinoscleromatis scleromatis* and *Klebsiella pneumonia* [10, 11].

More recently, Cerveny et al. (2002) [18] confirmed the therapeutic potential of bacteriophages as therapeutic agents against *V. vulnificus* in a mouse model. Park et al. (2003) [93] treated water-borne infection of *Pseudomonas plecoglossicida* in fish by impregnating phages in the feed. Ronda et al. (2003) [106] confirmed the therapeutic phage activity against *Streptococcus pneumoniae* also in fish. Sklar and Joeger (2001) [111], Fiorentin et al. (2004) [32] and Atterbury et al. (2007) [6] designed experiments for

reducing *Salmonella* colonization in poultry intestine, obtaining satisfactory results. Huff and co-authors have been reaching successful results in treating colibacillosis in chickens [58-64].

In Human Medicine, the safety and effectiveness in the use of phages for therapeutic purposes was demonstrated by Bruttin and Brussow (2005) [14] in a phage safety test run in healthy adult volunteers received in their drinking water a low dose of the well described T4 phage. No adverse events related to phage application were reported.

Nevertheless, despite all favorable reports, phages are still matter of controversy. One of the drawbacks of phage therapy is the arising of phage resistant bacteria. Mechanisms against phage infection might be developed by the host bacteria, and two main problems might come up: a negative influence in phage therapy efficacy as well as the mutants' propagation in the environment. In general, naturally occurring mechanisms of phage resistance, include mutations at the bacteria DNA level that allows the accomplishment of several defense resources: the prevention of phage infection by altering the cell surface carbohydrates that act as phage receptors, the blocking of phage adsorption or penetration systems, or the abortion of infections [46]. Considering that the surface component associated with bacterial virulence also seems to be the receptor for many phages attachment, there are evidences that bacteria resistant to phages usually present an attenuated virulence [9, 88, 95, 109, 113, 114]. Following this idea, Barrow et al. (1998) [9] state that the use of phages attaching to structures that are essential for virulence, such as, for example, the K1 antigen, may minimize the necessity of finding solutions to destroy resistant bacteria. In this particular case, most phage-resistant mutants that arise would be K1 negative, and thus less virulent. According to the same authors, this adaptation may contribute for successful phage therapy or control.

Other strategies might be carried out by phages, to evolve in the same sense as bacteria. These viral particles are also able to suffer mutations, some of which may overcome bacteria resistance [47, 81, 87].

A good strategy to overwhelm the phage resistant problem is to include in the therapeutic cocktail of phages with different bacterial receptors, which might delay the appearance of resistances, and on the other hand, broaden the therapeutic applicability of the product [18, 68]. In addition, when phage-resistance occurs, it should be possible to rapidly select a new phage active against the phage-resistant bacteria.

Another limitation being pointed out to phages is their narrow host range, if they are strain-specific rather than species-specific. Due to the high diversity of bacterial variants

to control, this characteristic could lead to some difficulties on preparing phage products and its therapeutic action might be restricted. Again, the usage of a cocktail of phages, including preferentially polyvalent phages (which are phages that can infect multiple species) can be a way to enlarge the lytic spectra and at the same instance delaying the resistance occurrence [15, 51, 61, 85, 112, 115, 120].

The development of phage-neutralizing antibodies is another potential problem which may obstruct phage effectiveness on treating recurrent infections *in vivo*. The prior exposure of a pathogen to this antimicrobial is likely to accelerate an immune reaction to therapeutic phage [54, 68, 88, 120]. In fact, the development of neutralizing antibodies after parenteral administration of phages has been well documented [67, 115]. It is indeed unclear how significant this problem may be for phage therapy, especially when phages are administered orally or locally [83]. According to Sulakvelidze (2001) [120], theoretically, the development of neutralizing antibodies should not be a significant obstacle during the initial treatment of acute infections, once the kinetics of phage action is much faster than the production of neutralizing antibodies by an organism. Furthermore, it is not clear how long the antibodies will remain in circulation and of which variables this depends. Thus, further studies are advised to be conducted to certify the validity of this concern[120].

Relatively to phages safety, there is also some apprehension on administering the phage lysate as a therapeutic product, without removing the host debris. As phage infection culminates on the bacteria disruption, the cell wall components are consequently released into the environment as cell debris. In Gram-negative bacteria lysates, also endotoxins (LPS) are released. Those structures easily pass through filters (0.22 μm) commonly used to remove the whole bacteria from phage suspensions (Williams, 2001b). The presence of these endotoxins in the lysates can lead to undesired side effects on phage therapy. The LPS toxicity is associated with the lipidic component of the molecule, the lipid A, while the immunogenicity is associated with the O-chain polysaccharide component, the O-antigen [12, 22, 101, 121, 129].

If it is true that small amounts of endotoxins can be advantageous for an organism, activating defense mechanisms to face infections, it must be said that, in larger amounts, these macromolecules may induce a variety of inflammatory responses being often part of the pathology of Gram-negative bacterial infections. Although individuals vary in their susceptibility to endotoxins, the sequence of pathophysiological reactions follows a general pattern: a latent period followed by physiological distress. Immunologic and

neurological system activation, induction of blood coagulation, general metabolic harmful effects, alteration of blood cell populations, pyrogenicity (fever induction), hypotension, hepatotoxicity, tissues necrosis and in more serious cases, endotoxic shock and death, are some of the known reactions to an endotoxin parenteral challenge [22, 130]. Some processes are being used to isolate phages from crude lysates, in order to get suspensions free of LPS. The density gradient ultracentrifugation (ex. cesium chloride gradients) [47], the ultrafiltration followed by size exclusion chromatography [12], specific "ready-to-use" column' systems based on affinity chromatography [102] are some of those processes, and are easily adaptable to phage small scale production. However, it becomes more difficult and less feasible to implement the existing solutions in larger scales, as required for the industry supply. It is thus important to adapt the purification level of these kinds of suspensions, to the purpose of the therapeutic product and to the variables of the therapeutic intervention. This includes, for example, the target specie (according to Culbertson and Osburn (1980) [22], there is a variable sensitivity to endotoxin among species, for example, chickens are more resistant to endotoxins effects compared to mammals) and the administration route (the oral or the spray administration of crude phage lysate shall have different approaches in terms of endotoxins effects on live organisms, comparing to the intramuscular or parenteral administration).

3.3 Motivations and expectations arising from bacteriophage technology

Scientific research groups have been improving the existing technology based on bacteriophages, and developing new approaches. Since the phage therapy successes and setbacks of the experiments in the former Soviet Union [15, 120], phages have been an object of interest. Several steps forward, even in an adverse epoch for phages due to the antibiotics rising, allowed the knowledge consolidation and the recognition of the research needs in this area. The trust on phages' high potential allowed a renewed hope for its use as antimicrobials tools, and the necessity to demystify those virus basic principles and mechanisms of action in order to get a better perception of phage Biology, consequently arise. The evolution of the knowledge on the phage Phylogeny, by describing statistically the similar
ity or differences between groups of species with an evolutionary tree, largely contributed for this intend. In another perspective, the phages genetic characterization

allowed the disclosure of the several protein functions. Indeed, the growth of interest in bacteriophage coincides with recent advances in Molecular Biology technologies. Since the 1980s the number of catalogued sequenced genomes maintained by the National Center for Biological Information (NCBI) has grown exponentially. From 2002 until now, the number of known phages grew from around 100 [80] to approximately 520 [91].

New phage applications emerged, and new generation phage products are being suggested [70]. Answers from fundamental research and proposals from applied investigation, consolidated important phage applications and drew attention to new ones. In fact, phage technology has been applied to a wide range of fields, as food safety, environmental technology, human and veterinary Medicine, Biotechnology, Immunology, Epidemiology, among others [15, 120]. Indeed, phages might play important roles, as the reduction of cross-contaminations through direct applications of bacteriophages or its enzymes in surfaces, manipulated food [16] or carcasses after slaughter [74]; the bio-recognition of bacterial pathogens as specific antigen molecules in diagnosis [77, 89]; its use as tracers, indicators of pollution or in the monitoring and validation of biological filters in the environment [81]; the treatment or control of bacterial infections in animals or humans [120]; the vaccination using phages as delivery vehicles of the antigen in the form of protein or DNA [104]; the development of laboratory techniques as protein/antibody library screening tools, like phage display or phage immobilization [81], etc.. Genetically engineering bacteriophages offers great possibilities to developed the above described applications and to enhance phage technology approaches [70].

From another perspective, phages present a continuous challenge for the fermentation industry in particular, dairy industry, where phage infections of bacterial stocks can be commercially disastrous [81].

In parallel to studies of phage Biology and biotechnological applications, mathematical models are being developed to facilitate a better understanding of how to improve phage value [69, 97-99].

Another interesting phage-based therapeutic advance, is centered in the use of phage-encoded enzymes, produced actively during the lytic cycle, which destroy the bacteria cell wall from the interior of the infected cell and enable the release of the phage progeny [106,96]. In 1995, Vincent Fischetti designated those substances as “enzibiotics”. Several patents arise from Fischetti and colleagues research on enzibiotics aiming practical applications [34-44], claiming, among others, the development of products containing phage lytic enzymes in chewing gums, eye drops, nasal sprays, vaginal

suppositories and tampons, oral syrups or bandages. The use of lytic enzymes purified from phage lysate as important tools in bacterial destruction have also been reported in several studies [33, 47, 48, 75, 76, 79, 93, 110].

The great diversity of phage technological applications and the added value of certain phage products is being the driving force of the creation of phage companies and the increase in research in this area. As examples, companies focused on the development, production and marketing of phage-based products might be mentioned: OmniLytics, Inc. (www.phage.com), EBI - Food Safety (www.ebifoodsafety.com), Biophage Pharma, Inc. (www.biophagepharma.net), Intralytix, Inc. (www. intralytix.com), Phage Biotech, Ltd. (www.phage-biotech.com), D&D Pharma (www.bakteriophag.com), Novolytics Ltd. (www.novolytics.co.uk), Controlvet, Segurança Alimentar (www.controlvet.pt), among many others.

3.4 Legislation for phages use

The use of phage as therapeutic agents in humans or animals still encounters a massive problem: the void in legislation. It is urgent to include in regulations, a specific edge for phages, avoiding the subjectivity of criteria that arises on adapting the inclusion of phage products on existing documents designed for other substances. Rigorous requirements for phage isolation, selection, characterization and production must be described as well as procedures for products validation. The knowledge of the bacteriophage Genetics and Ecology, might simplify the legislators task on defining procedures to guarantee phage safety control. Presently in EU, the phage commercialization approval is under criteria of already available legislation for other bio control substances, somewhat consistent with phages. Each member state has competencies to evaluate if the substances under approval are able to be commercialised and registered. For example, the Directive 98/8/CE of 16 February [1] establishes rules and procedures relative to the commercialisation of biocides and includes the viruses in the definition of “microrganism”. However, needs of new regulations are being recognised by the European Union. The regulation (EC) No 726/2004 of the European Parliament and of the Council of 31 March 2004 [2] set up Community procedures for the authorization and supervision of medicinal products for human and veterinary use and establishes a European Medicines agency. This agency will implement EC procedures regarding the commercialization of high-technology medicinal products, particularly

that resultant from Biotechnology research, aiming the maintenance of the high level of scientific evaluation in the EU and thus the preservation of the confidence of patients and medical professionals. This regulation specifies the importance of these measures for new therapies lacking legislation. However the term "bacteriophage" is never mentioned.

In 2006, the Food and Drug Administration (FDA) and the United States Department of Agriculture (USDA) approved a phage-based product to control *Listeria* in food products, the LISTEX P100™ (from EBI - Food Safety), conferring the GRAS (Generally Recognized As Safe) status to the product, and thus allowing its use in food in the US. SKAL, the designated Public Inspection Authority of The Netherlands, confirmed that in conformity with EU Regulation (EEC) nr. 2092/91, Annex VI, Section B, LISTEX™ had the "organic" status that, under EU law, allowed the product to be used in the EU in regular and organic products.

With regard to the Human Medicine, the entity that decides the marketing procedures for medicines in the EU is the Committee for Medicinal Products for Human Use (CHMP). CHMP is responsible for preparing the European Medicines Agency's opinions on all questions concerning medicinal products for human use, in accordance with Regulation (EC) No 726/2004. However, and citing Verbeke et al. (2007) [125], this Committee was established having classical drug products in mind, and the possibility to instigate the clinical studies that are required to generate the data demonstrating safety and efficacy of phage therapy, in the actual regulatory settings, will be very difficult or even impossible.

European regulation defines a medicinal product as 'any substance presented for treating or preventing disease in human beings'. According to this definition, from a therapeutic point of view, bacteriophages are medicinal products. However, researchers are not being able to document bacteriophages as such, once they cannot fulfill all the requirements to do clinical trials in humans (national notification, Eudract number, production license etc.).

In Poland, bacteriophages are already being used therapeutically. In the L. Hirszfeld Institute of Immunology and Experimental Therapy from The Polish Academy of Sciences, patients infected with antibiotic-resistant bacteria, can be treated with phages [53]. The regulatory basis for this therapeutic use on patients is the Declaration of Helsinki. In the Paragraph 32 of this Declaration, is stated:

"In the treatment of a patient, where proven prophylactic, diagnostic and therapeutic methods do not exist or have been ineffective, the physician, with informed consent from the patient, must be free to use unproven or new prophylactic, diagnostic and therapeutic measures. (…) these measures should be made the object of research, designed to evaluate their safety and efficacy (…) information should be recorded and, where appropriate, published".

However, the Declaration of Helsinki is only applicable when other therapeutic methods are not effective and thus is not a steady solution for phage therapy. As a long-term solution, it would be therefore vital the creation of a specific section for phage therapy under the Advanced Therapy Medicinal Product Regulation.

"We look forward to a time when phages, as both bacteria identifiers and biocontrol agents, are as ubiquitous in the clinic, on the farm, and even in the factory as Felix d'Herrele, over 85 years ago, so confidently hoped that one day they might be" (Goodrige and Abedon, 2003) [51].

Shall we believe that this time has just begun?

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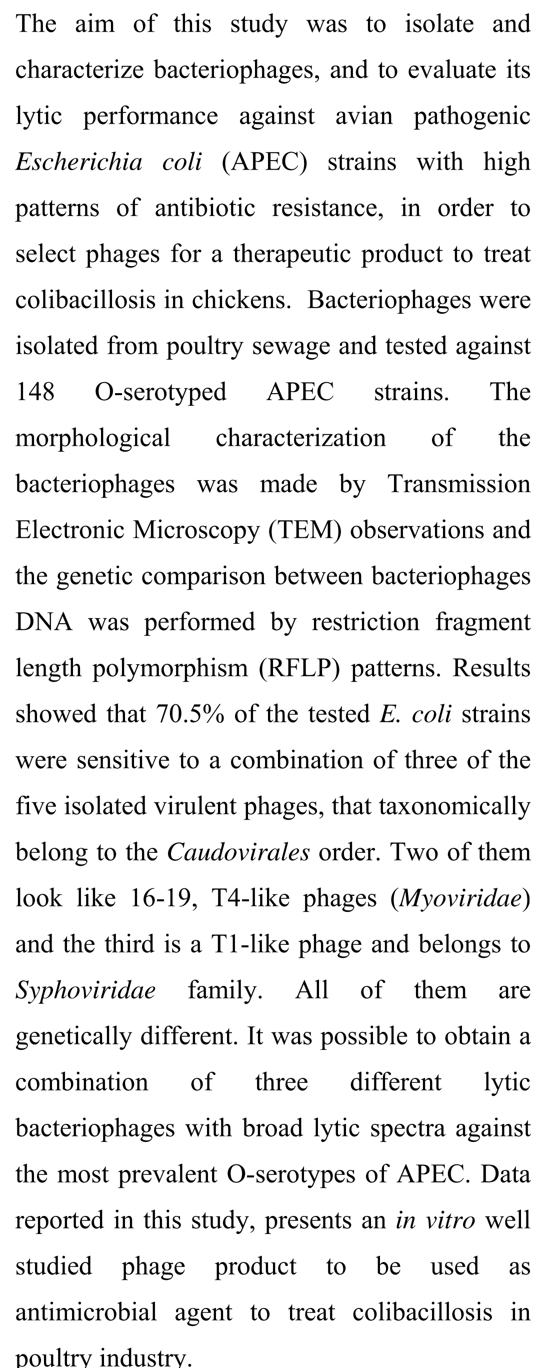

The aim of this study was to isolate and characterize bacteriophages, and to evaluate its lytic performance against avian pathogenic *Escherichia coli* (APEC) strains with high patterns of antibiotic resistance, in order to select phages for a therapeutic product to treat colibacillosis in chickens. Bacteriophages were isolated from poultry sewage and tested against 148 O-serotyped APEC strains. The morphological characterization of the bacteriophages was made by Transmission Electronic Microscopy (TEM) observations and the genetic comparison between bacteriophages DNA was performed by restriction fragment length polymorphism (RFLP) patterns. Results showed that 70.5% of the tested *E. coli* strains were sensitive to a combination of three of the five isolated virulent phages, that taxonomically belong to the *Caudovirales* order. Two of them look like 16-19, T4-like phages (*Myoviridae*) and the third is a T1-like phage and belongs to *Syphoviridae* family. All of them are genetically different. It was possible to obtain a combination of three different lytic bacteriophages with broad lytic spectra against the most prevalent O-serotypes of APEC. Data reported in this study, presents an *in vitro* well studied phage product to be used as antimicrobial agent to treat colibacillosis in poultry industry.

Keywords: bacteriophage, colibacillosis, *Escherichia coli*, poultry, therapy.

1. INTRODUCTION

Escherichia coli (*E. coli*) is present in the normal microflora of the intestinal tract of chickens. However, some of these *E. coli* strains are able to cause disease under certain conditions, like abnormal predominance over the other gut flora, host depressed immune system or adverse environmental exposure. Extra-intestinal pathogenic *E. coli*, termed avian pathogenic *E. coli* (APEC) possess specific virulence attributes causing invasive infections in poultry (chickens and turkeys), namely colibacillosis [48]. The pathogenesis of APEC infections include the colonization of the respiratory tract, the crossing of the epithelium and penetration into the mucosa of the respiratory organs, the survival and multiplication in the blood stream and in the internal organs, and the production of adverse effects and lesions in cells and tissues [16]. These bacteria can be typed according to the somatic cell-wall antigen (O-antigen), or the flagella antigen (H-antigen). In poultry, 10 to 15% of the serotypes are pathogenic, and are present in the poultry environment causing a variety of disease syndromes including colibacillosis [5].

Avian colibacillosis is a complex syndrome characterized by multiple organ lesions, typically pericarditis, aerosacculitis, perihepatitis and peritonitis, and in its acute form degenerates in septicaemia. The consequent chickens' high mortality rates and carcass rejection at slaughter causes significant economic losses in the poultry industry worldwide [14, 17]. The most important source of transmission seems to be faecal contamination through the inhalation of the microrganism into the respiratory tract [5].

E. coli isolates from poultry are frequently resistant to multiple drugs [29, 30]. An increased concern over the consequences of the mechanisms that bacteria have developed, to prevent the inhibitory effects of the antibiotics in the treatment of animal bacterial infections is widespread [25, 36]. The antibiotic capacity to select and allow proliferation of resistant bacteria is an important clinical problem with public health consequences. Antibiotic residues can be found in the environment for long periods of time after treatment [30]. These active ingredients affect the microbial community as long as they remain intact and at growth inhibitory levels [29].

World Health and Life Science institutions are concerned about a range of deleterious effects that antimicrobial resistant bacteria may have on human health, like increased duration of illness, treatment failure, and loss of therapeutic options as a consequence of human exposure to resistant bacteria through ingestion of animal derived food products. There have been three comprehensive reviews and reports on the problem of bacterial

antibiotic resistance, each of which comments on the use and abuse of antimicrobials in food animal production, and recommends application of alternative methods of reducing microbial pathogens loads [18, 26, 46]. Also in animal production, there is serious consideration being given to restrictions on the use of antibiotics [19].

Phage therapy is presented as an alternative to antimicrobial therapies. Bacteriophages or phages are viruses that exclusively infect bacterial cells. If they are obligate lytic phages, or virulent phages, multiply in the host bacteria and lyse it at the end of the cycle, after immediate replication of new phage particles. As soon as the cell is destroyed, the new phages can find new hosts. Like all viruses, phages are metabolically inert in their extra cellular form. These structures are only able to self-reproduce as long as the host bacteria is present, and thus are not toxic to non specific bacteria, animals or plants. In fact, their replication depends exclusively on the infection of a specific bacterial host and on the utilization of the host intracellular machinery to translate their own genetic code. Phages are part of both gastrointestinal and environmental ecosystems and are among the simplest and most abundant organisms on earth [13, 43]. Lytic phages are suitable for phage therapy in opposition to temperate phages. The former do not include the integrase genes on their genome, they lack the molecular basis for coexistence with the host and the potentiality to carry harmful genes from one host to another [11, 27, 38].

Recently, well-controlled animal models have demonstrated that phages can rescue animals (chickens, mice, calves, pigs, lambs, fishes,…) from a variety of harmful infections, like *E. coli* or *Salmonella* infections [6, 8, 10, 23, 24, 33, 37, 40-42].

In this study, *in vitro* efficiency of five phages was evaluated based on lytic spectra against 148 avian pathogenic *E. coli* (APEC) strains. The best lytic performance was obtained with a combination of tree phages. In order to characterize these phages, an effective phage sorting scheme based on phage life cycle, lytic efficiency rate, morphology and on phage DNA restriction endonuclease digestion profile (RFLP) was conducted.

2. MATERIALS AND METHODS

2.1 *Escherichia coli* isolation

E. coli strains were isolated from organs (liver, spleen, lungs) of infected commercial birds, with typical lesions of colibacillosis. Organs were emulsified in sterile saline solution 0.85% NaCl (Sigma, Osterode am Harz, Germany) and 0.1 ml of supernatant was plated in MacConkey agar (Biokar Diagnostics, Pantin Cedex, France), a selective medium for Gram-negative bacilli, which differentiates lactose fermenters (pink-red colonies) from non-fermenters bacteria. As approximately 95% of *E. coli* ferment lactose [31], pink red colonies were collected from plates and the specie confirmation of the isolates was conducted by using API strips according to manufacturer's instructions (Bio-Merieux, Marcy l'Etoile, France). *E. coli* isolates were stored in Nutrient Broth (Oxoid, Hampshire, United Kingdom) with 20% glycerol at -80ºC.

2.2 *E. coli* serotyping for the O-antigen

The O-antigen serotyping of *E. coli* strains was performed using a "kit for serotyping avian septicemic *E. coli* strains", supplied by the "Laboratorio de Referencia de *E. coli* (LREC)" of the Veterinary Faculty of Lugo, Spain. The kit included 26 antisera: O1, O2, O5, O6, O8, O9, O11, O12, O14, O15, O17, O18, O20, O35, O36, O45, O53, O78, O81, O83, O88, O102, O103, O115, O116 and O132. If the strain was negative for all these antisera, it was considered not typeable (N/T) with this kit. Samples were prepared and procedures were carried out according to the supplied protocol.

2.3 Antibiotic susceptibility testing of APEC

The isolated APEC strains were subjected to antibiotic susceptibility testing. The active ingredients with systemic action (relayed throughout the blood circulation) in poultry, generally used for colibacillosis treatment were selected to perform this antimicrobial test. In order to label strains as susceptible, intermediate or resistant, antibiotic discriminating concentrations were used: Ampicillin (AMP), 10 µg/disc, Doxycycline (DO), 30 µg/disc, Enrofloxacin (ENR), 5 µg/disc, Sulphamethoxazole/ Trimethropim

(STX), 25 µg/disc, Nalidixic acid (NA), 30 µg/disc, Pipemidic acid (PIP), 20 µg/disc, Tetracycline (TE), 10 µg/disc, Oxolinic acid (OA), 2µg/disc and Amoxycillin (AML), 30 µg/disc (Oxoid).
Each strain was plated in Mueller Hinton agar (Biokar Diagnostics), and the discs with the antibiotics were placed over the bacteria layer [7]. Plates were incubated at 37ºC overnight. After this period, the diameter of the clear zone was measured and strains classified according to the sensitivity to each antibiotic. An *E. coli* reference control culture (ATCC 25922) was used for quality control of the test.

2.4 Bacteriophage isolation and purification

Bacteriophages were isolated from samples of poultry sewage, collected randomly from Portuguese poultry houses. Under sterile conditions samples were emulsified in Luria Bertani (LB) broth (Sigma), and the decanted supernatant obtained from each emulsion was added to an early-log grown mixture of eight *E. coli* strains selected randomly, from different O-antigene serotypes. Suspensions were incubated overnight at 37ºC, with shaking (120 rpm) and were then centrifuged at 9 000 × *g* for 10 min (rotor 19776, Sigma 3-16k). The supernatant was then filtered through a 0.22 µm membrane, 33 mm Millex Filter Units, Durapore® (PVDF) (Millipore, Bedford, MA, USA). The spot test method was used as an initial test for the presence of phage. A procedure based on the double layer plaque technique was performed [12]. Layers of 3 ml of LB 0.6% agar (Sigma), previously inoculated with 100 µl of each *E. coli* strain used above, 6-8 h culture were spotted with 10 µl of the filtered suspension. This procedure was performed over LB 1.5% agar. Plates were incubated at 37ºC overnight. A clear zone in the plate, resulting from the lysis of host bacterial cells, indicated the presence of phage. In order to isolate phages from this clear lysis zone, serial dilutions in phage buffer (100 mmol l^{-1} NaCl (Sigma), 8 mmol l^{-1} $MgSO_4$ (Sigma), 50 mmol l^{-1} Tris (Sigma), pH 7.5) were done from the phage stocks obtained above. A colony of the respective hosts strains were grown 3-4 h (early-log phase culture) in 5 ml of LB broth. A volume of 100 µl of phage-containing sample and 100 µl of host culture were mixed with 3 ml of 0.6% LB agar, overlaid onto 1.5% LB agar plates and incubated overnight at 37ºC. Phages were purified by successive single plaque isolation, from the higher dilutions plates where plaques were still distinct. A single plaque was picked from the bacteria lawn,

inoculated into an early-log phase host culture, and the lysate plated as described above. After repeating the cycle three more times, lysates from single plaques were treated with chloroform 4:1 (v/v), mixed and centrifuged at 5 000 × *g* for 5 min. The phages were recovered from the upper phase suspension and filtered trough 0.22 µm. Phages stocks were stored at 4ºC.

2.5 Phage lytic spectra of the typed *E. coli* strains

Bacterial susceptibility to bacteriophage was assayed for the 148 isolated *E. coli* strains by adapting a modified procedure of the traditional double-layer technique [12]. Once the top agar was solidified at room temperature, 10 µl of the phage lysate suspension of about 10^7 PFU ml^{-1} was spotted, incubated at 37ºC overnight and examined for the presence of a clear zone of lysis.

2.6 Phage amplification

The amplification of each isolated bacteriophage was performed by inoculating 5 ml of the purified phage suspensions in 10 ml of a 3-4 h culture (in LB broth) of the respective *E. coli* hosts. It was incubated overnight at 37ºC, with shaking (120 rpm). The suspension was centrifuged at 9 000 × *g* for 10 min and filtered through a 0.22 µm membrane. This procedure was repeated again, by inoculating the resulting phage lysate volume in 100 ml of 3-4 h 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.

The number of phages present in this suspension was determined according to the Adams' method [3] with minor modifications. Successive dilutions of the phage suspension were performed in a saline solution (0.85% NaCl) 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.

2.7 Bacteriophages life cycle investigation by the induction of infected host strains with mitomycin C

In order to evaluate if phages selected based on the lysis efficiency were able to insert their genome into bacterial DNA remaining as a prophage, some tests were performed. Lambda (λ) phage (DSM 4499), a *Siphoviridae* temperate phage, and the respective *E. coli* host (DSM 4230) were used as positive controls. Reconstitution, propagation and storage of this phage and *E. coli* host strain were conducted according to the supplier instructions (DSMZ, Braunschweig, Germany).

Each of the host strains were early-log grown in LB broth and 20 µl of the respective phage were spotted on the lawns, as described above. After an overnight incubation at 37ºC, bacteria colonies change in the central lytic zone (resistant colonies) were picked (at least 5 colonies) and purified by successive sub-culturing in MacConkey agar, to remove attached phage particles. Phage resistance of those isolated strains was confirmed by the cross-streaking test and the spot lysis assay, and those phage resistant colonies were stress induced with mitomycin C (Sigma). The strains were grown in 200 ml of LB until an optical density at 600 nm of 0.2 was reached. The induction of phage release was attempted via overnight incubation at 37°C, in the presence of mitomycin C (1 $\mu g\ ml^{-1}$). A negative control, without mitomycin C was prepared. Bacteria lysate was centrifuged at 9 000 × *g*. The supernatant was filtered through 0.22 µm and tested against each phage-sensitive host strain (wild-type (WT)) [22, 28]. After an overnight incubation at 37°C, bacterial lawns were checked for clear zones.

2.8 Electron microscopy

Phage particles were sedimented at 25 000 × *g* for 60 min using a Beckman (Palo Alto, CA) J2-21 centrifuge with a JA 18.1 fixed-angle rotor. Phages were washed twice in 0.1 M ammonium acetate, pH 7.0 (Sigma), deposited on copper grids **(**Ernest F. Fullam, Clifton Park, NY) provided with carbon-coated Formvar films (Canemco & Marivac, Quebec, Canada), stained with 2% potassium phosphotungstate, pH 7.2 (Sigma) and examined in a Philips (Eindhoven, The Netherlands) EM 300 transmission electron microscope (TEM), operating 60 kV. Magnification was monitored with catalase cristals (performed by Dr. H. W. Ackermann, Laval University, Quebec, Canada).

2.9 Bacteriophage purification by CsCl precipitation

An ultracentrifugation method was performed based on a Cesium Chloride density gradient. Four different solutions were prepared in phage buffer: 1.70 g ml^{-1}; 1.50 g ml^{-1} and 1.30 g ml^{-1} CsCl (Sigma). After the volume of each phage suspension was measured, 0.5 g ml^{-1} of CsCl was added. These suspensions were ultracentrifuged (XL-90, Beckman) at 60 000 × *g* for 2 h at 4ºC. A bluish band indicative of phage particles was collected and placed in a microtube [35]. A Centricon 20 spin filter unit (Millipore) was used to reduce the volume of the recovered CsCl purified phage concentrate. The centrifugation was performed at 4 000 × *g* for 10 min at 4 ºC. The phage concentrate was then washed with the phage buffer 1:4 (v/v) and centrifuged with the same settings in the filtration module, three more times, to remove all the CsCl. The resulted suspension was stored at 4ºC.

2.10 RFLP pattern analysis

Differences between phages were confirmed by comparison between the individual restriction fragment length polymorphism (RFLP) patterns. A volume of 200 µl of the concentrated phage suspension by CsCl precipitation, was pre-incubated 30 min at 37ºC with 1 µl of RNase 20 mg ml^{-1} (Sigma) and submitted to DNA purification according to the protocol provided with a commercial kit, High Pure PCR Template Preparation Kit (Roche, Mannheim, Germany). Uncut phage DNA was run at 90 V for 45 min, in a 0.8% agarose gel (Qbiogene, Irvine, CA, USA) stained with ethidium bromide (Bio-Rad, Hercules, CA), to verify extraction yield and absence of bacterial genomic DNA. *Xap*I, *Bse*GI and *Sch*I restriction enzymes (Fermentas, St. Leon-Rot, Germany) were used in order to obtain phage DNA RFLP patterns. A concentration of 5 U μl^{-1} of each enzyme and the respective enzyme buffers 1 × diluted in RNase and DNase free water (Biological Industries, M.P. Ashrat, Israel) were added to 6 µl of phage DNA, with a final volume of the reaction mixture of 30 µl. Tubes were incubated at 37ºC for 3 h, according to supplier instructions. The loading buffer used to improve resolution was 1 × DNA Loading Dye & SDS Solution (Fermentas) and was added to the samples at 1:6 (v/v). Tubes were incubated at 65ºC for 10 min and chilled on ice. Samples were loaded in a 1 cm thick, 2.0% agarose gel stained with ethidium bromide. Electrophoresis was carried out at 45 V for 5 h in a dark place.

3. RESULTS

3.1 APEC O-serogroup and antibiotics susceptibility

The most common O-serotype of the 148 isolated APEC strains was the O78 with a frequency of 40.5%, followed by O2, O5 and O88 representing each from 5.2% to 6.9% of the isolated bacteria. It was not possible with the kit used to type 34.7% of the bacterial strains. *E. coli* serotypes as O1, O8, O11, O15, O20, O53, O86 and O103 were present at a low frequency (from 0.6 to 1.7%), while O6, O9, O12, O14, O17, O18, O35, O36, O45, O81, O83, O102, O115, O116 and O132 were not detected (Figure II.1

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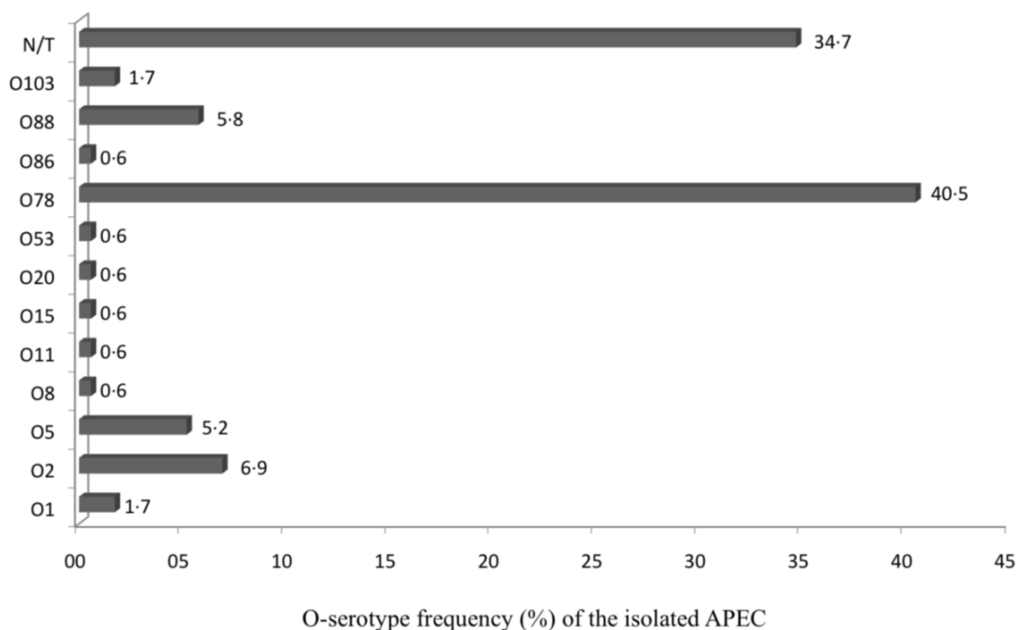

Figure II.1 Relative frequency (%) of the APEC O-serotypes.

E. coli strains were also grouped according to the respective O-serotype, by strain of birds, age or species, according to the source of isolation.

Figure II.2 refers to the relative distribution of O-serotypes per group of birds. It is possible to verify that different O-serotypes infected the same group of birds. For example, serotypes like O1, O2, O78 and N/T were isolated from laying hens, O8, O15, O78, O86, O88, O103 and N/T were isolated from broilers, O1, O2, O5, O11, O53, O78, O88, O103 and N/T from label chickens, O2, O5, O20, O78, O88 and N/T were found in chicks, O78 and N/T in breeders and O2, O5, O78 and N/T were isolated from

turkeys. It was observed that the isolated O-typeable strain more frequent in all the groups was the O78.

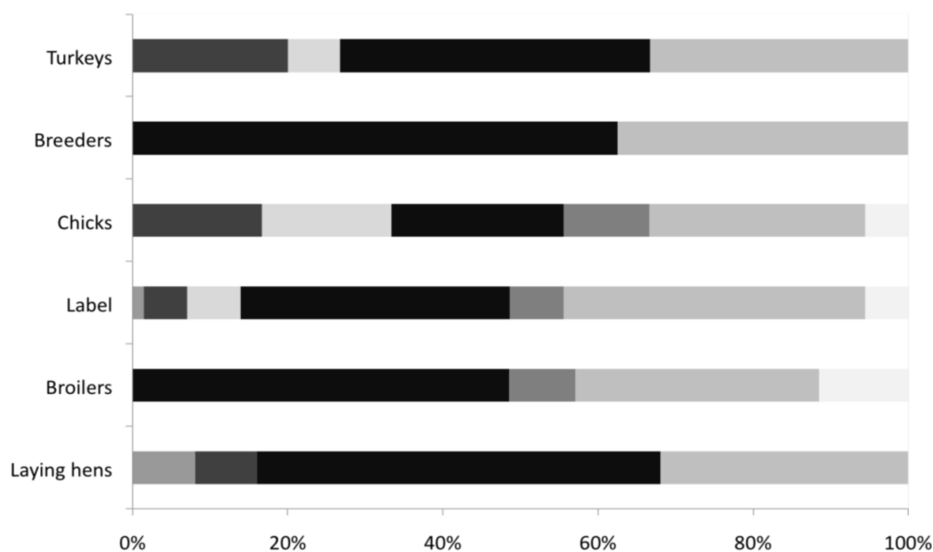

Figure II.2 Relative frequency (%) of *E. coli* main isolated serotypes, according to the birds' strain, specie or age. O-serotypes described as "others" are O8, O11, O15, O20, 053, 086 and O103, and were found in bacteria collected from broilers, 2.9% O8, 2.9% O15, 2.9% O86 and 2.9% O103, label chickens, 1.4% O11, 1.4% O53 and 2.8% O103 and chicks (5.6% O20) (■ O1; ■O2; □O5; ■O78; ■ O88; ■ NT; □ others).

The isolated strains were then subjected to an antibiotic sensitivity test and the percentages of susceptible, intermediate or resistant strains to each antibiotic are present in Figure II.3.

It was observed that 80 to 90% of the strains were resistant to TE, DO, OA and NA, 70 to 75% were resistant to AMP and PIP, 66.5% to AML, 61.6% to STX and 47.5% to ENR. The active ingredient with higher effectiveness to this group of strains was ENR, active against 50.8% of the APEC.

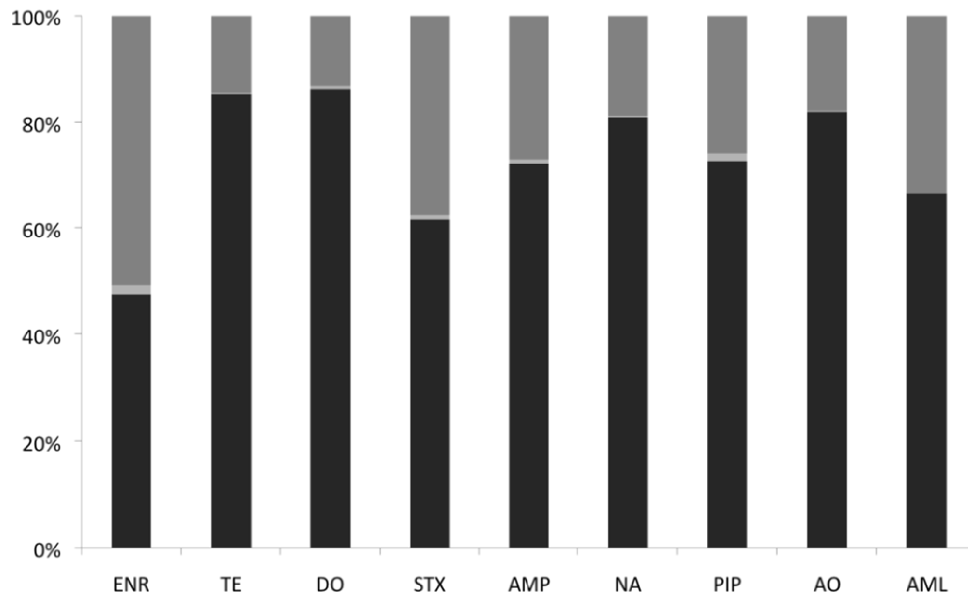

Figure II.3 Relative comparison (%) of the isolated strains according to susceptibility, intermediate susceptibility or resistance to a range of antibiotics commonly used for therapy in poultry industry. AMP - Ampicillin; DO - Doxycycline; ENR - Enrofloxacin, STX - Sulphamethoxazole / Trimethropim, NA - Nalidixic acid, PIP - Pipemidic acid, TE - Tetracycline, OA - Oxolinic acid; AML- Amoxycillin (resistant (■); intermediate (□) and susceptible (■)).

3.2 Bacteriophage lysis efficiency

Five phages were isolated: phi F78E, phi F258E, phi F2589E, phi F61E and phi F5318E. Phage lysis efficiency for the 148 O-serotyped strains is illustrated in Table II.1 and II.2. From Table II.1, it is apparent that all the phages were effective against O78, O5 and N/T *E. coli* strains and most of them were active for O2 and O88. From the low frequency O-typed strains (Figure II.1), O1, O20 and O53 were not sensitive to any of the tested phages.

Generally, observing the host lysis performance of each phage (Table II.2), phi F258E and phi F61E were found to have the broadest host range, 44.6% and 48.0% respectively.

Table II.1 Bacteriophages lytic score (%) by *E. coli* O-serotype.

		phi F78E	*phi F258 E*	*phi F2589E*	*phi F61E*	*phi F5318E*
O-serogroup	**O1**	0.00	0.00	0.00	0.00	0.00
	O2	63.64	9.09	0.00	18.18	9.09
	O5	35.29	5.88	5.88	47.06	5.88
	O15	100.00	0.00	0.00	0.00	0.00
	O20	0.00	0.00	0.00	0.00	0.00
	O53	0.00	0.00	0.00	0.00	0.00
	O78	8.33	30.73	14.06	23.96	22.92
	O88	26.32	0.00	26.32	47.37	0.00
	O103	100.00	0.00	0.00	0.00	0.00
	N/T	48.39	16.13	6.45	19.35	9.68

Table II.2 Bacteriophages sensitive strains (%).

Phage	*phi F78E*	*phi F258 E*	*phi F2589E*	*phi F61E*	*phi F5318E*
Sensitive strains (%)	35.14	44.59	23.65	47.97	33.11

Figure II.4 illustrates the best phage association according to the higher percentage of lysed strains, in groups of two, three, four and five phages. When combining them in groups of two, the strongest lysis association was between phi F78E and phi F61E (60.4%). Groups of three phages, phi F78E, phi F258E and phi F61E, presented a higher lysis percentage, 70.5%. Associations of four and five phages are able to lyse, respectively 71.8% and 72.5% and thus does not bring a relevant advantage for lytic spectra range when compared with an association of three phages. Based on these results, the phages selected for further characterization were phi F78E, phi F258E and phi F61E.

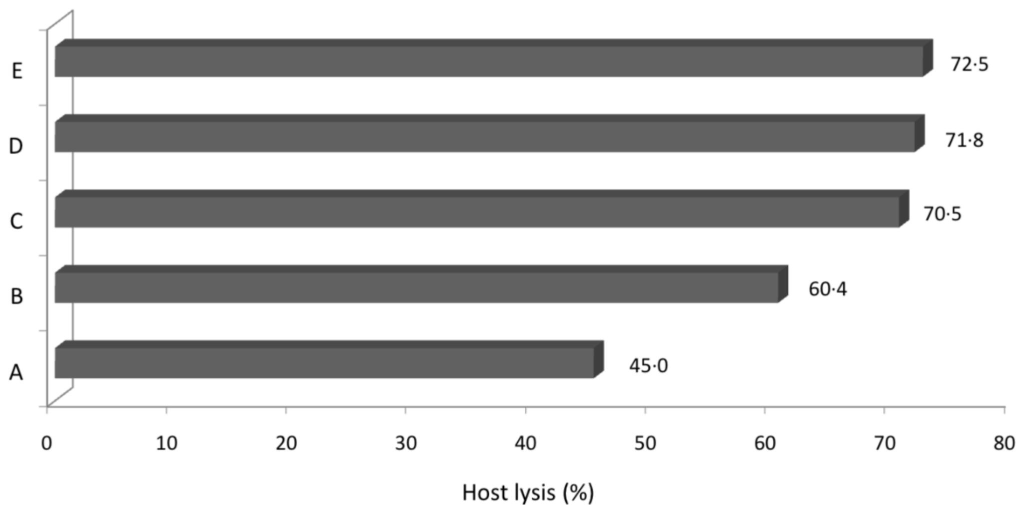

Figure II.4 Best phage associations according to the higher percentage of lysis of the tested *Escherichia coli* strains: A- phi F61E (45.0%); B- phi F78E + phi F61E (60.4%); C- phi F78E + phi F258E + phi F61E (70.5%); D- phi F78E + phi F258E + phi F61E + phi F5318E (71.8%); E- phi F78E + phi F258E + phi F2589E + phi F61E + phi F5318E (72.5%).

3.3 Characterization of phages phi F78E, phi F258E and phi F61E

Phages phi F258E and phi F61E formed very clear lytic zones on their hosts (H816E and H161E, respectively) exhibiting no resistant bacteria. Conversely, phi F78E induced the formation of resistant colonies on H561E lawns after subculture, which may be an indication of lysogeny. Temperate phages integrate into the DNA hosts and only lyse the cells under certain conditions. Stress induced infected cells with temperate phages usually results in the release of the phage. So, the mitomycin C assay with the phi F78E resistant bacterial cells was performed. Infected *E. coli* DSM 4230 with λ bacteriophage was used as a positive control. In the assay no clear zone was found after stress inducing phi F78E resistant bacteria, which indicated that phi F78E is not temperate.

Electron micrographs demonstrated that all phages do not possess any lipidic envelope. Phages phi F78E and phi F61E had a neck with a tiny collar and a contractile tail. Phi F78E has caudal fibers (20 × 2 nm) (Figure II.5A). Phi F78E and phi F61E capsids were 103 × 42 nm and the tails 100 × 17 nm (Figures II.5A and II.5C, respectively). Phi

F258E has a circular head with diameter of 62 nm and is characterized by a flexible tail of 160×8 nm (Figure II.5B).

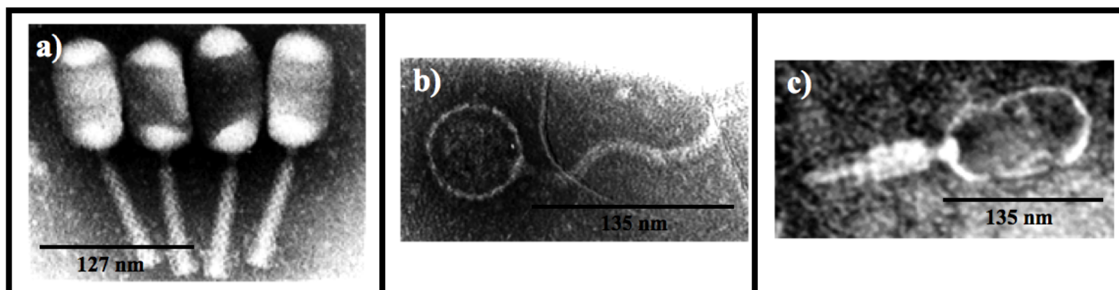

Figure II.5 Bacteriophage microphotograph obtained by TEM: (A) phi F78E (magnitude: 297 000 ×); (B) phi F258E (magnitude 297 000 ×); (C) phi F61E (magnitude: 148 500 ×).

It was possible to observe discriminatory bands in each phage DNA restriction pattern for a given enzyme (Figure. II.6). For example the digestion of the phi F78E DNA with *Bse*GI showed a distinct band between 2 300 and 2 000 bp, while phi F258E DNA digested with the same enzyme exhibited a strong band above 23 000 bp and others of lower molecular weight (< 2 000 bp). The digestion of phi F61E DNA with this enzyme gave rise to four distinct bands under 1000 bp. The same stands for the profiles obtained with *Xap*I: high molecular weight discriminatory bands only above 4000 bp were detected in phi F78E DNA, whereas in the case of phi F258E, there were more distinct bands under this molecular weight (4000 bp). The phi F61E digestion pattern was not very clear, probably due to overlapped bands. Also, divergent RFLP profiles were noticed for *Sch*I digestion: when comparing the two first profiles in Figure II.6 relative to this enzyme, it can be noticed that discriminatory bands of low molecular weight appeared in the gel, only for phi F258E. Phi F61E DNA did not present any discriminatory band for this enzyme.

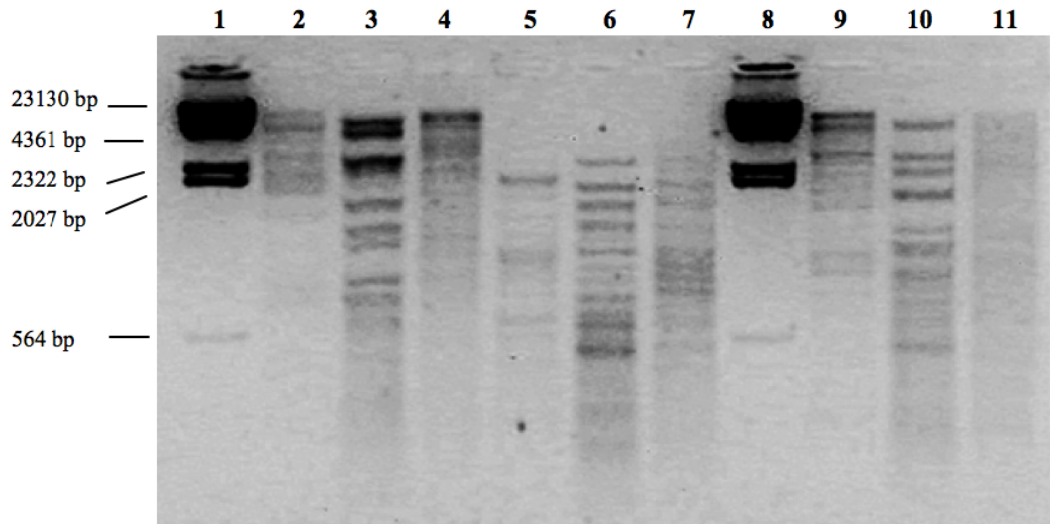

Figure II.6 Agarose gel 2% stained with ethidium bromide, 5 h run at 50 V: 1 and 8- λ DNA / *Hind*III; 2- phi F78E / *Xap*I; 3- phi F258E / *Xap*I; 4- phi F61E / *Xap*I; 5- phi F78E / *Bse*GI; 6- phi F258E / *Bse*GI; 7- phi F61E / *Bse*GI; 9- phi F78E / *Sch*I; 10- phi F258E / *Sch*I; 11- phi F61E / *Sch*I.

4. DISCUSSION

Avian pathogenic *E. coli* (APEC) possess specific virulence characteristics associated with colibacillosis. This is the primary cause of morbidity, mortality, and rejection of carcasses at slaughter in the poultry industry worldwide, with consequent high economical losses [14, 16, 17]. The incidence and severity of colibacillosis has been increasing and might become an even greater problem in the poultry industry [4, 9].

APEC can cause disease in birds of various ages and strains. The results obtained in the present work, suggests that the O- serotypes affected poultry, independently of specie, strain or age. The most common typeable *E. coli* O-serotype isolated from each defined group of birds was the O78, and generically, the O2, O5 and O88 were also found as common serotypes. This result is supported by several i*n vivo E. coli* pathogenicity tests performed in poultry, in which the same O-serotypes were found to be responsible for the most part of the colibacillosis infections [4, 9, 15, 21, 34, 48]. Data from the antibiograms demonstrated the high capacity of *E. coli* to acquire resistance to the most frequently used antibiotics (Figure II.3). Similar results have been reported by several authors [32, 44, 45].

Bacteriophages have several characteristics that make them potentially attractive therapeutic agents against bacterial infections. One of them is the high specificity and effectiveness in lysing targeted pathogenic bacteria. Due to phages high specificity, they are likely to have a relatively narrow host range, and so, the disease agent has to be isolated and a bacteriophage lysis test must be customized to the specific pathogenic bacteria [25].

From another perspective, the treatment of a disease with bacteriophage might benefit, if instead of one, a cocktail of phages effective against the most part of the bacteria that are known to cause the disease is used. From this point of view, it would be useful to develop a bacteriophage therapeutic product based on the best phage associations, increasing the antimicrobial range of the product [13, 20, 25, 39, 42, 43]. This was the underlying reason for testing the efficacy of several isolated *E. coli* phages against a pool of the isolated APEC strains resistant to the most common antibiotics. From the five phages isolated, two revealed broad lytic spectra, being phi F61E the most effective phage, lysing 48.0% of the bacterial strains. The association of phi F78E, phi F258E and phi F61E was effective for 70.5% of the strains. It is important to stress that with only three phages, a large range of APEC strains were covered, which is better than the most effective antibiotic, the ENR with 50.8% of efficacy (Figure II.3). A significant increment in the lysis efficiency combinations of four or five phages was not found compared to the efficacy observed with three phages (71.8% and 72.5% of lysed strains, respectively). In fact, an association of more than three phages would be even disadvantageous, because the economic recourses necessary to characterize and produce different phages would be higher. Based on this assumption, phi F78E, phi F258E and phi F61E were selected for further characterization.

The phages morphological characteristics observed by Transmission Electronic Microscopy (TEM) revealed that phi F78E and phi F61E belong to *Myoviridae* taxonomic family and seem to be 16-19 type phages, roughly like T4. The same phage types have already been isolated from sewage and characterized morphologically by Ackermann et al. [2], and later by Ackermann and Nguyen [1]. Similar to phi F78E and phi F61E, the two phages described by those authors showed contractile tails of 100×7 nm and 94×15 nm and elongated heads with 104×43 or 102×57 nm in diameter. The same authors described that in those phage types, heads resembled superficially those of T-even phages and appeared to be mostly oval. Tails of these phages were complex and consisted generally, of a neck, a base plate and tiny caudal fibres, similar to the phages

characterized in this work. Those phages were so far described for *Salmonella*, so, to the authors knowledge, this was the first time that 16-19 phages are described as being effective against *E. coli* strains. Phi F258E seemed to be a *Siphoviridae*, T1-like, already described for *E. coli* [47].

One of the major concerns in the use of phages for therapy purposes is to guarantee that the phages do not integrate into the DNA hosts. The morphological characteristics of these phages are similar to those described as lytic; nevertheless a mitomycin C stress inducement was performed to confirm that phage phi F78E is not temperate, because resistant colonies were recovered from the interior of the phage clear zone.

The three phages presented different structure and host range, and therefore are distinct. This was also corroborated by their different RFLP patterns.

In short, in this work three phages belonging to the *Myoviridae* and *Siphoviridae* families, isolated from poultry sewage, showed to be effective against 70.5% of the 148 isolated APECs, most of which were resistant to the majority of antibiotics tested. Morphological and genetic characterization of these phages suggests that they belong to different phage-types. Taking together all these results it can be suggested that these three phages combined in a therapeutic cocktail would be a more efficacious therapy over conventional antibiotic therapy.

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III. *IN VIVO* TOXICITY STUDY OF PHAGE LYSATE IN CHICKENS

In press in: *British Poultry Science*

Lytic bacteriophages (phages) are viruses that interact with bacterial cell walls, disturbing bacterial metabolism and promoting bacteria lysis. They are considered to be good candidates for antimicrobial therapy. Phage crude lysate of Gram-negative bacteria often contain bacterial debris, lipopolysaccharides found in the outer membrane of the cell wall potentially able to posses toxicity. In this study, an *in vivo* evaluation of the toxicity of a suspension of three phages, to control pathogenic *Escherichia coli* strains in poultry, was performed. For that, 18 commercial layers, 7-weeks-old, were intramuscularly injected with phage lysate (8.21×10^4 Endotoxin Units/dose). The control group, 18 layers, were injected with sterile Luria Bertani broth.

Bird prostration and decrease in body weight gain and water intake per gram of body weight were observed only in the day of the inoculation in the challenged group. In the following six days, no visual differences were found in chicken's activity.

These results represent a valuable contribution to support the safety of this product as a therapeutic tool for chicken.

Keywords: *Escherichia coli*; lipopolysaccharides; bacteriophage; toxicity; chicken.

1. INTRODUCTION

Lytic bacteriophages (phages) are viruses that infect and promote bacteria lysis through a multiple-step process: they multiply in the host bacteria and lyse it at the end of the cycle, after immediate replication of new phage particles. They are considered to be good candidates for antimicrobial therapy, as they provide an opportunity to control bacterial infections. Bacteriophages are safe once they exclusively infect bacterial cells, having no activity against animal or plant cells. Like all viruses, phages are metabolically inert in their extra cellular form and they are ubiquitous in nature [10]. Nevertheless, as phage infections culminate in lysis of bacteria, there is a consequent release of cell wall components to the environment as cell debris. In this process, Gram-negative bacteria release endotoxin into the environment, whose biological activity is associated with complexes of lipopolysaccharides (LPS), present in the outer layer membrane. This can lead to undesired side effects on phage therapy. The LPS toxicity is associated with the lipidic component of the molecule, known as “lipid A”, while the immunogenicity is associated with the polysaccharide component, known as “O-specific side chain” or “O-antigen” [2, 5, 18, 23, 24]). For that reason, LPS are present in the cellular debris in crude phage lysates, easily passing through filters used to remove whole bacteria from phage suspensions [24].

The endotoxins may induce a variety of inflammatory responses being often part of the pathology of Gram-negative bacterial infections. Although animals vary in their susceptibility to endotoxins, the sequence of pathophysiological reactions follows a general pattern: a latent period followed by physiological distress. Immunologic and neurological system activation, induction of blood coagulation, general metabolic effects, alteration of blood cell populations, pyrogenicity, production of endotoxic shock and hepatotoxicity are some of the known reactions to an endotoxin parenteral challenge, promoting symptoms like fever, diarrhoea, prostration and, in many cases, shock and death [5, 25]. The study of the way to remove endotoxins from solutions intended to be used in humans or animals is therefore an important area of study in applied Biotechnology. However the success of this procedure is greatly dependent on the initial composition of the mixture [2, 17]. Ultrafiltration and size-exclusion chromatography should theoretically provide a way of separating components differing in molecular mass. However, the application of these two down-stream processes in the

purification of phages only allows a partial removal of the contaminants present in the phage suspension. In fact, despite their relatively low molecular weight (4-20 kDa for LPS monomer), endotoxins are not effectively removed as they tend to aggregate forming structures **s**imilar to micelles and vesicles, ranging in molecular weight from 300 000 to 1 million, with diameters up to 0.1 μm [2, 24]. There have been reported other approaches to achieve the destruction or removal of endotoxins, like hydrolysis with acid or base, oxidation, alkylation, heat treatment and treatment with polymicin B [6]. However, all these approaches must be evaluated considering the economical viability of the scale-up of the process and the possibility of compromising the recovery rate of the desired product, in this case the phages. According to Petsch and Anspach (2000)[17], the question of how endotoxin removal can be carried out in an economical way has occupied many investigators and has been the reason for process rearrangements in many cases.
In this work, an *in vivo* trial with an *E. coli* phage crude lysate, administered intramuscularly to chickens, was conducted in order to evaluate the endotoxin effect and to assess the level of importance of endotoxin removal in ensuring the safety of this phage product for the target species.

2. MATERIALS AND METHODS

2.1 *E. coli* phage lysate

Crude phage suspensions were prepared by inoculating a single phage plaque of the phages phi F78E, phi F258E and phi F61E in 10 ml of the respective *E. coli* host H561E, H816E and H161E, mid-log grown (3-4 h) in Luria Bertani (LB) broth, (Sigma) (as described in Chapter II, Section 2.4). This was followed by an overnight incubation at 37ºC with shaking (120 rpm). The suspension was then centrifuged (9 000 ×*g*) for 10 min (rotor 19776, Sigma 3-16k), filtered through a 0.22 μm membrane (Millipore) and following the same procedure as previously described, inoculated again in 100 ml of mid-log grown culture of the respective *E. coli* host. The incubation was performed at 37ºC with shaking (120 rpm), the centrifugation was at 9 000 ×*g* for 10 min and the filtration was through 0.22 μm. The phage crude lysate was stored at 4ºC. The number of phages in this suspension was determined according to the Adams' method [1] with

minor modifications. Briefly, successive dilutions of the phage suspension were performed in phage buffer (100 mmol/l NaCl (Sigma), 8 mmol/l $MgSO_4$ (Sigma), 50 mmol/l Tris (Sigma), pH 7.5) and 100 µl of each dilution together with 100 µl of the respective bacteria 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 at 37ºC for 12 h and phages enumerated in the higher dilution with distinct plaques. Phage titration was performed in triplicate.

2.2 Measurement of endotoxin concentration

The concentration of LPS present in the *E. coli* phage lysate was measured using the Limulus Amebocyte Lysate assay (LAL) (Bio-Whittaker), which is based on the activation of Limulus Lysate by endotoxins [18]. The procedure was carried out according to the supplier instructions, using a spectrophotometer (Bio-TEK Synergy HT). This method was approved by FDA for detection and quantification of endotoxins [7].

2.3 Experimental Design

This study was conducted according to the Federation of European Laboratory Animal Science Associations (FELASA) principles of animal welfare, and the experiment was designed in accordance to the European Council Directive of 24 November 1986 (86/609/EEC) guidelines, on the approximation of laws, regulations and administrative provisions of the member States regarding the protection of animals used for experimental and other scientific purposes.
Thirty-six healthy 7-week-old growers (Rhode Island Red) were used. Two groups of 18 chickens were randomly selected and housed, 3 per cage, in a temperature and relative humidity controlled animal room, with a 12 h light/ 12h dark cycle. Birds were individually identified by leg rings. Feed and water were provided *ad libitum.* A volume of 1 ml of the previously prepared phage suspension and 1 ml of sterile LB broth were injected intramuscularly, respectively in the challenged group (CHG) and in the control group (CG), only in the first day. The chickens' body weight (BW) was recorded the day before and every day after challenge. In order to avoid unnecessary discomfort to the animals, the evaluation of chickens' reaction to challenge was done based on behavior observation, taking in account specific signs: healthy chickens were

recognized by their standing up position, with the neck strong and straight, flat feathers against the body and high frequency of seeking food and water. If, on the other hand, chickens were sitting, lying still, with the neck weak and shrunken, raised feathers, and not looking for food or water, they were reported as prostrated. Food and water consumption were recorded daily. On day 7, all chickens were euthanized by isofluorane (IsoFlo®, Abbott) inhalation and submitted to *post mortem* examination.

2.4 Statistical analysis

Statistical analysis was undertaken for each parameter assessed in the study: BW gain, feed and water intake per gram of BW. CHG means were compared with CG means at each data collecting period. Statistical variance analysis was performed using Kruskal-Wallis test in SPSS v15.00 software. Statistical significance was tested at $P = 0.05$.

3. RESULTS

3.1 *E. coli* phage lysate

The *E. coli* phage lysate is a mixture of three phages phi F78E, phi F258E and phi F61E, with the concentration of each phage being 1.67×10^8 PFU/ml, 2.5×10^8 PFU/ml and 3.0×10^8 PFU/ml, respectively. The LAL test revealed that the LPS concentration present in this suspension was 8.21×10^4 Endotoxin Units (EU)/ml.

3.2 *In vivo* challenge with phage lysate

A volume of 1 ml of phage lysate was administered intramuscularly to the chickens of the CHG, with the total amount of LPS being, in average, 2.32×10^5 EU/kg BW.
During the *in vivo* experiment, bird prostration was only observed during the day of the inoculation (day 1) in CHG. One bird died one hour after inoculation but in the *post mortem* analysis no macroscopic lesions were detected in internal organs. During the following six days, no visual differences were found in the chicken's activity between the two groups. Respecting to the BW gain (Figure III.1), it was observed that at day 1 and day 6, this parameter decreased significantly in CHG ($P = 0.043$ and $P = 0.010$,

respectively). The feed and water intake per gram of BW did not diverge significantly between groups (Figure III.2 and III.3). Apparently, there was a decrease in the water intake per gram of BW at day 1 in CHG, but with no relevant differences between groups (P = 0.065). At day 4, there was a significant decrease (P = 0.035) in CHG, however this tendency was no longer observed in the following days.

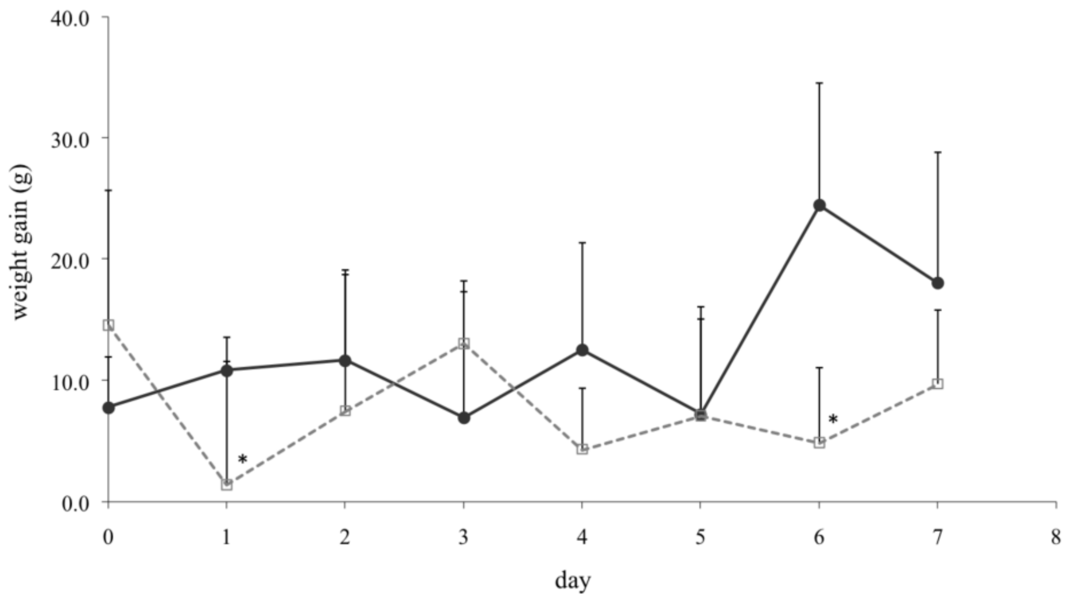

Figure III.1 Chickens' daily BW gain. In the figures, the solid line —●— represents CG variation in body weight gain and the dashed line –□– represents the CHG variation. Error bars represent standard deviations of experimental data from the six cages of three animals each. *Statistically different from CG.

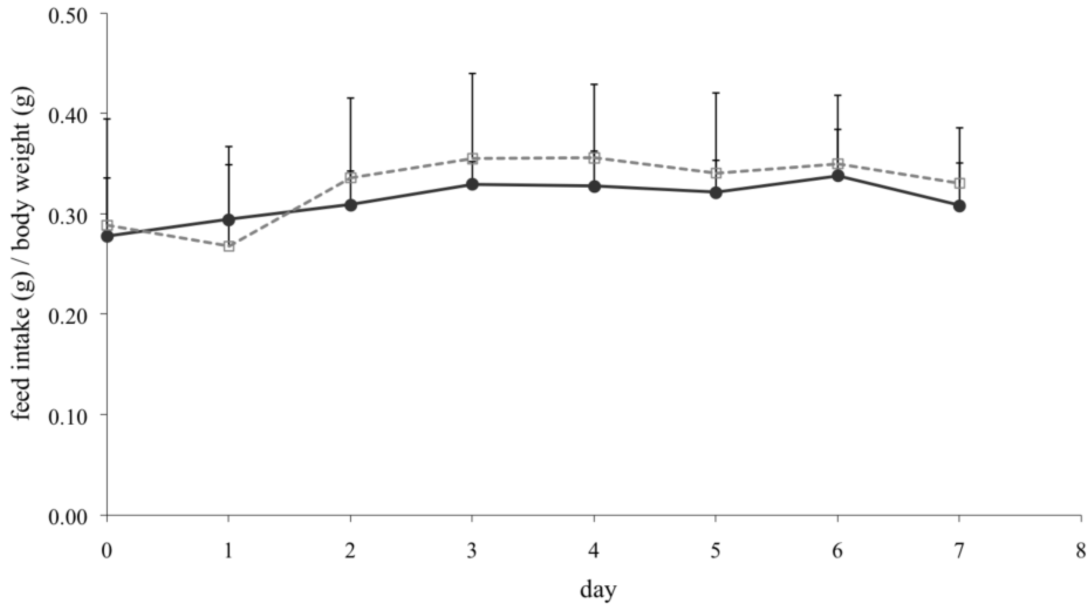

Figure III.2 Chickens' feed consumption per gram of BW. In the figures, the solid line —●— represents CG variation in body weight gain and the dashed line –□– represents the CHG variation. Error bars represent standard deviations of experimental data from the six cages of three animals each.

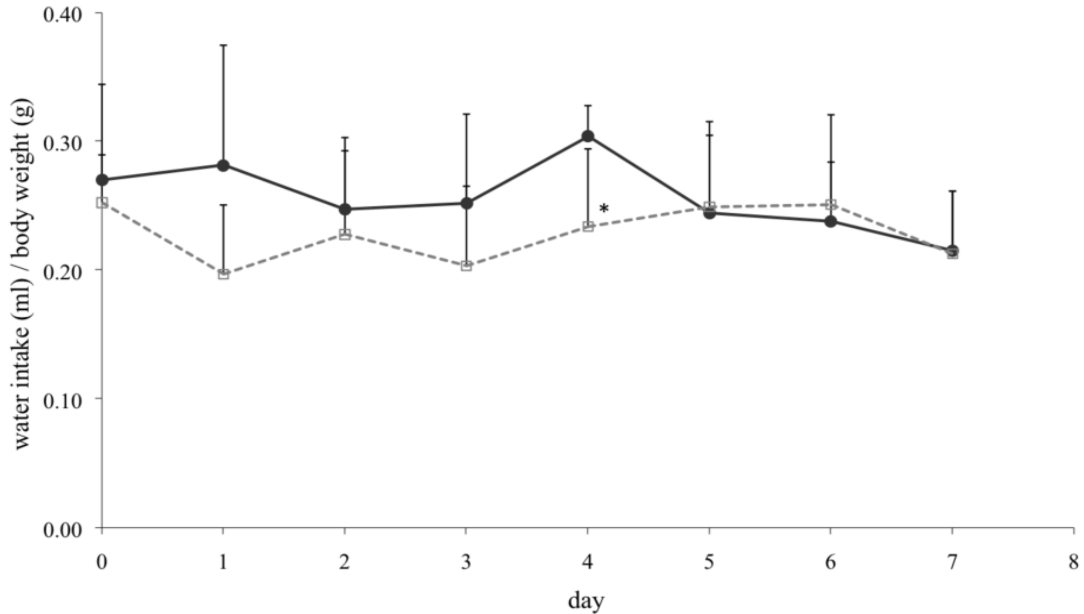

Figure III.3 Chickens' water consumption per gram of BW. In the figures, the solid line —●— represents CG variation in body weight gain and the dashed line –□– represents the CHG variation. Error bars represent standard deviations of experimental data from the six cages of three animals each. *Statistically different from CG.

4. DISCUSSION

Phage therapy has been considered an important alternative to the administration of antibiotics in the treatment of severe *E. coli* infections in birds [9, 10]. One of the main concerns of this approach is the presence of endotoxins in the phage crude lysate [19]. In the present work, 8.21×10^4 EU/ml LPS were found in the prepared phage cocktail used. Nevertheless, the chickens were challenged with 1ml of this phage lysate containing approximately 2.32×10^5 EU/kg BW, which was not supposed to be lethal, since, and according to Culbertson and Osburn (1980) [5], the lethal dose of *Escherichia coli* endotoxins to chickens is $\geq$ 50 mg/kg or $\geq 5\times10^8$ EU/kg (1 EU/ml $\approx$ 10 ng/ml). Kokosharov (2002) reported that little is known about LPS activity in chickens and sometimes experimental data are conflicting and divergent. It should be referred that the phage lysate administered to the chickens has 10 times the volume, and therefore might have about 10 times the LPS content of the phage cocktails used for therapeutic purposes as described by Huff and their colleagues (2004, 2005) [9, 10]. These authors did not observe any harmful effects on chickens' health and advise a phage concentration in therapeutic mixtures ranging between 10^7 and 10^9 PFU/ml [9, 10]. During the *in vivo* trial, the birds challenged with the phage lysate, as compared to the CG, exhibited prostration and decreased feed and water intake only during the day of inoculation. During the following days, chickens' behavior did not show visual differences between groups. Similar findings were reported by Smith et al. (1978) [22] during an experiment with endotoxins from *Salmonella enterica* serovar Gallinarum administered to 14-day-old chicks. Despite the observation of some clinical illness without mortality a few hours after intravenous injection of 1.5×10^7 ng/kg LPS, most of the responses returned to normal within 24 to 48 h. Also Kokosharov (2002) [12] observed illness in cockerels one hour after injection of 5.0×10^7 ng/kg LPS. Birds were described as standing in the corners of the cages with signs of depression, reluctance to move, somnolence, loss of thirst and appetite, and diarrhea, which all gradually disappeared. This author did not report any death among the cockerels challenged.

In the present work, one chicken died one hour after the intramuscular inoculation, probably due to an anaphylactic shock, as no visible lesions were found at necropsy. The statistically significant decrease of BW gain in CHG at day 1 was probably due to the chickens' prostration and apparent loss of appetite. At day 6, the differences

between groups might be explained by the occurrence of an unexpected factor during the experiment: some feeders of the CG were found empty in the morning, for the first time since the beginning of the housing. As feed had to be provided *ad libitum*, feeders were immediately refilled. This happened a few hours before birds weighing and thus might had contributed to the higher average weight in the CG. Concerning the water intake per gram of BW, results illustrate an apparent decrease at day 1 in CHG without statistic relevance, probably also due to chickens' prostration. A reasonable explanation for the significant decrease of this parameter at day 4 was not found. However, as in the following days CHG and CG presented the same water intake, this occurrence was not taken into significant account.

The absence of macroscopic lesions in the internal organs of the euthanized birds suggested that the phage lysate did not cause any visible internal injurious effect.

Many studies have already been carried out to evaluate endotoxin action in humans and other animals for several pharmaceutical purposes, like toxicity evaluation of antibiotic-induced endotoxin released in organism, water purification for dialysis, etc. [3, 8, 13-16, 20, 21]. The variation in sensitivity to endotoxin among species and the higher resistance of chickens to endotoxin effects as compared to mammals [4, 5, 11, 22], does not encourage the use of results from trials obtained with other animals to support the results obtained in this trial.

Summarizing, despite an initial prostration, no adverse effects were found in the chickens challenged by the phage crude lysate containing 8.21×10^4 EU/ml endotoxins, and thus, it was possible to conclude that phage crude lysate is not toxic for chickens.

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IV. THE INFLUENCE OF THE MODE OF ADMINISTRATION IN THE DISSEMINATION OF THREE COLIPHAGES IN CHICKENS

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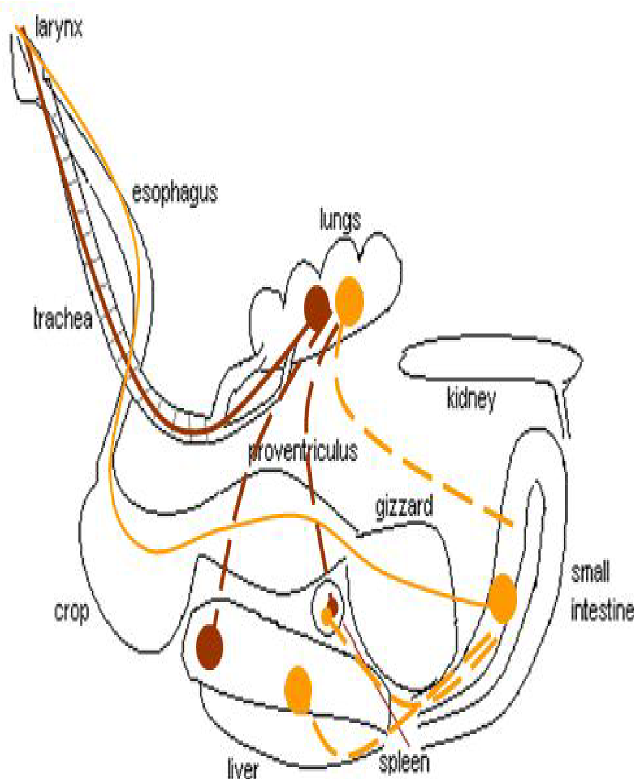

Escherichia coli (*E. coli*) can cause severe respiratory and systemic infections in chickens, and is often associated with significant economic losses in the poultry industry. Bacteriophages (phages) have been shown to be potential alternatives to the antibiotics in the treatment of bacterial infections. To accomplish that, phage particles must be able to reach and remain active in the infected organs. The present work aims at evaluating the effect of the route of administration and the dosage in the dissemination of three coliphages in the chicken's organs. *In vivo* trials were conducted by infecting chickens orally, by spray and intramuscularly with 10^6, 10^7 and 10^8 PFU/ml suspensions of three lytic phages: phi F78E (*Myoviridae*), phi F258E (*Syphoviridae*), and phi F61E (*Myoviridae*). Birds were euthanized 3, 10 and 24 h after challenge and the phage titre was measured in lungs and air sacs membranes, liver, duodenum and spleen. When administered by spray, the three phages reached the respiratory tract within 3 hours. Oral administration also allowed all phages to be recovered in lungs, but only phi F78E was recovered from the duodenum, the liver and the spleen. These differences can be explained by the possible replication of phi F78E in commensal *E. coli* strains present in the chickens gut, thus leading to a higher concentration of this phage in the intestines that resulted in systemic circulation of phage with consequent phage in organs. When phages were administrated intramuscularly, they were found in all the collected organs. Despite this better response, intramuscular administration is a non practicable way of protecting a large number of animals in a poultry unit. In general, the results suggest that oral administration and spray allowed phages to reach and to remain active in the respiratory tract and can, therefore, be considered promising administration routes to treat respiratory *E. coli* infections in the poultry industry.

Keywords: bacteriophage; *E. coli* respiratory infection; dissemination; chicken.

1. INTRODUCTION

The colibacillosis, caused by *Escherichia coli*, is a severe infection of farmed poultry leading to high morbidity and mortality [2]. The increasing incidence of antibiotic resistances in *E. coli* and the restriction of the use of antibiotics in animal production [20] emphasize the importance of the evaluation of alternative antimicrobial therapies.
Once bacteriophages (phages) are obligatory and exclusive bacterial parasites, they can act as antimicrobial agents, a fact that has encouraged researchers to test their potential as therapeutic agents. Phages are ubiquitous in nature and are known to inhabit animals and humans. Phages penetrate the blood stream and other tissues very freely upon their administration by different routes. The potential of phages as antibacterial agents lies on their ability to destroy bacterial cells at the end of an infectious cycle. The simultaneous releasing of the progeny leads to a concentration of phages in the places where bacterial infection occurs, retaining their full biological activity [10]. Moreover, phage therapy only needs to decrease the number of infecting bacteria to a level that allows the host defences to overcome the remaining infection [21].
However, phage therapy may fail if phages are unable to reach the target organs in the concentrations needed to trigger the infection cycle. Phages might be intolerant to the gastrointestinal (GI) tract conditions or inactivated by the immune system. Therefore, it is of utmost importance to understand the dynamics of phage dissemination in the target organism in order to predict the success of phage therapy. In this study, the dissemination of three different coliphages was assessed, taking into account the phage type, the administration route and the dosage.

2. MATERIALS AND METHODS

2.1 Bacteriophages Amplification

The phages used in this study were isolated from poultry sewage and screened against a pool of 148 avian pathogenic *E. coli* (APEC) strains. Phi F78E, phi F258E and phi F61E, were respectively lytic for 34.9%, 23.5% and 45.0% of APEC strains and the three phages associated were active against 70.5% of the strains. The morphological characterization of the phages revealed that phi F78E and phi F61E are 16-19 type phages, have capsids of 103 × 42 nm and contractile tails of 100 × 17 nm and both

belong to *Myoviridae*. Although morphologically similar, they were shown to be genetically different. The other phage, phi F258E, is a *Siphoviridae*, T1-like phage with a circular head of 62 nm diameter and a flexible tail of 160×8 nm (Chapter II). Phages replication was performed by inoculating 10 ml of each phage in 100 ml of the *E. coli* hosts H561E, H816E and H161E respectively for phages phi F78E, phi F61E and phi F258E and mid-log grown in Luria Bertani (LB) broth. This was followed by an overnight incubation at 37ºC with shaking (120 rpm). This suspension was then centrifuged at 9 000 × *g* for 10 min (rotor 19776, Sigma 3-16k), filtered through a 0.22 µm membrane and stored at 4 ºC.

The bacteriophage concentration was determined according to the plaque assay method described by Adams [1]. A volume of 100 µl of successive dilutions of the phage suspension was mixed with 100 µl of the respective bacteria host suspension (3-4 h culture) and 3 ml of LB 0.6 % melted agar. This suspension was poured onto a 1.5% LB agar plate and incubated at 37ºC, overnight. The suspensions volume was adjusted in order to obtain the desired phages concentration.

2.2 Bacteriophages viability under *in vitro* simulated chicken gastrointestinal tract conditions

The simulated conditions of the chicken Gastrointestinal (GI) tract were based on pH, enzyme activity and feed residence time on each gut compartment.

The phage buffer (NaCl, $MgSO_4$, Tris 1M, pH 7.5) was used as the control solution. This buffer was adjusted to different pH values by adding 1M of HCl, according to the pH defined for the respective segments of the chicken GI tract (adapted from Chang and Chen (2000)[7] and Gauthier (2002) [11]): crop and proventriculus (pH 4.5); gizzard (pH 2.5-3.5), small intestine (pH 5.8) and large intestine (pH 5.7). Each phage suspension, 1.0×10^8 PFU/ml, was added to the prepared buffers at 1:10 and incubated with slow shacking, at 42 ºC, anaerobically, for the following periods: 15 and 30 min for pH 4.5, (simulating respectively the crop and the proventriculus residence times), 90 min for pH 2.5 and 3.5, 90 min for pH 5.8, and 15 min for pH 5.7. The control solution was incubated for 90 min.

The susceptibility to the GI tract enzymes was performed by incubating the phage suspensions with enzymes, in the conditions previously described. For that, 3 210 U/ml

of pepsin (Sigma) [15] were used to simulate the proventriculus (pH 4.5) and the gizzard (pH 2.5) and 535 U/ml of trypsin (Sigma) to simulate the small intestine (pH 5.8). Following incubation, the phage concentrations were measured and compared by the plaque forming unit method, as described above.

2.3 Experimental design

Experiments were designed and conducted in accordance with the Federation of European Laboratory Animal Science Associations (FELASA) principles and the specific guidelines of animal welfare [31], based on the European Council Directive of 24 November 1986 (86/609/EEC) guidelines regarding the protection of animals used for scientific experimental purposes. According to those principles, the lowest number of animals necessary to reach the proposed goal in an *in vivo* experiment must be used. A total of 94 healthy 7-weeks-old growers (Rhode Island Red), obtained in a local poultry house, were housed in batteries and subjected to a 5-days acclimation period. The chickens were monitored for the presence of commensal enterobacteria sensitive to phi F258E, phi F61E and phi F78E. For that purpose, cloacae swabs were collected in triplicate, plated in MacConkey agar (selective and differential medium for gram-negative bacteria) and incubated at 37ºC. Five to eight pink colonies (micro-organisms that ferment lactose, as *E. coli*) were selected and picked from each plate, incubated separately in 10 µl of LB broth at 37ºC for 3 to 4 h, and spread in a lawn. Then, they were tested for phage sensitivity: 20 µl of phage were dropped on these bacteria lawns, and incubated at 37ºC overnight. Plates were then checked for clear zones.

Parallel trials were conducted to determine the efficacy of phage administration to chicken organism, concerning the route (oral, spray and intramuscular) and the dosage (1.0×10^6 PFU/ml, 1.0×10^7 PFU/ml and 1.0×10^8 PFU/ml). Feed and water was available *ad libitum*. Groups of 3 animals were challenged with 1 ml of the phage suspension at each of the indicated phage concentration, orally with a syringe, by spray directly to the beak or intramuscularly by injection in the chest muscle. One group, not challenged with the phages, was used as a control group. Birds were euthanized by isofloran
e inhalation (IsoFlo®, Abbott) [8], 3, 10 and 24 h after challenge. At necropsy, carcasses were dissected and different organs and tissues (lungs and air sacs membranes, liver, duodenum and spleen) were carefully excised, weighted and emulsified individually in LB broth at 1:10 (w/v). The supernatants were decanted, centrifuged at 9 000 × *g* for 10

min and filtered through 0.22 μm. The phage concentration was measured in each sample, as described above.

3. RESULTS

3.1 Bacteriophages susceptibility to *in vitro* GI tract conditions

Figure IV.1 presents the percentage of logarithmic reduction of each phage concentration when submitted to acidic and enzymatic conditions similar to those found in some segments of chicken GI tract, comparatively to the concentration at pH 7.5.
Among the three coliphages, phi F78E was the most affected to low pH values. In fact, the concentration of the other two phages only slightly declined when these phages were subjected to simulated gizzard conditions. The logarithmic concentration of phages phi F258E and phi F61E was reduced by 27.92 % and 26.38 % at pH 2.5 and by 1.94 % and 1.86 % at pH 3.5, respectively. Phi F78E lost all its activity (detection limit ≥ 1.0×10^{1} PFU/ml) at pH 2.5 and a logarithmic reduction of 4.48 % at pH 3.5. The pepsin added to the solution with pH 2.5 did not demonstrate any additional effect on the reduction of phages concentration (Figure IV.1B). Phi F78E was not significantly susceptible to the other simulated GI tract conditions, along with phages phi F258E and phi F61E (Figure IV.1A). The activity of pepsin at pH 4.5 and trypsin at pH 5.8 induced a phage log concentration reduction of 1.57 % and 2.65 % respectively (Figure IV.1B).

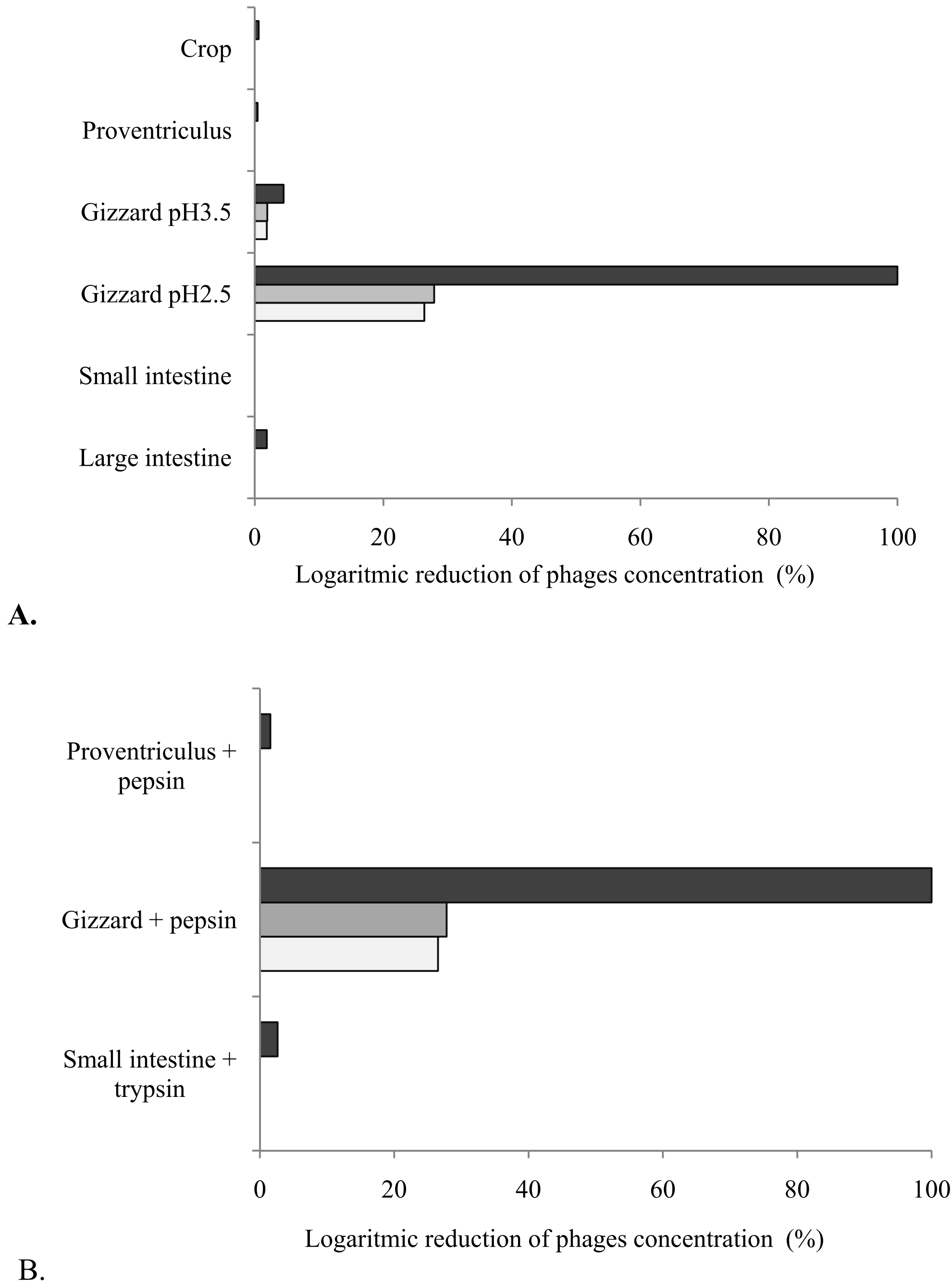

Figure IV.1 Logarithmic reduction (%) of phage concentration, after submission to simulated chicken GI tract pH conditions (A.) and pH + enzymatic conditions (B.), comparatively to pH 7.5 (■ phi F78E; ■ phi F258E; □ phi F61E)

3.2 Bacteriophages distribution in chicken organisms

Preliminary studies to detect host-susceptible strains to the three studied coliphages revealed the presence of a commensal *E. coli* strain susceptible to phi F78E.

The results of phages detection in the animal organs after oral and spray administration are presented in Tables IV.1 and IV.2, respectively. When administered by spray, all the three phages reached the respiratory organs. The oral administration also allowed phi F61E and phi F258E to reach the lungs and the air sacs membranes, being recovered from these organs at least at 10 h from challenge. The phage phi F78E remained in the same organs for the whole tested period when administered at 10^8 PFU/ml. Phi F78E was recovered from the duodenum at least 3 h after the oral administration of 10^7 and 10^8 PFU/ml suspensions; however, it was not possible to recover the other two phages (phi F61E and phi F258E) in this organ. Nevertheless, when spray administration was employed, all phages were found in the duodenum, and phi F78E titres were higher than the other phages (data not shown). Phi F78E could be isolated from the liver and spleen after oral and spray administration.

Table IV.1 Presence (+) or absence (−) of phages in organs and tissues after oral administration, according to the initial phage concentration and the time of slaughter (3, 10 and 24 h). A: lungs and air sacs; B: liver; C: duodenum; D: spleen.

	Phi F78E				Phi F258E				Phi F61E			
	A	B	C	D	A	B	C	D	A	B	C	D
Time (h)	3 10 24	3 10 24	3 10 24	3 10 24	3 10 24	3 10 24	3 10 24	3 10 24	3 10 24	3 10 24	3 10 24	3 10 24
PFU/ml 10^8	+ + +	— + —	+ — —	— — +	— + —	— — —	— — —	— — —	— — —	— — —	— — —	— — —
10^7	+ + —	+ — —	+ + —	+ — —	— + +	— — —	— — —	— — —	— + —	— — —	— — —	— — —
10^6	— — —	— — —	— — —	— — —	— — —	— — —	— — —	— — —	— — —	— — —	— — —	— — —

Table IV.2 Presence (+) or absence (-) of phages in organs and tissues after spray administration, according to the initial phage concentration and the time of slaughter (3, 10 and 24 h). A: lungs and air sacs; B: liver; C: duodenum; D: spleen.

	Phi F78E				Phi F258E				Phi F61E			
	A	B	C	D	A	B	C	D	A	B	C	D
Time (h)	3 10 24	3 10 24	3 10 24	3 10 24	3 10 24	3 10 24	3 10 24	3 10 24	3 10 24	3 10 24	3 10 24	3 10 24
PFU/ml 10^8	+ + +	— — —	+ + —	— — —	+ + —	— — —	+ — —	— — —	+ — —	— — —	+ — —	— — —
10^7	+ + —	+ — —	+ + —	— + —	+ + —	— — —	— — —	— — —	— — —	— — —	+ — —	— — —
10^6	+ + —	+ — —	+ — —	+ — —	— — —	— — —	— — —	— — —	— — —	— — —	+ — —	— — —

The presence of phages in organs following intramuscular injection is shown in Table IV.3. All the phages were recovered from the chicken lungs and air sacs membranes, the

liver and the spleen, at least 3 h after challenge (except for phi F61E, not found in the liver when administered at 10^6 PFU/ml). Phages remained in the spleen for all the experimental period. When injected at 10^8 PFU/ml, all the phages reached the intestine.

Table IV.3 Presence (+) or absence (-) of phages in organs and tissues after intramuscular administration, according to the initial phage concentration and the time of slaughter (3, 10 and 24 h). A: lungs and air sacs; B: liver; C: duodenum; D: spleen.

	Phi F78E				Phi F258E				Phi F61E			
	A	B	C	D	A	B	C	D	A	B	C	D
Time (h)	3 10 24	3 10 24	3 10 24	3 10 24	3 10 24	3 10 24	3 10 24	3 10 24	3 10 24	3 10 24	3 10 24	3 10 24
PFU/ml 10^8	+ + —	+ + +	+ + +	+ + +	+ — —	+ + —	+ + —	+ + +	+ — —	+ + —	— + —	+ + +
PFU/ml 10^7	+ + —	+ + +	— — —	+ + +	+ — —	+ — —	— — —	+ + +	+ — —	+ — —	+ — —	+ + +
PFU/ml 10^6	+ + +	+ + —	— + —	+ + +	+ — —	+ — —	— + —	+ + +	+ — —	— — —	— — —	+ + +

Figure IV.2 presents the concentrations of phi F78E, phi F258E and phi F61E recovered in the lungs and air sacs, the liver and the spleen, after intramuscular injection of 1.0×10^8 PFU. Data refers to phages enumeration at 3, 10 and 24 h of challenge.

In general, all the phages were rescued in the spleen, the liver and the lungs 3 h post administration, with the maximum concentration of phi F78E and phi F258E observed in the spleen and of phi F61E in the liver.

Concerning the phage titres measured in the chicken's lungs, it was observed that, for all administered concentrations and for all the phages, the higher phage titres were detected 3 h after phage administration. On the contrary, 10 h after challenge, no phage was detected in these organs.

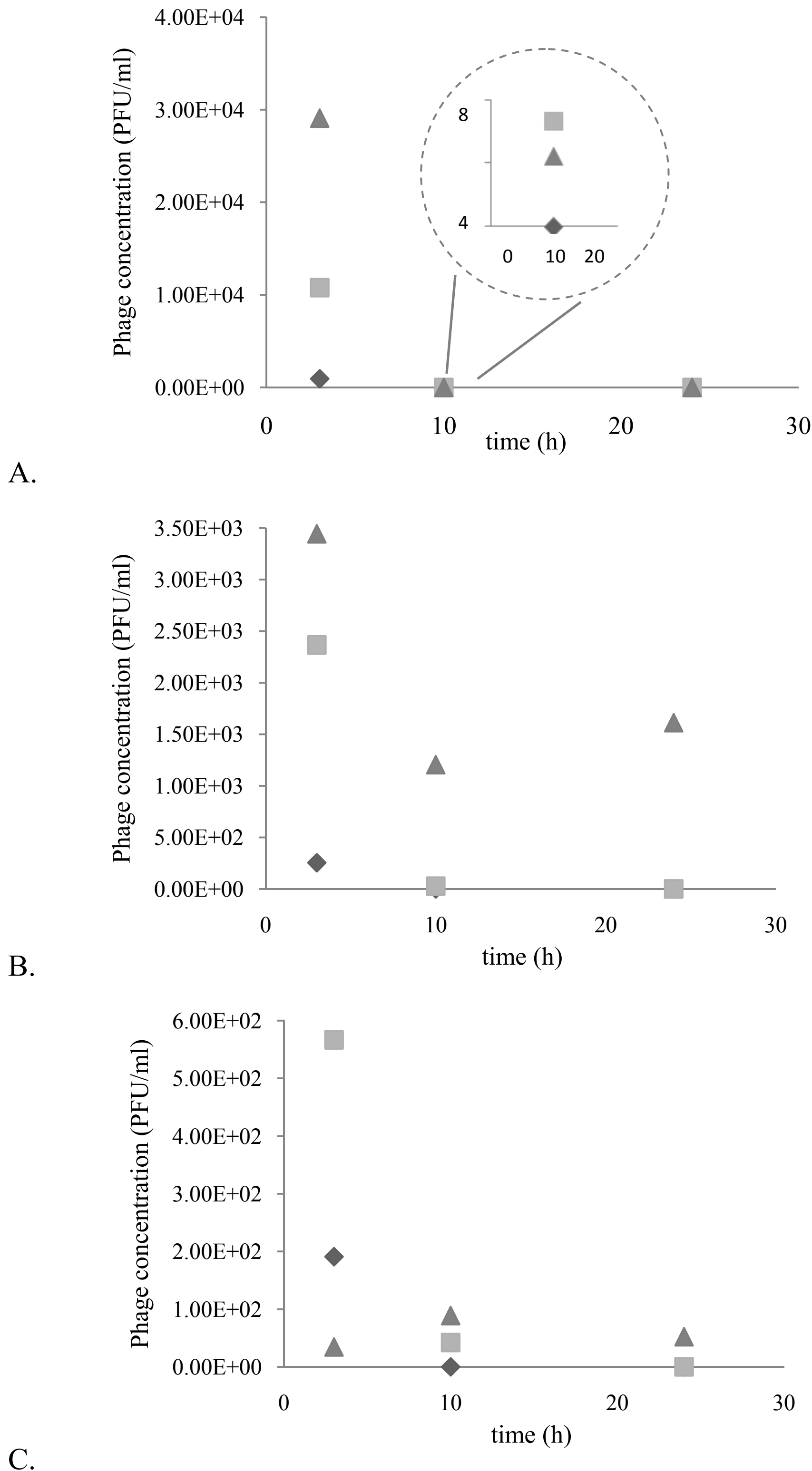

Figure IV.2 Concentration (PFU/ml) of phi F78E (A.), phi F258E (B.) and phi F61E (C.) found in lungs and air sacs, (◇) liver (□) and spleen (△) after 3, 10 and 24 h of the intramuscular administration of 1.0×10^8 PFU/ml. The inset in Figure IV.2 A illustrates, in an amplified scale, the values of phage concentration in organs at 10 h post-administration.

4. DISCUSSION

Extra-intestinal pathogenic *E. coli*, termed avian pathogenic *E. coli* (APEC) possess specific virulence attributes commonly causing respiratory and systemic infections in poultry (chickens and turkeys), namely colibacillosis [34, 2]. In a phage therapy perspective, phages must be able to reach the infected organs, a fact that might be dependent on the mode of administration, the administration titre and the phage itself. In fact, the overall results presented in this manuscript demonstrate that the phage type, the administration route and the dose delivered, were all factors contributing to variability of bacteriophage dissemination in tissues.

Phages have been administered orally, topically, by spray, directly into body tissues or systemically [3, 4, 16-20, 24, 26-29]. The method chosen for phage administration must guarantee the contact between phage particles and target pathogens. It is therefore important to ensure that, whatever route of administration, phage delivery to the infected organs will take place. In the particular case of avian respiratory infections caused by APEC, phages must be able to reach lungs and air sac membranes. On the other hand, from the practical point of view, some routes, like systemic ones, would be unfeasible, due to the large number of birds in a poultry unit. In this specific case, the most practical methods would be the oral inoculation in feed or water, or the aerosol (spray) delivery of phages. In fact, other management practices employ one or both of this routes for suspension delivery, like most of the vaccines application [6] or some antibiotic and probiotic administration [12]. Oral administration, however, could be considered an obstacle due to the potential phage inactivation during its passage through the acidic gut compartments [22].

Therefore, prior to the *in vivo* experiments, the survival of the three coliphages in the GI tract was assessed *in vitro* by submitting the phages to simulated gut conditions. The results revealed that, at the lowest pH that theoretically can be found in the gizzard and according to some authors in the proventriculus [11], a partial reduction in the concentration of the phages phi F258E and phi F61E occurred and a complete reduction of the concentration of F78E was observed. It would be therefore expected that the *in vivo* oral administration of this last phage would result on its inactivation. Nevertheless, the experiments herein reported revealed the presence of phi F78E in all the emulsified organs, after oral administration. This apparent absence of deleterious effects of the low pH on the phages, might be explained by the diluting effect of water/ feed intake (*ad*

libitum intake) on the digestive system that could raise the pH in gizzard and proventriculus [30]. Besides, it is important to emphasize that this phage was able to tolerate pH 3.5, a pH value more probably occurring during feed or water intake.

Another important aspect to consider is the fact that phi F78E was the phage recovered in higher amounts, compared to the other three phages, after oral or spray administration. This phage had a commensal *E. coli* host strain in the intestine, and might have replicated there. This could be the reason why this phage reached and remained in the studied organs at higher titres for longer periods, being the only one that apparently reached the blood stream. Based on this result, it can also be speculated that there might be an advantage of administering a non-pathogenic bacterial host together with the phage in order to ensure its amplification in the gut.

The ability of this phage to infect commensal *E. coli* strains can be advantageous in a phage therapy context because high internal titres of the phage are obtained. On the other hand by infecting commensal strains the phage might impair the flora equilibrium.

The presence of phages phi F258E and phi F61E in the respiratory tract after oral administration cannot be explained by their penetration into the blood stream through the intestinal mucosa after reaching the duodenum, since they were not rescued by liver or spleen, filters against foreign organisms that enters the bloodstream. Thus, phages might have reached chickens' lungs and air sacs probably due to the inhalation of aerosols or suspension droplets during the administration. Relative to phi F78E, aerosols might have been formed and breathed as well from the dust of the cages, where the concentration of this particular phage should be higher (due the presence of an intestinal host strain). Conversely to the other two phages, phi 78E was found in the liver and spleen when given orally to chickens, as well as in the duodenum, the segment of the small intestine with a higher absorption rate, indicating its absorption trough the intestinal mucosa. Some researchers [9, 13, 14, 32] reported that orally administered phages can reach the peripheral blood and migrate to the infection sites. The phage occurrence in the blood is also supported by several authors [9, 14, 23].

Spray administration allowed all phages to reach the respiratory tract. This may be a promising route of administration allowing phages to reside in the tissues and membranes where the pathogenic bacteria are located. Huff et al. (2003) [19] also reported the presence of phages in the respiratory tract after aerosol administration. The fact that with this route of administration phages reached the chicken duodenum is probably due to the spray swallowing. This route allowed phi F78E to circulate in the bloodstream,

reaching all organs. Three hours after challenging the chickens intramuscularly, it was possible to find the three phages in all organs including lungs and air sacs. This is an important indicator for therapy, once phages can rapidly reach the target organs of infection for pathogenic *E. coli*. However, these results indicate that although phages rapidly disseminated in the animal organs (at least 3 h after challenge) reaching the infected tissues, they were quickly cleared by the chicken organism. In fact, all the phages were cleared from lungs after 10 h. So, for practical purposes, it can be hypothesized that in this particular case, phage therapy of respiratory infections is only efficient immediately after phage administration and the fact that phages would not confer protection against *E. coli* after 10 h might compromise their use as prophylactic agents.

Whatever the route of administration, as expected, the phage dosage seemed also to be an important factor for phage therapy *in vivo* efficiency. Results suggest that the initial concentration of phages administrated intramuscularly, was directly proportional to the quantity of phages that reached the potentially affected organs (data not shown). A dose-dependence effect was reported by several authors in phage efficacy studies and modelling [5, 25, 33].

Summarizing, phage dissemination into the chickens' organs is highly dependent on the dosage and route of administration. The presence of commensal bacteria might also play an important role in phage spreading. Spray and oral phage administrations enables phages to reach the chickens respiratory tract and therefore can be consider important administration routes to control *E. coli* respiratory infections.

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V. *IN VIVO* PHAGE PERFORMANCE EVALUATION TO CONTROL SEVERE RESPIRATORY *E. COLI* INFECTIONS IN POULTRY

Infections caused by *Escherichia coli* (*E. coli*), namely colibacillosis, cause important losses in poultry industries due to high mortality rates and high carcasses rejection at slaughter. The increased resistance of *E. coli* strains to antibiotics, triggered the search of new therapeutic solutions to control those infections. Bacteriophages (phages) have been suggested as a valuable alternative to antibiotics in animal production. Studies intending the *in vivo* phage performance evaluation on treating severe respiratory *E. coli* infections in commercial birds are presented. Three coliphages exhibiting strictly lytic characteristics, phiF78E (*Myoviridae*), phi F258E (*Siphoviridae*) and phi F61E (*Myoviridae*), were individually administered to chickens, orally and by spray. The results revealed that the success of phage therapy was dosage dependant. In fact 10^7 PFU/ml was not enough to treat the infected chickens whereas a concentration of 10^9 PFU/ml allowed a decrease in 25 % and 43% in chickens' mortality and morbidity, respectively.

Keywords: bacteriophages; therapy; colibacillosis

1. INTRODUCTION

Escherichia coli (*E. coli*) is a commensal bacterium of chickens' intestine. However, some *E. coli* strains, extra-intestinal pathogenic *E. coli*, are by their own, able to cause disease. In fact, avian pathogenic *E. coli* (APEC) possess specific virulence attributes causing invasive infections in poultry [35]. The pathogenesis of APEC infections includes the colonization of the respiratory tract, the crossing of the epithelium and penetration into the mucosa of the respiratory organs, the survival and multiplication in the blood stream and internal organs, and the production of adverse effects and lesions on the chicken's cells and tissues [13]. For these reasons, colibacillosis is a serious problem for poultry production. High morbidity and mortality levels and lesions on chicken's tissues lead to carcass rejection at slaughter, causing important economic losses in avian industry [17].

Antibiotics are being used to prevent and treat this disease, but as a consequence of its overuse, multiple antibiotic resistances are emerging. This fact constitutes a great public health threat worldwide [25]. Moreover, few new active ingredients with novel chemotypes have entered the market over the past 30 years and new classes of agents are being developed [3]. Bacteriophages (phages) are bacterial viruses, obligate intracellular parasites of bacterial cells. The ability of lytic phages to invade and disrupt bacterial metabolism causing the bacteria lysis and its own new progeny release, makes from these viral particles good candidates to perform as therapeutic agents. Phages are highly host specific, preventing the destruction of the most part of the healthy flora in the intestine, and they replicate inside the pathogenic bacteria, growing exponentially at the site of infection, where it is needed. Phages are harmlessness for animals and plants, and therefore, also for the environment [10, 32].

In this work, *in vivo* trials were performed in order to determine the efficacy of three phages, phiF78E (*Myoviridae*), phi F258E (*Siphoviridae*) and phi F61E (*Myoviridae*) administered orally and by spray in treating chickens from severe colibacillosis.

2. MATERIALS AND METHODS

2.1 Isolation of APEC strains

Escherichia coli strains were isolated from poultry carcasses with colibacillosis, exhibiting typical *post mortem* lesions as perihepatitis, pericarditis, aerosacculitis and enteritis. Livers, spleen and lungs samples were collected from carcasses, emulsified in sterile saline solution (0.85% NaCl) and 0.1 ml of supernatant was plated in MacConkey agar, a selective medium for Gram-negative bacilli. Plates were incubated overnight at 37ºC. As *E. coli*, is a lactose fermenter, the specie confirmation of the isolates were conducted by selecting pink-red colonies from the referred media and using API E20 strips, according to manufacturer's instructions (Bio-Merieux). *E. coli* isolates were stored in Nutrient Broth (Oxoid,) with 20 % glycerol, at -80 ºC.

2.2 Bacteriophage isolation and amplification

The bacteriophages used in this study, phi F78E, phi F258E and F61E were isolated from samples of sewage from Portuguese poultry houses, as described in Chapter II., section 2.4. Briefly, the isolation assay comprised an overnight incubation (37ºC) of these samples with the isolated *E. coli* strains, 3-4 h culture in Luria Bertani (LB) broth, the supernatant centrifugation at 9 000 × *g* for 10 min and filtration through 0.22 µm, and the searching for clear zones after spotting the resultant suspension over the respective bacterial strain lawn. The phage replication was performed by inoculating 10 ml of 10^7 PFU/ml of each phage suspension in 100 ml of the respective host strain, 3-4 h culture in LB broth, followed by an overnight incubation at 37ºC with shaking (120 rpm). The resultant suspension was centrifuged at 9 000 × *g* for 10 min, filtered through a 0.22 µm membrane and stored at 4 ºC.

The phage concentrations were determined based on the plaque assay method described by Adams (1959) [1]. A volume of 100 µl of successive dilutions of the suspension of each phage, mixed with 100 µl of the host strain (3-4 h culture) and 3 ml of LB 0.6 % melted agar, was poured onto 1.5 % LB agar plates and incubated overnight at 37ºC.

2.3 Welfare, housing and handling

The *in vivo* tests were performed in healthy growers (Rhode Island Red), acquired from a local poultry house. Preliminary experiments entailed the pathogenicity evaluation of the isolated *E. coli* strains sensitive to phages phi F78E, phi F258E and phi F61E. Subsequent trials were conducted to determine the efficacy of these three phages on treating chickens with experimentally induced colibacillosis. Phages were administered orally and by spray, in a single dose (administration routes discussed in Chapter IV), immediately after chickens have been challenged with an avian pathogenic *E. coli* suspension.

All tests were designed and conducted in accordance with principles and specific guidelines of animal welfare of the Federation of European Laboratory Animal Science Associations (FELASA) [33], and based on the European Council Directive of 24 November 1986 (86/609/EEC) guidelines, regarding the protection of animals used for scientific experimental purposes. In all the designed experiments, there was a great concern to minimize the number of animals used in the experiments.

Chickens were housed in batteries, in two experimental rooms, with forced air exhaustion: the birds not exposed to phages were placed in a separated room (named phage-free room) from that of chickens subjected to phage treatment. Temperature and relative humidity were measured and controlled during the experiments, in order to ensure the optimal environmental conditions. A 5-days acclimation period preceded the challenging. Feed (commercial grower feed) and water were available for *ad libitum* consumption. The birds were weighted at the day of arrival and at the beginning of the experiment.

The efficacy of the treatments was evaluated based on mortality, morbidity and severity of the colibacillosis lesions [34]. Chickens that died during the challenging period, and the ones euthanized at the end of the trial through isoflurane (IsoFlo®, Abbott) inhalation [12] were submitted to the *post mortem* examination. The severity of the lesions was evaluated and scored as follows: 1- no macroscopic lesions or thickening and opacity of the inoculated air sac; 2- non severe lesions in the internal organs, not interfering in carcass quality; 3- severe and generalised colisepticemia injuries, as fibrinous aerosaculitis, pericarditis and perihepatitis; 4- death before euthanasia. The pathology score of each group was calculated: (∑ (number of birds with the same score × score)) / total number of birds.

Before the beginning of the trials, chickens were monitored for the presence of other phages active against the challenging *E. coli* strains. For that purpose, feces were collected from each battery, emulsified in LB broth, inoculated in a 3-4 h grown culture of the selected APEC strain, and incubated at 37ºC overnight with shaking (120 rpm). After centrifugation at 9 000 ×*g* for 10 min and filtration through 0.2 μm, the suspension was spotted on the bacteria lawns (LB agar), incubated at 37ºC overnight, and checked for clear zones. This procedure was repeated daily in the phage-free room. The screening of phage-sensitive commensal enterobacteria was also performed. Cloacae swabs were collected, seeded in several MacConkey agar plates and incubated at 37ºC. Eight to ten pink colonies were picked from each plate, separately incubated in 10 ml of LB broth at 37ºC for 3 to 4 h, and each one was spread in a lawn for phage sensitivity test: 10μl of phage were dropped on plate bacteria lawns, and incubated at 37ºC overnight. Plates were then checked for clear zones.

2.4 *In vivo* pathogenicity tests of phage-sensitive *E. coli* strains

i) Phi F61E-sensitive strain

The *in vivo* virulence of a phi F61E-sensitive strain, H161E, was evaluated. Six-weeks-old chickens with 332.9 g Body Weight (BW) in average were divided in two groups of four. One of the groups was challenged with *E. coli* (0.2 ml of a bacterial suspension of 2.3×10^8 CFU/ml) injected in the chickens' left air sacs and the other received LB broth as a placebo, by the same way.

The euthanasia and *post mortem* examination was carried out 5 days after inoculation.

ii) Phi F258E and phiF78E-sensitive strains

The same procedure described for the phi F61E-sensitive strain, H161E, was performed. However, in this case chickens were challenged with the *E. coli* strains using two different ways: i) injections of the bacterial suspensions in the chickens' left air sac; ii) inoculation directly into chickens' trachea, through a syringe fitted with an adapted blunt ended needle (intratracheal inoculation). A volume of 0.2 ml (5.0×10^8 CFU/ml) of each bacteria suspension was used in both ways.

In this test, 8 weeks-old chickens, weighting in average 732.5 g (BW) were housed in batteries, in 14 groups of four chickens each. Two of the groups received sterile LB

broth as a placebo intratracheally and intra sacs, respectively. The other groups (challenged groups) were inoculated with the six *E. coli* strains (H280E and H856E, both F258E-sensitive, and H757E, H924E, H839E and H1094E, phi F78E-sensitive) by each of the two routes of infection.
The euthanasia and *post mortem* examination was carried out 5 days after inoculation.

2.5 *In vivo* evaluation of phages efficiency to treat colibacillosis

i) PhiF61E

In this study, 25 chickens of 6 weeks-old, weighing 332.9 g (BW) in average, were divided in three groups: two groups of 11 birds were injected in the left air-sac with 0.2 ml, $1x10^8$ CFU/ml, of a H161E suspension; one of these groups received orally 1ml of $3.3x10^7$ PFU/ml of a phi F61E suspension and 1ml by spray. One group of 3 birds had sterile LB broth as placebo by intra-sacs injection. The euthanasia was performed 7 days after challenging.

ii) Phi F258E alone and in combination with antibiotic

Nine weeks-old chickens with 767.1g (BW) in average were used. The selected strain to challenge the birds, H280E was submitted to an antibiogram (performed as described in Chapter II) and the Amoxicillin (AML) was the selected as the active agent. Forty seven chickens were housed and divided in five groups: four groups of 11 chickens were challenged with H280E (0.2 ml of $7.5x10^8$ CFU/ml) by intratracheal inoculation, whereas one group of 3 chickens was used as the negative control, receiving sterile LB broth, also intratracheally. One of the infected groups was not treated whereas the other three groups were treated, respectively, with phages, with an antibiotic and with phages and antibiotic simultaneously. The antibiotic treatment was performed by diluting AML (0.2 g/l) in the drinking water (this is the usual antibiotic treatment procedure) during the whole experiment. The phage treatment was performed by administrating phi F258E ($5.7x10^7$ PFU/ml) orally and by spray (1 ml by each route). The treatment with phage and antibiotic was performed by giving AML (0.2 g/l) in the birds' drinking water, in the same described conditions, and phages orally and by spray.
The euthanasia was carried out 7 days after challenging.

iii) Phi F78E at different titres alone and in combination with antibiotics

(a) Low phage titre suspension

In this trial, the phi F78E efficacy to control APEC infections was also determined in association with an antibiotic. The challenging *E. coli* strain selected above, H839E was submitted to an antibiogram (performed as described in Chapter II) and the active agent used was the AML. A total of 40 growers of 12 weeks-old weighing 1055.1 g, were randomly divided in groups and placed in batteries. Four groups of 9 birds were challenged by injection with 0.2 ml of a 3-4 h grown culture of *E. coli* containing 5.0×10^8 CFU/ml, in the left air-sac. Immediately after being challenged, two of the four groups were treated with a suspension of 5.2×10^7 PFU/ml phi F78E, orally (1ml) and by spray (1ml). One of these two groups received as well, 0.2 g/l AML in the drinking water, during the whole experiment. The third challenged group was treated only with the antibiotic (same prescription). A group of 4 birds was set as the negative control, receiving sterile LB broth as a placebo, injected in the air sacs.

(b) High phage titre suspension

In the subsequent trial, the concentration of phage administered was 1.5×10^9 PFU/ml. A total of 28 chickens of 10 weeks-old, weighting in average 883.9 g (BW), were divided in three groups and placed in batteries. Two groups of 12 chickens were challenged, as previously described, with 0.2 ml of a 3-4 h grown culture of 5.0×10^8 CFU/ml H839E by intra-sacs injection. One of the groups was treated with 1 ml of phi F78E orally and 1 ml by spray. The negative control, comprised of 4 birds was treated as in (a). In both trials, the euthanasia and *post mortem* examinations were performed 7 days after being challenged with *E. coli*.

iv) Post mortem *screening for the presence of host resistant strains*

At the *post mortem* examination of each efficiency trial, infected livers were collected from the phage-treated groups. The organs were emulsified, separately, in LB broth. A volume of 0.1 ml of the supernatant was plated in MacConkey agar. Plates were incubated at 37ºC overnight. Pink-red colonies were picked, sowed in the same selective media and incubated at 37ºC overnight, being this procedure repeated three more times.

In each of the experiments, in order to test if the strain isolated from the carcasses remained sensitive to the respective infecting phage, about 10 pink-red colonies were

picked from the previously incubated MacConkey agar plates, inoculated separately in 0.6 % LB agar, and poured onto the wells of a 24-well microplate. This procedure was performed in duplicate and 5 µl of phage suspension were dispensed in each bacteria lawn. The original *E. coli* strain was used as positive control.

2.6 Statistical analysis

A two-sided Student´s *t*-test was used, with a significance level of 5% and a statistical power of 90% ($\alpha = 0.05$ and $\pi = 0.90$). The experimental unit was considered to be the chicken. The estimation of the number of experimental units needed for was based on Beyen et al. [7] statistical assumptions, in which the sample size was function of the difference considered meaningful between groups from a physiological point of view, expressed as multiples of the standard deviation that was estimated from an anticipated individual variation ($(\mu_1-\mu_2)/\sigma$).

The individual variation was estimated previously as 10% in average, and the meaningful difference between groups ($P>0.05$) was variable according to the trial.

In the *E. coli* pathogenicity tests, 30% of differences were considered meaningful between groups' results, and thus, 4 experimental units per group were used. In the first phage efficiency trial, to get 20% of differences between group's responses as meaningful, it was necessary to use at least 7 experimental units per group and in the second phage efficiency tests (in which the dose of treatment was increased), a minimum of 11 experimental units allowed to compare groups with 15 % of accepted biological difference.

3. RESULTS

3.1 *In vivo* pathogenicity tests of phage-sensitive *E. coli* strains

i) Phi F61E-sensitive strain

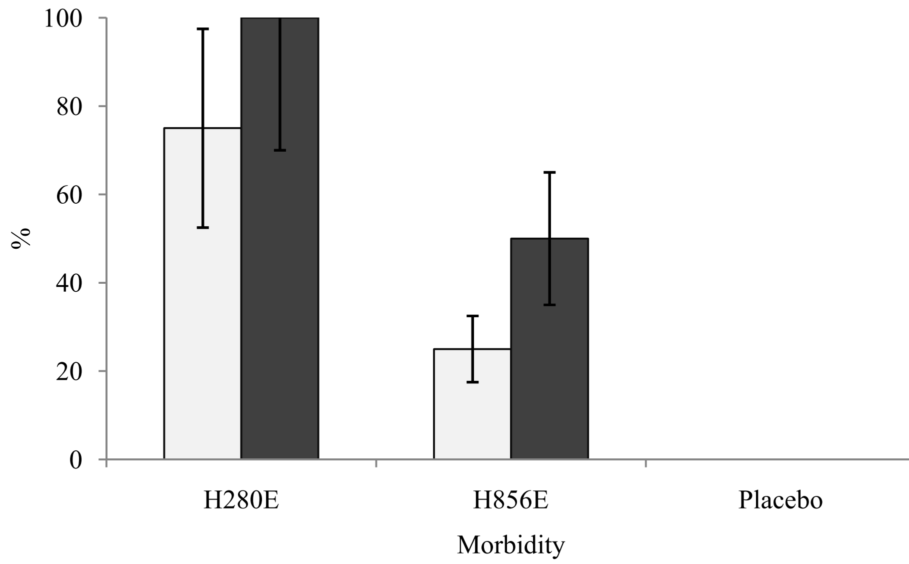
In vivo virulence tests of H161E injected in chickens' left air sacs resulted in no mortality recorded in the challenged group. The morbidity was 100% and the pathology score 2.3 (data not shown). This strain was used in the phage phi F61E efficiency trials.

ii) Phi F258E-sensitive strains

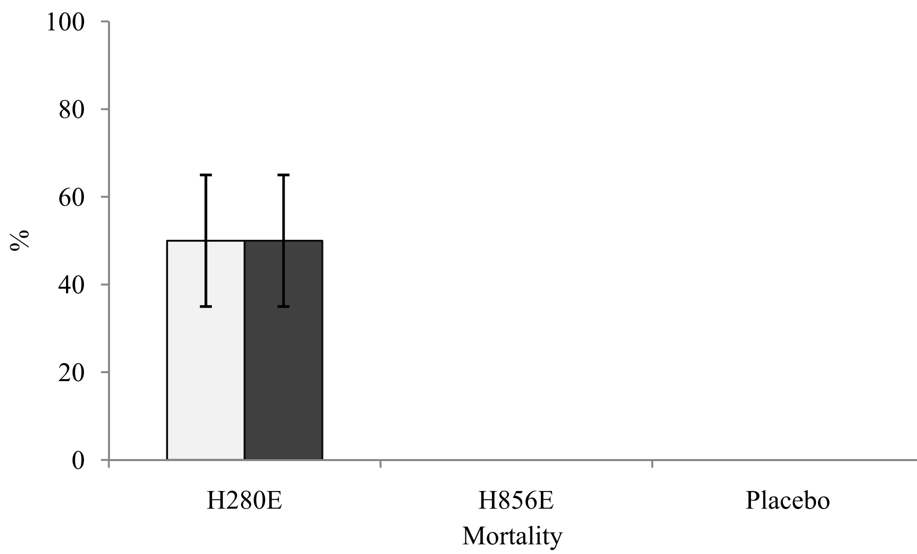
This *in vivo* experiment, allowed the selection of the most pathogenic phi F258E-sensitive *E. coli* strain and the challenge route able to induce colibacillosis in chickens. Results are illustrated in Figure V.1. In the group injected intra-sacs with H280E, all the chickens got sick, scoring 3.25. When chickens received the bacterial suspension in the trachea, neither the morbidity nor the pathology score were significantly lower than in the group injected in the air-sacs with the same suspension ($P<0.05$) (Figure V.1A and C). In both groups infected with H280E, the mortality was 50% (Figure V.1B). Chickens challenged with H856E by air-sac injection, presented a higher percentage of morbidity compared to chickens inoculated intratracheally. Nevertheless, the severity of lesions was not statistically different between birds infected by these two routes. Mortality caused by this strain was reported by none of the referred routes. Chickens infected with the placebo didn´t get any injuries.

According to these results, H280E seemed to be more virulent than H856E, so, this strain was selected to induce colibacillosis in the phage efficiency trials. Concerning the challenging route, after H280E intra-sacs injection birds got prostrated and two birds died in the inoculation day (data not shown), suggesting that the infection was rapidly spread and installed. Conversely, the intra-tracheal inoculation led to a less severe infection. In this case, bird prostration only occurred from the second day on, and the first bird died in the 3rd day. Based on these results, intra-tracheal challenging route was used to assess phage efficacy because it reproduces in a more realistic way the progression of the infections in aviaries. In fact, since colibacillosis doesn´t occur in all flock simultaneously or with the same level of severity (the morbidity varies and mortality ranges from 5 to 20% [22]), birds cohabit in different stages of infection, from earliest to systemic stages. Consequently, phage efficacy on therapy could vary. It might

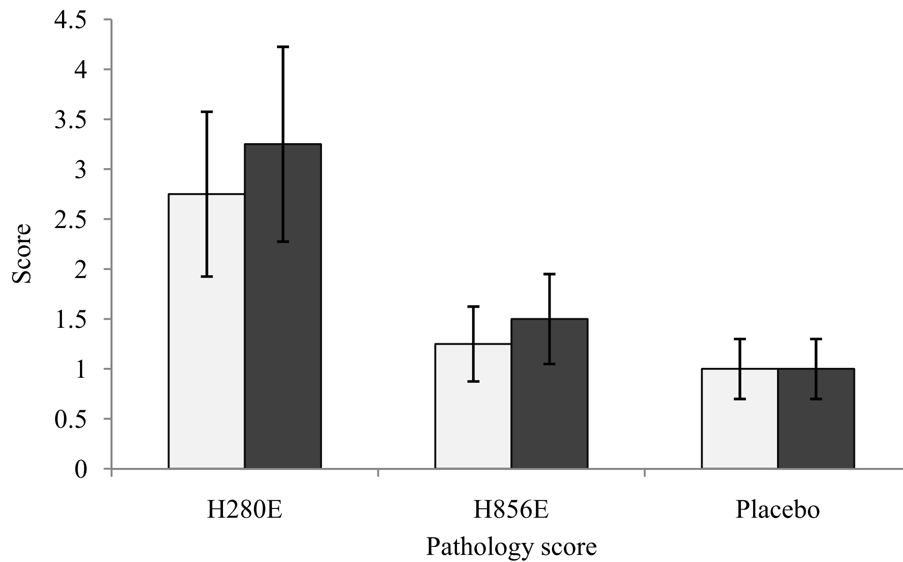
thus be important that in experimental conditions, birds develop the infection in diverse stages.



A.



B.

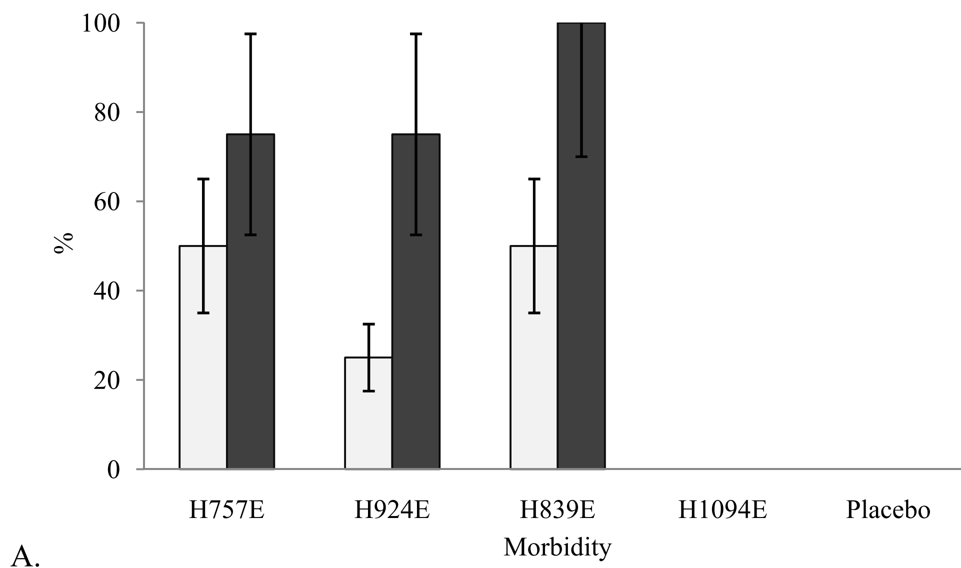


C.

Figure V.1 Morbidity (%) (A.), mortality (%) (B.) and pathology scores (C.) observed in each group of chickens (n=4) challenged with APEC strains, H280E and H856E, and with sterile LB broth (placebo), by intratracheal inoculation (□) or injected in the left air sac (■). Scores - 1: no injuries; 1 to 2: non severe lesions of colibacillosis; 2 to 3: generalised lesions of colibacillosis; 3 to 4: acute colisepticemia. Error bars indicate a meaningful difference of 30%.

iii) Phi F78E-sensitive strains

Injuries caused by the APEC strains, namely H757E, H839E, H924E and H1094E, in challenged chickens were registered and scored, after *post mortem* examination (Figure V.2).



A.

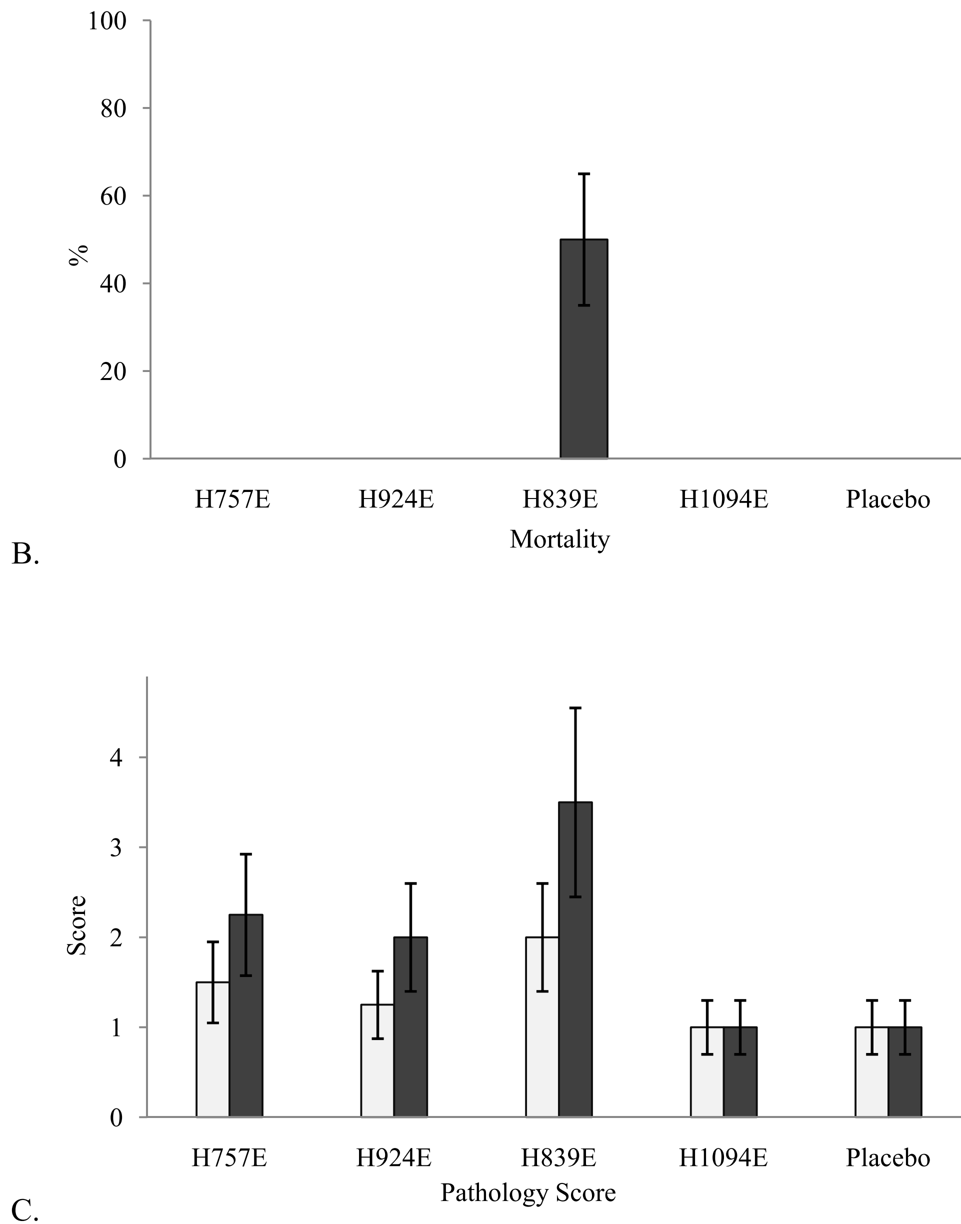


Figure V.2 Morbidity (%) (A.), mortality (%) (B.) and pathology scores (C.) observed in each group of chickens (n=4) challenged with APEC strains, H757E, H924E, H839E, H1094E, and sterile LB broth (placebo), by intratracheal inoculation (□) or injected in the left air sac (■). Scores - 1: no injuries; 1 to 2: non severe lesions of colibacillosis; 2 to 3: generalised lesions of colibacillosis; 3 to 4: acute colisepticemia. Error bars indicate a meaningful difference of 30%.

Regarding the morbidity and the lesions scores, no statistical difference (P>0.05) occurred among the groups of animals challenged by intra-sacs injection with H757E, H924E and H839E strains, being detected in most of the birds severe and generalised

lesions consistent with colibacillosis. Conversely, when the birds were challenged with H1094E, no lesions were detected in carcasses and the scores were similar to the negative control ($P<0.05$).
When inoculated in the trachea, the strains H757E and H839E caused higher morbidity in chickens than H924E and H1094E, being the lesions caused by H839E the most severe ($P<0.05$). Particularly, H924E and H839E originated more illness when administered in the air sacs than in the trachea (Figure V.2 B.). Concerning mortality, only the strain H839E injected in the air sacs caused chickens deaths (Figure V.2A.).
Taking into account these results, it seemed that the most pathogenic *E. coli* strains tested was the H839E, and the air sac injection the most effective way of causing a severe infection. These results were considered in the subsequent experiments.

3.2 *In vivo* evaluation of phages efficiency in treating colibacillosis

i) Phi F61E

The microbiological control of the birds at housing revealed no phage particles active against the inoculated host and no phi F61E-sensitive strains were found in the chickens' feces. The daily phage screening in the "phage-free" experimental room revealed a total absence of this phage during the trials.
Results from this experiment are presented in Figure V.3. The treatment with phi F61E slightly decreased the morbidity ($P>0.05$) from 100% to 72.7%. However, there were no significant differences in the lesions scores between the treated and the untreated groups, and mortality wasn't reported in none of them (data not shown). Birds from negative control did not exhibit any injuries. In these conditions, this phage was not considered to be efficient in treating the induced colibacillosis.

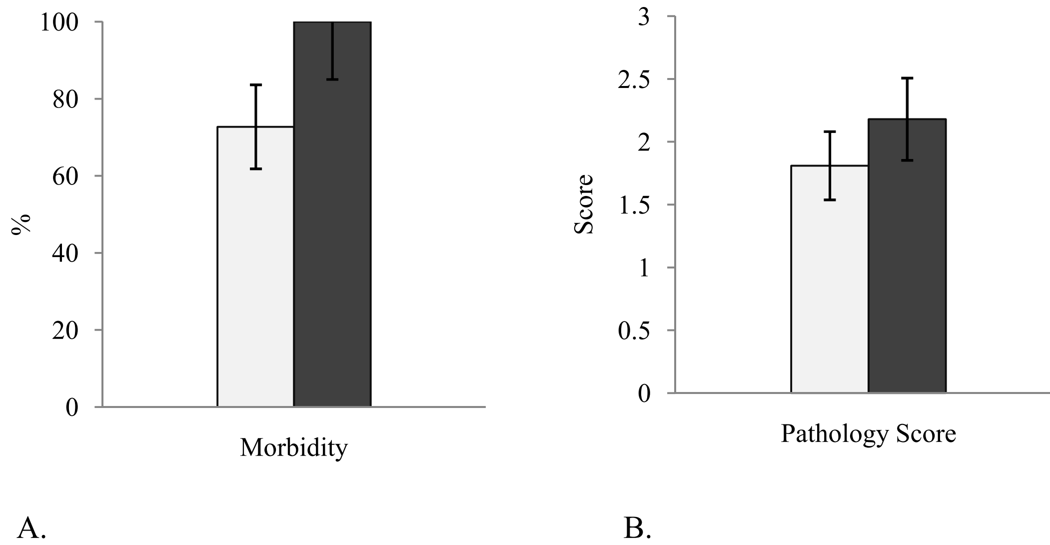
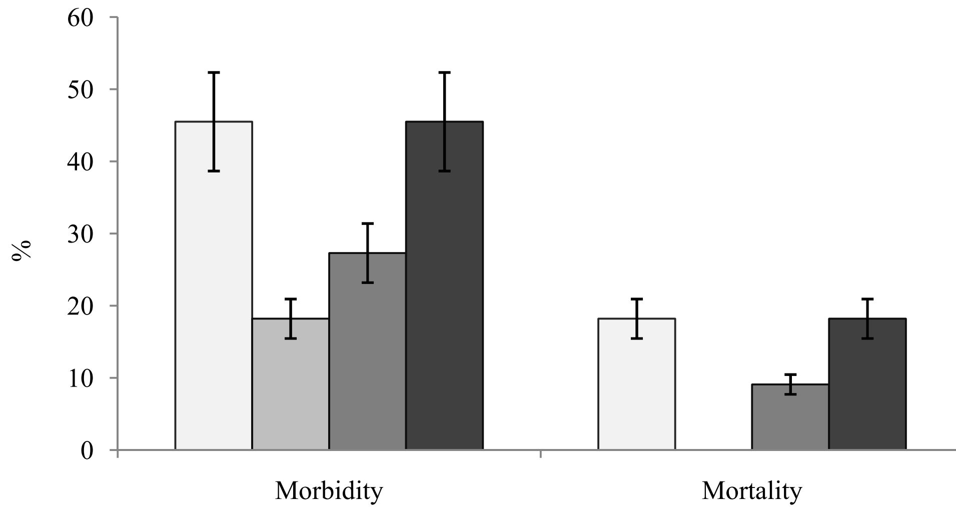


Figure V.3 Morbidity (%) (A.) and pathology scores (B.) obtained for each group of chickens (n=11). Groups: phi F61E+ H161E (□) - challenged with H161E and treated with phi F61E; H161E (■) - challenged with H161E. Scores - 1: no injuries; 1 to 2: non severe lesions of colibacillosis; 2 to 3: generalised lesions of colibacillosis; 3 to 4: acute colisepticemia. Error bars indicate a meaningful difference of 15%.

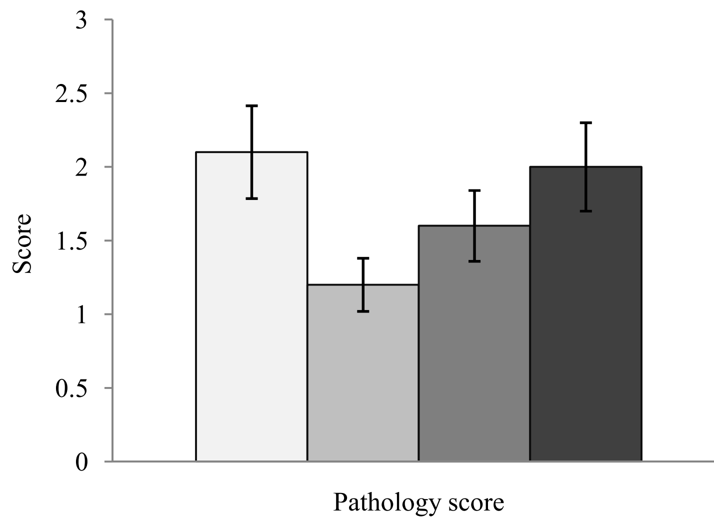
ii) Phi F258E

In this trial, the microbiological control of the birds at housing revealed absence of phage particles active against the inoculated host and no phi F258E-sensitive strains was found in the chickens' feces. The daily phage analysis in the "phage-free" experimental room was always negative.

Mortality and morbidity, as well as lesions scores recorded in each group are present in Figure V.4. (A and B).



A.



B.

Figure V.4 Morbidity and mortality (%) (A.) and pathology scores (B.) obtained for each group of chickens (n=11): Groups: phi F258E+H280E (□) - challenged with H839E and treated with phi F258E; AML + H280E (□) - challenged with H280E and treated with Amoxicillin; phi F258E+AML+H280E (□) - challenged with H280E and treated with phi F258E and Amoxicillin; H280E (■) - challenged with H280E. Scores: 1- no injuries; 1 to 2- non severe lesions of colibacillosis; 2 to 3- generalised lesions of colibacillosis; 3 to 4: acute colisepticemia. Error bars indicate a meaningful difference of 15%.

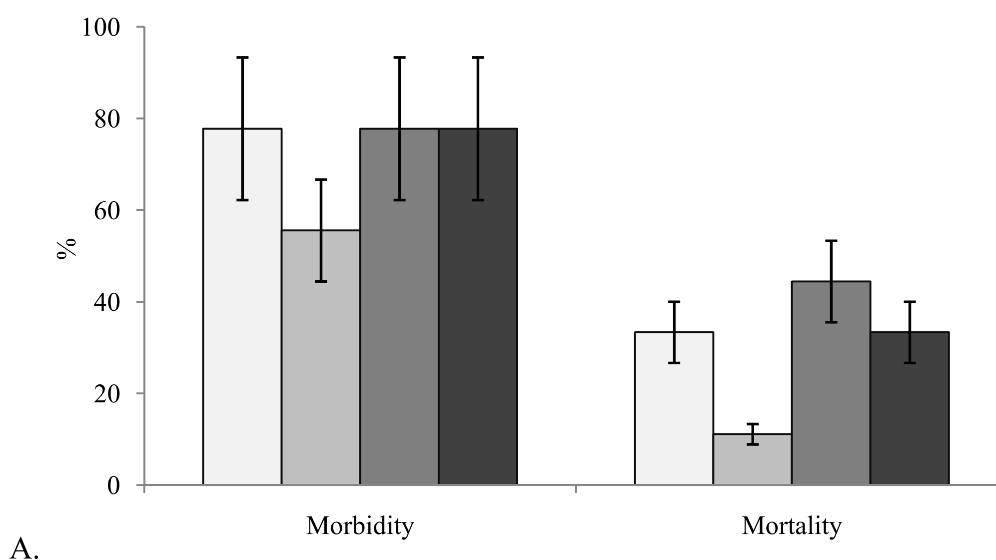
In this experiment, no significant differences (*P*>0.05) in mortality, morbidity or pathology scores were obtained between the untreated group and the group treated with phi F258E. In the groups treated with AML and with the association of the antibiotic and the phage, the mortality and morbidity were significantly lower than in the other groups (*P*<0.05). In the former group these parameters were still lower than in the latter. Carcasses from the group treated only with the antibiotic showed the less severe lesions. Thus, from these results it can be inferred that phi F258E was not effective in controlling the infection with H280E.

iii) Phi F78E

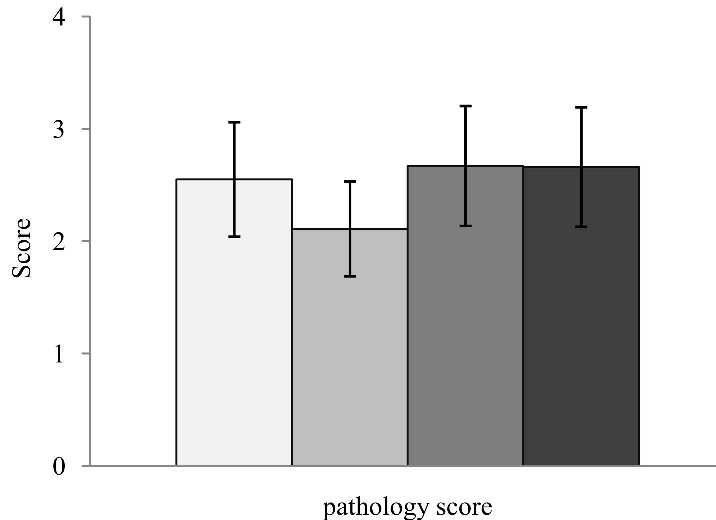
(a) Low phage titre suspention

The microbiological control of the birds at housing revealed no phage particles active against the inoculated host and no phi F78E-sensitive to *E. coli* strains present in the chickens' feces. The daily phage presence control in the "phage-free" experimental room revealed a total absence of this phage during trials.

This *in vivo* efficiency experiment was performed by infecting chickens with 5×10^6 CFU/ml H839E and immediately after, by administering 5.2×10^7 PFU/ml phi F78E orally and by spray, with and without the simultaneous administration of AML in the drinking water . The pathology scores and the morbidity and mortality recorded in this experiment, for the four challenged groups, are present in Figure V.5. Chickens from the negative control did not develop any lesion detectable at the *post mortem* examination.



A.



B.

Figure V.5. Morbidity and mortality (%) (A.) and pathology scores (B.) obtained for each group of chickens (n=9). Groups: phi F78E+H839E (□) - challenged with H839E and treated with phi F78E; AML+H839E (▢) - challenged with H839E and treated with Amoxicillin; phi F78E+AML+H839E (■) - challenged with H839E and treated with phi F78E and Amoxicillin; H839E (■) - challenged with H839E. Scores: 1- no injuries; 1 to 2- non severe lesions of colibacillosis; 2 to 3- generalised lesions of colibacillosis; 3 to 4: acute colisepticemia. Error bars indicate a meaningful difference of 20%.

No meaningful differences ($P<0.05$) were observed between groups, relatively to the scores of birds lesions and the morbidity (Figure V.5A and V.5B). The mortality percentage did not differ between the groups phi F78E+H839E, phi F78E+H839E+AML and H839E ($P>0.05$), being however in these cases statistically higher ($P<0.05$) than in the group AML+H839E (Figure V.5 B). In all groups, mortality occurred in the inoculation day.

For these trial settings, neither the phage preparation nor the antibiotic (acting individually or in association) was efficient in treating chickens from the infections caused by *E. coli*.

(b) High phage titre suspension

The results from the microbiological control at housing and the daily phage screening in the “phage-free” room were identical to those previously described.

In this *in vivo* trial, in which the concentration of phage administered was 1.5×10^9 PFU/ml, the pathology score, the morbidity and the mortality were significantly lower ($P<0.05$) in the group phage-treated than in the untreated group (H839E) (Figure V.6A). Lesions found in carcasses were also less severe in the phage-treated group (Figure V.6B).

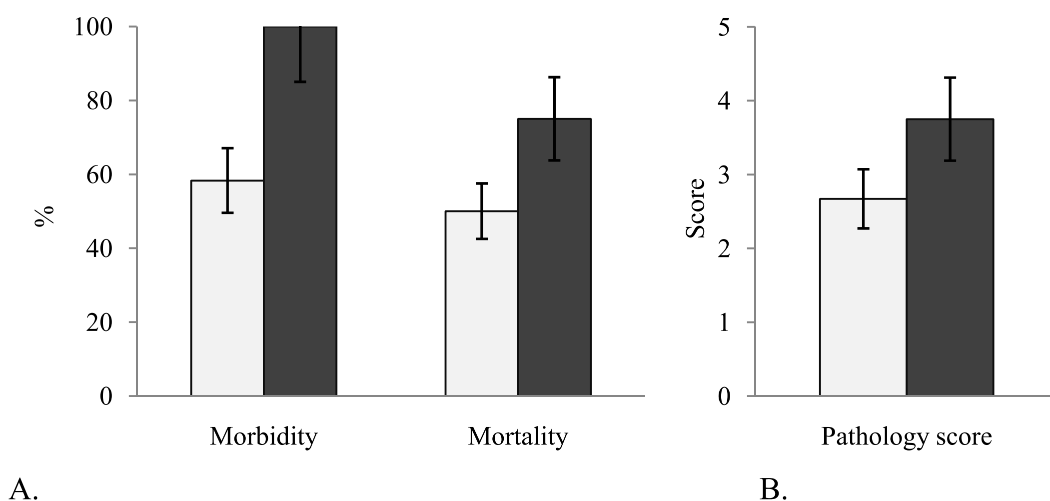


Figure V.6 Morbidity and mortality (%) (A.) and pathology scores (B.) obtained for each group of chickens (n=12). Groups: phi F78E+H839E (☐) - challenged with H839E and treated with phi F78E; H839E (■) - challenged with H839E. Scores: 1- no injuries; 1 to 2- non severe lesions of colibacillosis; 2 to 3- generalised lesions of colibacillosis; 3 to 4: acute colisepticemia. Error bars indicate a meaningful difference of 15%.

With this highly concentrated phage suspension, a decrease, in average, of 25.0% on chickens' mortality and of 41.7% on morbidity was obtained.
In the negative control group, no chicken died and no lesions were detected in the *post mortem* analysis.

iv) Post mortem *screening for the presence of host resistant strains*

In all the phage-treated groups in study, the *E. co*li isolated from chickens receiving the phage preparations remained sensitive to the phages even after being in contact with it in the organism.

4. DISCUSSION

It has been suggested that phages might be able to reduce the densities and of dissemination of the infecting populations of bacteria to levels at which they are possible to be controlled by the host immune system [21]. Furthermore, by replicating in the infected areas, phages shall be able to control localized infections that are relatively inaccessible via the circulatory system as, for example, the air sacs in chickens. Theoretically, if a bacteriophage reaches the site of a bacterial infection, it should be effective in eliminating the infection [20 10, 11, 27, 32]. These attributes make phages powerful antimicrobials alternatives. This idea is shared by several phage researchers who postulate that these viral particles, as antibiotics, are effective in treating bacterial diseases. Indeed, successful trials are being reported, with phages conferring high protection levels against infections [4, 6, 8, 15-19, 24, 28-31].

In this work, the *in vivo* efficiency of phi F68E, phi F78E and phi F258E in treating chickens with colibacillosis was evaluated. As previously described (Chapter II) a cocktail of these phages are able to cover about 70.5% of the most common APEC strains causing colibacillosis in Portuguese poultry farms. Phages were tested independently in order to evaluate its individual performance.

Prior to the *in vivo* phage efficacy trials, preliminary experiments were conducted in order to select the strain or strains and the challenging routes able to cause chickens colibacillosis. For this purpose, chickens were submitted to intra-sacs or intratracheal injections of APEC strains sensitive to the phages, and mortality, morbidity and severity of lesions were recorded. Typical signs of colibacillosis are caracterised by multiple organ lesions, typically pericarditis, aerosacculitis, perihepatitis and septicemia [2, 14].

Apparently all strains except one, H1094E, caused typical symptoms of collibacilosis. However some of them did not cause any mortality. The exception was H280E and H839E. Chickens infected with H161E, H280E and H839E exhibited clear signs of colibacillosis, so, these strains were selected to be used in the phi F61E, phi F258E and phi F78E efficiency experiments, respectively. Two inoculation routes were tested, and for the most virulent strains, intra-sacs injections induced a rapid and severe disease. Conversely, intratracheal inoculation seemed to promote a more gradual evolution of the infection which is more close to the real conditions.

It was curious to notice that, despite all the *in vivo* tested *E. coli* strains are isolates of chickens suffering from colisepticemia, they promoted different levels of pathological

signs. Those variations might happen, since the infections occurring in the natural environment might be often aggravated by extrinsic and intrinsic conditions affecting birds, as the exposure to other infectious agents, the levels and duration of exposure to bacteria, the route of infection, the vulnerability of the management conditions, among others [26]. Those are important variables affecting birds' susceptibility in Nature that are not possible to mimic in controlled experimental rooms.

In the *in vivo* phage efficacy trials, phi F61E was able to reduce the morbidity, in average, 27.3%. However the severity of the lesions in carcasses observed on the *post mortem* analysis were not significantly different from the control group. This fact might indicate that some of the birds that effectively got sick in the phage-treated group had more severe pathological signs in organs than in the untreated group. So, it might be speculated that, at in this experimental conditions phages were only able to treat the chickens that were in an early stage of the infection.

Phage phi F258E was ineffective in controlling the induced *E. coli* infection. Conversely, the antibiotic significantly decreased the morbidity and mortality, as well as the pathology scores. It must be noticed that, while the antibiotic was being continuously administered for the whole experiment, the phage was given as a single dose, only at the beginning.

Respecting to the phi F78E efficiency performance, when it was administered at 10^7 PFU/ml, this phage was not able to control the infection. No meaningful differences were noticed between morbidity and pathology scores in the untreated and in the phage-treated groups. In this case, the antibiotic was also ineffective in controlling the disease. These results might be explained by the severity and rapid progression of the infection (in groups challenged with H839E, mortality occurred in the day of the inoculation) and on the other hand, with an administered phage concentration which was probably too low to control such a severe infection.

It is important to reiterate that, unlike experimental conditions, in natural-occurring infections the bacteria transmission happens gradually and horizontally from one chicken to another, and birds are not synchronized at the same stage of infection [19]. Therefore, despite these unsuccessful results, it might be speculated that the tested phage titre could be efficient in treating natural colibacillosis, by controlling the earlier stages of the infection and avoid the progressive transmission to the flock.

The importance of the phage administered concentrations was demonstrated in the *in vivo* phage performance evaluation. Experiments have been shown that the effectiveness

of therapy with phages highly depends whether the phage titre administered provides or not the sufficient number of particles to the site of the infection [15, 27, 17, 20]. Moreover, it is reported that in a systemic infection, the treatment efficacy would be improved by ensuring that sufficiently large numbers of phage are available in the blood stream. In a previous study (Chapter IV), we have demonstrated that phi F78E at 10^7 PFU is able to reach the lungs and air sacs when administered orally and by spray, so, reinforcing what was said above, the amount of phage was not probably enough to control such a severe infection.

Indeed, a phage concentration of 5.2×10^7 PFU/ml was not efficient to control the infection, but higher concentration of phage (1.5×10^9 PFU/ml) was able to decrease 25.0% the mortality and 41.7% the morbidity of the treated birds.

Encouraging results were also obtained in other works describing phage administration by an aerosol spray, in which significant although not complete protection to chickens form severe colibacillosis was obtained [15, 17, 19]. Huff et al. (2005) reported that, once the infection become systemic, the spray doesn´t seem to be very effective on treating the disease [19]. The protecting capacity of phages after oral administration has been documented in other species [5, 9, 23, 24].

It must be stressed that some differences on mortality and morbidity (caused by the same strain) between control groups from different experiments were noticed. This might be due to differences on chicken's age, between experiments. Nevertheless, for each trial, conditions were uniform, and chickens under study were always from the same batch. Thus comparisons between groups in the same trial are considered to be trustfully.

Overall, the results of the phage *in vivo* performance, demonstrated that the efficacy of phage treatment might be dosage dependant. The failure of some of the phage treatments reported is probably due to the fact that, phages administered orally and by spray in a low dosage, were not able to control systemic infections. In fact, colibacillosis was artificially induced by inoculating high amounts of APEC strains in the bird's respiratory tract. When phages were administered, the animals were suffering already from a severe *E. coli* infection and only a high phage dosage was able to control the infection.

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VI. THE EFFICIENCY OF A PHAGE COCKTAIL IN CONTROLLING COLIBACILLOSIS IN EXPERIMENTAL POULTRY HOUSES

Phage therapy experiments have been demonstrating the elevated potential of phage in controlling bacterial infections. However, *in vivo* trials confined in experimental rooms do not mimic faithfully an infection occurring in large scale, as in an intensive rearing animal production. Therefore, the aim of this study was to evaluate the performance of a phage cocktail in controlling colisepticemia, scaling up the *in vivo* experiments in confined rooms to pilot trials in experimental poultry houses.

Naturally infected chicken flocks with *E. coli*, refractive to antibiotherapy received a phage cocktail, composed by 5.0×10^7 PFU/ml of three different bacteriophages (phi F61E, phi F78E and phi F258E), in the drinking water and by spray, in a single application.

In most part of the flocks, the mortality rate felt below 0.5% in no more than three weeks, with no recidivism.

The results obtained showed a remarkable efficacy of this phage cocktail in controlling *E. coli* infections, in large scale poultry production.

Keywords: Bacteriophage therapy; *E. coli*; large scale; poultry.

1. INTRODUCTION

Escherichia coli are part of the common microbial flora of the poultry intestine. However, despite most of the isolates are harmless, about 10 to 15 % of the serotypes are pathogenic [1], the avian pathogenic *E. coli* (APEC), causing systemic disease in poultry (avian colibacillosis) [4]. This infection is responsible for important economic losses in the poultry industry worldwide, due to lowered production, high treatment costs, carcass rejection at processing and mortality [16, 27]. Losses occur at all ages. Depending on the virulence grade of the strain, on host susceptibility or influence of external predisposing factors, the infection manifests as an initial septicemia, followed by either rapid death or by a diverse display of lesions as perihepatitis, aerosacculitis and pericarditis, among others [1]. Morbidity varies, in average, between 5 to 20% in intensive raised flocks [13]. The antibiotic therapy is being used to control colibacillosis, however a significant increase in drug-resistant strains of *E. coli* has also become a problem in the poultry industry [17]. In fact, the use of antibiotics is considered the most important factor promoting the emergence, selection and dissemination of antibiotic-resistant microorganisms [26], limiting its own therapeutic effectiveness.

The efficacy of bacteriophage therapy has been reported by numerous authors. *In vivo* confined experimental trials have been performed to establish the proof of principle of bacteriophage therapy to treat different animals against different types of bacteria [6-11, 14, 15, 19-24]. However, *in vivo* confined trials do not reproduce all real condition that influences the outcome of any antimicrobial therapy.

In the present manuscript the efficacy of phage therapy in treating broiler chicken flocks from colibacillosis is reported. A phage cocktail was administered to flocks naturally infected with *E. coli* and refractive to antibiotherapy.

2. MATERIALS AND METHODS

2.1 Therapeutic phage cocktail composition

Bacteriophages were isolated from samples of poultry sewage, collected randomly from Portuguese poultry houses (Chapter II). Three genetically different virulent phages, phi F78E, phi F258E and phi F61E, active against 70.5% of APEC strains, were selected to

compose a therapeutic cocktail. Taxonomically, phi F78E and phi F61E seemed to be 16-19 type phages, belonging to *Myoviridae* family and phi F256E, a T1-like, *Syphoviridae* phage.

The phage cocktail used in this study was composed by 5.0×10^7 PFU/ml of each phage, in LB broth 20% NaCl. The phage production was performed by inoculating 50 ml of 10^7 PFU/ml of each phage suspension in 500 ml of the respective host strain (3-4 h culture in LB broth) followed by an overnight incubation at 37ºC with shaking (120 rpm). The resultant suspension was centrifuged at 9 000 × *g* for 10 min, filtered through a 0.22 µm membrane and stored at 4 ºC. In order to determine the phage concentration, a volume of 100 µl of successive dilutions of the suspension of each phage, mixed with 100 µl of the host strain (3-4 h culture) and 3 ml of LB 0.6 % melted agar, was poured onto 1.5 % LB agar plates and incubated overnight at 37ºC. Distinct phage plaques detectable in the higher dilutions indicated the phage concentration.

2.2 Large scale experiments

E. coli infected flocks with high mortality rates even after antibiotic treatment, were the experimental units of these experiments (n=11). Dead chickens from each of these flocks was submitted to *post mortem* analysis, and after confirmation that death occurred due to colisepticemia, through lesions macroscopic evaluation (perihepatitis, pericarditis, aerosacculitis, enteritis...), samples of infected organs - livers, spleen and lungs - were collected from carcasses. Those samples were emulsified (1:10 (v/v)) in sterile saline solution (0.85% NaCl) and 0.1 ml of supernatant was plated in MacConkey agar, a selective medium for Gram-negative bacteria. Plates were incubated overnight at 37ºC and, pink-red colonies (indicative of *E. coli* presence) were selected. API E20 strips (Bio-Merieux) were used to specie confirmation of the isolates.

For each isolated *E. coli*, *in vitro* phage lytic tests were performed, by spotting 10 µl of the cocktail suspension over the respective bacterial lawn in LB agar. Plates were incubated overnight at 37ºC. Clear zones indicated phages *in vitro* efficacy to lyse the bacteria causing the infection, in the respective flock. In these cases, the phage mixture was administered to all flock, as a single application. A volume of 500 ml for 10 000 birds was prescribed: half of the dose was diluted in the drinking water to be consumed in half day, and the other 250 ml were administered by fine drop spray , by adding a

volume of 500 ml of water for each 1000 birds. The water used in this trial was free of disinfectants or other phage inhibitors. Mortality was recorded at the beginning of the trial, and for three weeks on.

2.3 Statistical analysis

A two-sided Student´s *t*-test was used to compare groups, with a significance level of 5% and a statistical power of 90% (α = 0.05 and π = 0.90). The estimated number of experimental units needed to the trials (the flocks) were obtained based on Beyen et al. [2] statistical assumptions. The estimated coefficient of variation between flocks was 10% and the difference considered meaningful between groups was 15%.

3. RESULTS

E. coli strains isolated form 11 flocks of broiler chickens (Rhode Island Red) with 7 weeks-old in average, were shown to be *in vitro* sensitive to the prepared phage cocktail. Flocks had between 5 000 and 10 000 birds. The mortality before and after the phage cocktail administration was registered and is presented in Figure VI.1. The infection was considered to be controlled once the mortality was 0.5% or less (usual in healthy flocks), and in most cases, reaching this condition, no further data was collected.

One week after the cocktail administration, the mortality was controlled in five flocks (≤0.5%). In the following week, one more flock achieved the regular levels of mortality, and at the third week, all the flocks except one, were controlled for colibacillosis. The exception was relative to one case (indicated with an arrow in Figure VI.1), in which the mortality decreased consistently since phage administration, from 1.52% to 0.68%. No recidivism in the mortality rate was observed in any flock until slaughter.

One case study of a broiler's flock (Cobb) was also studied under the described conditions, and results showed that, besides a mortality decrease during the experiment (from 0.6 to 0.08 %), the rejections at slaughter were reduced as well with the phage administration, from 4.8 % to 1.82 % (data not shown).

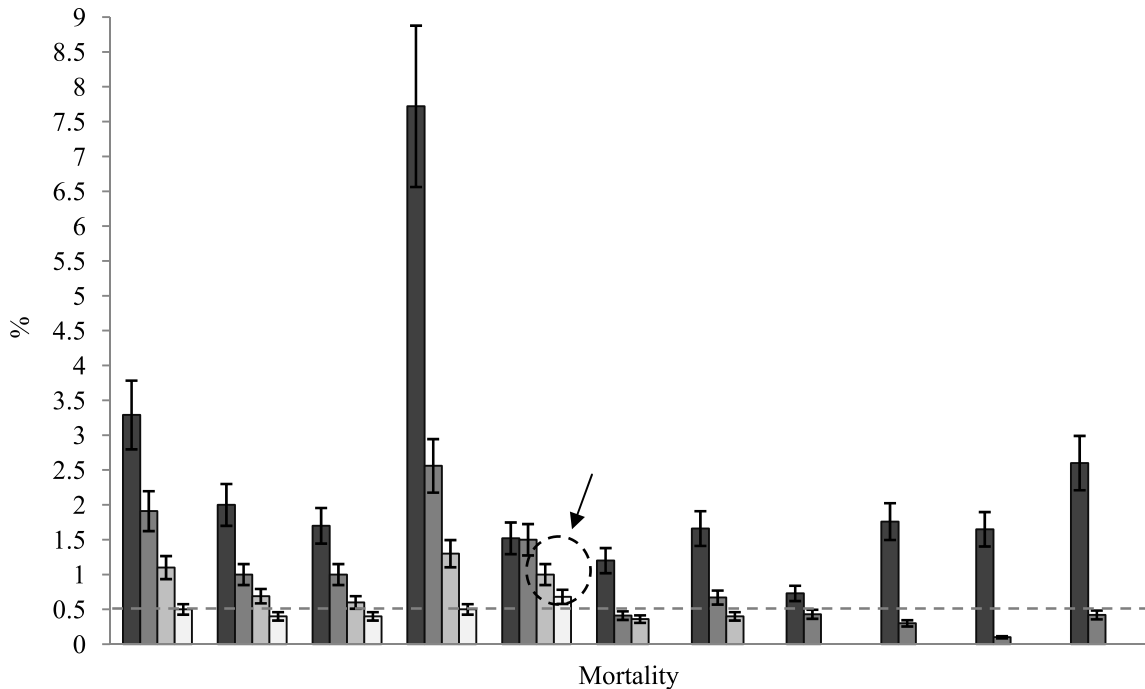

Figure VI.1 Mortality rate (%) measured in 11 *E. coli* naturally infected flocks, previously treated with antibiotics. Records were taken at the phage administration and repeated weekly, for 3 weeks: week before phage administration (■); 1st week (■); 2nd week (■); 3rd week (□). Error bars indicate a meaningful difference of 15 %.

4. DISCUSSION

A three phage cocktail was used in this study as a therapeutic product to control colibacillosis in poultry. Many authors recognize benefits on having different phages on the same product, enlarging the lytic spectra and delaying the resistances occurrences to phages [3, 5, 10, 18, 21, 25].

In this work, a low titre phage product was administered, in a single application, orally and by spray to flocks naturally infected with pathogenic *E. coli*, and results showed a mortality reduction in no more than three weeks. The gradual decrease revealed that the number of chickens that reached acute septicaemia and consequently died diminished and this might be due to the phages action, by destroying bacteria on early stages of infection. Also the probability of bacteria propagation from bird to bird might have been prevented. So, despite the recognized importance of a high phage concentration for successful therapy[12], it is possible that, in natural occurring infections in which chickens

are in different stages of colibacillosis evolution, a lower titre phage product becomes effective as well. The product must, nevertheless, be administered as soon as possible after the infection diagnosis.

The administration of a low phage titre is also advantageous since it is more feasible to produce large volumes of low concentrated phage suspensions.

As a main conclusion it can be said that phages are able control colibacillosis by avoiding chickens' losses before severe lesions or septicemia occur, and that they might be able to act therapeutically in early stages of infection. This last assumption can explain the potential decrease of carcasses rejection at slaughter.

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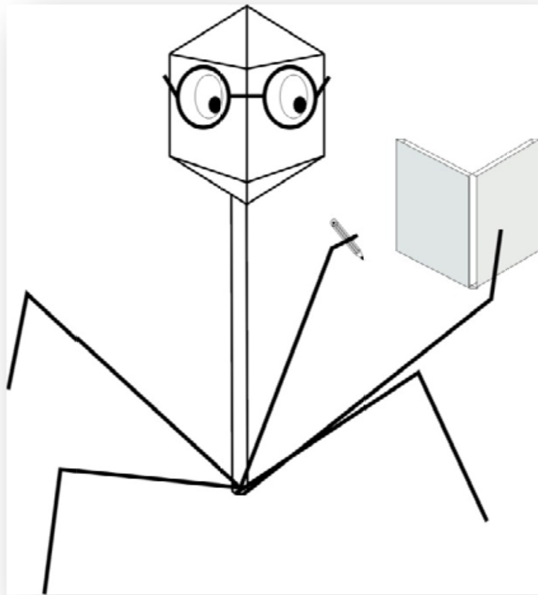
VII. CONCLUSIONS AND FINAL REMARKS



FINAL CONCLUSIONS

The studies presented in this thesis were designed aiming the development of a phage product, constituted by no more than five phages, to be used as an antimicrobial alternative in the poultry industry. This product showed to be able to control colibacillosis propagation within flocks and consequently, the evolution of this infection into colisepticemia. The work was conducted in collaboration with Controlvet-Segurança Alimentar S.A., a company that provides consulting services for the poultry industry, mostly based on microbiological data obtained from samples recovered from the field. This company identified the need of having alternatives to the antibiotics, and proposed the analysis of the effectiveness and feasibility of phage therapy on the poultry daily management.

Bacteriophages were isolated from aviaries. The cocktail was expected to cover the widest range of *E. coli* strains with the minimum quantity of phages, and based on this assumption, the *in vitro* lytic spectra evaluation was performed. Three phages were selected for further studies, phi F61E, phi F78E and phi F258E. Taxonomically, they belong to the *Caudovirales* order. Two of them, phi F61E and phi F78E, belong to the *Myoviridae* family and are 16–19 phages (T4-like). Phi F258E is a Syphoviridae , and looks like T1. All of them are genetically different and apparently lytic (according to their morphology). Nevertheless, this assumption was confirmed by a stress-induction prophage release test.

In vivo experiments were designed and carried on in confined experimental rooms, in order to assess the safety and efficacy of this three-phage product. For the experimental designs, the coefficient of variation between the experimental units [1] (Rhode Island Red chickens) was estimated based on the weight of commercial growers (data not shown). This parameter was set to be 10%.

In a first trial, after producing the phage cocktail in a concentration of 10^8 PFU/ml, the quantity of endotoxin (LPS) present in the suspension was determined. Chickens were injected (i.m.) with the phage lysate. As no abnormal behavior was detected in chickens, like prostration or reluctance to move, depression, somnolence, loss of thirst and appetite or loss of weight, except in the day of the inoculation, and as no toxicity effects were notice in organs at *post mortem* examination, the product was considered to be safe for the birds. In a following experiment, the best administration mode and concentration was identified. Two of the tested routes, the oral and the nasal, showed to

be efficient in delivering the phages to the target organs involved in colibacillosis and furthermore the bloodstream. These routes are very feasible for the management of flocks with large number of birds. Concerning phage concentration, (10^6 PFU/ml, 10^7 PFU/ml and 10^8 PFU/ml), 10^7 PFU/ml seemed to be enough to provide phages to the organs. Besides, it might be economically viable for the scale up production.

In the last *in vivo* trial, the aim was to test the phages efficiency on treating chickens challenged with APEC strains. The phages were tested separately. Preliminary experiments were designed to select the most suitable phage-sensitive APEC strain to infect chickens, and to determine how the birds should be inoculated. The phages were administered orally and by spray in a concentration of 10^7 PFU/ml, as suggested by the results of the previous experiment. The parameters evaluated during the trial were the mortality and morbidity, as well as the pathology score of the organs observed *post mortem*. The results of this trial showed that none of the 3 phages was able to efficiently control the induced infection. The effect of the phage association with an antibiotic was also tested for two of the phages, phi F78E and phi F258E, but the results didn't demonstrate any advantage on this alliance. However, in a subsequent experiment the concentration of phi F78E was increased to 10^9 PFU/ml, it was possible to observe effectiveness in reducing the infection effects. Chickens' mortality decreased in average, 25.0 % and morbidity, 41.7 %. Nevertheless, even though results from the phages efficiency apparently revealed that low phage concentrations were not effective on controlling colibacillosis, experiments performed in APEC naturally infected flocks revealed very promising results. The flocks used in these large scale experiments were experiencing high mortality rates even after the antibiotic treatment, and a 5×10^7 PFU/ml phage cocktail was administered, orally and by spray, in a single dose. The mortality rate was controlled to regular levels (≤0.5%) in one week in 46% of the cases, in two weeks in 9%, and in three weeks in 36% of the flocks. No recidivism in the mortality rate was observed in any flock until slaughter. The gradual decrease in mortality might have revealed that the number of chickens that reached acute septicaemia and consequently died was diminishing, and that this effect was probably due to the phages action, by destroying bacteria on early stages of the APEC infections. In the same extent, the probability of bacteria propagation from bird to bird might have been prevented.

For these reasons, the lack of efficiency obtained in experimental conditions with *E. coli* challenged chickens, might not necessarily means that phages were not effective in destroying the pathogens, but instead that they were not able to control the infection on its severe state.

All these results were presented to the Portuguese Veterinary Authorities (DGV), and Controlvet requested permission to launch the product. A provisional authorization was conceded until the product is registered. The form required for this process, entitled "Application for approval of special use of Veterinary drugs", is shown in Annex 1. Figure VII.1 presents a picture of the phage product named "Colifagos" (A.), as well as the information enclosed in the label (written in Portuguese) (B.). This information describe the product and its composition, to witch specie and strain it is targeted, the therapeutic indications as well as the contraindications, the side effects, the dosage and mode of administration, the interactions with other medicines, the safety interval between administration and the carcass commercialization, the packaging and the advised storing conditions.

Figure VII.1 A. "Colifagos": Therapeutic cocktail composed by 3 coliphages directed to colibacillosis in poultry. B. Label of the product.

CONCLUDING REMARKS

The planning of this thesis enclosed studies assumed as necessary to the development, testing and validation of a therapeutic product constituted by bacteriophages, aiming its commercialization.

On the course of the experiments entailing this main goal, the necessity and will of accessing more expeditious techniques and methods was experienced. In fact, it would be important to shorten the development period of a phage product for similar applications, overcoming the laborious and time consuming phage handling methods. Therefore, the development of expeditious techniques, for example to ensure phage safety (meaning a strictly lytic phage not encoding toxins) or to assess phage genome integrity upon replication, seems to be definitely necessary.

Relatively to the evaluation of phages as antimicrobials in Veterinary Medicine, it is important, firstly, to emphasize that it might not be possible to infer about phage efficiency, only by knowing the *in vitro* hosts lysis rate, or even the phages burst size. It is essential to guarantee that the experimental design of *in vivo* experiments allow reaching all the proposed aims. Tests shall be performed preferentially in the target animals, and the parameters under study have to be carefully selected, taking in account the disease effects in their organisms and the animals' behavioural alterations. Moreover, if, as happened in this study, the researcher is dealing with livestock, normally raised in numerous groups and intensive systems, the way of induce the infection and its severity must be carefully considered, in order to mimic as faithfully as possible the natural occurring disease. It is determinant to the success of the therapy that bacteriophages have the opportunity to meet bacteria, before septicemia is installed.

For the future success of the phage therapy in Veterinary Medicine, there is still much work to be undertaken in order to optimise the effectiveness of phages for each kind of animal and infection. Much of it was already carried on and all the successes and setbacks that have been reported encourage further studies and give confidence to believe that, in a near future phages will be currently used in animal production as a regular antimicrobial treatment.

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ANNEX

Direcção de Serviços de Medicamentos e Produtos de Uso Veterinário

(A preencher pela DGV)
Decisão do Director Geral de Veterinária :
1. Autorizado em __ -__-____
___/___

_____/___AEVPT

2. Não autorizado em __-__-______
Justificação:

Ex.mo Senhor
Director-Geral de Veterinária
Lg. da Academia Nacional de Belas Artes nº 2
1249-105 Lisboa

Faxe: 21 3239565

Assunto	PEDIDO DE AUTORIZAÇÃO DE UTILIZAÇÃO ESPECIAL DE MEDICAMENTOS VETERINÁRIOS	Data

Nome do Médico Veterinário | Nº da Cédula Profissional

Local de exercício da actividade profissional

Tel: Telemóvel: Fax: e-mail:

Ao abrigo da alínea h) do nº 2 do Decreto-Regulamentar n.º 11/2007, de 27 de Fevereiro, solicita autorização para aquisição do seguinte medicamento veterinário:

Nome do Medicamento veterinário		Apresentação	Forma Farmacêutica
Substância activa	Via de Administração	Distribuidor	Número de Embalagens

Justificação clínica e protocolo terapêutico

Outros documentos:

Cópia da autorização de introdução no mercado obtida no país de origem, da qual constem a composição qualitativa e quantitativa do medicamento.

Cópia do resumo das características do medicamento, do qual constem as espécies alvo e a indicação do intervalo de segurança.

Assinatura do médico veterinário (deverá ser identificada sob a forma de carimbo e/ou vinheta):

Figure 1 Application form needed for the approval of special use of Veterinary drugs by DGV.