In vivo investigation of the preventative and therapeutic potential of isolated bacteriophages (PBM-1, PBM-2, & PBM-3) against Colibacillosis in poultry

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Islamabad
Pakistan
2022

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This thesis is submitted to the Atta-ur-Rahman School of Applied Biosciences in partial fulfillment of the requirements for the degree of Bachelor of Applied Biosciences

Atta-ur-Rahman School of Applied Biosciences

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Thesis Acceptance Certificate

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30 May, 2022

Acknowledgement

We would like to express our honest gratitude to **Dr. Fazal Adnan** for his invaluable support, patience, and guidance as our supervisor on this project. Our sincerest regard to **Dr. Muhammad Tahir** and **Dr. Saadia Andleeb** for their assistance. We want to appreciate the efforts of **Mr. Fida Hussain** and **Mr. Danish** for their help at the lab animal house. We are grateful to the incredible faculty members at Atta-ur-Rahman School of Applied Biosciences for believing in our potential and encouraging us to be the best version of ourselves. Our project was made manageable due to the special efforts of **Ms. Andleeb Khizar**, **Ms. Hira Niaz, Ms. Saleha Masood,** and **Mr. Murtaz Aziz Ahmad** who were there for us at every step. Finally, we want to thank our friends and family for their immense support and prayers.

This thesis is dedicated to our 101 chicks who sacrificed their lives for the sake of science.

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List of Abbreviations

AC	Antibiotic Control
AMC	Amoxicillin clavulanic acid
AML	Amlodipine besylate
AMP	Ampicillin
AMR	Antimicrobial Resistance
APEC	Avian Pathogenic *Escherichia coli*
ASAB	Atta-Ur-Rahman School of Applied Biosciences
AST	Antimicrobial/Antibiotic Susceptibility Testing
AT	Antibiotic Treatment
CAZ	Ceftazidime
CC	Cocktail Control
CFU	Colony Forming Unit
CIP	Ciprofloxacin
CN	Gentamycin
CTC	Combination Therapy Control
DNA	Deoxyribonucleic acid
EMB	Eosin-Methylene Blue
Ex-PEC	Extraintestinal Pathogenic *Escherichia coli*
Etc.	Et Cetera
FDA	Food and Drug Authority
FEP	Cefepime

FOX	Cefoxitin
GDP	Gross Domestic Product
Hrs.	Hours
IM	Intramuscular
IPM	Imipenem
LB	Luria Broth
LEV	Levofloxacin
MEM	Meropenem
NC	Negative Control
NOR	Norfloxacin
NUST	National University of Sciences and Technology
OD	Optical Density
OFX	Ofloxacin
PC	Positive Control
PFU	Plaque Forming Unit
PPM	Parts per million
SC	Subcutaneous
SXT	Sulfamethoxazole/ Trimethoprim
TOB	Tobramycin
TZB	Tazobactam
USA	United States of America
Vs.	Versus

Abstract

Poultry is the second largest industry of Pakistan, with a 3.1% contribution to the Gross Domestic Product. Colibacillosis is a widespread poultry disease caused by the Avian Pathogenic *Escherichia coli* bacteria (APEC). Emerging antimicrobial resistance against Colibacillosis and the ban on the antibiotic Enrofloxacin, presents the need to develop alternative treatment strategies. The study aimed to investigate the preventative and therapeutic potential of bacteriophages against Colibacillosis. One hundred poultry birds, divided into fifteen groups, were infected with APEC, and treated with Enrofloxacin, bacteriophage cocktail, and a combination of antibiotics and phage cocktail. The treatment doses were administered via subcutaneous, nasal, and intramuscular routes. Survivability, lesion scoring of the organs, and weight gain trends were used to determine the efficiency of the bacteriophage cocktail. Test subjects of the positive control showed a drastic weight loss trend as compared to healthy birds of the negative control. Test birds given the phage cocktail as a prophylactic, and those given it in combination with antibiotics both showed a survival rate of 100% as compared to 17% of the positive control. Intramuscular injection of the cocktail proved to be the most effective mode of administration with a 67% survival rate. Phage therapy, as cocktails used for prophylaxis, and in combination with antibiotics, can prove to be an invaluable tool in the ongoing fight against anti-microbial resistance (AMR).

Introduction

It's hard to imagine a world without chicken. In the last century, poultry has emerged as the chief source of meat in various parts of the world, becoming front and centre because of its simple, economically feasible production. (*National Chicken Council*, 2022) This explosive growth can be attributed, almost exclusively, to the development of the broiler chicken.

A broiler is any chicken (*Gallus gallus domesticus*) that has been bred, fed, and raised with the sole purpose of producing meat for consumption (*U. S. Broiler Industry Structure*, 2013). Tracing its origin leads to one Mrs. Wilmer Steele of Delaware, USA. She is cited for pioneering the first broiler farm, by raising 500 chicks intended to be sold for meat in 1923, having expanded the operation to 10,000 chicks by 1926. (*National Chicken Council*, 2022) Internationally, Pakistan is the 11th largest poultry producer in the world, with an industrial investment of more than Rs. 750 billion. It is the second largest industry in Pakistan and contributes about 3.1% to the GDP (Ahmad, 2020) (Memon, 2012). The present-day broiler, or at least the most predominant breed, was produced as a cross between the New Hampshire, Langshans, Jersey Black Giant, Plymouth Rock, Brahmas, and Cornish Game, among other breeds. The result is a typically white-feathered bird, with a red comb and an astonishing rate of growth. Modern-day broilers can reach a slaughter weight of 2.5kg in 38 days compared to 63 days in the 1960s (*Farmers Weekly*, 2014), or 120 days in 1925 (Bessei, 2006). But the exclusive selection of genetic factors for such accelerated growth has led to the development of several structural dysfunctions, such as that of the cardiovascular system, skeletal system (lameness), and ocular abnormalities. Perhaps most significantly, it has led to the loss of 'rare alleles', those normally present in their wild counterparts, causing a consequential decrease in resistance to infectious diseases. (Muir et al., 2008)

Colibacillosis is a bacterial infection caused by the Avian Pathogenic *Escherichia coli* (APEC) bacteria. It is one of the most commonly occurring bacterial diseases in poultry and has thus proven itself to be a major threat to poultry worldwide. It can manifest as both a localized and systemic infection. Causing the infected bird to develop symptoms such as acute fatal septicemia (blood poisoning), subacute pericarditis (inflammation of the pericardium), airsacculitis (inflammation of air sacs), salpingitis (inflammation of fallopian tubes), peritonitis (inflammation of the peritoneum), and cellulitis (bacterial skin infection). (Nolan, 2019) The

causative agent - APEC is a rod-shaped, gram-negative, non-acid-fast, non-spore-forming, bacillus bacterium. Clinical signs of an APEC infection include respiratory distress, diarrhoea (enteritis), high embryonic mortality, decreased performance in older birds, and a high mortality rate in younger birds. (Nolan, 2019) Its mode of transmission is through contaminated feed and drinking water, going from one bird to the next through either the faeco-oral route or by droplets in the aerosol route. (Kathayat et al., 2021)

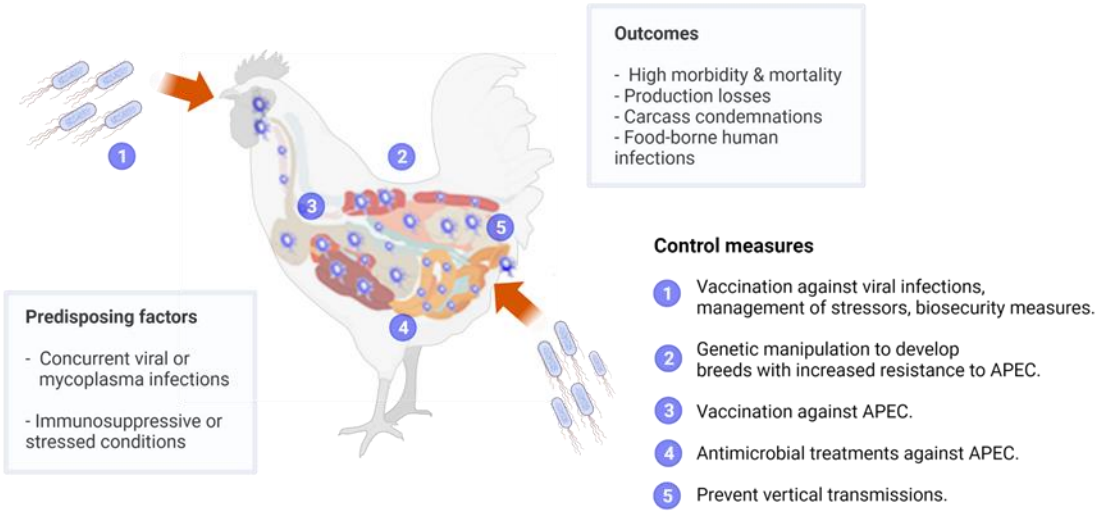

Figure 1 Schematic Diagram of APEC infection in Chicks (Modified from Kathayat et al., 2021)

Poor environmental conditions such as an overcrowded space, or high levels of dust or ammonia and stressors, in the form of a suppressed immune system, production-related stress, and already have been suffering from a primary infection, are all factors that enhance the susceptibility to an APEC infection.

Colibacillosis has had a major impact on the global poultry industry, being one of the most infectious disease in birds of all ages. It has led to economic losses not only by causing high mortality but also by causing decreased productivity in all affected birds. Egg production too is greatly reduced, and it is one of the few diseases that result in complete carcass rejection. (Abalaka et al., 2017) The only currently available treatment for an APEC infection is antibiotics.

Antibiotics, also known as antimicrobials, are a class of typically low-molecular-weight naturally occurring or synthetic compounds. They affect bacteria by either killing them directly, slowing down, or stopping their growth. (Ferri et al., 2017) Over the past century, antibiotics have predominantly become the way to treat bacterial infections. This has led to the development of antimicrobial resistance.

Antimicrobial resistance, AMR, is defined as the development of the ability of microorganisms to both survive and stay viable under the influence of antimicrobial agents. (Abushaheen et al., 2020) A natural consequence owing to the accumulation of mutations because of rapid division and the short life cycle of bacteria.

APEC demonstrates broad-range antibiotic resistance. Antibiotic susceptibility tests against various antimicrobial agents show that APEC is a multidrug-resistant bacterium. The highest resistance is observed against ampicillin (98.6%), then tetracycline (97.3%), followed by ciprofloxacin (72%). Sulfa drugs, streptomycin, etc. are also facing resistance by APEC. Colistin, widely considered the last resort due to it being linked to permanent renal dysfunction, is being used in a desperate attempt to fight the infection (Azam et al., 2019).

One of the few antibiotics that APEC is not yet resistant to, is Enrofloxacin. The FDA banned the use of Enrofloxacin in 2005, due to the growing antibiotic resistance in *Campylobacter spp.* bacteria against fluoroquinolones, the antibiotic class of Enrofloxacin. *Campylobacter* does not cause disease in poultry but is a human pathogen, and fluoroquinolones are an important class of antibiotics used against it (Nelson, 2004). The rising resistance, having reached critical levels, has highlighted the need for an alternative treatment against bacterial infections.

Bacteriophages are viruses that infect bacteria. Entering a host bacterial cell and replicating, causing it to burst and die. They are highly specific, each type of bacteriophage or ‘phage’, only attacks one species, or sometimes only one strain of a certain bacterium (Kasman & Porter, 2022). This quality of bacteriophages can be harnessed to treat bacterial infections, and it has been, for almost one hundred years - it has come to be known as phage therapy.

An unexpected advantage of bacteriophage therapy is the fitness trade-off. Any accumulation of mutations that develops resistance to bacteriophages, consequently, causes loss of antibiotic resistance genes, leaving bacteria once again vulnerable to antibiotics (Keen, 2014). Combined,

this puts bacteriophages as a strong bet, in our fight against antibiotic-resistant bacterial infections.

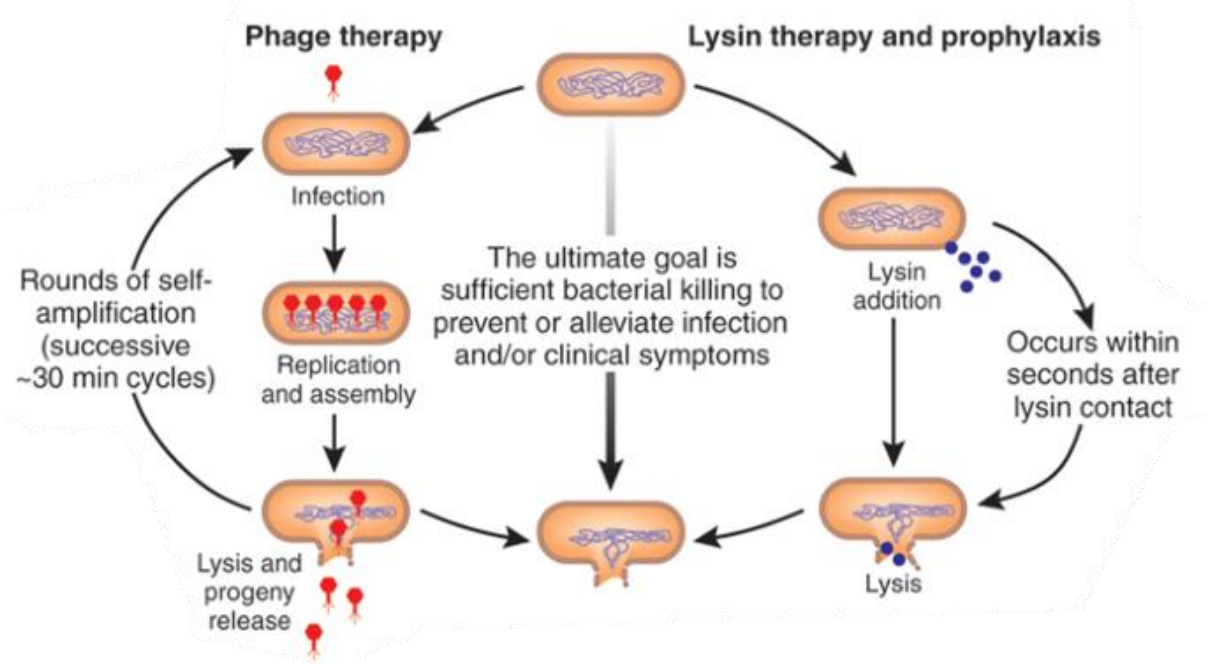

Figure 2 Bacteriophage Therapy of Bacterial Infections (Modified from Fischetti et al., 2006)

We aim to investigate the use of a cocktail of bacteriophages; PBM-1, PBM-2, and PBM-3 in combination with the antibiotic Enrofloxacin, as a treatment against APEC O1 infection in poultry. In addition to this, the prophylactic nature of the phage cocktail is also to be studied through the *in vivo* trials including the role of administration via different routes and after various time intervals to determine the best method.

1.1. Rationale

With an investment of Rs. 760 billion, the poultry industry remains the second largest industry in Pakistan. Colibacillosis infections pose a threat to the birds every year resulting in huge economic losses. Antimicrobial resistance to all commercially available antibiotics leaves Enrofloxacin, banned by the FDA, as the only effective drug to treat Colibacillosis. This requires research into alternative treatment and preventative methods to control the spread of APEC. This study investigates the preventative and treatment potential of bacteriophages against Colibacillosis and its most effective mode of administration.

1.2. Research Objectives

The objectives of the research were:

- Optimizing a phage cocktail against APEC
- Using the cocktail in conjunction with antibiotic Enrofloxacin to determine the effects of Combination therapy *in vivo*
- Comparing the efficacy of the treatment through different modes of administration
- Determining the prophylactic effect of the phage cocktail

2. Literature Review

2.1. Prevalence of Colibacillosis in poultry

Avian Colibacillosis is an infectious disease of birds, caused by Avian Pathogenic *Escherichia coli* (APEC) bacterium. APEC is a subset of extra-intestinal *E. coli* (Ex-PEC) (Dho-Moulin & Fairbrother, 1999). Colibacillosis is considered one of the poultry's most significant infectious diseases worldwide due to its significant economic impact. Infections caused by APEC in poultry result in severe losses due to morbidity, mortality, low production, and carcass condemnation. Infectious colibacillosis affects birds of all ages, causing high mortality in younger birds and low productivity in older birds (Panth, 2019).

The clinical manifestation of Colibacillosis ranges from salpingitis (inflammation of the oviduct in egg-laying hens), airsacculitis (respiratory tract inflammation), pericarditis (lesions in pericardium), perihepatitis (inflammation of the liver), to omphalitis (yolk-sac infection) which is a major cause of mortality in young chicks (Apostolakos et al., 2021). Sporadic lesions that are observed irregularly or mostly only in certain locations include arthritis (swelling of joints), peritonitis (inflammation or swelling of the abdominal tissue), pneumonia (lung inflammation with congestion), and osteomyelitis (swelling or inflammation in bone) (Ask et al., 2006).

2.2. Risk factors of Colibacillosis

The spread of Colibacillosis is linked to the conditions and environment of the area where poultry birds are kept. Poor hygiene conditions and overcrowding are major factors in the spread of the disease. Birds also become more susceptible when they are already suffering from primary infection or an immunosuppressive disease. The respiratory system is majorly affected due to inhalation of contaminated dust or ammonia resulting in declination of the upper respiratory tract and making the respiratory system more susceptible to APEC. Faecal contamination of eggs can result in penetration of APEC through the shell which spreads after hatching. Other risk factors include breed, immunity of the birds, exposure, and virulence of the infecting strain (Kabir, 2010). Its mode of transmission is mainly through contaminated

feed and drinking water, nasal and cloacal routes, and direct contact with infected birds (Kathayat et al., 2021).

2.3. Effect of Colibacillosis on Broiler Chickens

Chickens in the first week of their life have some level of natural passive immunity that has been passed down from their mothers in the form of antibodies circulating in their system. But since the immune system of the bird itself has not yet developed, this immunity is extremely short-lived. From the ages of day 6 to day 13 a broiler chicken's immunity is at its lowest after which it starts developing. And the full maturation of the immune system of these birds occurs when the birds reach about day 30 to day 34 of age. This is in accordance with a study conducted in the year 2021, where 106 one-day-old broiler chickens were reared in cages and euthanization was done for sampling on days 1, 6, 13, 20, 27, and 34. To determine the development of the immune system; cytokines, immune indicators, immune cell ratios, expression of certain genes, cell proliferation, and immune organ indices were studied (Song et al., 2021).

2.3.1. Immune System and Clinical Signs

The development of the immune system and the varying levels of immunity in different age groups of broiler chickens leads to the clinical signs being non-specific and per the age of the affected birds. Usually, it is seen that young birds that die of acute septicemia or sepsis (commonly known as blood poisoning) have less area of abnormal tissue and fewer lesions on their body organs. Birds dying in this way mostly simply display a hyperemic liver that is also enlarged i.e., an abnormal amount of blood is present in it, and an increased amount of fluid is also present in the body cavities. Chickens that are older or survive the blood septicemia stage develop lesions on body organs and tissues even leading to scarring. The liver, the heart, the lungs, the spleen, the kidneys, etc. display the greatest number of such lesions and abnormal tissue development. In addition to this acute airsacculitis, lymphocytic depletion, pericarditis,

and perihepatitis are also widely observed in such birds. The lesions do start to heal once the birds start to recover (Abu Daud et al., 2014).

2.4. Antimicrobial resistance

Antimicrobials are small molecular weight compounds classified on the basis of their chemical origin and their ability to interact with the micro-organisms. They can either stop bacterial growth or kill the microorganism altogether. The first antibiotic was developed in 1929 by Alexander Flehming and no new antibiotics have been developed since 1987. This is a major contributing factor toward the new bacterial strains becoming resistant to the existing antibiotics. Bacteria have many ways to obtain resistance against antimicrobials (Ferri et al., 2017).

Bacterial strains becoming resistant to antibiotics is directly linked to the excessive use of antibiotics. Bacteria either change their pre-existing DNA or acquire new genetic material to become resistant to the antibiotics. The three major methods of DNA transfer are conjugation, transformation, and transduction (Bennett, 2008).

2.5. Multidrug Resistant APEC

Antibiotic resistance of a particular bacterium is measured using antibiotic susceptibility testing or antibiotic sensitivity testing (AST). AST is a widely used method that shows that bacterial or fungal growth can be inhibited by which possible antimicrobial. It tells us which antibiotic will prove to be most effective to treat a specific infection and which antibiotics the bacteria or fungi are resistant to so that they can be avoided (Khan et al., 2019).

APEC demonstrates broad-range antibiotic resistance. Antibiotic susceptibility tests against various microbial agents show that APEC is a multidrug-resistant bacterium. The highest resistance is observed against ampicillin (98.6%), then tetracycline (97.3%), followed by ciprofloxacin (72%). Sulfa drugs, streptomycin, etc. are also facing resistance by APEC. Colistin is used as a last resort approach (Azam et al., 2019). According to another study

conducted in Bangladesh, APEC showed the highest resistance to ampicillin (90.91%), nalidixic acid (90.11%), tetracycline (83.72%), and nitrofurantoin (65.12%). An antibiogram is an overall profile of a microorganism to a number of antimicrobial drugs done through antimicrobial susceptibility testing and the results are compiled. Antibiogram profiling of APEC performed *in vitro* revealed that 100% of the isolates were resistant to at least 3 antibiotics, with the majority being resistant to more (Kabir, 2010).

One of the few antibiotics that APEC is not yet resistant to is enrofloxacin, a fluoroquinolone drug. The FDA banned enrofloxacin in 2005, due to the growing antibiotic resistance in *Campylobacter* against fluoroquinolones (FDA, 2007). Campylobacter does not cause disease in poultry but is a human pathogen, and fluoroquinolones are an important class of antibiotics used against it (Nelson, 2004).

APEC demonstrates a wide range of antibiotic resistance due to a number of factors. Treatment with antibiotics including the route of administration of them, and the widespread presence of the bacterial strain and its fitness greatly affect the prevalence of the antibiotic-resistant strain. In addition to this, even without the presence of antibiotics, antibiotic-resistant genes can transfer from one bacterium to another. This is known as horizontal transfer and the prevalence of this phenomenon increases when bacteria are exposed to antibiotics. The increased use of cephalosporin - a third-generation antibiotic as a preventive measure in broilers and hatcheries has also led to widespread antibiotic resistance against this class of antibiotics (Christensen et al., 2021).

2.6. Bacteriophages

Bacteriophages are viruses that infect bacteria i.e., bacteria eaters. They invade and disrupt the bacterial systems and metabolism in turn leading to bacterial cell lysis. Bacteriophages are sometimes referred to as the deadliest beings on the planet. They are the most abundant living organisms on Earth because they are found wherever the bacteria they prey upon are present in nature. Bacteriophages are highly specific; usually, each phage only attacks a certain bacterium (Kasman & Porter, 2022).

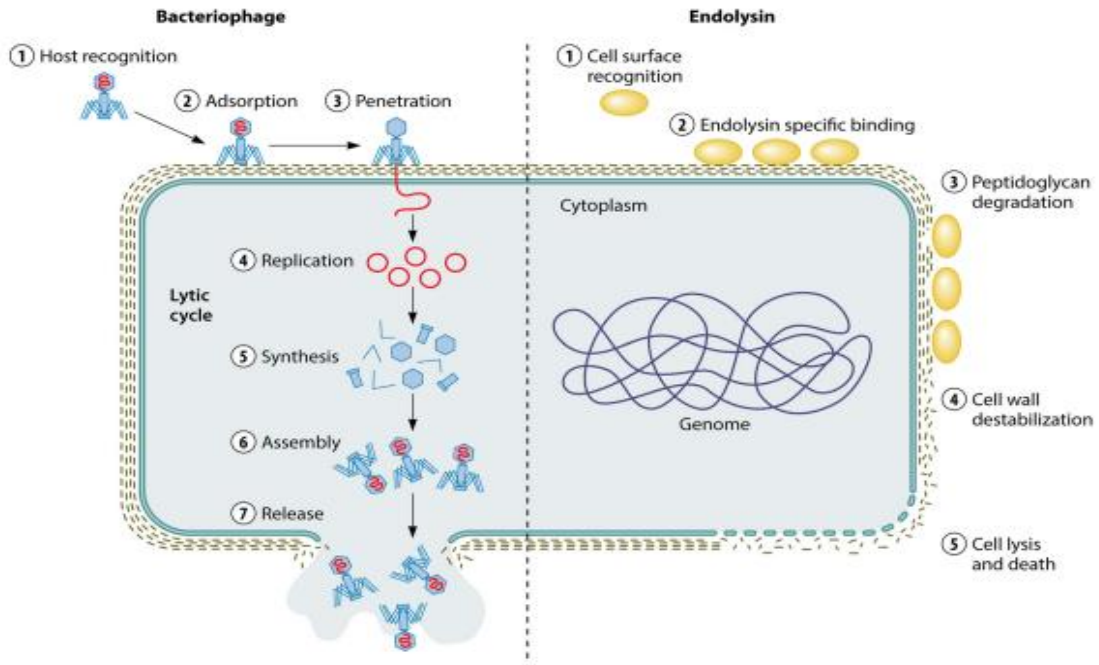

Figure 3 Lytic Activity of Bacteriophages and Activity of Endolysins against Bacterial Cells (Adopted from Álvarez et al., 2019)

2.7. Phage Therapy

Since bacteriophages are highly specific against certain bacteria or bacterial strains only, they can be used to help us to fight bacterial infections. This is known as phage therapy i.e. using bacteriophages for therapeutic purposes. They do not have any effect on the animal, human, or plant cells due to their highly specific nature and lead to bacterial cell lysis which can be used in fighting infections (Lin et al., 2017).

Phage therapy is employed in many ways including the conventional mode of phage therapy in which bacteriophages specific to the bacterium are employed directly, the use of enzymes derived from bacteriophages that result in cell wall lysis, using bacteriophages that have been bioengineered, and using phages in a way that they function using the synergy between them, the immune system, and the antibiotics employed (Gordillo Altamirano & Barr, 2019).

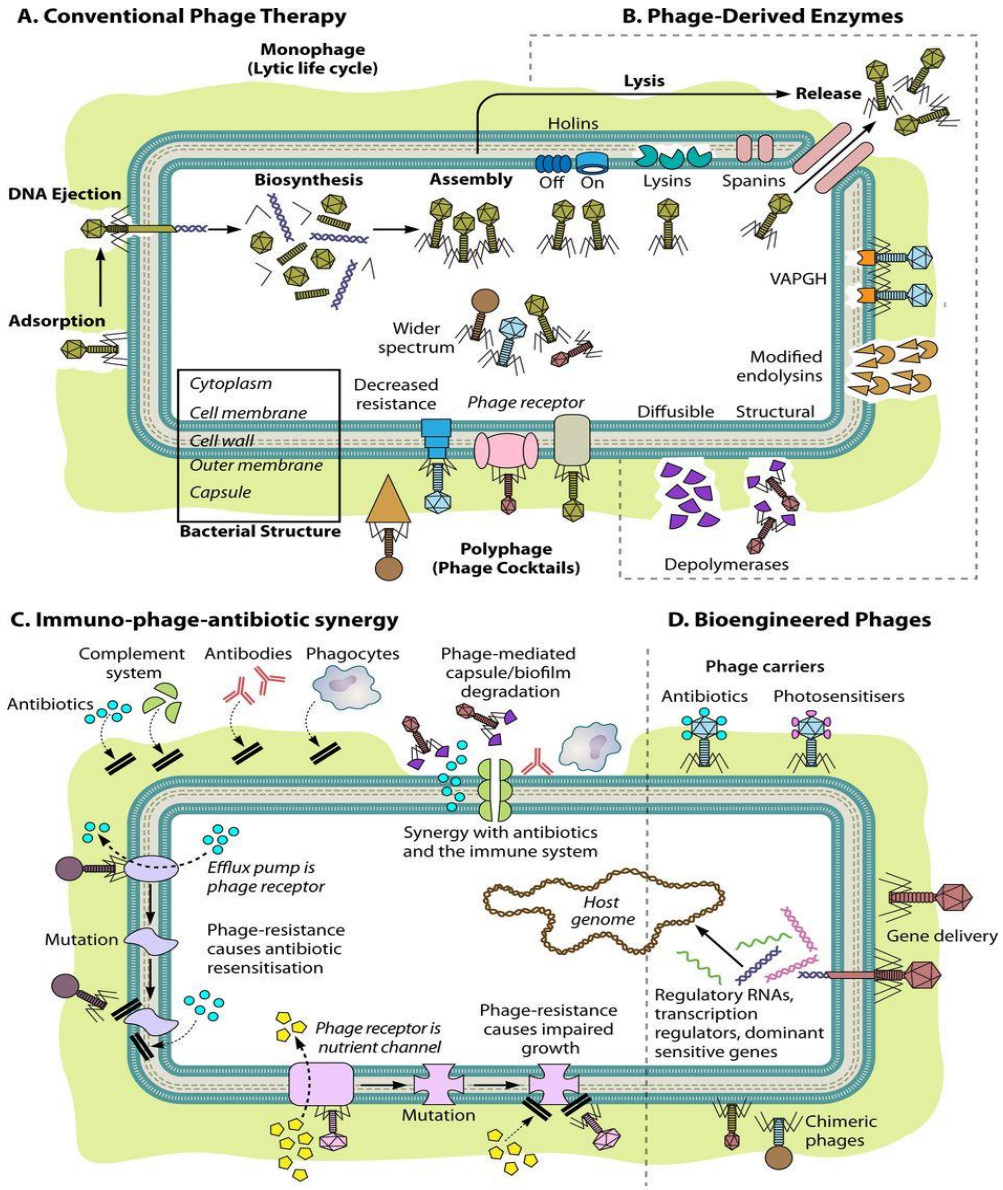

Figure 4 Modes of Phage Therapy: A. Conventional Phage Therapy, B. Phage-Derived Enzymes, C. Immuno-phage-antibiotic synergy, D. Bioengineered Phages (Adopted from Gordillo Altamirano & Barr, 2019)

Lytic phages are preferred to temperate phages in phage therapy. This is because temperate phages contain the integrase enzyme and can incorporate into the host genome, therefore, co-existing with it. In addition to this, they may also carry harmful genes from one bacterium to another. Lytic phages lack the integrase and cannot integrate into the host genome (Skurnik & Strauch, 2006).

It is important to note that the bacteriophages are cleared from the body system by the natural activity of the kidneys. In addition to this, the innate immune system also generates some level of immune response to the phages. Therefore, an understanding of these roles and the spread of induced bacterial infection is to be developed to effectively administer phage therapy (Tsonos et al., 2013).

2.8. Prophylactic effect of Phage therapy

Prophylaxis is defined as the measure or treatment given for disease prevention. Bacteriophages have been shown to have therapeutic as well as prophylactic effects against pathogenic bacterial strains in poultry and other animal models. Intramuscular administration of bacteriophages in birds could cross the blood-brain barrier resulting in the prevention and treatment of colibacillosis (W. Huff et al., 2002). The prophylactic effect of bacteriophages on natural microflora is rendered safe due to their highly specific nature against bacterial strains (Ahmadi et al., 2016).

A study that included animal trials was conducted to check the prophylactic as well as the therapeutic effect of bacteriophages on 7-day old chicks, in the year 2008. Phages were given in the form of aerosol spray 1 and 3 days prior to the challenge with *E. coli.* A significant reduction in mortality was observed as compared to untreated groups. The intramuscular route of phage administration proved to be effective as therapeutic as well as preventative but is not feasible to be used on large flocks on poultry farms. However, it has been demonstrated that a combination of injecting phages into hatching eggs and using the aerosol spray in a hatchery could significantly prevent an outbreak of Colibacillosis. It is important to note that the prophylactic effect of bacteriophages is significant only when a high number of phages is present at the time of exposure (Johnson et al., 2008).

2.9. Phage Cocktail

The concept of the Phage cocktail refers to a mixture of several phages used to formulate a concoction. The combined efficacy of which is more than individual phages or in certain

circumstances, even the sum of its parts. This also slows down or even prevents the formation of phage-resistant bacterial strains since a combination of various different bacteriophages is present. (Yang et al., 2020) The use of a phage cocktail (multi-phage) is also known as polyphage therapy as it utilizes multiple phages. (Chan & Abedon, 2012)

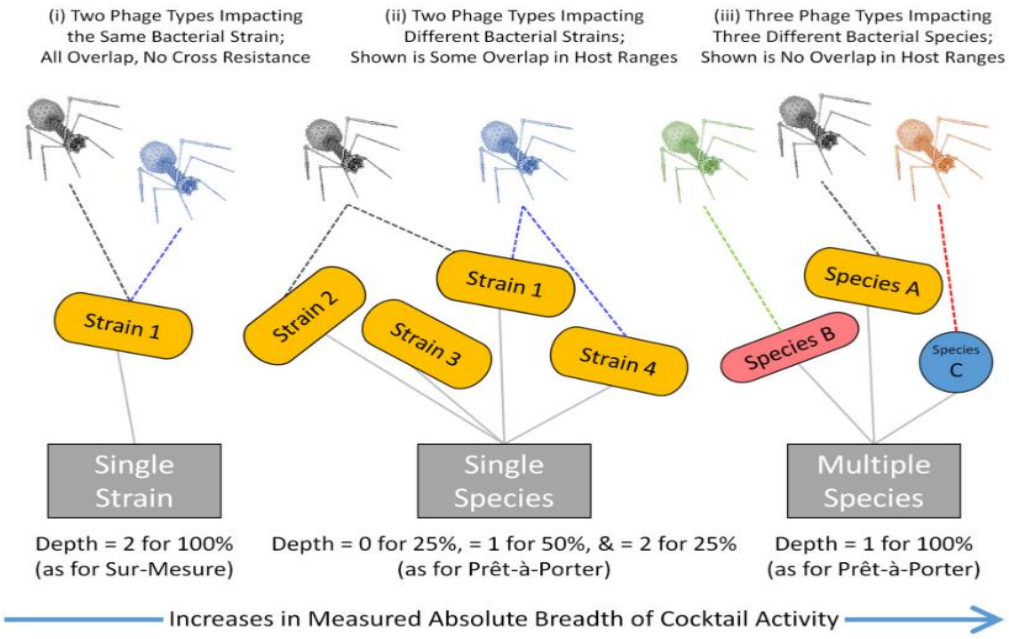

Figure 5 Different Types of Phage Cocktails and the Measure of their Activity (Adopted from Abedon et al., 2021)

A bacteriophage cocktail may consist of a number of different phages acting against the same bacterial strain so that complete overlap will exist in the host range. On the other hand, the different bacteriophages present in a cocktail may be acting against different bacterial strains of the same species thereby increasing the host range. In addition to this, a bacteriophage cocktail may consist of different bacteriophages all having a bacterial host from completely different species which will maximize the host range and no overlap will exist amongst the phages' activity (Abedon et al., 2021).

When analyzing a phage cocktail *in vitro*, it was found that phage-resistant variants of bacteria arose when challenged with a single phage, but not when challenged with a cocktail of four to six different bacteriophages (Korf et al., 2020).

2.10. Combination Therapy

Combination therapy is the use of both antibiotics and bacteriophages together to treat diseases. The trade-off principle comes into play here. According to the trade-off principle, for a bacterium to become resistant to phages it must first give away the antibiotic resistance that it has acquired. (Keen, 2014) Although the evolution of bacteria to develop resistance to phages might be inevitable, combination therapy still has potential through this phenomenon. Selection pressure leads to the bacteria evolving and developing such resistance to bacteriophages. Such a fitness trade-off might lead to reduced virulence of the bacterium or becoming re-sensitized to antibiotics in order to gain resistance against the bacteriophages (Mangalea & Duerkop, 2020).

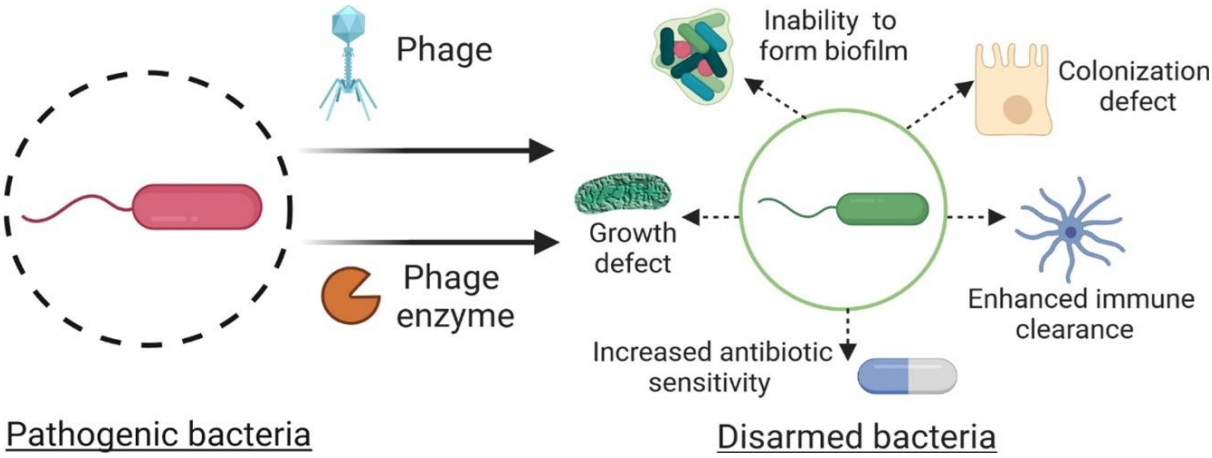

Figure 6 The effect of Bacteriophages and Phage Enzyme Activity on Pathogenic Bacteria (Adopted from Shen & Loessner, 2021)

When bacteriophages act on bacteria they interact with and target certain bacterial surface structures. These bacterial surface structures may be involved in virulence. The modification or loss of these molecules may occur when a bacteriophage challenge is administered which may lead to the development of phage resistance and certain trade-offs. These trade-offs that might lower the fitness of the bacteria include reduced virulence, enhanced immune clearance, inability to form a biofilm, colonization defects, growth defects, increased antibiotic sensitivity i.e., loss of antibiotic resistance, etc. (Shen & Loessner, 2021).

The synergy of bacteriophages and antibiotics has great efficacy against APEC-induced Colibacillosis. This suggests that combination therapy is a significant treatment option in treating Colibacillosis in poultry (W. E. Huff et al., 2004).

2.11. *In vitro* Analysis

Before carrying out *in vivo* clinical trials, phages must first be analyzed *in vitro* to determine their efficacy against a particular bacterial strain. The *in vitro* analysis includes bacteriophage isolation, bacteriophage purification, phage amplification, phage categorization, determination of host range, phage titer, phage cocktail formation, and antimicrobial activity of phage cocktails, etc. (Chan et al., 2013).

For the antibiotic susceptibility testing, the disk diffusion method is used. According to a study published in 2022, antibiotic susceptibility testing against *E. coli* showed the most sensitivity to enrofloxacin and ciprofloxacin. While amoxicillin, oxytetracycline, and neomycin were resistant against *E. coli* isolates. In order to avoid drug resistance and economic loss, it is advisable to perform an antibiotic susceptibility test prior to starting the treatment (Tanveer Ahmad & Umer, 2022).

2.12. *In vivo* testing against Colibacillosis

In vivo studies or trials refers to a study involving experiments conducted on living organisms which may be animal models, plants, whole cells, or in the case of clinical trials, humans. After *in vitro* analysis of bacteriophages, the next step is to determine the *in vivo* efficacy by conducting animal trials.

2.12.1. Mode of treatment administration

Mode of administration refers to the approach through which, the treatment; be it antibiotics or phages is provided entry into the body of the organism. Enrofloxacin, the antibiotic under

consideration, is dissolved in drinking water and administered through the peroral route (W. E. Huff et al., 2004).

For the therapeutic administration of bacteriophages, a few different modes have shown to be of prominence. According to a meta-analysis and systematic literature review that was conducted, it was found that the therapeutic effect of phages administered via feed was significantly greater than those administered phages via drinking water or aerosol spray (Mosimann et al., 2021). Another paper found the intramuscular administration of phages into the left thigh of chickens at 7 days of age, resulted in a decrease in mortality from 68% in the untreated control to 15% in the treated group (W. E. Huff et al., 2004). In another study, they administered their bacteriophages through intratracheal injection and reported a decrease in mortality from 83.3% in the untreated birds to 13.3% in the group that received therapeutic treatment intratracheally (Lau et al., 2010).

2.12.2. *In vivo* Trials

Experimental trials were conducted to study the treatment of Colibacillosis in broilers using bacteriophages and antibiotics both individually and in combination. The experimental design contained 320 birds total with 10 birds in 4 replicate pens each and 8 treatments being studied. The birds were challenged at 7 days of age with 10^4 CFU of 0.1 ml *E. coli* injected into the thoracic air sac. The study continued for 3 weeks after the birds had been challenged after which euthanization was carried out of any remaining birds. The bacteriophage treatment was administered immediately after the administration of the challenge by means of a single intramuscular injection in the left thigh region it contained two different isolated bacteriophages of 10^9 PFU per ml each, DAF6 and SPR02. The challenged group receiving antibiotic treatment was given the antibiotic in drinking water consecutively for 7 days following the challenge at 50 ppm. The antibiotic being used was enrofloxacin and was given immediately following the challenge administration. A combination of both treatments was also administered to one group to study the synergy of the two treatments i.e., phage therapy and antibiotic therapy. A significant reduction in morbidity and mortality was observed in all treatment groups. The mortality observed in the group that received no treatment but was challenged with *E. coli* was 68%, while antibiotic treatment, phage therapy, and combination

therapy showed 3%, 15%, and 0% mortality, respectively. Lesion scores for airsacculitis were also calculated. The lesion incidence of surviving birds at 3 weeks was 38%, 3%, 15%, and 5% for the untreated challenged group, antibiotic therapy group, bacteriophage therapy group, and combination therapy group, respectively. This study concluded that combination therapy proved to be an effective treatment against APEC-induced Colibacillosis infection (W. E. Huff et al., 2004).

For a critical evaluation of the prevention and treatment of Colibacillosis, a series of *in vivo* animal trials was conducted using two isolated bacteriophages. The birds were injected with 6.0×10^4 CFU per ml of *E. coli* in the left thoracic air sac. To observe prophylaxis bacteriophages were given in two ways. One group received the bacteriophages to prevent the colibacillosis infection as a nasal spray prior to the administration of the *E. coli* challenge. In another case, prevention was observed by mixing the bacteriophages with the *E. coli* challenge culture and then administering it to the birds (G. R. Huff et al., 2009). Two different doses were used in two groups to determine the efficacy of mixing bacteriophages with the *E. coli* bacterial culture and then administering it to the birds. A low dose group received 10^4 PFU per ml phages while a high dose group received 10^8 PFU per ml phages, mixed with 10^4 CFU per ml of E. coli. Mortality observed in the birds that were simply only challenged with *E. coli* was 85%, while it was reduced to 35% in the low dose group, and no mortality was observed in the group receiving a high dose of phages mixed with bacterial cell culture (W. Huff et al., 2002). The birds which were to be studied for bacteriophage treatment were given bacteriophages as an intramuscular injection in the thigh region after the bacterial challenge culture had been administered in the thoracic air sac. The challenge was administered at 7 days of age. One group received treatment on the same day, the second group after 24 hours, and the third group after 48 hours i.e., 2 days. Multiple doses of bacteriophages given as a treatment were also studied. A significant reduction in morbidity and mortality was observed in all the groups that were administered bacteriophages (G. R. Huff et al., 2009).

A study was conducted in the year 2010 to determine the efficacy of a lytic bacteriophage EC1 against *Escherichia coli* O78:K80 which causes Colibacillosis in poultry. Four treatment groups were established containing 4 pens of 30 one-day-old birds each, a total of 480 birds were used. The bacteriophage-treated group was first challenged with 10^8 CFU per ml of *E. coli* O78:K80 and was then treated 2 hours post-infection with 10^{10} PFU/ml of bacteriophage

EC1. The mode of administration for all the materials was intratracheal inoculation. Over the 3 weeks experimental period of this study, the total mortality rate was reduced from 83.3% in the untreated, *E. coli*-challenged group to 13.3% in the group that was administered bacteriophages. On completion of the three-week period, the body weights of the bacteriophage-treated group were 15.4% higher than the untreated, *E. coli*-challenged group, although no significant difference was observed from the unchallenged controls. The infection too was observed to be less severe. On days 1 and 2 post-infection, the mean total viable cell counts of *E. coli* identified in the lungs were significantly lower in the treated birds compared to the untreated challenged birds. The cell counts were identified on Eosin Methylene Blue agar (EMB). The isolation frequency of EMB + *E. coli* in the treated birds was also found to be lower. In the treated birds, *E. coli* were isolated from body organs only on day 1 post-infection, no other sampling day showed any isolation frequency. *E. coli* were detected at a low ratio of 2/6, 1/6, and 3/6 from the liver, the heart, and the spleen, respectively from the treated chickens. No detectable levels of *E. coli* were observed in the blood samples on any of the sampling days. Based on these results they concluded that to control colibacillosis caused by APEC in poultry, phage therapy is a valuable approach and gives beneficial results (Lau et al., 2010).

According to a 2010 study which was published in the Veterinary Microbiology journal, bacteriophages were used to treat APEC infections in both naturally infected broiler flocks and experimentally contaminated birds. Phages were administered in a single application both orally and through a spray. In naturally infected APEC flocks displaying severe Colibacillosis that were not responding to antibiotic treatment, a cocktail of 3 bacteriophages combined to make 5.0×10^7 PFU/ml was used. The three lytic coliphages combined to make the cocktail were, phi F78E (Myoviridae), phi F61E (Myoviridae), and phi F258E (Siphoviridae). The mortality of the birds treated with bacteriophages in these large-scale experiments was reduced to less than 0.5% in less than 3 weeks of study time with no signs of recidivism (tendency to relapse into the disease). The birds that were experimentally infected received the treatment at two different phage titers. The bacteriophage phi F78E was administered at 10^7 PFU/ml and 10^9 PFU/ml. The results indicated that in experimentally infected birds the reduction in mortality and thereby the success of the phage therapy being administered was dosage-dependent. 10^7 PFU/ml led to a decrease of 25% morbidity and mortality of the chickens while the decrease with 10^9 PFU/ml was 43% (Oliveira et al., 2010).

In a study conducted in 2020 to evaluate both the therapeutic and preventative potential of bacteriophages against Colibacillosis in broiler chickens, *Escherichia coli* O119 bacteriophages were used. The study consisted of 210 one-day-old birds being divided into 7 groups each group containing 10 birds in triplicates (30 birds in total) kept in a biosafety level 2 room for animal use. Before the grouping of chicks, swabs were taken from the experimental room to test for any preexisting Colibacillosis infection. In addition to which internal organs were taken from 10 birds and tested for *E. coli* infection. Faecal testing was also done to determine faecal shedding of *E. coli* both before the start of the experiment and regularly throughout the trial period. *E. coli* O119 challenge was administered to 1-day-old chicks in the form of 0.1 ml oral inoculation of 10^9 CFU per ml. *E. coli* O119 bacteriophages too were administered orally at 0.1 ml of 10^7 PFU per ml. The preventative role was studied by administering phages to the broilers before the administration of the challenge. While the group that was being studied for the therapeutic role was given the bacteriophages as a treatment after the bacterial challenge of *E. coli* had been administered. The mortalities observed in the untreated challenged group, preventative group, and therapeutic group was 30%, 0%, and 10%, respectively. The severity of signs, symptoms, and lesions observed in the treated groups was reduced significantly. No clinical signs were observed in both the therapeutic and preventative group, although within 3 days post-infection the positive control showed signs of respiratory distress, pasty vent, and soft stool. The positive control group observed severe gross lesions including perihepatitis, pericarditis, and airsacculitis. While the groups receiving bacteriophages showed mild lesions which disappeared by the end of the three-week experimental period. This study suggests that bacteriophages have efficacy both as prevention and therapy against Colibacillosis (Sorour et al., 2020).

A study published in the Veterinary Microbiology journal in the year 2013 concluded that although their bacteriophages had shown great potential during *in vitro* testing, the in vivo results were not promising that bacteriophages could be used to treat Colibacillosis infections effectively. PhAPEC2, PhAPEC5, PhAPEC7, and PhAPEC9 bacteriophages were combined to form a phage cocktail after they were proved to be effective against APEC *in vitro*. For the *in vivo* trials, 59 one-day-old birds were divided into 6 groups and were administered with 500 µl of APEC serotype O78 and strain CH2 at 4-weeks of age after being weighed. This administration was done using a feeding needle intratracheally. The cocktail was administered 2 hours post-infection to group 1 intratracheally, group 2 received the cocktail

intraesophageally, and group 3 was administered the cocktail through drinking water. The trials were concluded 7 days post-infection when the birds were euthanized after being weighed. The treated groups did not show a sufficient decrease in morbidity, mortality, or lesion scores. Although the phages could be re-isolated from the dissected birds' body organs, like the heart and the liver. The re-isolated bacteriophages still showed sensitivity to the phage cocktail which showed that resistance had not developed. In addition to this, there was no significant difference in the weights between the untreated challenged group and the groups receiving phage treatments (Tsonos et al., 2013).

3. Methodology

3.1. Phage Spot Assay

In order to check the bacterial sensitivity against the phages PBM 1, 2, & 3, a phage spot assay was performed. Nutrient agar plates were prepared for streaking of APEC O1 bacteria. After overnight incubation of plates at 37°C, a single colony of APEC was picked and added to 30 ml Luria Broth (LB) in a flask. The flask with bacterial colonies was kept overnight i.e. 24 hours in a shaking incubator at 37°C and 120 rpm. After incubation, 100 µl of the bacterial culture was added to a test tube containing 4 ml of semi-solid agar and poured and spread onto LB agar plates. Plates were incubated for 5 minutes at room temperature. 5 µl each of all the phage lysates was spotted on the bacterial lawn by using a pipette. The spots were allowed to dry for 10-15 minutes followed by overnight incubation of plates at 37°C.

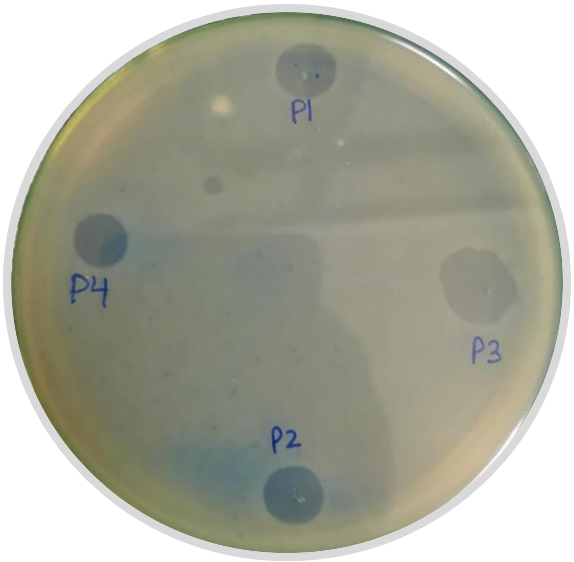

Figure 7 Spots of PBM-1, PBM-2, PBM-3, and PBM-4 on APEC O1 Bacterial lawn grown on LB agar plate to conduct phage spot assay to determine phage lytic viability

3.2. Performing AST

The bactericidal activity of different antibiotics against APEC O1 was checked by the Disk-diffusion method. Isolated APEC O1 colonies grown on Eosin-Methylene Blue (EMB) Agar were collected and suspended in LB Broth. The bacterial suspension was kept in a shaking

incubator at 37°C overnight to let the bacteria multiply. Simultaneously, nutrient agar plates were prepared. The next day APEC suspension was poured on the plates and the antibiotic discs were added. Five discs were added and lightly pressed onto the agar on each plate to prevent displacement of the discs. The plates were then incubated for 24 hours. After the completion of incubation, zones of inhibition also known as the clearance zones were measured using a metal ruler for each antibiotic disc. The larger the zone of inhibition i.e., the diameter of the spot, indicated that the antibiotic was most effective in its activity against APEC O1. The results were recorded (Reller et al., 2009).

3.3. Cocktail Preparation

A phage cocktail was prepared by combining the purified bacteriophages PBM-1, PBM-2, and PBM-3 in equal volume. They were suspended in LB. The density of plaque-forming units (PFU) in the cocktail was determined by calculating the phage titer. A double layer agar assay was performed to calculate the phage titer. The titer of PBM-1 was 1.37×10^{10} PFU/ml, PBM-2 was 2.4×10^{9} PFU/ml, and that of PBM-3 was 5.3×10^{12} PFU/ml. The cocktail was stored at 4°C and used later.

The phage titer was calculated by first forming serial dilutions from 10^{-1} to 10^{-10} of the cocktail in LB. These serial dilutions were then plated. The plates were kept in an incubator for 24 hours at 37ºC. The next day the plaques were counted using a colony counter.

The PFU/ml was calculated by using the following formula:

PFU/ml = No. of plaques / (Volume plated x Dilution factor)

$= 270 / (0.1 \times 10^{-5})$

$= 2.7 \times 10^{8}$ PFU/ml

0.1 ml dose of the above-calculated titer was given per bird

3.4. Bacterial Challenge Dose

According to pre-existing literature, the bacterial challenge to be administered was 10^8 CFU/ ml which is obtained on 0.6 optical density (OD). To obtain an APEC dose equivalent to this, APEC O1 was grown on an agar plate. The inoculum was then transferred from the plate to liquid LB culture media and was put in the shaking incubator at 37ºC. OD was taken on a spectrophotometer repeatedly. After 3.5 hours when a desirable OD was obtained, serial dilutions of the culture were made from 10^{-1} to 10^{-10}. After pouring 0.1 ml onto a plate each, the plates were kept for 24 hours in an incubator at 37ºC. The next day the colonies were counted using a colony counter.

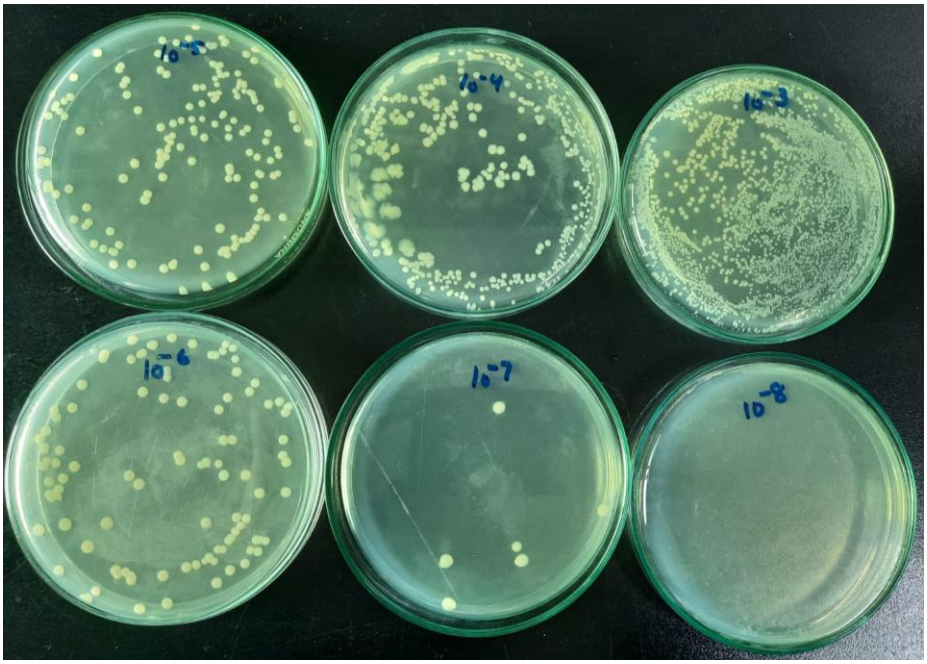

Figure 8 The white dots on the agar plates show APEC O1 colonies ranging from serial dilutions 10^{-3} to 10^{-8}. This was obtained by pouring 0.1 ml of the LB media of the different serial dilutions onto the plates and keeping in a 37°C incubator for 24 hours

The CFU/ml was calculated using the following formula:

CFU/ml = No. of colonies / (Volume of culture plated in ml x Dilution factor)

$= 89 / 0.1 \times 10^{-6}$

$= 8.9 \times 10^8$ CFU/ml

3.5. Antibiotic dose

The commercially used dose of the antibiotic was scaled down according to the number of birds present in the groups and their average weights using the dilution equation (C1V1 = C2V2)

Therefore, 4.5 ml antibiotic was added to 500 ml water and given to the birds present in the groups which were to receive this treatment.

3.6. Experimental Design

A total of 100 birds were divided into 15 groups and kept separate from each other. The conditions of each group were kept constant including humidity, temperature, feed, water, bedding, etc.

The following groups were formed:

1. Negative Control (**NC**) – Untreated and Unchallenged Birds (no bacterial challenge given, and no treatment administered)
2. Antibiotic Control (**AC**) – Unchallenged but given Antibiotic Treatment in water
3. Cocktail Control (**CC**) – Unchallenged but given Phage Cocktail through injection
4. Combination Therapy Control (**CTC**) – Unchallenged, Administered Phage Cocktail through injection and Antibiotic Treatment in water
5. Positive Control (**PC**) – Untreated but Challenged (bacterial challenge administered)
6. Antibiotic Treatment (**AT**) – Challenged, Antibiotic Treatment in water
7. Combination Therapy Both in Water (**Comb [Both H]**) – Challenged, Antibiotic Treatment and Phage Cocktail both given in water
8. Combination Therapy Antibiotic Water, Cocktail Injection (**Comb [Both H + I]**) – Challenged, Antibiotic Treatment in water and Phage Cocktail administration through injection
9. Preventative (**Preventative**) – Challenged, Phage Cocktail given before the administration of the bacterial challenge
10. Subcutaneous 0 hours (**SC 0hrs**) – Challenged, Subcutaneous Phage Cocktail administered immediately after challenge administration

11. Subcutaneous 24 hours (**SC 24hrs**) – Challenged, Subcutaneous Phage Cocktail administered 24 hours after challenge administration
12. Nasal Drops 0 hours (**Nasal 0hrs**) – Challenged, Nasal Drops Phage Cocktail administered immediately after challenge administration
13. Nasal Drops 24 hours (**Nasal 24hrs**) – Challenged, Nasal Drops Phage Cocktail administered 24 hours after challenge administration
14. Intramuscular 0 hours (**IM 0hrs**) – Challenged, Phage Cocktail administered through intramuscular injection immediately after challenge administration
15. Intramuscular 24 hours (**IM 24hrs**) – Challenged, Phage Cocktail administered through intramuscular injection 24 hours after challenge administration

3.6.1. Mode of Administration

The bacterial challenge was administered through subcutaneous injection on the head. The subcutaneous injection was administered on the head of the chicks, while the intramuscular injection was given in the muscle of the thigh region. Nasal drops were administered in liquid form through the nose of the birds. While the antibiotic was added directly to the drinking water.

3.7. Faeces Testing

To check the presence of APEC in uninfected birds before the start of the animal trials, 10 fresh faecal samples of test birds were collected. Individual samples were added to falcon tubes and suspended in LB broth. Falcon tubes containing the samples were incubated overnight in a shaking incubator at 37°C. Incubated faecal samples were streaked on EMB. The streaked plates were incubated overnight and checked for bacterial growth the next day. *E. coli* gives a distinctive green metallic sheen on EMB which was not observed on streaked plates showing the absence of APEC in the uninfected test birds.

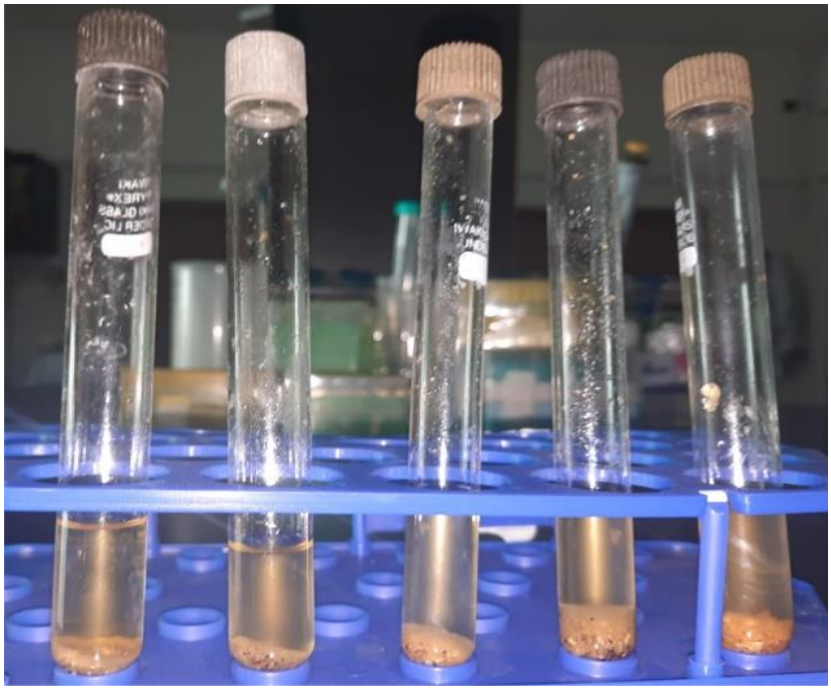

Figure 9 Falcon tubes containing the faeces inoculated in Luria Broth. The faeces were collected before the start of the animal trials, to check for the presence of APEC in the uninfected birds

3.8. Weight Measurement

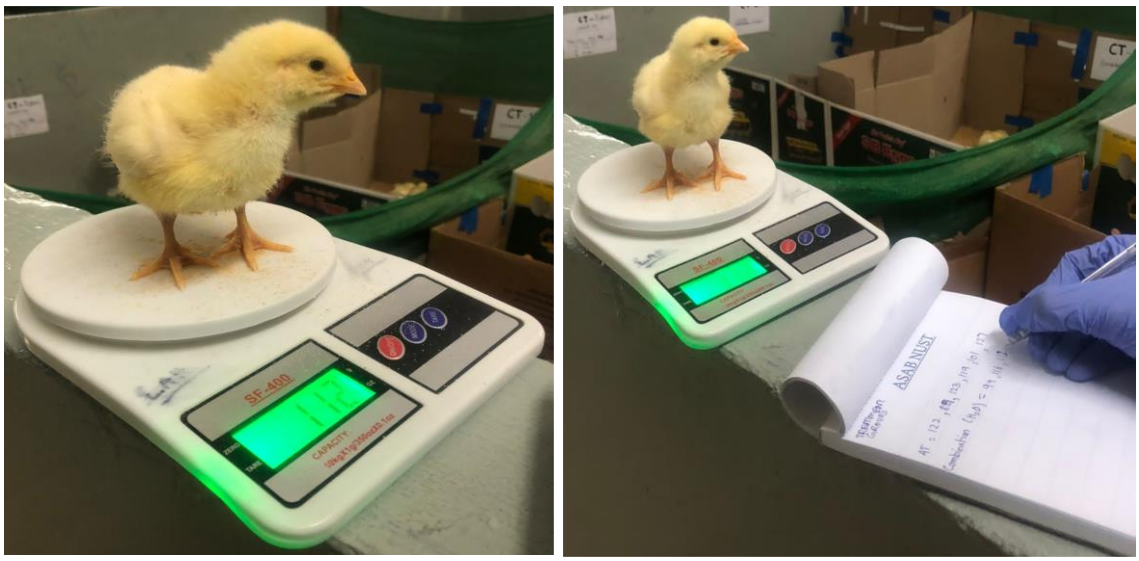

Figure 10 Measuring the Weight of the Chicks on a digital weighing scale, starting from the controls and then moving on to the experimental birds to prevent any cross-contamination

The birds were weighed individually every morning throughout the whole experimental period. The weighing process started with the Control groups to avoid any cross-contamination. The digital weighing scale was then disinfected with ethanol and the experimental birds were weighed. Every bird in each group was weighed daily. Averages of each day for each group were calculated and have been shown graphically in the Results section of this document.

3.9. Dissections

To study organ damage and disease progression, dissections of the test birds were carried out regularly when any mortality was observed. The major organs that were observed were the trachea, the heart, the liver, and the lungs. The ideal time to dissect a dead bird is before rigor mortis sets. A dissection kit consisting of scalpels and blades, dissecting scissors, forceps, probes and pins. A dissection board or tray, gloves, and masks are required.

The bird is laid on its back and its limbs are pinned to the board. The trachea is the first organ to be observed. Use forceps to hold out the skin and cut with dissecting scissors laterally to observe if the trachea is clear or not. It is held out and a picture is taken with the label of the bird. The next step is to cut the dermal layer on the chest of the bird and expose the heart under the muscle. The heart is checked for pericarditis or any other morphological signs of disease. The liver is also checked for yellowing and lesions. The lungs are the very last organ that is checked and removed. A large falcon is filled with formalin and all organs are suspended in it. The falcons are kept in an icebox until they can be stored in a refrigerator. It is important to label all the samples correctly for future histopathological analysis or the streaking for bacteria. The dissected bird is wrapped in airtight foils and put in a biohazard red bag to be properly discarded away. The dissecting station is cleaned with 70% Ethanol. The tools were cleaned and then autoclaved for the next use.

3.10. Lesion Scoring

Lesion scoring of the dissected organs including the trachea, the heart, and the liver was performed to access the severity of Colibacillosis. The dissected organs were observed with

the naked eye as well as with the help of a magnifying glass. Each of the three organs was given a score between 0 to 2, depending on the presence and absence of lesions. A score of 0 was given when no lesions were observed. A score of 1 indicated mild cloudiness and the presence of minor fibrinous exudate. A score of 2 was given when a large amount of fibrinous exudate was observed over the hepatic surface and the pericardium. A very cloudy air sac was given a score of 2. An average of the total scores of the birds in all the groups was calculated and graphs were made accordingly.

The method adopted for the lesion scoring is summarized in the following table:

Table 1 Lesion Scores corresponding to the observable state of the Trachea, the Heart, and the Liver

Organ	Score	Lesions
Trachea	0	No Lesions
	1	Mild Cloudiness
	2	Very Cloudy or Inflammation
Heart	0	No Lesions
	1	Small amount of fibrinous exudate over pericardium surface
	2	Large amount of fibrinous exudate over pericardium surface
Liver	0	No Lesions
	1	Small amount of fibrinous exudate over hepatic surface
	2	Large amount of fibrinous exudate over hepatic surface

4. Results

4.1. Phage Spot Assay

A phage spot assay was performed to check the viability and the bactericidal activity of the isolated phages PBM-1, PBM-2, and PBM-3 against the APEC O1 strain by placing a 5 µl drop of phages on a Petri plate inoculated with the bacterium. Phage spots were assessed based on the magnitude of their activity against the bacterial culture. Full activity is indicated by no bacterial growth within the spot, partial clearance is indicated by a visibly turbid spot or individual colonies within a clear spot. No visible clear spots are regarded as No activity. Significant clearance zones or zones of inhibition were observed for all phages indicating full activity of PBM-1, PBM-2, and PBM-3 against APEC-O1.

PBM-4, an isolated phage not utilized in our experiment, was also checked for its viability and bactericidal activity through phage spot assay.

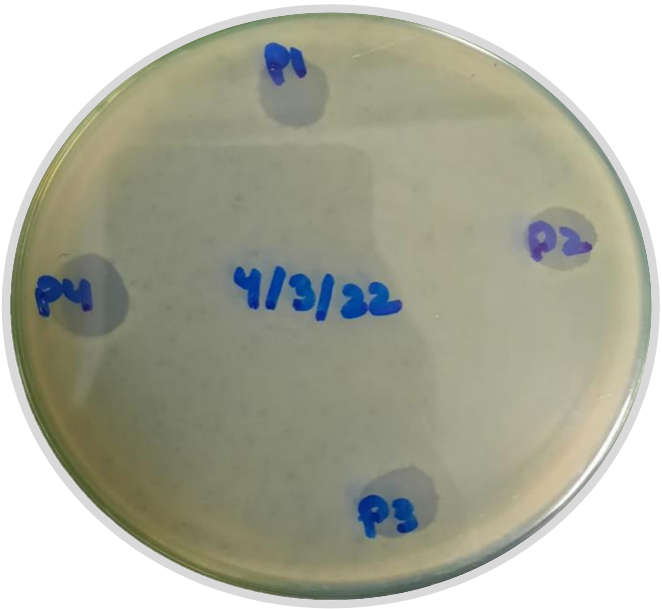

Figure 11 Spots showing the zones of inhibition of PBM-1, PBM-2, PBM-3, and PBM-4 obtained by performing phage spot assay on an APEC O1 bacterial lawn.

4.1.1. Phage Cocktail Spot Assay

The two phage cocktails formed were named ‘A’ and ‘B’ for the sake of *in vitro* spot assay and phage titer determination. Cocktail ‘A’ included phages PBM-1, PBM-2, and PBM-3. While phage cocktail ‘B’ was a combination of the phages PBM-1, PBM-2, PBM-3, and PBM-4 in equal ratios (volumes). Our spot assay and phage titer results indicated that cocktail ‘A’ worked better, therefore, this was the one that was taken further for the analysis and *in vivo* trials.

Figure 12 Spots ‘A’ and ‘B’ showing the zones of inhibition of the two cocktail combinations. The spot obtained from Cocktail ‘A’ shows a wider and clearer zone of inhibition than that of Cocktail ‘B’

4.2. Antibiotic Susceptibility Test

Antimicrobial testing of the APEC O1 bacterium was performed using the disk diffusion method to determine its antibiotic resistance and susceptibility. Zone of inhibition diameters were measured using a metal centimetre scale to determine the diameter which shows antibiotic activity.

Larger zones of inhibition or clearance zones show that the antibiotic is effective against the bacterium and APEC O1 is not resistant to it. While no zone of inhibition shows that the bacterial colonies continued to grow in that region, so the bacterium is resistant to that

particular antibiotic, and it has no inhibitory effect. While a clearance zone or zone of inhibition having a small diameter shows that the antibiotic has an intermediate effect on the bacterial colonies present.

Table 2 Zones of Inhibition/Clearance Zones of various Antibiotics against APEC O1

Antibiotic	Clearance Zone
AMP-10	No Zone
TE-30	7 mm
DO-30	9 mm
AMC-30	No Zone
C-30	25 mm
CRU-30	17 mm
ETP-10	30 mm
MEM-10	14 mm
TZP-110	25 mm
TOB-10	15 mm
CN-10	18 mm
AK-30	21 mm
IPM-10	15 mm
F300	19 mm
S10	1 mm
CIP-5	18 mm
NOR-10	11 mm
LEV-5	17 mm
SXT-25	No Zone
CTX-30	19 mm
CAZ-30	30 mm
FOX-30	20 mm
FEP-30	27 mm
OFX-5	13 mm
E-15	8 mm
AML-10	No Zone

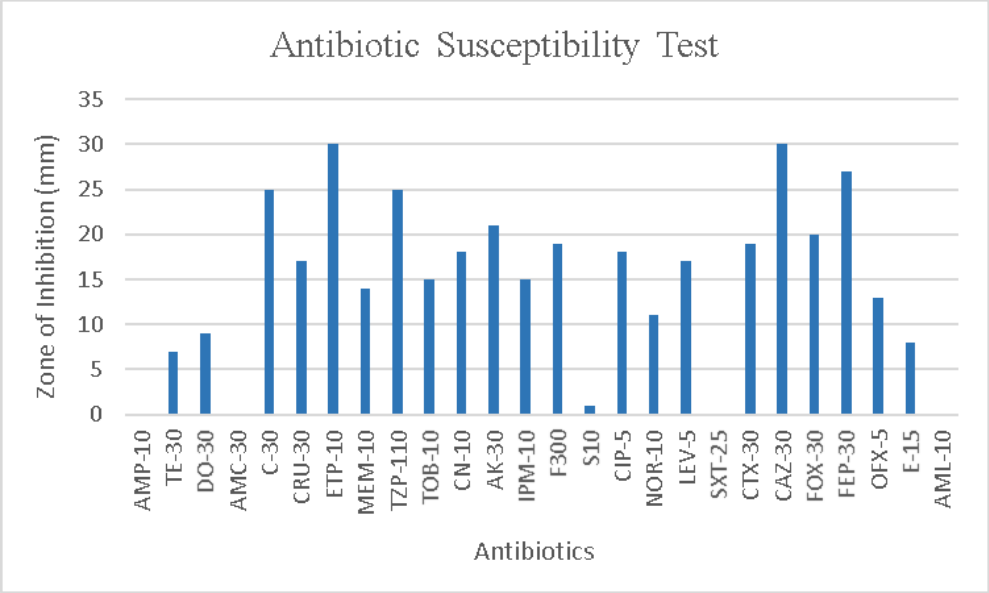

Figure 14 Clearance Zones (mm) of different Antibiotics observed on the APEC O1 lawn grown on an agar plate measured using a centimetre metal scale to determine the antibiotic susceptibility of APEC O1 to various commercially used antibiotics

Our assay showed that the APEC O1 bacterium was completely resistant to Sulfamethoxazole/Trimethoprim 25 mg (SXT-25), Amoxicillin/Clavulanate 30 mg (AMC-30), Ampicillin 10 mg (AMP-10), and Amlodipine besylate 10 mg (AML-10) antibiotics since no clearance zone or zone of inhibition was formed. APEC O1 also showed resistance to Streptomycin 10 mg. It also demonstrated significant resistance against Tetracycline 30 mg, Doxycycline 30 mg, and Erythromycin 15 mg.

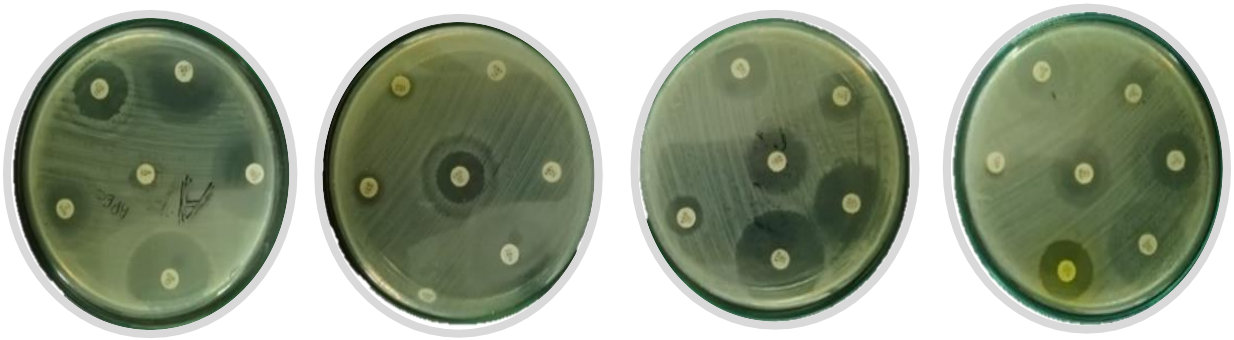

Figure 13 Antibiotic Susceptibility Test Results obtained using the Disc Diffusion method showing the zones of inhibition of various antibiotics against APEC O1

4.3. Weight Gain

The weights of the birds measured across the experimental period indicated that the birds, once challenged, underwent weight loss, or did not show any significant weight gain. During the early growing period the weight of the birds normally doubles every day, therefore, the effectiveness of the treatment was also analyzed by observing the weight trend. The challenge was administered on day 14.

4.3.1. Control Groups - Average Body Weight

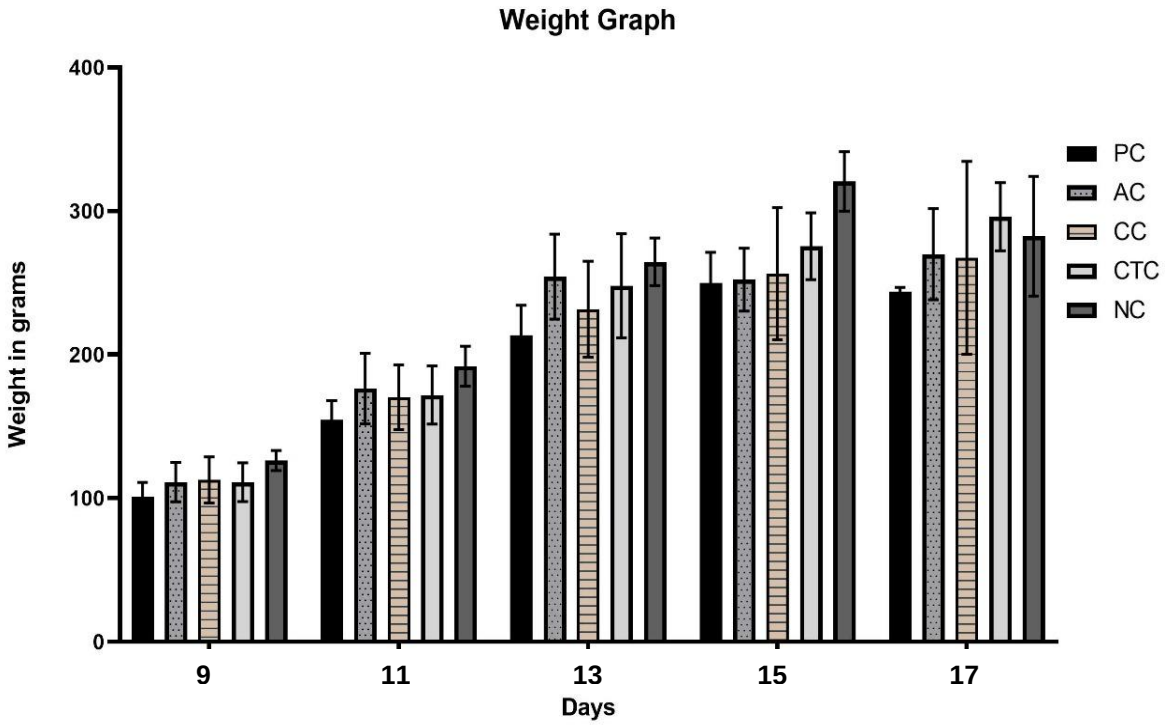

Figure 15 The Average Body Weight Graph of All Control Groups. The weight is displayed in grams on the x-axis and the days are shown on the y-axis. The challenge was administered on day 14

As the negative control was administered with no bacterial *E. coli* challenge and also did not receive any treatment, it depicts the average body weight of birds that exists naturally. The remaining groups show significant variation. The positive control, receiving bacterial challenge but no treatment, has the most drastic average body weight trend. As the mortality within the

first 24 hours was 83.333% and the average body weight of the remaining decreased significantly and the weight gained per day afterwards was also very low.

4.3.2. Preventative Group compared with Antibiotic Treatment

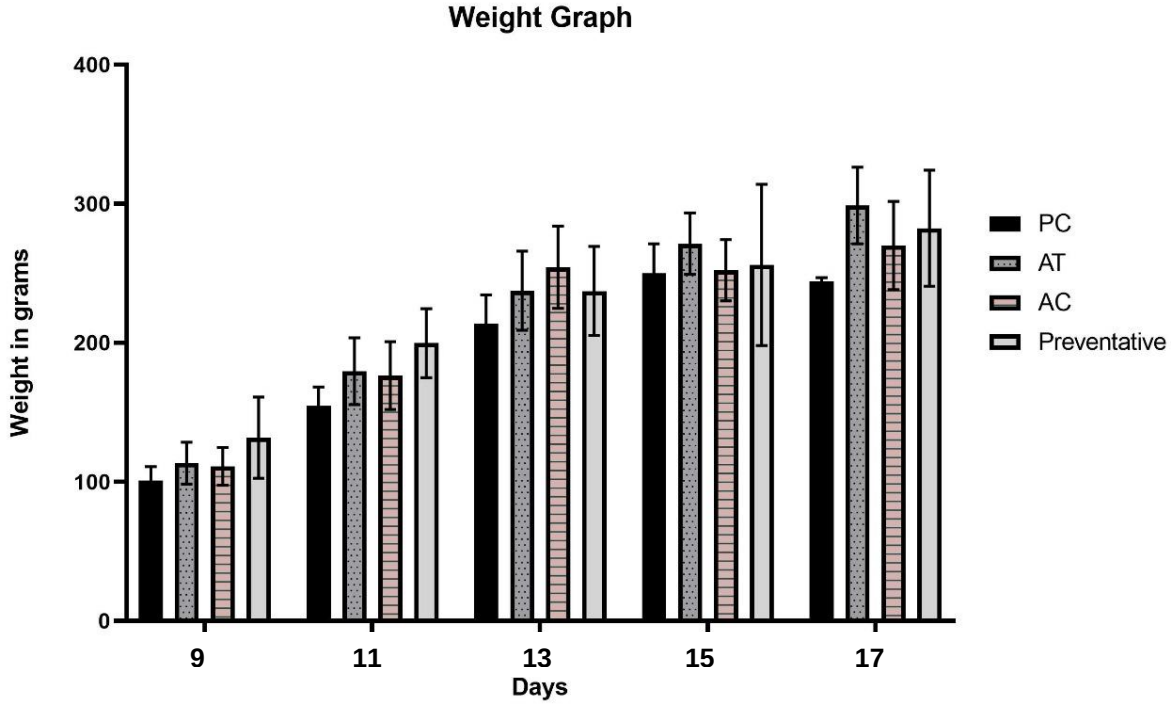

Figure 16 Average Body Weight Graph showing the Prophylactic effect of the Phage Cocktail compared to the Antibiotic Treatment. The Positive Control and the Antibiotic Control are also shown for reference

A significant overlap was observed between the average body weights of birds in our preventative group and the antibiotic treatment group. Both followed the same trend after the administration of the bacterial challenge. This suggests that the phage cocktail when administered for prophylactic effect had the same efficacy as antibiotic – enrofloxacin treatment that is already in use. The group administered a phage cocktail as a preventative measure i.e., to prevent Colibacillosis from occurring post administration of *E. coli*, also showed a gain in average body weight indicating that the ability to fight the infection was significant and the birds were healthy enough to gain weight normally.

4.3.3. Combination Therapy Groups

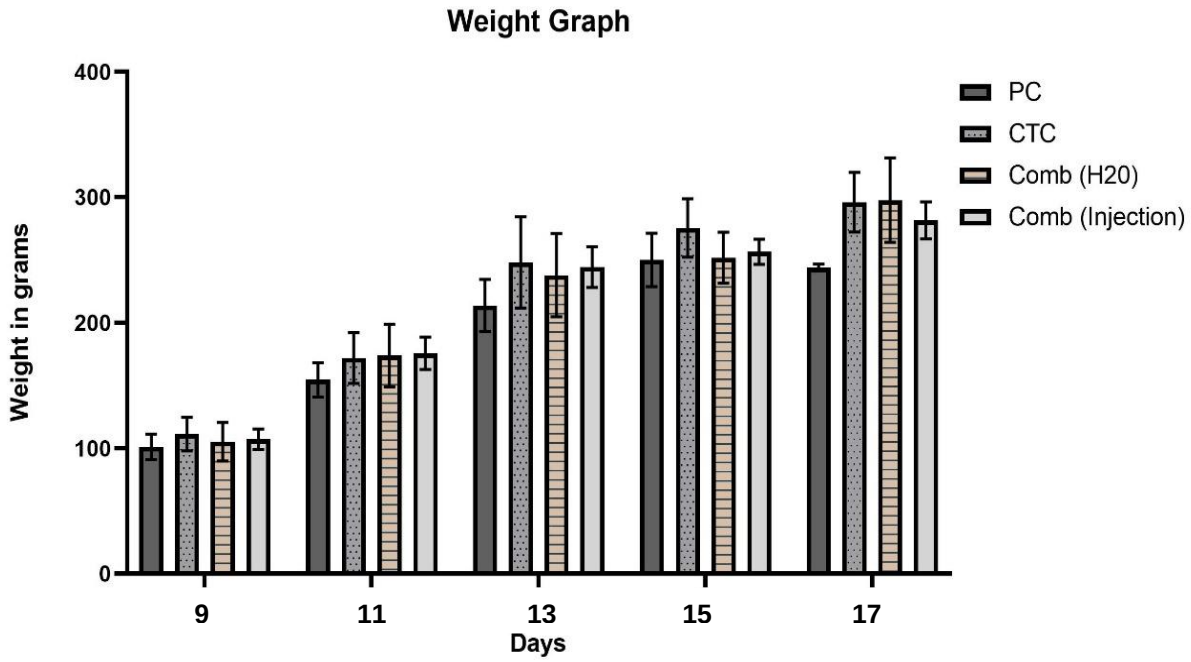

Figure 17 Average Body Weight Graph of the Combination Therapy (Phage Cocktail + Antibiotic) given through injection and through water at day 6 after administration of bacterial challenge of APEC O1. The x-axis shows the days, while the y-axis displays the weight of the chicks measured in grams

A similar trend was observed for the combination therapy control and both the combination therapy experimental groups where a cocktail was administered through injection and antibiotic in water and where cocktail and antibiotic were both given in water. There was a complete overlap between the three groups and no loss in the average body weight which indicates that combination therapy, both administered orally through water and through injection, is effective in treating an APEC-induced Colibacillosis infection.

4.3.4. Phage Cocktail Administered via Different Routes

Amongst the groups that were administered the phage cocktail treatment through various routes, Intramuscular 0 hours proved to be the most effective as the average weight gain increased significantly post-infection which can be seen in the graph. This indicates that this treatment method significantly decreased bacterial infection. On the other hand, this graph

shows that the phage cocktail when administered after 24 hours via the subcutaneous route had the least efficacy and there was no increase in the average body weight of the group.

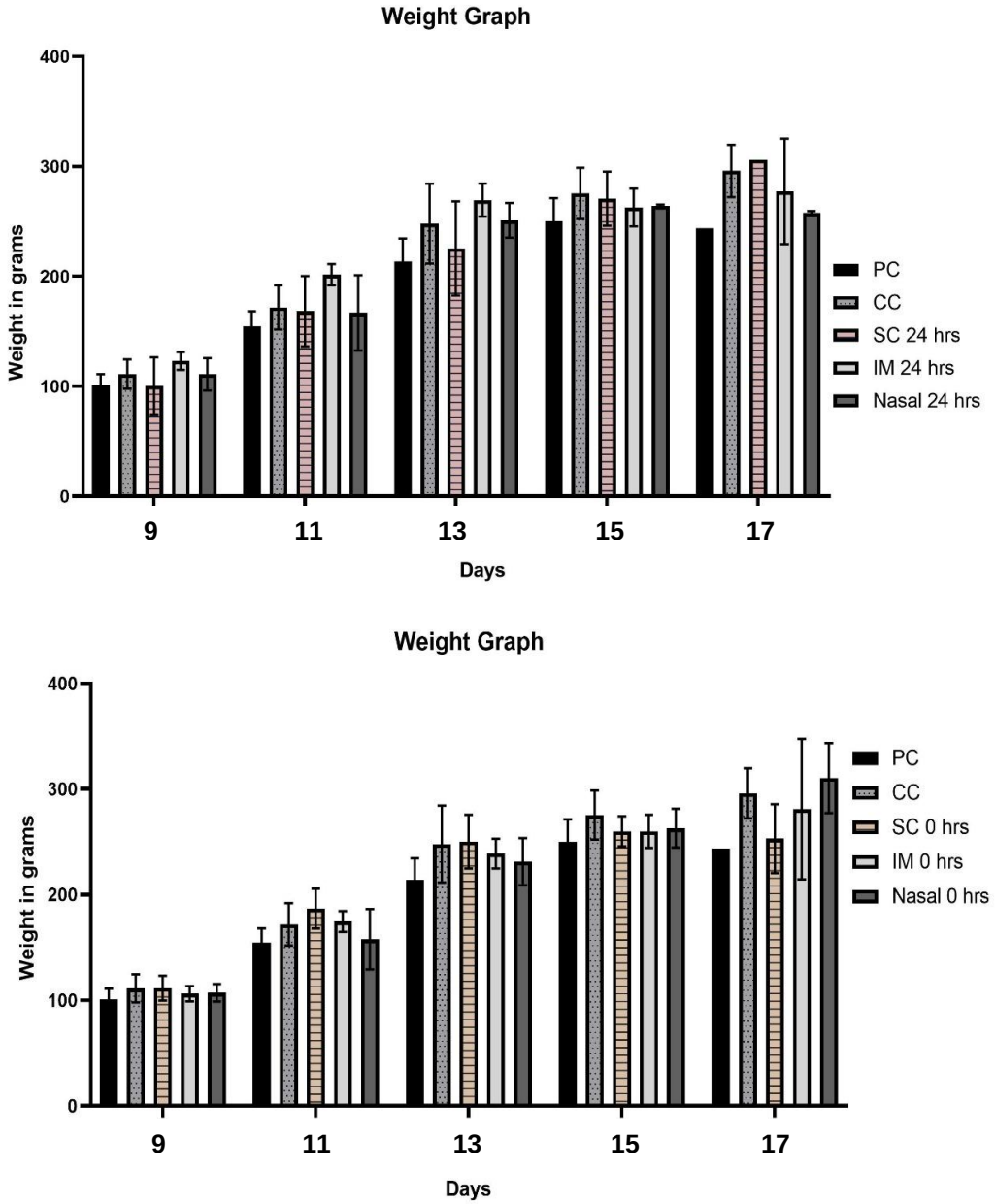

Figure 18 Average Body Weights in grams of the Groups Administered Phage Cocktail Treatment via Different Routes at different time intervals including the Cocktail Control and the Positive Control groups shown for reference

4.4. Mortalities

At the end of the experimental period, the positive control showed 83% mortality while no mortalities were observed in the remaining control groups i.e., the negative control, the antibiotic control, the cocktail control, and the combination therapy control groups.

4.4.1. All Groups Survival Percentage

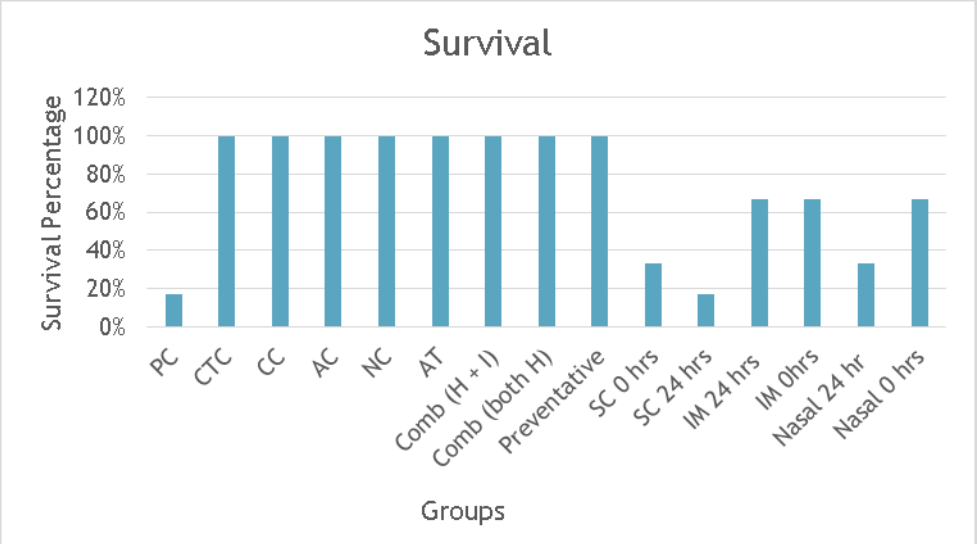

Figure 19 Survival Percentage of All Groups with the groups displayed on the x-axis and the percentage on the y-axis

Amongst the experimental groups that were challenged, and treatment was also administered, no mortalities were observed in the antibiotic treatment group, the combination therapy group that had both treatments administered through water, the combination therapy group that was given a cocktail as an injection while the antibiotic was given in water, and the preventative group.

Percentage of Survival in Cocktail Treatment Groups

Amongst the 6 groups administered cocktail treatment via different routes, Intramuscular injection proved to be the most effective with the mortality being reduced to 33% for both the injection at 0 hours and 24 hours. The mortality percentage in the group given nasal drops at 0 hours was reduced to 33%, while for the group given nasal drops 24 hours after the administration of challenge was reduced to 67% when compared to the 83% mortality in the positive control (challenged but untreated group). Subcutaneous injection administered immediately following administration of challenge reduced the mortality to 67% but when administered to a group at the 24 hours i.e., one day mark did not manage to reduce the mortality even slightly and the mortality was observed to be 83% which was the same as the positive control.

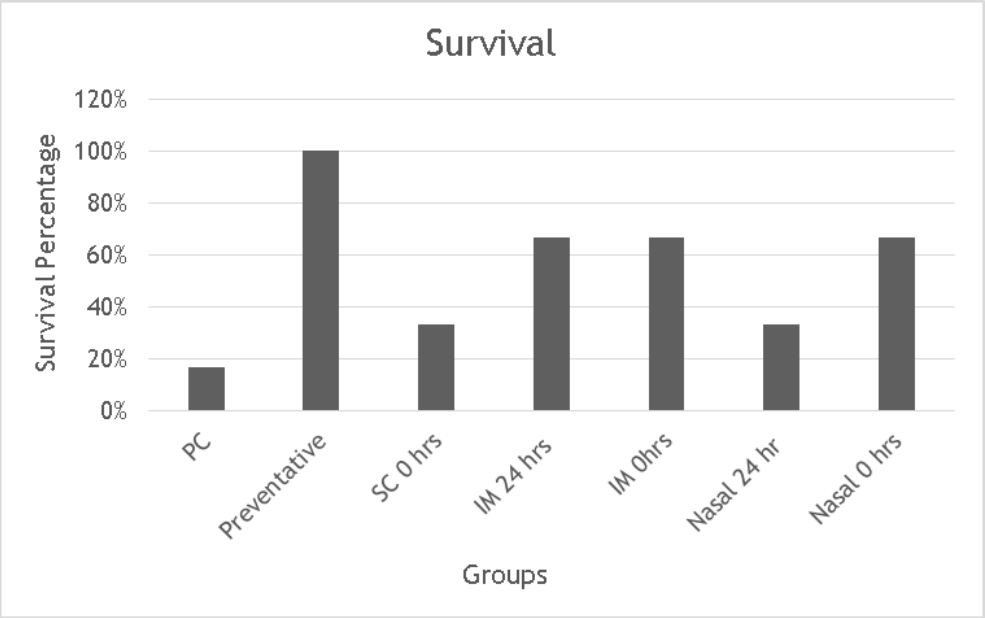

Figure 20 Percentage of Survival in Groups Administered Cocktail Treatment via Different Routes including subcutaneous injection, intramuscular injection, and nasal drops at two different time marks i.e., 0 hours and 24 hours. The name of the groups mentioned on the x-axis and the percentages on the y-axis

4.5. Gross Lesions

The lesion scores for the trachea, the heart, and the liver were calculated that demonstrated air sacculitis, pericarditis, and perihepatitis, respectively. With 6 being the highest possible score and 0 being the lowest. All control groups excluding the positive control had an average lesion score of 0.

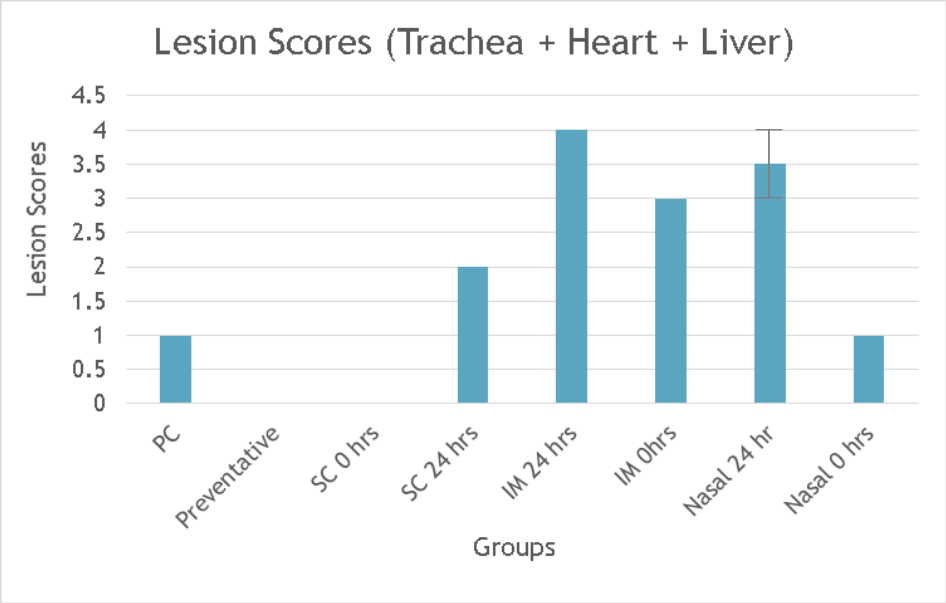

Figure 21 The Lesion Scores of The Trachea, The Heart, and The Liver. The y-axis shows the group names while the x-axis shows the average lesion scores

The average lesion score of the positive control was 1. The Intramuscular injection 24 hours group had an average lesion score of 4, followed by Nasal drops 24 hours at 3.5, and Intramuscular 0 hours at 3. These were high lesion scores and most birds demonstrated all three conditions i.e., pericarditis, perihepatitis, and air sacculitis. Subcutaneous injection 24 hours had an average of 2, and Nasal drops 0 hours with an average lesion score of 1. The subcutaneous injection administered at 0 hours group showed no gross lesions and the average was 0. Similarly, the group administered the phage cocktail as a preventative before the administration of the challenge too showed an average lesion score of 0 with no gross lesions observed.

4.5.1. Lesion Scoring of different groups

Figures 6 to 13 show the dissected organs of different groups on which the lesion scores were observed.

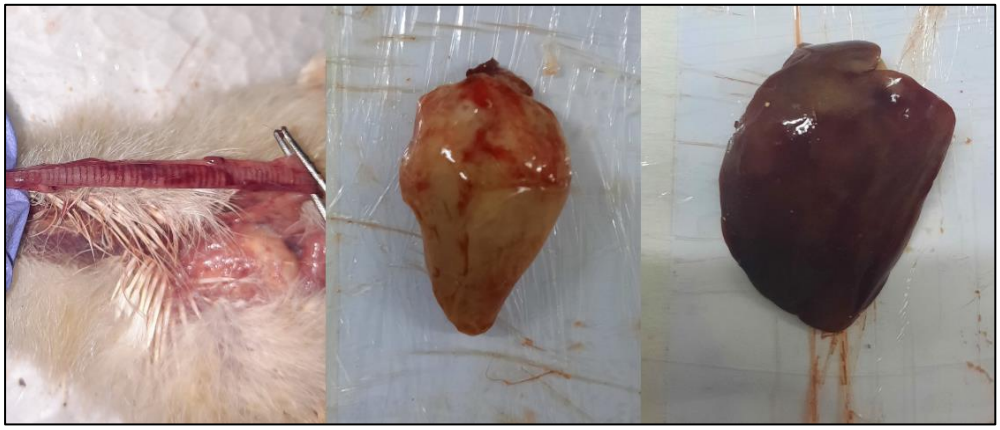

Figure 22 Dissected Organs (Trachea, Heart, and Liver) of the Positive Control

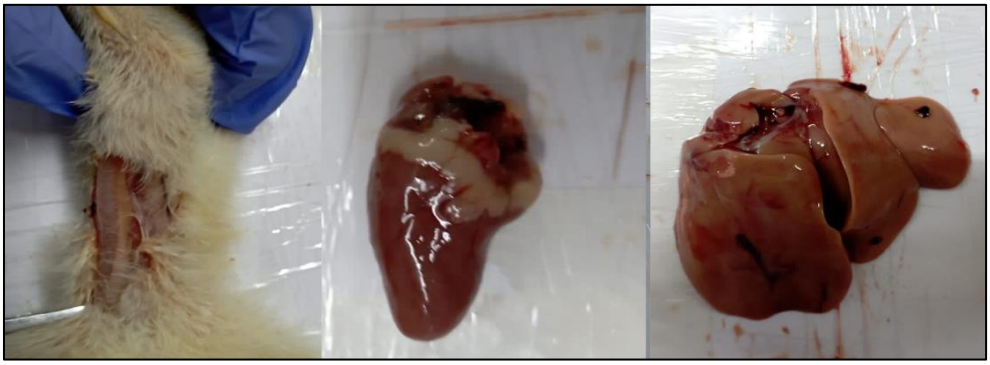

Figure 24 Dissected Organs of the Preventative group

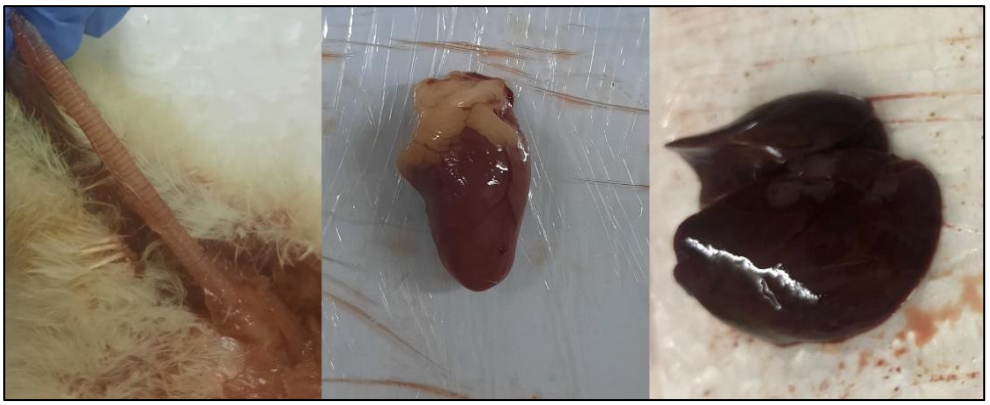

Figure 23 Dissected Organs of SC 0 hrs.

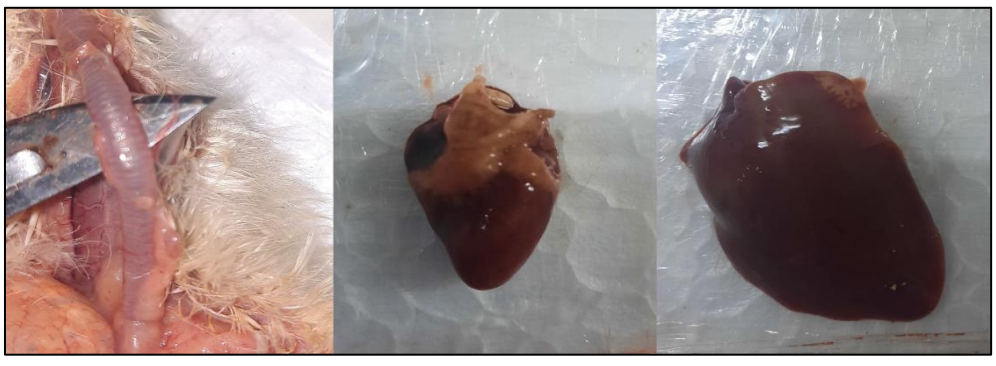

Figure 25 Dissected Organs of SC 24 hrs.

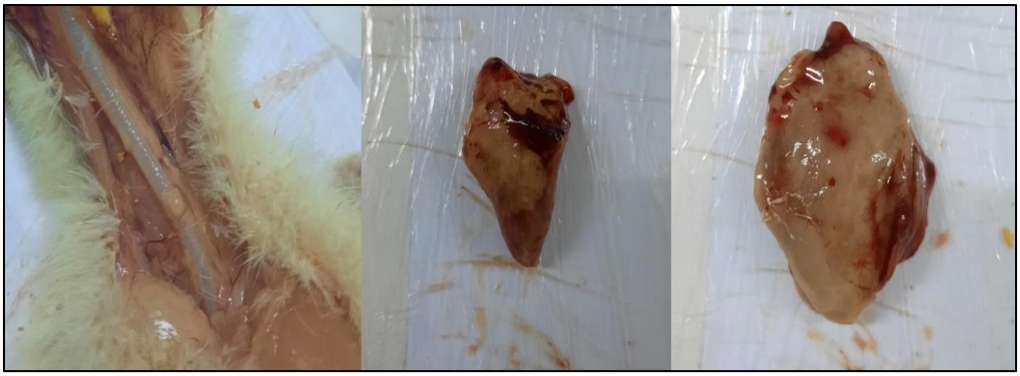

Figure 28 Dissected Organs of IM 0 hrs.

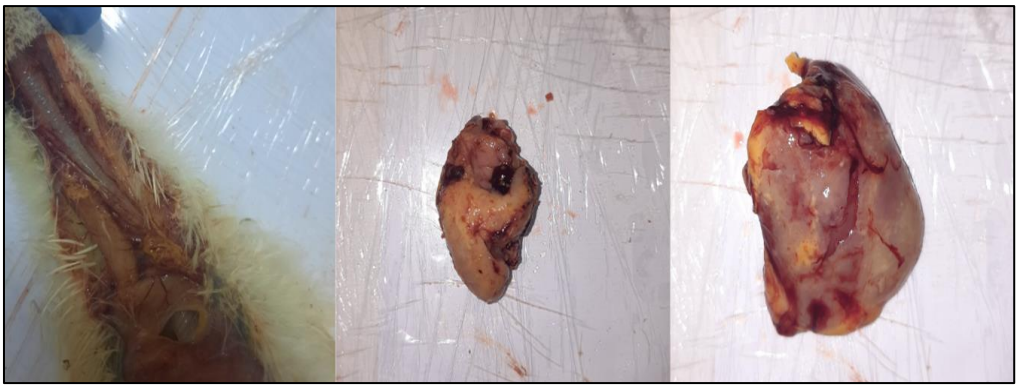

Figure 26 Dissected Organs of IM 24 hrs.

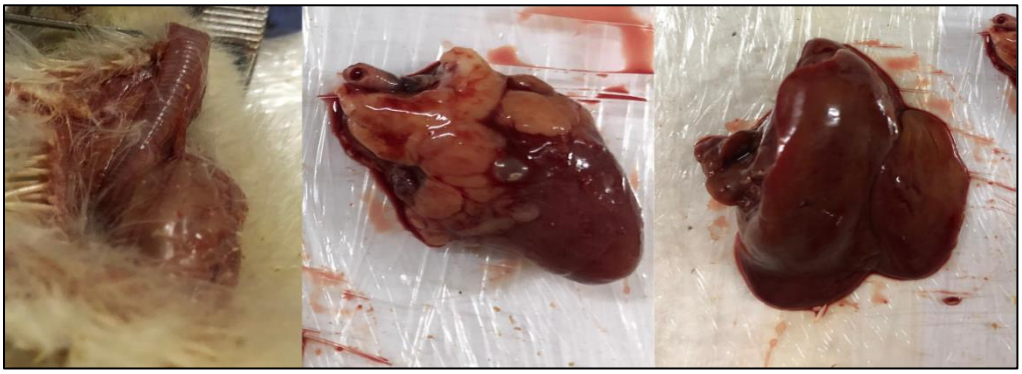

Figure 27 Dissected Organs of Nasal Drops 0 hrs.

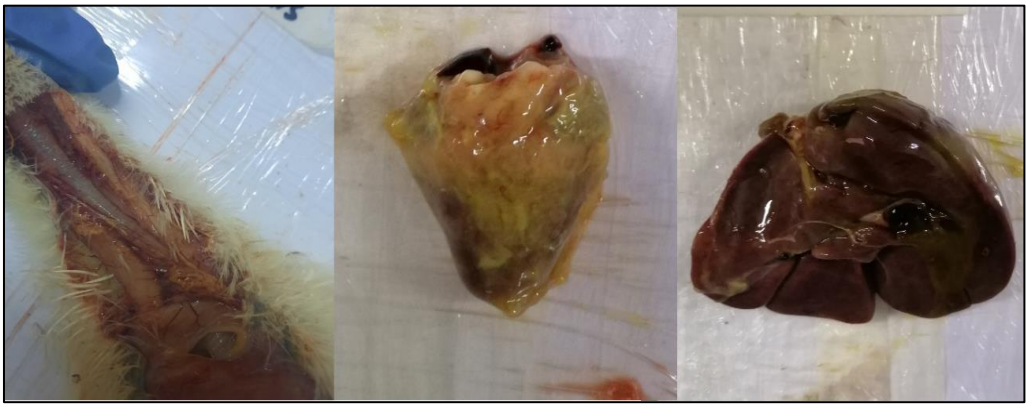

Figure 29 Dissected Organs of Nasal Drops 24 hrs.

4.5.2. Survivability and Lesion Scores

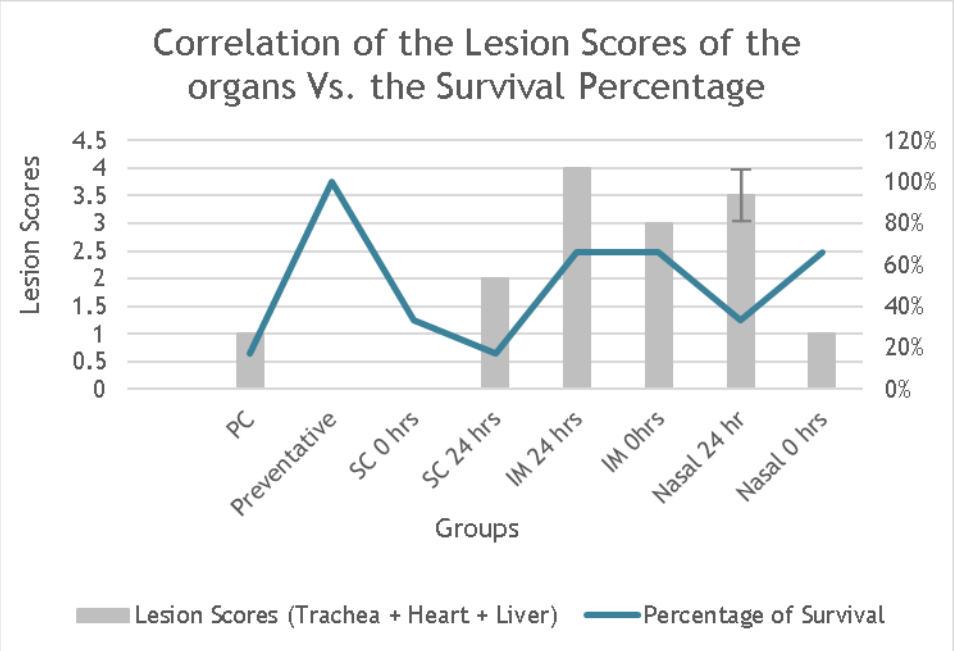

Figure 30 Relationship between the Lesion Scores and the Percentage of Survival. The lesions scores of different groups are shown as bars with the percentage of survival shown as a line graph

The survival percentage observed in the positive control was 17% with an average gross lesion score of 1. Compared to this it was observed that the survival percentage was high in the preventative group and the nasal drop 0 hours group being 100% and 66%, respectively. This was in accordance with their low average lesion scores. Although the average lesion score for the subcutaneous 24 hours group was 2 which is a low score, the group had a survival percentage of only 17% with the mortality percentage as the untreated positive control at 83%. The subcutaneous 0 hours group showed an average lesion score of 0 but had a survival percentage of 33% only which was low as compared to other treatment routes. While both the intramuscular 0 hours and 24 hours showed a high number of survivors with the percentage being 66% for both, although they had shown high average lesion scores of 3 and 4, respectively. Nasal drops 24 hours showed a high average lesion score of 3.5 and had a low survival percentage of only 33%.

5. Discussions

Antibiotic susceptibility of APEC-O1 in response to multiple commercially available and used drugs was tested. The results of Antibiotic susceptibility testing confirmed the resistance of APEC-O1 reported in literature against Sulfamethoxazole/Trimethoprim (SXT-25), Amoxicillin/Clavulanate (AMC-30), Ampicillin (AMP-10), and Amlodipine besylate (AML-10) demonstrated by no clearance zones on the bacterial cultures (Kabir, 2010). The full bacterial clearance shown by Enrofloxacin and PBM-1, PBM-2, and PBM-3, individually, shows their bactericidal activity significant enough to be used against APEC O1.

The average body weight of all groups calculated over the span of the entire experimental period indicated that post-administration of bacterial challenge birds do not gain any significant weight which was seen clearly in the positive control. While the negative control showed the average body weights of unchallenged untreated birds. The increase in the body weight should have been 130g/day which was clearly not observed in the Positive control (Zuidhof et al., 2014).

Our results indicated that the phage cocktail when administered as a preventative against APEC-induced Colibacillosis infection was as effective as the enrofloxacin antibiotic treatment which can be seen by the overlap between the two in the graph. Combination therapy, administered both through water and through subcutaneous injection with antibiotics in water, had a similar weight trend to the preventative and antibiotic treatment groups. This shows that combination therapy, too, is an effective treatment in controlling APEC-induced Colibacillosis. The efficacy of combination therapy has been reported by Huff et al. (W. E. Huff et al., 2004). Its working is backed by the tradeoff principle whereby bacteria can stay resistant to antibiotics or bacteriophages, even losing their virulence (Yang et al., 2020).

Among the experimental groups that were administered phage cocktail via various routes, Intramuscular 0 hours was the most effective which was seen by the significant average weight gain post-infection similar towards the end to what was seen in the negative control and other control groups that had not been administered a bacterial challenge. The therapeutic effect of phage cocktails through intramuscular injections has been reported in the literature. Different studies have also reported that phages administered by water have also shown to be significant in their therapeutic potential (W. E. Huff et al., 2004). The subcutaneous route of administering

the phage cocktail proved to be ineffective in treating the bacterial infection as the graph levelled out and there was no significant average weight gain. It resembled the most to the positive control average weight graph with subcutaneous 24 hours having the least effectivity. Nasal drops 0 hours showed a similar curve to the Intramuscular 24 hours group indicating that they both had similar efficacy in treating the infection. While nasal drops 24 hours although depicting some effectiveness, did not show a significant enough trend to be classified as an effective treatment based on the group's average body weight (Lau et al., 2010).

The data obtained over the period of the animal trials showed that no mortalities occurred in the following four groups i.e., Preventative, Combination therapy (Phage cocktail administered via subcutaneous route), Combination therapy (Phage cocktail administered through water) and Antibiotic therapy. The highest mortalities were observed in Positive control and subcutaneous 24 hours treatment groups. From the results and prior studies conducted to determine the prophylactic potential of bacteriophages, we can suggest that Intramuscular injection shows to be the most effective mode of administration (Johnson et al., 2008).

The results obtained from treatment administered after various periods of time of the challenge administration suggest that Preventative therapy works most efficiently. No mortality was observed in birds that were given bacteriophages as prevention prior to infection with APEC O1. Huff et al. and Barrow have independently reported the successful use of bacteriophages to prevent *E. coli* respiratory and systemic infections (W. Huff et al., 2002). Nasal drops administered at 0 hours and intramuscular injections at 0 hours and 24 hours showed fewer mortalities as compared to subcutaneous injections given at 0 and 24 hours (Barrow, 2001).

Antibiotics are extensively used as prophylactics in poultry. The extensive use of antibiotics leads to the bacteria becoming resistant to the drugs. Alternative strategies to keep the mortality low and yield high have been explored. Among these alternatives has been the use of bacteriophages. Bacteriophages showed equally effective antimicrobial activity against APEC-O1 *in vitro*. Their efficacy was confirmed in animal testing and reported in the literature (W. Huff et al., 2002). It was observed that the prophylactic activity of phages and antibiotics was equally efficient to prevent Colibacillosis in poultry. No mortalities were observed in the groups given phages for prevention.

Using bacteriophages in tandem with antibiotics to treat Colibacillosis proved to be effective. Antibiotic Enrofloxacin was given in water while the phages were tested through different routes i.e., water and injection. No mortality was observed in any group that was administered with a combination therapy of antibiotics and bacteriophages. This can prove critical in countering Anti-Microbial Resistance against Colibacillosis because of the trade-off principle whereby the bacteria can be resistant to either of two antimicrobials: bacteriophages or antibiotics.

The degree of organ damage by Colibacillosis was determined using lesion scoring. Birds in positive control showed low lesion scores and a 100% mortality rate within 24 hours. Similarly, both subcutaneous groups 0 and 24 hours showed scarce lesions on body organs but high mortality rates. Low lesion scores and sudden deaths in subcutaneous and positive control are a result of extremely acute septicemic infection which occurs when APEC enters the bloodstream. These results can be backed by two previous studies reported in the Journal of Poultry Science (Lau et al., 2010) (El-Gohary et al., 2014).

The groups with the intramuscular mode of injection at 0 and 24 hours had lesions scores of 3 and 4 respectively. High lesion scores and low mortality rate indicates that the birds did not develop acute septicemia. Despite the high lesion scores, the birds survived with no physical symptoms of Colibacillosis. This suggests that the phage cocktail administered through the intramuscular route prevented sudden deaths due to blood septicemia. The prophylactic group had zero lesion score and no mortality suggesting that the phage cocktail shows the best results when given before infection. The results of prevalent and prior animal testing have shown that prevention and treatment of Colibacillosis through bacteriophages can be a promising alternative to counter AMR (Lau et al., 2010).

Conclusion

Data obtained from survivability, lesion scoring, and weight gain of the test birds suggests that using bacteriophages for prevention and in combination with antibiotics for treatment is the most effective way to control the spread of Colibacillosis as no mortalities were observed in these groups. Phages administered intramuscularly treated APEC infection better as compared to other routes of administration i.e., nasal, and subcutaneous. The significant efficacy of these treatments can be used as a tool to fight emerging APEC Antimicrobial Resistance.

Future Prospects

No new antibiotics have been developed since 1987 and keeping in view the emerging antimicrobial resistance, the poultry industry can potentially face the challenge with Enrofloxacin. This study used three isolated bacteriophages, PBM-1, 2, and 3, in the cocktail to prevent and treat Colibacillosis. Intratracheal mode of administration for bacteriophage cocktail can be put into *in vivo* trials for therapeutic potential against APEC along with the intramuscular and nasal routes (Lau et al., 2010). More bacteriophages against APEC can be isolated and optimized into a cocktail. Being prepared for the looming danger of AMR against Enrofloxacin can help save the second largest industry of Pakistan.

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