United Arab Emirates University
Scholarworks@UAEU

Theses | Electronic Theses and Dissertations

6-2014

Bacteriophage Therapies as Alternatives to Antibiotics in Chicken Feed In the United Arab Emirates

Wafa Awad Al Shamsi

Follow this and additional works at: https://scholarworks.uaeu.ac.ae/all_theses

Part of the Environmental Sciences Commons

Recommended Citation

Al Shamsi, Wafa Awad, "Bacteriophage Therapies as Alternatives to Antibiotics in Chicken Feed In the United Arab Emirates" (2014). *Theses*. 418.
https://scholarworks.uaeu.ac.ae/all_theses/418

This Thesis is brought to you for free and open access by the Electronic Theses and Dissertations at Scholarworks@UAEU. It has been accepted for inclusion in Theses by an authorized administrator of Scholarworks@UAEU. For more information, please contact fadl.musa@uaeu.ac.ae.

United Arab Emirates University

Collegeof Science

Department of Biology

BACTERIOPHAGE THERAPIES AS ALTERNATIVES TO ANTIBIOTICS IN CHICKEN FEED IN THE UNITED ARAB EMIRATES

Wafa Awad Al Shamsi

This thesis is submitted in partial fulfillment of the requirements for the Degree of Master of Science in Environmental Sciences

Under the direction of Dr. Khaled El Trabily and Dr. Abdulmajeed Al Khajeh

June 2014

DECLARATION OF ORIGINAL WORK

I, Wafa Awad Al Shamsi, the undersigned, a graduate student at the United Arab Emirates University (UAEU), and the author of the thesis titled as "Bacteriophage therapies as alternatives to antibiotics in chicken feed in the United Arab Emirates", hereby I declare that this thesis is an original work done and prepared by me under the guidance of Dr. Khaled A. El-Tarabily, and Dr. Abdulmajeed S. A. AlKhajeh, in the College of Science at UAEU. This work has not previously formed as the basis for the award of any degree, diploma or similar title at this or any other university. The materials borrowed from the other sources and included in my thesis have been properly acknowledged.

Student's Signature..... **Date**.....

UAEU LIBRARIES
1000491385
UAEU
LIBRARIES DEANSHIP
U.A.E UNIVERSITY
مكتبات الجامعات بالامتلام
M. ICLAM LIBRARIES

Copyright ©2014 by Wafa Al Shamsi
All Rights Reserved

Approved by

Thesis Examining Committee:

1) Advisor (Committee Chair): Dr. Khaled A. El-Tarabily

Department of Biology

College of Science

Signature Khaled El Tarabily Date: 3-6-2014

2) Member: Dr. Abdulamjeed S. Al-Khajeh

Department of Biology

College of Science

Signature _____ Date: _____

3) Member: Dr. Ibrahim Hassan Belal

Department of Arid land Agriculture

College of Agriculture

Signature _____ Date: 3-6-14

4) Member (External Examiner): Prof. Dr. Mohamed A badawi

Division of Soil Microbiology

Institution: Emirates Environmental Solutions

Signature _____ Date: 3/6/2014

Accepted by

Master`s Program Coordinator:

Signature ..... Date 15/6/2014

Dean of the College:

Signature ..... Date June 22, 2014

Copy .....of .....

ABSTRACT

Two bacteriophages were isolated from untreated raw waste water from Al-Ain sewerage treatment plant. These two phages were active against *Salmonella typhimurium* and *Escherichia coli* which known to cause food poisoning diseases in chicken feed. The optimum concentration of the two phages used was found to be 10^{10} plaques forming unit (PFU ml^{-1}). Bacteriophage 1 (O_1) was isolated using *S. typhimurium* as a propagation host and was found to be monovalnet. On the other hand, bacteriophage number (O_2) isolated using *E.coli* as a propagation host was found to be polyvalent and lysed Gram positive bacteria including *Bacillus subtilis*, *Staphylococcus aureus*, *Staphylococcus epidermidis* and *Enterococcus faecalis* and Gram negative bacteria including *Proteus vulgaris*, *Pseudomonas aeruginosa* and *Shigella dysenteriae*. Bacteriophage (O_1) formed small circular clear plaques (0.5 mm). However, bacteriophage (O_2) formed small circular clear blue plaques that lack the reddish color observed on the bacterial lawn(0.9 mm). Negatively stained particles of the bacteriophage (O_1) active against *S. typhimurium* and bacteriophage (O_2) active against *E. coli* fitted the *Siphoviridae* (B1) morphotype which consisted of icosahedral heads and base plate.

yeast extract agar, nutrient agar and tryptic soy agar was found to increase the number of PFU ml^{-1} on all the three media types compared to the same medium but without the addition of calcium nitrate. Bacteriophage (O_1) was found to be more sensitive to the treatments with physical agents that including (freezing, refrigeration, heating, boiling, ultrasonic treatment andUltra Violet (UV) radiation), and chemical agents including: (chloroform, thymol, hydrogen peroxide, phenol, ethyl alcohol, clorox, iodine, and merthiolate) than bacteriophage (O_2).Fewer bacteriophage (O_1)

were obtained when propagation hosts were incubated for 0-3 h, in comparison to those incubated for 5-10 h. Meanwhile, fewer bacteriophages (O_2) were obtained when propagation hosts were incubated for 0-3 h, in comparison to those incubated for 5-10 h. Greater levels of host inoculum resulted in greater phage titers for bacteriophage (O_1). Also, greater levels of host inoculum resulted in greater phage titers for bacteriophage (O_2). Greater phage inoculum size resulted in greater phage output for bacteriophage (O_1). Also, greater phage inoculum size resulted in greater phage output for bacteriophage (O_2).The application of bacteriophage (O_1) in corn-soy diet infested with *S. typhimurium*, or the application of bacteriophage (O_2) in corn-soy diet infested with *E. coli*, or the application of bacteriophage (O_1) and bacteriophage (O_2) in corn-soy diet infested with *S. typhimurium* and *E.coli* significantly ($P < 0.05$) reduced the population densities of *S. typhimurium* and the population densities of *E.coli* compared to the treatments which included the application of living *E. coli* alone, living*S. typhimurium* alone or living *E. coli* + living *S. typhimurium*. The treatment which received the mixture of the two phages or the application of bacteriophage (O_1), or the application of bacteriophage (O_2) was almost as effective as the antibiotic oxytetracycline applications in reducing the incidence of *S. typhimurium* and *E. coli* in the chicken feed. In conclusion, the isolated bacteriophages have great potential in the field of bacteriophage therapy and chicken feed industry because they are self-replicating and can be targeted against bacterial receptors that are essential for bacterial pathogenesis. The two phages obtained in the present study can be used as a safe and alternative technique to eradicate bacterial infections and chronic polymicrobial biofilm caused by antibiotic resistant bacteria.

Acknowledgement

All Praise is due to Allah, and peace and blessings be upon our Prophet Muhammad and upon his family and his companions. I would like to express my sincere thanks and gratitude to His Highness, Sheikh Khalifa Bin Zayed Al- Nahyan President of the United Arab Emirates, may God protect him and to my country UAE

Special thanks to Mr. Saeed Tareq from faculty of Medicine and Health Science, UAEU, for his cooperation, in using electron microscope.

I would like to express my gratuities to all who give me there advice, and helped me to continue this thesis.

Furthermore I would like to thank all technicians and assistants in Biology Department, College of Science, UAE University during the period of my work in microbiology laboratory.

All thanks to my family and friends who remained beside me and give me their great support and help.

Dedication

I dedicate this thesis to my parents, my brother and my sister whomplayed a great role to encourage me to continue my higher education, and gave me the strength to do my best in order to be a successful scientist.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

CHAPTER I: INTRODUCTION

1.1 Poultry market in the United Arab Emirates (UAE)

The poultry market in the United Arab Emirates (UAE) is a fast growing and demanding market. According to the Global Agricultural Information Network (GAIN) report, the total domestic production of poultry meat in the UAE had raised to 41,000 Tons of Meat (TM) in 2013 compared to 37,000 TM in 2012. The estimated increase is attributed to the increase in demand for poultry products driven by the growth in UAE population and the establishment of a new production facility in many emirates (Simon *et al.*, 2013). Generally, poultry producers do not receive any support from the UAE federal government. However, Abu Dhabi Emirate gives a subsidy of 25% on feed to its poultry farmers (Simon *et al.*, 2013).

Poultry producers do not use growth hormones in their production, since the UAE has outlawed the use of growth hormones in the production of poultry, and this sector geared to breeding stock is the most ardent user of newer technologies. However, the farmers do use antibiotics to get safe poultry meat that meet the high standards (Simon *et al.*, 2013).

Along with the increase in production, the local consumption remains stable at about 290,000 TM of both imported and locally produced fresh poultry. Fresh locally produced chicken are generally consumed by the UAE nationals, consumers with high disposable income and Moslems who prefer to consume products with guaranteed Halal slaughter (Simon *et al.*, 2013).

The UAE government has continuously played an important role in guiding and assuring consumers of the safety of consuming locally produced and imported

chickens. All locally produced chicken is subject to strict supervision and inspection to ensure fitness for human consumption (Simon *et al.*, 2013).

1.2 Food poisoning in chickens

Zoonotic diseases are common in many parts of the world and despite great improvements in food safety, food poisoning remains a problem in Europe and USA, with numerous deaths each year caused by food-borne bacteria (Alisky *et al.*, 1998). Flocks of chicken are frequently infected with bacteria, like *Escherichia coli, Salmonella* and *Campylobacter* which considered to be the most severe infective bacteria, which cause a high proportion of cases of serious illness and fatalities especially when intensively raised for food (Projan, 2004). These bacteria can survive in chicken meat or eggs, transmitting the diseases to the people who consume them, however an experimental techniques show some promise in combating those three pathogens (Thacker, 2003; Miller *et al.*, 2010).

1.2.1 *Salmonella* in chickens

Salmonella food poisoning is a bacterial food illness caused by the *Salmonella* bacterium (McCarthy *et al.*, 2007; Harrison *et al.*, 2008) which results in the swelling of the lining of the stomach and intestines (gastroenteritis). Domestic and wild animals, including poultry, pigs, cattle, and pets such as turtles, iguanas, chicks, dogs, and cats can transmit this illness and most people become infected by ingesting foods contaminated with significant amounts of *Salmonella* (Harrison *et al.*, 2008).

Enteric *Salmonella* infection is a global problem both in humans and animals, and has been attributed to be the most important bacterial etiology for enteric infections worldwide (McCarthy *et al.*, 2007). Salmonellosis is an endemic disease everywhere

and its importance, as potential zoonosis needs no emphasis (Barrow *et al.*, 1998; Harrison *et al.*, 2008).

Depending upon the species of individual *Salmonella* serovars, these are either grouped as host adapted (e.g., *S. typhi* in humans, *S. choleraesuis* in pigs) or non-host adapted (e.g., *S. typhimurium*, *S. enteritidis*) (Miles *et al.*, 1991; McCarthy *et al.*, 2007).

Flocks of intensively raised chickens often carry these infections, which can be passed with chicken meat and eggs on to humans (Capita *et al.*, 2003). Because of the dramatic increase of human salmonellosis and the growing numbers of cases with serious symptoms, resulting from the enhanced invasiveness of the dominant species *S. enteritidis*, more effective defense reactions against the spread of *S. enteritidis* are required. The most important measure would be the general decontamination of the mixed feed just before sending it to the farmer. Contaminated feed is the primary source of *Salmonella* infection to livestock and to man (Miles *et al.*, 1991; Guard-Petter, 2001; Modi *et al.*, 2001).

1.2.2 *Escherichia coli* in chickens

Enteritis is an inflammation of the small intestine caused by *Escherichia coli*. It is the most common cause of travelers' diarrhea.

flora of intestines of humans and animals without causing any infection most of the time. However, certain strains of *E. coli* can cause food poisoning, such as *E. coli* strain (O157:H7) that can cause a severe case of food poisoning (Craig *et al.*, 2009).

Bacteria may get into the food in different ways like; (i) poultry meat that may come into contact with normal bacteria from the intestines of an animal while it is

being processed, (ii) food that is handled in an unsafe way during transport or storage. Although not common, *E. coli* can be spread from one person to another (Schiller *et al.*, 2010).

Symptoms occur when *E. coli* bacteria enter the intestine, after incubation time of 24 to 72 hours. The most common symptom is sudden, severe diarrhea that is often bloody, also Fever, floating, loss of appetite, vomiting and stomach cramping are other symptoms that result from *E. coli* food poisoning (Sodha *et al.*, 2009). goals of treatment are to relief the infected person from the symptoms and avoid dehydration. Getting enough fluids and managing the symptoms are steps followed to treat the food poisoning from *E. coli* (Sodha *et al.*, 2009).

1.2.3 *Campylobacter* in chickens

Campylobacter species are bacteria that commonly infect a broad range of livestock species, pets and wild animals. In poultry they tend to multiply in large numbers in the hindgut, principally in the caecae.
cause of enteritis in humans. Infected poultry are a potential reservoir of this zoonosis. *Campylobacter jejuni* is the commonest species found in poultry. All *campylobacter* species are delicate organisms that survive for relatively short periods outside the host unless be protected by organic material, biofilm or engulfed by protozoa (Carvalho *et al.*, 2010).

In principle, housed poultry can be maintained free of *Campylobacter* infection by consistent application of excellent bio-security. Key aspects of this include effective sanitation of drinking water, sourcing of water from high quality supplies, avoidance

of contact with pets and other farmed species, good hand hygiene by stockmen, and changing of overalls and boots on entering bird areas (Carrillo *et al.*, 2005).

In practice the success of reduction of *Campylobacters*, will also depend upon the degree of environmental contamination by the organism. For this reason it may be difficult to stop the spread of infection between houses once it becomes established in one house (Carrillo *et al.*, 2005).

Many infections are introduced during thinning or other forms of partial depopulation. Insects and rodents may act as a means of transfer of the infection from the general environment into the poultry buildings. Research is ongoing on the development of vaccines, phage treatments and competitive exclusion approaches, as well as processing plant technologies to reduce carcase contamination (Carvalho *et al.*, 2010).

1.3 Antibiotics and their applications in the prevention and treatment of diseases

Antibiotics are widely used for human health to treat infections; moreover they are used for animals and plants in order to prevent diseases or to treat them against other diseases which help growth promotion in animal farming (Quiberoni *et al.*, 2011). In the USA about 80% of total antibiotic production is used in agriculture with a substantial portion of this is used for non-therapeutic growth promotion (Nigam *et al.*, 2014; Miller *et al.*, 2010).

Pathogens or bacteria are able to transfer from animal to humans through the food chain and if the bacterium has an antibiotic resistant gene, this gene can be transferred to other bacteria by transformation, conjugation or transduction (Bradbury, 2004). In order to achieve that link between antibiotic use in animals and

the extension of resistance to human beings, an understanding of the nature of antibiotic resistance is required (Adamia *et al.*, 1990; Bradbury, 2004). Because of its effect on the intensive animal production, antibiotics are now extensively used for both prevention and treatment of animal diseases (Toro *et al.*, 2005).

Antibiotics are employed in animal feed for many reasons; to enhance growth, to eliminate pathogens, to treat infections, to increase production performance and to increase the efficiency of the use of feed for growth or production output, which will lead to the production of healthy animals. In return antibiotics will increase growth rates and therefore the economic benefits (Miller *et al.*, 2010).

Using antibiotics as feed additives have become more common in animal husbandry, nevertheless, these feed additives must be of specific conditions such as; they must have a positive result on livestock production, they must be safe for animal or human health and to be easy to handle and control (Alisky *et al.*, 1998).

1.4 Antibiotics in chickens feed

Chicken feed has been routinely dosed with antibiotics as a process of infection control. However this has not provided a lasting solution, as bacteria readily mutate to acquire resistance to these antibiotics (Bradbury, 2004). As new antibiotics are developed, and new resistant strains emerge, therefore many of the developed countries now limit the use of antibiotics in animal feed. However antibiotics still remains effective for treating human infections (Johnke *et al.*, 2014).

There are many examples of antibiotics which are commonly used in the animal feed; these include oxytetracycline, bambermycin, avilamycin and bacitracin (Alisky *et al.*, 1998).

Oxytetracycline is used for human treatment against diseases caused by bacteria such as rickettsiae, mycoplasma, and spirochetes (Wegener *et al.*, 2003). Oxytetracycline falls under the tetracycline group of antibiotics and is added to animal feed. It is utilized for prophylaxis and medication of different types of diseases, used in veterinary medicine to prevent and control diseases and added to animals feed such as; cattle, pigs, sheep, poultry and fish farming (Arikan *et al.*, 2007). Oxytetracycline has low activity and toxicity and good diffusion into the tissues so it is utilized for treatment of respiratory, urinary and alimentary tract infections (Kowalski *et al.*, 2007).

Agricultural Research Service Researchers in Britain has studied the breakdown of oxytetracycline in manure and found that this breakdown slowed with increased saturation of the manure because of a decrease in oxygen levels. This shows oxytetracycline effects in animal feed on antimicrobial resistance, bacteria and the environment. It might also damage organs that are rich in calcium like bones and teeth. In addition, it sometimes causes nasal cavities to erode (Butaye *et al.*, 2003).

Bambermycin is a phosphoglycolipid antimicrobial antibiotics formed by different strains of the actinomycete *Streptomyces*. It acts primarily against Gram positive bacteria because of the inhibition of transglycosylase (Faller *et al.*, 2006). Furthermore, it is employed as a feed additive growth promoter in cattle, pigs, chickens, and turkeys but has no healing application in humans or animals and it does not kill beneficial bacteria that form part of the normal intestinal microflora. It is also reported that bambermycin reduces the colonization and shedding of bacterial pathogens such as *Salmonella* spp. (Faller *et al.*, 2006).

Avilamycin belongs to the orthosomycin group of antibiotics. It is approved for use as a growth promoter within the European Union (EU) in swine and poultry industries but not allowed for therapeutic applications. Its mechanisms in growth are not clear and it is used as a postulated to involve a reduction of the intestinal bacterial load (Sunderland *et al.*, 2004).

Bacitracin is a polypeptide antibiotic and is active against Gram positive and some Gram negative bacteria. In addition it has been used as an additive in animals feed (Murphy *et al.*, 2007). Bacitracin is available as a lyophilized powder to administer in food and drugs and it is applied in treatment of infections (Faller *et al.*, 2006).

1.5 Disadvantages of antibiotics in chickens feed

Antibiotics have been used in a dramatic way since the 1940s, when it was discovered that the by-products of antibiotics can be added to animal feed because they have a high level of vitamin B12, which is considered to be a growth promoter, also it is widely used in poultry for this reason, and to treat and prevent infections (Wright *et al.*, 2009). Many scientists claimed that this is considered as a risk because bacterial strains gain stronger resistances (Wright *et al.*, 2009; Nigam *et al.*, 2014). Eventually risks of using antibiotics were discovered, after seven decades of heavy use of antibiotics and arguments are increasing rapidly about these risks to human, and actual examples are hard to come by. However, the risk with slaughtered chickens is that pathogenic bacteria may be transmitted to humans who consume them (Bradbury, 2004). For example, in October 2000 the U.S. Food and Drug Administration (FDA) discovered two antibiotics that are not useful in treating diseases in factory-farmed chickens. The FDA pulled one from the market, but the

other one, called Baytril was not withdrawn until 2005 (Häusler, 2006; Wright *et al.*, 2009; Lim *et al.*, 2011).

1.6 Probiotic metabolites in chickens feed

Over the years the word probiotic has been used in several different ways. It was originally used to describe substances produced by one protozoan which stimulated by another, but it was later used to describe animal feed supplements which had a beneficial effect on the host animal by affecting its gut flora (Weld *et al.*, 2004). A probiotics is "a culture of specific living microorganisms (primarily *Lactobacillus* spp.) which implants in the animal to ensure the effective establishment of intestinal populations of both beneficial and pathogenic organisms". Fuller later gave a unique definition of probiotics as "a live microbial feed supplement which beneficially affects the host animal by improving its intestinal microbial balance" (Fuller, 2001; Bradbury, 2004).

The US National Food Ingredient Association presented, probiotic (direct fed microbial) as a source of live naturally occurring microorganisms and this includes bacteria, fungi and yeasts (Alisky *et al.*, 1998).

According to the currently adopted definition by FAO/WHO, probiotics are: "live microorganisms which when administered in adequate amounts confer a health benefit on the host" (FAO/ WHO, 2001). More precisely, probiotics are live microorganisms of non-pathogenic and non-toxic in nature, which when administered through the digestive route, are favorable to the host's health (FAO/ WHO, 2001).

It is believed by most investigators that there is an unsteady balance of beneficial and non-beneficial bacteria in the tract of normal, healthy, non-stressed poultry

(Chibani *et al.*, 2004). When a balance exists, the bird performs to its maximum efficiency, but if stress is imposed, the beneficial floras, especially lactobacilli, have a tendency to decrease in numbers and an overgrowth of the non-beneficial ones seems to occur. This occurrence may predispose Frank disease, i.e., diarrhea, or be subclinical and reduce production parameters of growth, feed efficiency and others (Parker *et al.*, 1979).

The protective flora which establishes itself in the gut is very stable, but it can be influenced by some dietary and environmental factors (Häusler, 2006). The three most important are excessive hygiene, antibiotic therapy and stress. In the wild, the chicken would receive a complete gut flora from its mother's faces and would consequently be protected against infection (Bradley, 1967). However, commercially reared chickens are hatched in incubators which are clean and do not usually contain organisms commonly found in the chicken gut. There is an effect of shell microbiological contamination which may influence gut microflora characteristics (Janez *et al.*, 2013).

Moreover, also HCl gastric secretion, which starts at 18 days of incubation, has a deep impact on microflora selection (Bradbury, 2004). Therefore, an immediate use of probiotics supplementation at birth is more important and useful in avian species than it is in other animals. The chicken is an extreme example of a young animal which is deprived of contact with its mother or other adults and which is, therefore, likely to benefit from supplementation with microbial preparations designed to restore the protective gut microflora (Bradbury, 2004).

The species currently being used in probiotic preparations are varied and many. These are mostly *Lactobacillus bulgaricus*, *Lactobacillus acidophilus*, *Lactobacillus casei*, *Lactobacillus helveticus*, *Lactobacillus lactis*, *Lactobacillus salivarius*, *Lactobacillus plantarum*, *Streptococcus thermophilus*, *Enterococcus faecium*, *Enterococcus faecalis*, *Bifidobacterium* spp. and *Escherichia coli*. With two exceptions, these are all intestinal strains. The two exceptions, *Lactobacillus bulgaricus* and *Streptococcus thermophilus*, are yoghurt starter organisms. Some other probiotics are microscopic fungi such as strains of yeasts belonging to *Saccharomyces cerevisiae* (Fuller *et al.*, 1989).

1.7 A renewal of interest in bacteriophages

The use of antibiotics is currently being reduced in animal production so alternative methods are needed to combat bacterial diseases in animals feed and to control transmission to humans of the pathogens responsible for foodborne illnesses. The excessive use of antibiotics in agro-food and in animal husbandry, contributes to the occurrence and persistence of antibiotic resistant pathogens for humans and animals through the food chain (Nigam *et al.*, 2014).

There is an urgent need for alternative methods to combat bacterial diseases of agriculturally important animals. Of the present possibilities, bacteriophages (phages) therapy shows promise (O'Flaherty *et al.*, 2009).

In the 1980s, a British bacteriologist, William Smith, had conducted a series of experiments in both mice and farm animals. Bacteriophages were shown to be more effective than a variety of antibiotics in reducing the mortality of mice with

generalized and itracerebral *Escherichia coli* infections and bacteriophages were shown to be multiplying in the tissue (O'Flaherty *et al.*, 2009; Nigam *et al.*, 2014).

Very low doses of phages were found to be almost effective than high doses. Alisky *et al.* (1998) evaluated the treatment of experimentally induced diarrhea in calves, piglets and lambs due to several different strains of *E. coli*, and were able to successfully treat and prevent infections; even when low doses of bacteriophages were used (Alisky, *et al.*, 1998).

These demonstrations of the potential of the bacteriophages therapy, combined with the growing urgency of the antibiotic crisis, led to renewed interest in this approach in the 1990s (Alisky *et al.*, 1998). Janez *et al.* (2013) showed that bacteriophages could prevent the destruction of skin grafts in guinea pigs and could protect mice against lethal levels of bacterial infection (Janez *et al.*, 2013).

Combined with the continuing increase in antibiotic resistance, work showing that bacteriophages could be effective as a medical treatment. This led several groups to look at the possibility of bringing the therapeutic bacteriophage technology back from the former Soviet bloc, which was now accessible following the fall of the old communist regimes several years earlier (O'Flaherty *et al.*, 2009; Harper, 2012).

Unfortunately, despite the apparent promise of this technology, and despite the widespread use including a very large body of work involving the treatment of many thousands of patients in local studies, the work which had been done was not enough to prove the case for the bacteriophage therapy for western use (Häusler, 2006). Under the different pressures that applied in the Soviet era, particularly the desperate need for treatment options, this work had not involved the detailed documentation,

and double-blind controls required for progress within a modern regulatory framework. The evidence was supportive, but not sufficient (Lim *et al.*, 2011).

As a result, much of the excitements over access to former Soviet science in the mid 1990s proved unsupportable. While clinics in Poland and Georgia offered bacteriophages treatment to those motivated enough to travel to them for treatment, these could not be used in Western Europe or in the United States (Bradbury, 2004). Those companies that managed to survive in the West generally did so on the basis of other kinds of applications, in particular the somewhat less complex areas of food and agricultural applications (Wong *et al.*, 2014).

By 2007, five bacteriophage products were likened in the United States. One was to prevent bacterial infections of tomatoes and peppers, two to destroy bacteria associated with food poisoning on food animals before slaughter, and two for the control of *Listeria monocytogeses* on ready-to eat food (Chibani *et al.*, 2004). In this latter use, exposure of humans to the bacteriophages was accepted by the FDA which was an important step forward (Johnke *et al.*, 2014).

Clinical work in humans was slower to develop, not least due to the relatively high cost of such work. Some groups have focused on investigating the use of genetically modified (GM) bacteriophages, often with enhanced abilities to destroy their bacterial target but with reduced ability to replicate, essentially a gene vector approach (Lim *et al.*, 2012). However, any use of GM technology brings with it very significant additional regulatory requirements (Johnke *et al.*, 2014).

In addition, many researchers considered the ability of bacteriophages to replicate and thus to expand their numbers at precisely those points where they are needed to

be a major strength of the approach, and thus question the use of non-replicating bacteriophages or bacteriophages derived vectors. Unlike gene therapy vectors, where replication appears to have the potential to cause problems, bacteriophages can only replicate in their prokaryotic target cells (Thacker, 2003)

1.7.1 Bacteriophages as therapeutic agents

The emergence of pathogenic bacteria resistant to most, if not all, currently available antimicrobial agents has become a critical problem in modern medicine, particularly because of the concomitant increase in immunosuppressed patients (Abdul-Hassan *et al.*, 1990; Harper *et al.*, 2008). The concern that mankind is re-entering the pre-antibiotics era has become very real and the development of alternative anti-infection modalities has become one of the highest priorities of modern medicine and biotechnology (Krylov, 2014).

Bacteriophages were discovered before effective antibiotics and so it was hoped that they would be able to control bacterial disease (Projan, 2004). In 1919, there were reports of the apparently successful treatment of typhoid in chickens and of dysentery in five humans. In 1921, bacteriophages were used against *Staphylococcus aureus* in skin disease (Nigam *et al.*, 2014).

During the 1920s, both localized and large-scale experiments were undertaken in many countries, including the treatment of over a million patients in India (Wright *et al.*, 2009). A wide range of commercial preparations were sold in Europe and the USA. The novel (1925) and film (1931) Arrowsmith presented a fictionalized account of their use (Nigam *et al.*, 2014).

Unfortunately, understanding of the nature of bacteriophages was extremely limited at the time. It was not until 1939 when the use of early electron microscopes helped to settle an ongoing argument as to whether they were viruses or some form of chemical toxin. In y work using bacteriophages was deeply flawed because: (i) bacteriophages were used against diseases with no bacterial component; (ii) it was assumed that bacteriophages were able to destroy a wide range of bacteria, whereas they are highly specific.

They were thus used against inappropriate bacterial targets; (iii) inappropriate growth conditions or preservatives were used that limited or prevented the inclusion of infectious bacteriophages; and (iv) methods of administration were used that inactivated any bacteriophages present (Projan, 2004; Hauer *et al.*, 2010).

In 1934, the Journal of the American Medical Association published the results of a large study carried out by the US Council on Pharmacy and Chemistry. It concluded that, apart from few restricted uses, there was no good evidence that bacteriophages actually worked in the therapeutic uses that had been evaluated (Alisky *et al.*, 1998).

It was further noted that most of the work carried out was deeply flawed, lacking proper controls, adequately purified therapeutic substance, or sufficient numbers of patients. Despite this, therapeutic use of bacteriophages continued through World War II. The German and Soviet armies used many bacteriophage preparations, notably against dysentery (Harper, 2012).

1.7.2 Commercial bacteriophage products for the treatment of human diseases

Bacteriophages have a long history of safe therapeutic use in the former Soviet Union particularly at the Eliava Institute in Georgia, where they have been used since 1923. Patients travelled from all over the Soviet Union and bacteriophages became a routine part of treatment in clinics and hospitals (Wright *et al.*, 2009). Phages have been applied in ointments for the skin, pills, drops, and rinses and tons of phage products have been produced per week. patients today, but on a reduced scale. Phage therapy is also available in a very limited form in some European countries (such as Poland) for compassionate use, in patients who have not responded to antibiotic treatment (Johnke *et al.*.

However, none of the phage applications mentioned above were tested in full clinical trials, to the standards required for Western Medicinal products. In the United States, certain phage products have been given a "Generally Recognized as Safe" (GRAS) rating by the FDA, for use in food treatment (Bradbury, 2004). However, for phage therapeutic treatments in humans, full clinical trials are still required. The process of producing phage cocktails to cGMP (current Good Manufacturing Practice) standards must be developed and validated before safety and efficacy studies in humans can begin (Monk *et al.*, 2010).

In order to fully exploit these natural, biological alternatives to antibiotics, Novolytics, is a company that had a goal of exploiting the therapeutic potential of the bacteriophages, in a way that is commercially viable and attractive to its investors (Häusler, 2006). Novolytics is planning such a series of clinical trials. Their first

product is a phage cocktail targeting Methylene resistant *Staphylococcus aureus* (MRSA) (Tsonos *et al.*, 2013).

The use of bacteriophages was of wide range in many fields, including agriculture, aquaculture, veterinary, food safety and human health which also include identification and detection of disease and therapeutics. Some examples are Novolytics for MRSA, GangaGen for Staphylococcal infections, Phico Therapeutics and Ampliphi Biosciences for systemic use against *Pseudomona aeruginosa* (Monk *et al.*, 2010; Tsonos *et al.*, 2013).

1.7.3 Issues relating to the use of bacteriophage to replace antibiotics

The first modern clinical trial of a bacteriophage therapeutic was carried out in 1999 in London, UK, though this used healthy volunteers without the targeted infection, and thus only addressed safety issues (Tsonos *et al.*, 2013).

The intended target was vancomycin resistant *Enterococcus* infections. After this, additional safety trials were carried out, but it was not until 2007 that the first modern clinical trial of the efficacy of a bacteriophage therapeutic was begun targeting *Pseudomonas aeruginosa* infections of the ear. The trial completed in 2007 and larger, phase 3 trials are now planned. It will be necessary for these trials to be completed successfully and approved by the relevant regulators, such as The European Medicines Agency in Europe and the Food and Drugs admistration (FDA) in the USA, before any bacteriophage therapeutic can proceed to market. However it seems that progress is being made (Tsonos *et al.*, 2013).

1.7.4 Advantages of using bacteriophage in medicine

There are many advantages of using the bacteriophage in the medical field as suggested by Johnke *et al.* (2014), these include; (i) bacteriophages are highly specific, helping to avoid side effects. This is in line with an increasing trend in antibiotic usage to avoid drugs with broad specificity; (ii) the use of replicating bacteriophages also allowed for the use of very low input levels that can multiply up at need. This can reduce both cost and toxicity; (iii) bacteriophages are unaffected by antibiotic resistance, and there is evidence that they may be able to work synergistically with some conventional antibiotics; (iv) bacteriophage preparations appear free of gross toxicity. Potential complications arising from the release of bacterial endotoxins from lysed cells but this do not seem to be an issue at the dosing levels now in use; (v) bacteriophages are relatively simple to manufacture, although the highly regulated manufacturing process required for clinical use adds to costs and; (vi) bacteriophages themselves may be able to adapt to counter bacterial resistance (Johnke *et al.*, 2014).

1.7.5 Disadvantages of using bacteriophage in medicine

Moreover there are also several disadvantages of using the bacteriophages in medicine as suggested by Johnke *et al.* (2014), these include; (i) high specificity means that it is important to identify the pathogenic bacteria present and to ensure that they are responsible for the disease. Multiple bacteriophages may be required to obtain useful levels of coverage, though this can in turn reduce the chance of resistance; (ii) bacteriophages have limited suitability for systemic use since they can generate immune responses which can reduce efficacy. This can be avoided by the

use on infected positioned on the body surface or oral administration; (iii) the mobilization of bacterial genetic material that may be able to moderate virulence is to be avoided. For this reason, only lytic rather than lysogenic, bacteriophages are used, and regulators require careful monitoring of all forms of transduction or transfer of bacterial genes (Johnke *et al.*, 2014).

1.7.6 Active and passive bacteriophage therapy

There are two types of bacteriophages therapy; (i) active bacteriophage therapy and (ii) passive bacteriophage therapy (Wright *et al.*, 2009). The approach that relies on bacteriophage multiplication to generate therapeutic doses of the bacteriophages against the far larger numbers of bacteria that are present is called the active bacteriophage therapy. On the other hand the use of higher doses sufficient to produce antibacterial effects without relying on bacteriophage replication is known as passive bacteriophage therapy (Janez *et al.*, 2013).

1.7.7 Application of bacteriophages in chickens feed

It was always a matter of urgency to find alternative ways of preventing and treating the chicken infections that are also a risk to humans. Of the present possibilities, bacteriophage (phage) therapy shows promise (Häusler, 2006). Bacteriophages are highly specific in killing certain strains of bacteria, just like antibiotics, but with the added benefit of multiplying as they consume the host, so they spread rapidly. Recent work has shown that bacteriophages are highly effective at clearing pathogens from poultry carcasses and at killing them in the intestines of live birds and in their eggs (Häusler, 2006).

Barrow *et al.* (1998) also proved that a lytic bacteriophage, which was previously isolated from sewage and which attaches to the capsular antigen, has been used to prevent septicemia and a meningitis-like infection in chickens caused by a bacteremic strain of *E. coli*. Protection was obtained even when administration of the phage was delayed until signs of disease appeared. The phage was able to multiply in the blood. In newly born colostrums-deprived calves given the *E. coli* orally, intramuscular inoculation of phage delayed appearance of the bacterium in the blood and lengthened life span (Barrow *et al.*, 1998).

Fiorentin (2005) stated that reducing *Salmonella* contamination in poultry is of major importance to prevent the introduction of this microorganism into the food chain. *Salmonella* may spread during storage time (shelf life) whenever pre-harvest control fails or post-harvest contamination occurs. Therefore, preventive measures should also be used in the post-harvest level of poultry production in order to control *Salmonella*. Chicken skin samples were experimentally contaminated by immersing whole legs (thighs and drumsticks) in a suspension containing 10^6 colony forming units per milliliter (cfu ml^{-1}) of *Salmonella enteritidis* phage type 4 (SE PT4) at the slaughter day. One day later, samples from one group were immersed in a suspension pool containing 10^9 cfu ml^{-1} of each of three wild *Salmonella*-lytic bacteriophages previously isolated from feces of free range chickens (Fiorentin, 2005). *Salmonella* counting was performed at three-day intervals in the chicken legs stored at 5°C and showed a significant reduction ($P<0.05$) of SE PT4 in bacteriophage-treated cuts on days 3, 6 and 9 post-treatment (Fiorentin, 2005).

These findings suggest that the use of bacteriophages may reduce SE PT4 in chicken skin. Further studies are encouraged and might demonstrate the potential of this approach as an efficient and safe technique to be routinely used for *Salmonella* control in chicken products (Fiorentin, 2005).

Carvalho *et al.* (2010) concluded that poultry meat is one of the most important sources of human campylobacteriosis, acute bacterial enteritis which is a major problem worldwide. *Campylobacter coli* and *Campylobacter jejuni* are the most common *Campylobacter* species associated with this disease. These pathogens live in the intestinal tract of most avian species and under commercial conditions they spread rapidly to infect a high proportion of the flock, which makes their treatment and prevention very difficult (Carvalho *et al.*, 2010).

Carvalho *et al.* (2010) tested the efficacy of a phage cocktail composed of three phages for the control of poultry infected with *C. coli* and *C. jejuni*. They evaluated the effectiveness of two routes of phage administration (by oral gavage and in feed) in order to provide additional information regarding their future use in a poultry unit (Carvalho *et al.*, 2010).

The results indicate that the birds had no signs of disease even at the highest dose of *Campylobacter* administered. The phage cocktail was able to reduce the titre of both *C. coli* and *C. jejuni* in faeces by approximately 2 log10 cfu g^{-1} when administered by oral gavages and in feed (Carvalho *et al.*, 2010).

Miller *et al.* (2010) observed that several lytic bacteriophages effective at destroying a genetically diverse population of *Clostridium perfringens* were isolated from the environment, extensively characterized, and used to formulate a multivalent

bacteriophage cocktail designated "INT-401". Two *in vivo* studies were conducted to determine the cocktail's efficacy in controlling necrotic enteritis (NE) caused by *C. perfringens*. The first study investigated the efficacy of INT-401 and a bacteriophage-derived, toxoid-type vaccine in controlling NE in *C. perfringens*-challenged broiler chickens. The study was designed as a proof-of-concept battery cage study with birds reared until 28 days old (Miller *et al.*, 2010).

Compared with the mortality observed with the *C. perfringens*-challenged but untreated chickens, oral administration of INT-401 significantly ($P < 0.05$) reduced the mortality of the *C. perfringens*-challenged birds by 92%. Overall, INT-401 was more effective than the toxoid vaccine in controlling active *C. perfringens* infection. The second study was conducted to investigate the effectiveness of the cocktail when administered via oral gavage, feed, or drinking water (Miller *et al.*, 2010)

The study was conducted with birds reared to 42 days old. INT-401 administered by all three methods significantly ($P < 0.05$) reduced mortality. Weight gain and feed conversion ratios were significantly better in the *C. perfringens*-challenged chickens treated with INT-401 than in the *C. perfringens*-challenged, and phage-untreated control birds (Miller *et al.*, 2010). The data indicated that delivering INT-401 to broiler chickens via their drinking water or feed may be an effective means for controlling Necrotic Enteritis (NE) caused by *C. perfringens* and may improve weight gain and feed conversion ratios in birds with clinical or subclinical NE (Miller *et al.*, 2010).

Lim *et al.* (2011) also stated that *Salmonella gallinarum* (SG)-specific bacteriophage isolated from sewage effluent was used to prevent horizontal

transmission of SG in commercial layer chickens. Six-week-old chickens, each challenged with 5 x 10^8 colony forming units of SG, cohabited with contact chickens treated with 10^6 plaque-forming units kg^{-1} of bacteriophage, prepared in feed additives, for 7 days before, and 21 days after challenge with SG. Mortality was observed for 3 weeks after challenge and SG was periodically re-isolated from the liver, spleen, and cecum of chickens. SG re-isolation from organs was decreased and a significant ($P < 0.05$) reduction in mortality was observed in contact chickens treated with the bacteriophage, as compared to untreated contact chickens, indicating that bacteriophage administration in feed additives significantly prevented the horizontal transmission of SG. These results provide important insights into prevention and control strategies against SG infection and suggest that the use of bacteriophages may be a novel, safe, and effectively reasonable alternative to antibiotics for the prevention of SG infection in poultry (Lim *et al.*, 2011).

Lim *et al.* (2012) proved that bacteriophage ΦCJ07 with broad host ranges for *Salmonella* strains isolated from sewage effluent was used to reduce *Salmonella enteritidis* (SE) infection in chickens. One-day-old chicks challenged with 5×10^7 colony forming units per bird of SE were cohabitated with contact chicks and treated with three concentrations (10^5, 10^7 and 10^9 plaque forming units (pfu) g^{-1}) of bacteriophage prepared as a feed additive for 21days after challenge. *Salmonella* in the intestine was quantified and environmental contamination level was examined at 1, 2 and 3 weeks after challenge (Lim *et al.*, 2012).

In the work carried out by Lim *et al.* (2012), all treatments reduced intestinal SE colonization in challenged and contact chickens and reduced the environmental

contamination level, but the reductions produced by 10^7 and 10^9 pfu g^{-1} of bacteriophage were significant ($P<0.05$) as compared with untreated controls. In addition, seven out of 10 (70%) contact chickens treated with 10^9 pfu g^{-1} of bacteriophage had no detectable intestinal *Salmonella* at 3 weeks after treatment, suggesting that bacteriophage therapy significantly prevented the horizontal transmission of SE. These results provide important insights into preventive and control strategies against SE infection in poultry and indicate that the use of bacteriophage could reduce the incidence of *Salmonella* food poisoning (Lim *et al.*, 2012).

Janez *et al.* (2013) demonstrated that since the ban on antibiotic use for growth promotion, bacterial infections of the intestines are an emerging problem in poultry and pig farming. In addition, there is increasing pressure to find better alternatives to antibiotics in the battle against bacterial infections. The use of bacteriophages in animal feed might be such an alternative. In pigs and poultry a relatively small group of bacteria exists, which form the major threat to intestinal health. Therapeutic phage/endolysin treatment of some of these intestinal pathogens; i.e. *Brachyspira* ssp, *C.perfringens*, *Clostridium difficile* and *Escherichia coli* might be an option (Janez *et al.*, 2013).

However, bacteriophages are not equally applicable for all these infections. Various phages have been isolated, characterized and tested *in vitro* and sometimes *in vivo*. They vary greatly in, specificity, virulence, lytic potential, sensitivity (for light, pH-value) per individual phage against the pathogenic bacteria. Therefore, Janez *et al.*, (2013) suggested that each phage has to be considered independently for its

potential to survive residency in animal feed, and the oral application route and finally its efficacy to reduce or eliminate the specific pathogen must be examined (Janez *et al.*, 2013).

Furthermore, the distinctive properties of targeted bacteria affect the suitability of the therapy. Overall, the limited research, and not the possibility, has hindered the use of phages for such therapeutically application. While bacteriophage treatment will probably never fully replace antibiotics, they can be a good addition to and might be used in combination with them, to combat the increasing bacterial infections in pigs and poultry (Wong *et al.*, 2014).

Table 1.1 The applications of bacteriophages to compete *E. coli* and *Salmonella* infections.

Table 1.1.1 Pre-harvest *E. coli* O157: H7 phage applications.

Year	Animal/product	Phage(s)	Strategy	Main outcome	Reference
Pre-harvest application					
2002	Poultry (broiler chicken)	SPR02	Air sac or drinking water	Air sac inoculation prevented mortality. Drinking water offered no protection	(Huff *et al.*, 2002)
2002	Poultry (broiler chicken)	SPR02 and DAF6	Sprayed	Significant decrease of mortality but not complete protection	(Huff *et al.*, 2002)
2003	Poultry (broiler chicken)	SPR02 and DAF6	Sprayed and i.m. injection	Aerosol spray effective only when applied immediately after bird challenge with *E. coli*. A single i.m. injection reduced mortality when applied immediately and 24 and 48 h after challenge	(Bach *et al.*, 2003)
2003	Ruminant (lamb)	DC22	Oral delivery	No reduction of fecal shedding over 30 days	(Bach *et al.*, 2003)
2004	Meat	e11/2, e4/1c, pp01	Applied on top	Eradication in seven of nine samples	(O'Flynn *et al.*, 2004)
2006	Poultry (broiler chicken)	SPR02 and DAF6	i.m. injection into the left thigh	Only high phage titers (108) reduced mortality	(Raya *et al.*, 2006)

2006	Ruminant (sheep)	CEV1	Oral delivery	2 log cfu reduction within 2 days	(Huff *et al.*, 2006)
2006	Ruminant (cattle)	Phage cocktail	Oral/rectal delivery	No reduction of cfu when applied orally. Combined oral/rectal treatment reduced cfu but did not eradicate it	(Sheng *et al.*, 2006)
2008	Fresh produce (tomato, spinach) and meat	Phage cocktail (ECP-100)	Applied on top/sprayed	94% and 100% reductions in cfu after 120 h and 24 h in tomato and spinach; 95% reduction in ground meat after 24 h at 10°C	(Abduladze *et al.*, 2008)
2009	Ruminant (steer)	Phage cocktail	Oral/rectal delivery	Small fecal shedding reduction of oral/rectal compared to the rectal treatment and control	(Rozema *et al.*, 2009)
2009	Fresh produce (lettuce, cantaloupe)	Phage cocktail (ECP-100)	Sprayed	Significant cfu reductions after 2 days at 4°C	(Olieviera *et al.*, 2010)
2010	Ruminant (steer)	Phage cocktail (wV8, rV5, wV7, wV11)	Oral delivery (gelatin capsules and in feed)	No reduction of fecal shedding of nalidixic acid-resistant *E. coli* O157:H7, but duration of shedding was reduced by 14 days	(Rivas *et al.*, 2010)
2010	Poultry	Phage cocktail	Oral delivery and spray	Significant reduction of mortality in large scale animal experiments	(Stanford *et al.*, 2010)
2010	Ruminant (cattle)	Phage cocktail (e11/2, e4/1c)	Oral delivery	Rapid cfu decrease within 24 to 48 h, but no decrease in fecal shedding levels	(Raya *et al.*, 2011)
2011	Ruminant (sheep)	Phage cocktail (CEV1, CEV2)	Oral delivery	Cocktail eradicated (>99.9%) the pathogen and is more effective than CEV1 alone	(Raya *et al.*, 2011)
2011	Fresh produce (lettuce, spinach)	Phage cocktail	Added to foods together with trans-cinnamaldehyde (TC)	No survivors detectable after 10 min of phage combined with the TC treatment	(Raya *et al.*, 2011)
2011	Food surfaces (spinach blades)	Phage cocktail	Sprayed	4.5 log reduction cfu after 2 h of phage	(Raya *et al.*, 2011)
2011	Food surfaces (steel, ceramic chips)	Phage cocktail (BEC8)	Applied on top	Eradication after 10 min at 37°C and after 1 h at 23°C	(Raya *et al.*, 2011)

Table: 1.1.2: Pre-harvest and post-harvest *Salmonella* phage applications

Year	Animal/product	Phage(s)	Strategy	Main outcome	Reference
Preharvest application					
2001	Poultry (chicken)	Phage cocktail	Oral delivery (direct and via feed)	Reduction of cfu in cecal counts between 0.3 and 1.3 log compared to controls birds	(Huff *et al.*, 2002)

2001	Swine (pig)	Felix01	Oral delivery and i.m.	Reduction of cfu in the tonsils and cecum	(Huff *et al.*, 2002)
2005	Poultry (broiler chickens)	CNPSA1, CNPSA3, CNPSA4	Oral delivery	Reduction of cfu by 3.5 orders of magnitude after five days	(Huff *et al.*, 2006)
2005	Poultry (chickens)	Phage cocktail (Sa2, S9, S11)	Oral delivery phage/competitive exclusion	Reduction of cfu in cecum and ileum after phage cocktail and/or competitive exclusion treatment	(Raya *et al.*, 2006)
2007	Poultry (broiler chickens)	Φ151, Φ25, Φ10	Oral delivery (antacid suspension)	Reduction of 4.2 log and 2.19 log with phages Φ151 and Φ25 within 24 h compared with control	(Abduladze *et al.*, 2008)
2007	Poultry (broiler chickens)	Phage cocktail (CB4ϕ, WT45ϕ)	Oral delivery	Reduction of cfu in cecal tonsils after 24 h. No significant differences at 48 h compered to controls	(Rozema *et al.*, 2009)
2008	Poultry (chickens)	Phage cocktail	Oral delivery (coarse spray/drinking water)	Reduction of intestinal colonization of ten-day-old experimentally contaminated birds	(Stanford *et al*, 2010)
2010	Swine (pig)	Phage cocktail		Reduction of colonization by 99.0 to 99.9% in the tonsils, ileum, and cecum	(Stanford *et al.*, 2010)
2011	Swine (weaned pigs)	Phage cocktail	Oral delivery	Significant reduction of cfu in the rectum	(Raya *et al.*, 2011)
2011	Poultry (chickens)		Oral delivery (via feed)	Phage prevented horizontal transmission on six-week-old infected chickens	(Raya *et al.*, 2011)
Postharvest application					
2001	Processed food (ripened cheese)	SJ2	Added to milk	No survival during 89 days in pasteurized cheeses containing phages (MOI 104)	(Huff *et al.*, 2002)
2001	Fresh produce (fresh-cut melon and apple)	Phage cocktail	Added to foods	Significant cfu reduction on melon but not on apple	(Huff *et al.*, 2003)
2003	Meat (chicken skin)	P22, 29C	Applied on top	MOI 1 caused less than 1 log reduction in cfu; MOI 100-1,000 caused 2 log reductions in cfu and eradicated resistant strains	(Bach *et al.*, 2003)
2003	Meat (chicken frankfurters)	Felix O1		Approx. 2 log reduction with a MOI of 1.9×104	(Bach *et al.*, 2003)
2004	Fresh produce (sprouting seeds)	A, B	Applied by immersion	Phage-A reduced cfu by 1.37 logs on mustard seeds. Cocktail resulted in a 1.5-log reduction in cfu in the soaking water of broccoli seeds	(Bach *et al.*, 2003)

2005	Meat (broiler. turkey)	PHL 4	Sprayed	Phage treatments reduced frequency of *Salmonella* recovery as compared with controls	(Rozema *et al.*, 2009)
2008	Meat	P7	Applied on top	Reduction in cfu of 2-3 log at 5°C and approx. 6 log at 24°C	(Rozema *et al.*, 2009)
2009	Fresh produce (tomatoes)	Phage cocktail	Phage + E. asburiae JX1 added to food	Prevalence reduction of internalized S. Javiana, although the major suppressing effect was via antagonistic activity of E. asburiae JX1	(Rivas *et al.*, 2010)
2010	Fresh produce (mung bean sprouts and alfalfa seeds)	Phage cocktail	Phage + E. asburiae JX1 added to foods	Combined biocontrol with E. asburiae and phage suppressed pathogen growth on mung beans and alfalfa seeds	(Rivas *et al.*, 2010)
2011	Meat	Phage cocktail	Applied on top	Above 99% reduction in cfu for MOI of 10 or above at 4°C for 96 h	(Raya *et al.*, 2011)
2011	Ready-to-eat foods and chocolate milk	FO1-E2	Added to foods and mixed in milk	At 8°C no viable cells. At 15°C reduction of cfu by 5 logs on turkey deli meats and in chocolate milk and by 3 logs on hot dogs	(Raya *et al.*, 2011)

1.7.8 The current ongoing projects for bacteriophages as an alternative to antibiotics in chicken feed and poultry industry

There is a current project funded by the European Union (EU) in order to produce massive amounts of bacteriophages to be used in the poultry industry to replace the application of antibiotics. This project is called the Phage Vet project.

1.7.8.1 Phage Vet project

The Phage Vet project is a European-led multinational project to examine the effectiveness of bacteriophages at fighting infections to replace the applications of antibiotics in chicken feed.

The Phage Vet project aim is to establish that bacteriophages can reduce or eliminate *Salmonella* and *Campylobacter* from small flocks of live chickens. The other significant aim is to ensure that such birds then provide poultry products that

are fit for human consumption and have greatly reduced levels of contamination by these two pathogens. The Phage Vet project will isolate and produce highly lytic phages specific to *Salmonella* and *Campylobacter* and establish the most effective way to use them to treat birds and chicken feed as alternative treatments to antibiotics (http://ec.europa.eu/agriculture/antimicrobials/phagevet). Controlling the spread of bacterial resistance to antibiotics will benefit human health as well as animal welfare. This innovative technique could provide new research techniques, as well as new business opportunities (http://ec.europa.eu/agriculture/antimicrobials/phagevet).

The outcomes of the Phage Vet project would be; (i) the establishment of a promising method for preventing bacterial infection of food that could be cheaper than other antibiotic-free alternatives; (ii) the selection and production of highly lytic specific phages, and (iii) establishment of effective phage administration to live poultry (http://ec.europa.eu/agriculture/antimicrobials/phagevet).

1.8 Biology of bacteriophages

Bacteriophages are highly specific viruses that can target, infect and if correctly selected destroy pathogenic bacteria (Harper *et al.*, 2008). Antibacterial activity was first observed in the waters of the Gangs and Jumna rivers by Ernest Hankin in 1896. The causative agent was discovered independently in 1915 by Fredrick Twort and in 1917 by Flelix d'Heerelle who named them bacteriophages and who then expanded on his initial finding to establish many of the techniques that form the basis of modern virology (Guttman *et al.*, 2005; Oliveira *et al.*, 2014).

Bacteriophages are believed to be the most numerous types of viruses accounting for the majority of the 10^{31} viruses present on Earth. They can be found at high

concentrations in water, with over 10^8 per milliliter being recorded from some other sources. More than 90% of characterized bacteriophages are classified in the order Caudovirales (Häusler, 2006). These are the tailed bacteriophages, with large double-stranded DNA genome in the range of 33000 to 170000bp or even larger. Other families of bacteriophages also exist, with range of morphologies, genome types, and genome sizes (Harper, 2012).

From the 1940s onward, bacteriophages became one of the basic tools of molecular biology. A vastly increased understanding of the nature and activities of the bacteriophages came along with an understanding of the processes of the cell (Janez *et al.*, 2013).

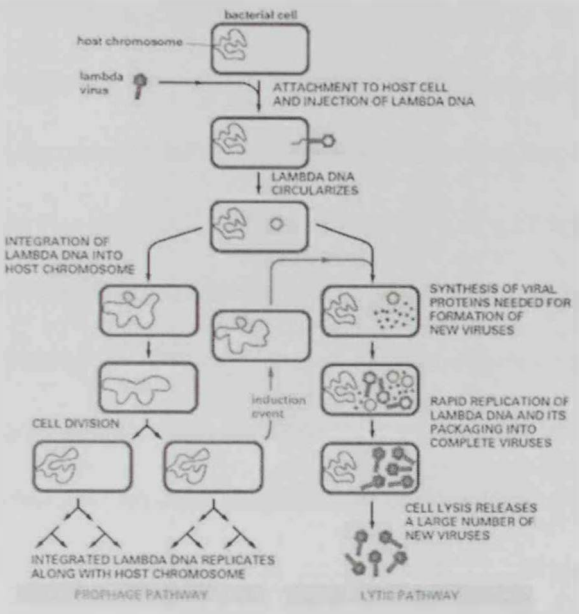
It soon became clear that very large numbers of bacteriophages existed, and that the vast majority were specific to a single host species (and indeed usually restricted to a limited range of strains within that species) (Lacey *et al.*, 2007).

While many bacteriophages produced rapid lysis of the host cell, other could integrate into the host chromosome, entering a latent state known in this context as lysogeny. The inserted bacteriophage DNA is then known as a prophage (Harper *et al.*, 2008). Reactivation may occur in response to a variety of stimuli, and is directly analogous to the chromosomal insertion/ reactivation cycle seen with the Retroviridae in eukaryotic cells (Tsonos *et al.*, 2013).

The ability to enter the lysogenic state is associated with a range of characterized genetic functions within the bacteriophage genome including a repressor of lytic gene function (that prevents killing of the host bacterial cell) and DNA integrates (that insert the viral DNA into the bacterial genome). When lysogenic bacteriophages

emerge from their latent state they may pick up and transfer bacterial DNA as part of this process. In some cases these may be associated with bacterial virulence, but this is not universal (Häusler, 2006).

Figure 1.1 Lytic and prophage pathways of the bacteriophages.



1.9 Isolation of bacteriophages from the environment

Purdy *et al.* (1984) showed that phages can be recovered from large volumes of sewage water by adding suspensions of their host bacteria, allowing adsorption to occur and harvesting cells by centrifugation. The resuspended cells are then incorporated into agar overlays and on inoculation; they produce lawns containing plaques of the bacteriophages (Purdy *et al.*, 1984).

The process of isolation of viruses by infecting host exploiting the specificity of the viruses is also employed in the isolation of bacteriophage (Armon *et al.*, 1993). The experiment determined to isolate bacteriophages from waste or sewage water by introducing them to 18-24 hours old pre-grown desired specific phage pathogen like

Escherichia coli. Further steps include addition of chloroform for 1 hour then transferring the suspension tubes of soft (0.7%) nutrient agar that would be poured to nutrient agar plates (Flint *et al.*, 2000). Double layer method to trap the bacteriophage in between the two layers is well known and effective method (Mahony *et al.*, 2010).

Most recent methods to isolate bacteriophages is the use of selective kits from specific companies, an example would be the Easy Phage kit developed by Scientific Methods, Indiana, USA. The new Easy Phage medium is used to enumerate bacteriophages present in food or in a water sample. The Easygel media contains ingredients to stimulate the growth of *E. coli* species susceptible to specific coli phages. The medium also include bacterial staining solution to produce good contrast between plaques and host bacterial lawn.

Easy Phage kit system produces better recoveries than the usual double layer method because it does not need the use of molten agar. EasyPhage is also simpler than traditional Single Agar Layer method (www.Scientificmethods.com).

1.10 Other beneficial uses of viruses

While viruses are primarily known as pathogens, they can be used for the benefit of mankind. The most obvious benefit is in their role in increasing our understanding of biology. The first genome sequences to be revealed were those of viruses (RNA bacteriophage M2S and DNA bacteriophage ΦX 174) (Harper, 2012).

The basic technology of genetic manipulation was developed from studies with bacteriophages and viral elements are still widely used in such work. The reverse

flow of genetic information (RNA to DNA) was discovered from work with viruses from the family including HIV virus *Retroviridae* (Harper, 2012).

There are also a number of ways in which viruses may produce direct benefit for human health. The most obvious of these are the production of vaccines and vaccine vectors (Harper, 2012). Vaccines do not simply protect against infection with the same virus. Relatively harmless viruses are used to provide protection from their more dangerous relatives (for example, the use of vaccinia virus to protect against smallpox, or Shope fibroma virus to protect against myxomatosis) (Hauer *et al.*, 2010). The viral vectors may be used to develop candidate vaccines against a range of diseases both viral and non-viral in nature (Hauer *et al.*, 2010).

There are four major beneficial applications of viruses, which are (i) Gene therapy, (ii) Cancer control and prevention, (iii) Control of harmful or damaging organisms and (i) bacteriophage therapy in both agricultural and medical field (Templeton, 2008; Harper, 2012).

1.10.1 Beneficial viruses in the field of gene therapy

The key element of gene therapy is the introduction of functioning genes into the cells of human patient, to express desired functions or to correct defective or non-operational genes within those cells (Hauer *et al.*, 2010).

Delivery systems must be able to introduce DNA into appropriate target cells. *Ex vivo* gene therapy involves removal of the cells from the body, followed by their treatment and reintroduction. *In vivo* gene therapy is more challenging and involves targeting of target cells within the body (Yata *et al.*, 2013; Krylov, 2014).

Beneficial viruses are routinely used in the genetic modification of model organisms for research purposes. The production of transgenic plants and animals in agriculture has also been established, but germ line modification of humans has not been attempted for technical and ethical reasons. However, genetic manipulation of somatic cells of individuals has been under investigation for many years. This is known as gene therapy (Lim *et al.*, 2011; Yata *et al.*, 2013).

1.10.2 Virus vector system

Virus vectors are used for *ex vivo* gene therapy, but are particularly useful where the target cells are in less accessible areas of the body. Viruses provide highly efficient systems for getting foreign nucleic acid into cells, and they are also highly suited to protect a nucleic acid while transporting it to the required area of the body (Harper, 2012).

1.10.3 Complications of gene therapy

While the principles of gene therapy are apparently straight forward, there are many issues that complicate the successful use of gene therapy. Specializations of cells (differentiation) may result in variant gene expression (Hauer *et al.*, 2010). For example, lung cells (targeted in attempts to treat cystic fibrosis) are highly specialized. The limited numbers of cases where a single gene defect produces serious illness and is amenable to correction using such methods, are likely to provide the first examples of human use (Tsonos *et al.*, 2013).

1.10.4 Beneficial viruses in cancer control and prevention

A number of viruses are associated with cancer in humans. These viruses have provided the first instances of the prevention of cancers by vaccination; however,

viruses can also have beneficial applications in the control of cancer (Oliveira *et al.*, 2014).

1.10.5 Vaccines

The most direct approach of using viruses to prevent cancers is simply that of vaccination against viruses that are associated with cancer. Vaccines for hepatitis B virus (Hepadnaviridae: associated with hepatocellualr carcinoma, and human papilomavirus (Papillomaviridae: associated with cervical cancer, are already available and are in widespread use (Häusler, 2006).

Although many studies have been carried out, these are the only vaccines approved to date by the US Food and Drug Administration (FDA) for the prevention of cancer (Johnke *et al.*, 2014).

1.10.6 Virotherapy

It is also possible to use the cell-killing effects of viruses directly, rather than relying on the immune system. A range of viruses have been used in efforts to produce targeted killing of cancerous cells, and the approach as a whole is known as virotherapy (Wong *et al.*, 2014).

1.10.7 Virus-directed enzyme prodrug therapy (VDEPT)

Viruses may also be used in virus-directed enzyme prodrug therapy (VDEPT) to insert into target cell an enzyme that can activate an inactive precursor of a cytotoxic drug (prodrug) that is administered systemically (Hauer *et al.*, 2010). For example, an adenovirus expressing the thymidine kinase (TK) enzyme of herpes simplex virus can be combined with systemic administration of ganiclovir, which is converted by TK to its active form only in cells where this enzyme is present (Borie *et al.*, 2009).

1.10.8 Control of harmful or damaging organisms in agriculture and medicine

The use of biological organisms to control damaging pests is broadly known as biological control, or biocontrol (Harper, 2006). Traditionally this has been used in agriculture, but application exist in the control of agents important to human health as well (Harper, 2012).

1.10.9 Beneficial viruses to control insect pests

Baculoviruses (Baculoviridae) are a large group of viruses that infect insects and other arthropods (Harper, 2006). All tend to be quite specific in the species that they will infect. Baculoviruses show a high level of environmental stability due to their formation of thick protein shell. They cause infection in the larvae of butterflies and moths and usually results in death in 4-5 days, although this can take longer under field conditions (Nigam *et al.*, 2014).

1.10.10 Beneficial viruses to control rabbits

Viral agents also provided the most successful approach to control the devastating numbers of European rabbits in Australia. The myxoma virus is a member of the *Poxviridae*, and it resulted in killing 90% to 100 % of European rabbits in infection known as myxomatosis (Meader *et al.*, 2010).

1.10.11 Beneficial insects viruses to control red palm weevil

Palm trees had always been the most common plant in many countries as it tolerates the hot and dry climate. Being a source of living for many decades, the palm trees draws a lot of attention to its health and well being. The red palm weevil *(Rhynchophorus ferrugineus)* attacking palm trees trunk is a great challenge against maintaining the palm trees in a good health (Lacey *et al.*, 2007). The management of

the red palm weevil had been with many methods including chemical and biological control. Viruses were used to control the red palm weevil and they showed promising results and outcomes (Kalha *et al.*, 2014).

1.10.12 Integrated pest management (IPM)

Integrated pest management or IPM is refered to the use of multiple, often low-potency, controls that together can reduce pest numbers to acceptable levels (Harper, 2006). In this approach, chemical pesticides can also be used in combination with biological agents, provided that the biological agents itself is not harmed by the chemical treatment used which makes viruses a good agent for this approach (Meader *et al.*, 2010).

1.11 Aims of the study

The major aim of the present thesis was to search for a safe method to reduce the risk of *Salmonella typhimurium* and *Escherichia coli* in chicken feed in the United Arab Emirates (UAE) poultry farms. The bacteriophages will then be cultivated and used to treat chicken feed in order to eliminate or reduce the application of antibiotics in the chicken feed which affect the food safety.

The objectives and aims of the current thesis were to:

1. Isolate super lytic bacteriophage that can kill *Salmonella typhimurium* and *E. coli in vitro*.
2. Test the effectiveness of the isolated bacteriophages in chicken feed.
3. Compare the isolated bacteriophages with the currently in use antibiotics in chicken feed.

CHAPTER II: MATERIALS AND METHODS

2.1 MATERIALS

2.1.1 Untreated raw waste water samples

Untreated raw waste water samples were collected from Abu Dhabi Sewerage Services Company (ADSSC) Al Ain, United Arab Emirates (UAE). The two samples were obtained from two different sewerage treatment plants in Al Ain, which are Al Saad Plant and Seih Lehma Plant in February 2013.

2.1.2 Bacterial strains and propagation hosts

Type strains of *Staphylococcus aureus* (ATCC 29213), *Staphylococcus epidermidis* (ATCC 12228 D-5), *Enterococcus faecalis* (ATCC 51299), *Escherichia coli* (ATCC 25922), *Proteus vulgaris* (ATCC 33420), *Pseudomonas aeruginosa* (ATCC 27853), *Salmonella typhimurium* (ATCC 14028) and *Shigella dysenteriae* (ATCC 13313) were obtained from the Microbiology Laboratory, Tawam Hospital, Al Ain. UAE. *Bacillus subtilis* (DSM 1088) was obtained from the culture collection of Dr. Khaled El-Tarabily, Biology Department, Faculty of Science, UAE University. The culture of *B. subtilis* was obtained from Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, (DSMZ), Braunschweig, Germany.

2.1.3 Media

Nutrient broth, nutrient agar, tryptic soy broth, and tryptic soy agar were all obtained from Lab M Limited, Moss Hall Road, Heywood, Lancashire BL96 7jj, United Kingdom. All these media were prepared according to the manufacturer recommendations. The Peptone-yeast extract calcium nitrate (PYCa) was prepared as described by Bradley *et al.* (1961) (Appendix 1).

2.1.4 Easy phage-100 medium kit for the isolation of coli phage (*E. coli* phage)

A kit of easy phage 100-medium for the isolation of coli phage (*E. coli* phage) was obtained from Scientific methods Inc., Beckley Street, Granger, Indiana, USA. This kit was used only to isolate the bacteriophages active against *E. coli* from untreated raw waste water samples.

2.1.4.1 Ingredients of the easy phage-100 medium kit

The easy phage-100 medium kit consisted of

(i)- Tryptic soy broth medium single strength (1 X).

(ii)- Culture of *E. coli*. In the current study *E. coli* (ATCC 25922) was used.

(iii)- Sterile easy phage medium (100 ml per bottle). The medium was kept in the fridge at 4°C until use. The medium was warmed up to room temperature and was mixed well before use.

(iv)- Sterile bacterial stain (0.7 ml of bacterial stain for every bottle of 100 ml easy phage medium).

(v)- Sterile pretreated Petri dishes provided by the Scientific methods Inc., Granger, Indiana, USA.

(vi)- *E. coli* phage MS2 was used as a positive control. The MS2 *E. coli* phage was used to spike the positive control water sample.

2.1.5 Transmission electron microscopy

Transmission electron microscopy (TEM) grids and uranyl acetate were carried out in the Electron Microscopy Unit, Faculty of Medicine and Health Sciences, Al-Ain, United Arab Emirates University, UAE with the assistance of Mr. Saeed Tariq.

2.1.6 Chicken feed

The corn-soybean diet was prepared in the Faculty of Food and Agriculture/Al Foha Farm, UAE University, Al-Ain, UAE. The chemical composition of the diet is described in Table 2.1

The diet was prepared as follows: all feed ingredients were ground to a suitable size and mixed in a commercial mixer (Spar mixer, 3HP, Taiwan) for 20 minutes. Vitamins and minerals premixes, fish meal and oil were gradually added with continuous mixing. The wet mix was then passed through a commercial mixer (Silla, 350L, Italy) for 15 minutes for a homogenous distribution of the nutrients and particle sizes. The corn-soy diet was placed in large Erlenmeyer flasks and was autoclaved at 121°C for 30 minutes on three consecutive days.

Table 2.1: Composition of the corn-soybean chicken feed diet

Ingredient Name	Corn soybean diet (Kg)
Yellow corn	59.4
Soybean meal	32
Sodium chloride	0.4
Limestone	1.1
Di-calcium phosphate	1.56
Vitamin + Mineral Premix	1
Methionine	0.24
Lysine	0.02
Corn oil	2
Fish meal	2.3

2.1.7 Antibiotic in chicken feed

The antibiotic used in the chicken feed experiment with and without the application of bacteriophages was oxytetracycline which is a broad-spectrum antibiotic, active against a wide variety of bacteria. It was used in the concentration of (50g/100Kg).

2.2 METHODS

2.2.1 Waste water samples

The untreated raw waste water samples were firstly centrifuged at a speed of 2000 rpm for 15 minutes in order to precipitate all large particles. The supernatant was then filtered sterilized through sterile Millipore membranes (Pore size 0.8 µm, 0.4 µm and 0.2 µm, respectively, Millipore Corporation, MA, USA) and were collected in sterile bottles. The bacteriologically filtered waste water samples were then used as a source for the isolation of bacteriophages in the easy phage-100 medium kit (Burm *et al.*, 2010). The raw waste water samples were stored in the fridge at 5°C until use (Clark, 1965).

2.2.2 Propagation hosts

All the type strains were cultivated on nutrient broth and nutrient agar medium. The plates and flasks were incubated at 37°C for 48 hours in a dark incubator.

2.2.3 Isolation of *S. typhimurium* phage from waste water sample

Erlenmeyer flasks (250 ml) containing 20 ml of sterile peptone-yeast extract calcium nitrate broth (PYCa) (Bradley *et al.*, 1961) were inoculated with 1 ml of *S. typhimurium* (ATCC 14028) suspension and with 25 ml of raw-untreated bacteriologically filtered waste water samples. This PYCA was chosen since the yeast extract supports phage reproduction (Walton, 1951) and the divalent cation (Ca^{++}) is required for adsorption of phage to the receptors (Lomovskaya *et al.*, 1972), and it has also been found to increase the size and number of phage plaques obtained (Gold, 1959). The flasks were then incubated in a gyratory shaker (Model G76, New Brunswick Scientific-Edison, N.J., USA) at 200 rpm at 37°C for 48 hours.

After incubation, the suspensions from each flask were centrifuged for 10 minutes at 4000 rpm and the supernatant were filtered three times through sterile Millipore membrane filters (Millipore Corporation, MA, USA) (0.8 µm, 0.4 µm and 0.2 µm), respectively, and were collected in sterile tubes.

Aliquots (0.3 ml) of *S. typhimurium* (ATCC 14028) (host) were inoculated separately onto PYCa agar plates (Bradley *et al.*, 1961). The plates were dried for 30 minutes in a laminar flow (Vickers and Williams, 1987). After drying the plates, 0.2 ml of the raw-untreated bacteriologically filtered waste water samples was spotted onto the plates as a centrally placed droplet (Bradley *et al.*, 1961). The plates were then incubated in the dark for 48 hours at 37°C and examined for lytic zones (plaques) (Williams *et al.*, 1980).

2.2.4 Isolation of *E. coli* phage from waste water sample using the easy phage-100 medium kit method

The protocol for the detection of bacteriophages in waste water samples using easypahe-100 medium developed by Scientific methods Inc., Beckley Street, Granger, Indiana, USA was used in the current study.

Erlenmeyer flasks with tryptic soy broth were sterilized by autoclaving at 121°C for 20 minutes. These flasks were inoculated with *E. coli* (ATCC 25922) and the flasks were incubated at 37°C for 24 hours in the dark. Each 100 ml of the Millipore filtered raw waste water sample were inoculated with 3.5 ml of the *E. coli* culture. Accordingly 40 ml of tryptic soy broth were sufficient to analyze 10 X 100 ml raw waste water samples.

The easyphage medium and the tryptic soy broth (TSB) were warmed to 35°C prior the start of the experiment. The Millipore filtered waste water sample (100 ml) was added into a bottle of easy phage medium. Then 0.7 mL of bacterial stain and 3.5 ml of log phase *E. coli* (ATCC 25922) culture were added for every 100 ml of the easy phage medium. The solution was mixed by swirling the bottle several times. A 20 ml of the mixture was transferred into each pretreated Petri dish using sterile syringes for a total of 10 Petri dishes.

The Petri dishes were swirled gently several times to settle the bubbles on the edge of each pretreated Petri dish. The medium mixture was left to solidify on a horizontal bench for 1 hour. All the pretreated Petri dishes were transferred to the 37°C incubator and the pretreated Petri dishes were incubated in the upright position. The pretreated Petri dishes were incubated at 37°C in the dark for 48 hours.

After the end of the incubation period, the blue plaques on the background of red bacterial lawn were counted.

2.2.5 Quality assurance procedures in the easy phage kit method

In order to demonstrate that the reagents employed during the sample run of the easy phage kit were performing as expected; a negative control and a positive control were also processed and analyzed.

For the negative control, a 100 ml of sterile water containing no coli phage was processed the same way the waste water sample was processed. This sample produced no plaques and demonstrated that the procedural run employed in the current study was valid and correct.

For the positive control, 100 ml water sample containing viable coli phage (100 ml water sample spiked with MS2 pure *E. coli* phage) was used as a positive control.

Sterile water (100 ml) was added to a 100 ml easy phage bottle. Then sufficient amount of stock solution of the *E. coli* phage MS2 was spiked to the easy phage medium mixture to produce a countable number of plaques as recommended by the scientific methods protocol for the isolation of coli phages.

The bacterial stain (0.7 ml) and log phase *E. coli* (ATCC 25922) culture (3.5 ml) were then added. The solution was mixed by swirling the bottle several times. A 20 ml of the mixture was transferred into each pretreated Petri dish using sterile syringes for a total of 10 dishes.

The positive control sample produced plaques and this demonstrated that the analytical run was valid. This positive control result was associated with all samples analyzed during the analytical run.

Among the benefits of using the easy phage kit were, it was capable of producing quantitative results and quality control schemes for investigating the precision and accuracy of the microbiological assays.

2.2.6 Purification of the bacteriophages from the phage isolation plates

After incubation, a single plaque (single individual lysis zone) on the lawns of the bacterial hosts (from experiments 2.2.3 and 2.2.4) was aseptically removed with a scalpel and re-suspended in 100 ml of host-inoculated (5 ml) PYCa broth and incubated for 48 hours at 37°C in the dark. The broth was filtered through Whatman paper No. 1 (Whatman International Ltd, Maidstone, U.K.) and filtered again through sterile 0.22 μm Millipore membrane filters. The purified phage suspensions were

stored at 4°C (Williams *et al.*, 1980). A sample of this purified phage suspensions were then filtered and spotted on the PYCa agar plates previously inoculated with every prospective host alone in order to determine the concentration of the phage in the suspension.

To obtain higher concentrations, the same procedure was repeated by increasing the soaking period in PYCa broth for an additional 24 hours at 37°C in the dark. The procedure was repeated until a high titre of 10^{10} plaque-forming units (pfu) ml^{-1} of phage suspension was obtained. The purified phage suspension was stored at 4°C in order to minimize the bacterial growth which would impede filtration (Williams *et al.*, 1980). These phages were used immediately in each experiment to avoid a decrease in the concentration of the phage suspension.

2.2.7 Phage assay

Phage assays were carried out using the serial tenfold dilution (up to 10^{12}) prepared in PYCa broth. Aliquots (0.3 ml) of *E. coli* or *S. typhimurium* host suspensions were inoculated onto PYCa agar plates and dried in a laminar flow cabinet for 30 minutes. Bradley *et al.* (1961) showed that excessive moisture on an assay plate can create false plaques or cause smearing and streaking of valid plaques.

A (0.2 ml aliquot) of phage dilutions were then placed onto a plate and spread carefully with a glass rod. Three replicate plates were used for each dilution. All plates were incubated in the dark at 37°C for 48 hours. After incubation, pfu were counted in order to know the titre of the phage suspension.

2.2.8 Plaques morphology

Purified phage suspension of *E. coli* (0.2 ml) or purified phage suspension of *S. typhimurium* (0.2 ml) each containing 10^{10} pfu ml^{-1} were spotted onto PYCa agar seeded with the *Eshericia coli* host species (X 10^{10} C ml^{-1}) or *S. typhimurium* host species (X 10^{10} cfu ml^{-1}). The plates were then incubated for 48 h at 37°C and plaque morphology in the Petri dishes was noted.

2.2.9 Host range (Activity spectra of the phage)

All bacterial hosts were grown on nutrient agar plates and the bacterial cells were harvested by scraping the surface of the plates into 20 ml of sterile 20% (v/v) glycerol and stored at -20°C.

The bacterial hosts studied were the Gram positive bacteria *B. subtilis* (DSM 1088), *S. aureus* (ATCC 29213), *S. epidermidis* (ATCC 12228 D-5) and *E. faecalis* (ATCC 51299). The Gram negative bacteria tested included *E. coli* (ATCC 25922), *P. vulgaris* (ATCC 33420), *P. aeruginosa* (ATCC 27853), *S. typhimurium* (ATCC 14028), and *S. dysenteriae* (ATCC 13313).

The host range of *E. coli* phage or *S. typhimurium* phage were studied by spotting 0.2 ml of each phage suspensions containing 10^{10} pfu ml^{-1} onto PYCa agar plates each previously seeded with a glycerol suspension (X 10^{10} cfu ml^{-1}) of one of the type strains mentioned above. Each phage suspension was added to the dried host seeded plates, 30 minutes after seeding the plates. The plates were then incubated at 37°C for 48 hours and examined for lysis and plaques formation.

2.2.10 Electron microscopy

In order to determine the morphology and the morphological features of *E. coli* phage or *S. typhimurium* phage, using the transmission electron microscopy (TEM), the negative staining technique using uranyl acetate (Sigma Chemical Co, St. Louis, USA) as described by Ackermann and Heldal (2010) was employed.

The negative staining method (Ackermann and Heldal, 2010) depends on some stain that remained around the edges of a virus and accordingly the details of the virus were clearly defined. However, the positive staining method stains the virus itself and accordingly the viruses appeared as dark objects against a lighter background.

All TEM photomicrographs taken in the current study were all taken by the use of the negative staining method. This was the easiest staining method to be employed and yields results suitable for analyzing the morphological diversity of each virus as described by Burm and Steward (2010).

Briefly, the uranyl acetate solution was filtered through Millipore membrane syringe filters (Pore size 0.22 µm, Millipore Corporation, MA, USA) into a 2 ml screw cap tube. This removed any particles of uranyl acetate that had not fully dissolved.

A drop of each phage suspension (10^{10} pfu ml^{-1}) was placed on 200-mesh copper grids with carbon-coated formvar films and the excess drawn off with sterile filter paper. A saturated solution of filtered uranyl acetate was then placed on the grids and the excess drawn off as described by Ackermann and Heldal (2010). The grids were left for 1 hour to dry and then placed in the grid box in a desiccator until examination.

Specimens were then later observed with a TEM (Philips CM10, The Netherlands) operating at 80 KV at a magnification of X 205,000.

2.2.11 Effects of medium composition on phage propagation and viability

In order to study the effect of medium chemical composition on *E. coli* phage or *S. typhimurium* phage, three different media were used. These three media were peptone-yeast extract, nutrient agar and tryptic soy agar supplemented with and without calcium nitrate $Ca(NO_3)_2$ (0.05%) (Sigma Chemical Co, St. Louis, USA) and with two different concentrations of sodium chloride (NaCl) (0.1 M and 0.01 M) (Sigma) (Brownell *et al.*, 1967). These media were tested in order to determine the effects of these complex media on phage viability and propagation.

2.2.12 Effects of physical and chemical agents on phage propagation and viability

The effects of selected physical and chemical agents on the phage viability and propagation were tested according to the methods described by Brownell *et al.* (1967).

To determine the effects of physical treatments on phage propagation and viability, PYCa broth-grown preparations of each phage was diluted 1 to 10 in PYCa broth. A (0.1 ml) sample was added to 0.9 ml PYCa broth, the physical treatment applied, and viable phage numbers were estimated immediately after the treatment as pfu ml^{-1}. A similarly diluted but untreated PYCa broth preparation served as a control (Brownell *et al.*, 1967).

The physical treatments examined included (i) freezing the phages for (30 minutes, one hour, two hours, three hours, four hours, five hours, six hours, 12 hours, 24 hours, and 48 hours), (ii) refrigerating the phages at 4°C for (1 day, 7 days, 14 days, 30 days, 45 days and 60 days), (iii) heating the phages at 45°C for (15 minutes

and 30 minutes), heating the phages at 55°C for (15 minutes and 30 minutes), heating the phages at 65°C for (15 minutes and 30 minutes), (iv) boiling the phages at 100°C for (5 minutes and 10 minutes), (v) ultrasonic treatment for (60 seconds and 120 seconds), (vi) exposing the phages to Ultra Violet radiation (UV) for (30 seconds, 60 seconds, 120 seconds, 180 seconds and 240 seconds) as described by Brownell *et al.* (1967).

To determine the effects of the chemical agents on phage propagation and viability, PYCa broth grown preparations of each phage was diluted 1 to 10 in water. A (0.1 ml) sample was added to 0.9 ml of the reagent to be tested at the specified concentration in water. After 2 hours incubation at 37°C in the dark, the mixtures were assayed for phage pfu ml^{-1}. A similarly diluted but untreated PYCa broth preparation served as a control as described by Brownell *et al.* (1967).

The chemical treatments examined included (i) chloroform (0.5%, 1% and 3%), (ii) thymol (0.5%, 1% and 3%), (iii) hydrogen peroxide (0.5%, 1% and 3%), (iv) phenol (0.5%, 1% and 3%), (v) ethyl alcohol (30%, 70% and 100%), (vi) Clorox (0.5%, 1% and 3%), (vii) iodine in the form betadine (0.5%, 1% and 3%), (viii) merthiolate (0.5%, 1% and 3%) as described by Brownell *et al.* (1967).

2.2.13 Effects of host age, phage inoculum size and the host inoculum size on phage propagation and viability

The effect of host age on phage propagation was tested by adding suitable concentrations of phage to the host which had been incubated for various times as described by Brownell *et al.* (1967). The numbers of pfu ml^{-1} were counted as described by Brownell *et al.* (1967).

The effect of phage inoculum size on phage propagation was examined by mixing phage inocula at different concentrations with a standard host concentration and counting the numbers of PFU ml^{-1} as described by Brownell *et al.* (1967).

The effect of the host-inoculum size on phage proliferation was determined by varying the host inoculum concentration and assaying pfu ml^{-1}.

For all the above experiments the numbers of pfu ml^{-1} were determined after 48 hours at 37°C as described by Brownell *et al.* (1967).

2.2.14 Determination of adsorption rate constant, latent period and burst size

The adsorption rate constants of the two phages (*E. coli* phage and *S. typhimurium* phage) were determined by measuring residual plaque-forming ability in membrane-filtered samples of an attachment mixture as described by Dowding (1973). Briefly, a 250 ml Erlenmeyer flask containing 50 ml of PYCa broth was inoculated with a host suspension. The host was incubated with shaking at 37°C for 3 hours to allow growth to occur. Each phage was added separately at low multiplicity of infection (0.1), and the incubation was continued.

At various times, samples were removed, membrane filtered, diluted and plated and the numbers of pfu ml^{-1} was counted. The ratio of the number of phages to the host was termed as the multiplicity of infection and values much less than one are commonly used in kinetic studies on phage growth in order to ensure that each cell was infected by a single virus (Dowding, 1973).

The adsorption rate constant, K ml min^{-1}, was calculated as described by Sykes *et al.* (1981) by using the equation $K = 2.3 / Bt \times \log_{10} (P_0/Pt)$ where B is the host

concentration (cfu ml^{-1}); P_0 is the initial phage concentration (pfu ml^{-1}); Pt is the phage concentration at t min (pfu ml^{-1}); and t is the period of adsorption.

In order to determine the latent period, rise period and the burst size for each phage, a one-step growth experiment was determined as described by Dowding (1973). A suspension (1×10^{10} cfu ml^{-1}) of each propagation host (*Eshericia coli* or *S. typhimurium*) was incubated in PYCa broth for 3 hours to allow growth. A predetermined quantity of each phage was then added to give a low multiplicity of infection (approximately 0.1) and the incubation was continued for 20 minutes. A (10 ml) sample of the attachment mixture was removed and membrane-filtered. Un-adsorbed phages were removed from the infected host cells on the filter by passing the 10 ml of the broth through the filter to wash them. The filter was transferred to a flask containing 50 ml of PYCa broth at 37°C (first growth flask) and the infected cells were re-suspended by agitating the flask. A 50-fold dilution was made to another flask held at 37°C (second growth flask) and the two flasks were re-incubated at 37°C. Samples (0.1 ml) were removed (from the first growth flask until time 35 min and then alternately from the two flasks until 120 min), plated immediately and the numbers of pfu were counted after incubating the plates at 37°C for 48 hours as described by Dowding (1973).

2.2.15 The effect of incorporation of bacteriophages on the population densities of *S. typhimurium* and *E. coli* in the chicken feed

The aim of this experiment was to examine the effect of the incorporation of *S. typhimurium* phage or *E. coli* phage or both *S. typhimurium* and *E. coli* phages on the

population densities (bacterial counts) of *S. typhimurium* and *E. coli* in a chicken diet contaminated with these bacteria.

2.2.15.1 Preparation of the stock phage suspension

The host *E. coli* or *S. typhimurium* was grown on nutrient agar plates and incubated in the dark for 3 days at 37°C. Cells were harvested by scraping the surface of 20 plates into 1 liter of sterile 20% glycerol and storing at -20°C. Five large Erlenmeyer flasks (5 liters capacity) were each filled with 2 liters of sterile PYCa broth into which 250 ml of propagation host suspension (x 10^{10} cfu ml^{-1}) and 250 ml of phage suspension (x 10^{10} pfu ml^{-1}) were inoculated. The suspension was incubated in the dark for 4 days at 37°C. After incubation, the suspension was centrifuged for 1 h at 2000 rpm and serially filtered through decreasing pore size filters culminating with a 0.22 μm tubular membrane filter. After filtration, the phage suspension was calculated to be (x 10^{10} pfu ml^{-1}) by serial dilution. A total of 15 liters of every bacteriophage suspension was produced and stored at 4°C.

2.2.15.2 Preparation of *E. coli* or *S. typhimurium* inoculum

The host *E. coli* or *S. typhimurium* inoculum was prepared by scraping 20 plates of bacterial cultures, grown on nutrient agar plates, into 1 liter of sterile distilled water. Two hundred ml of this suspension was added to 5 liters of sterilized (121°C for 20 min) nutrient broth and incubated for 3 days in the dark at 37°C. Inoculum was used immediately. Serial dilutions were prepared and five 0.2 ml aliquots were inoculated separately onto nutrient agar plates before use to confirm the inoculum density. The inoculum density for *E. coli* or *S. typhimurium* was calculated to be (x 10^{10} pfu ml^{-1}) by serial dilution.

2.2.15.3 Mixing the host inoculum and the bacteriophage suspension and their amendments in the chicken feed

In the current study, individual phage of *E. coli* and individual phage of *S. typhimurium* each of about 10^{10} pfu ml^{-1} were used to inoculate the chicken feed individually alone in order to study their effects on the reduction of the population densities of *E. coli* or *S. typhimurium*.

In addition a stock phage suspension of about 10^{10} pfu ml^{-1} was prepared by mixing each of the two individual high-titer phage suspensions. The resulting titer of the stock phage suspension was 10^{10} pfu ml^{-1}. This stock phage suspension was also used to treat chicken feed in order to study the effects of the combined two bacteriophages on the reduction of the population densities of *E. coli* or *S. typhimurium* in the chicken diet.

The chicken corn-soy diet was prepared by adding 300 g of the chicken corn-soy diet and 40 ml of distilled water into 1 liter Erlenmeyer flasks. The flasks were autoclaved at 121°C for 30 minutes on three consecutive days.

For the treatments which included only the addition of the bacterial hosts alone, the corn-soy diet were then aseptically inoculated with 40 ml of *E. coli* (x 10^{10} cfu ml^{-1}) or 40 ml of *S. typhimurium* (x 10^{10} cfu ml^{-1}) or 40 ml of a mixture of *E. coli* and *S. typhimurium* (x 10^{10} cfu ml^{-1}) under aseptic conditions.

For the treatments which included the addition of the bacteriophages alone, the corn-soy diet were aseptically inoculated with 40 ml of *E. coli* phage (x 10^{10} pfu ml^{-}

$^{-1}$) or 40 ml of *S. typhimurium* phage (x 10^{10} pfu ml^{-1}) or 40 ml of a mixture of *E. coli* phage and *S. typhimurium* phage (x 10^{10} pfu ml^{-1}).

For the treatments which included the combination of bacteriophages and the bacterial hosts, the corn-soy diet were then aseptically inoculated with 20 ml of *E. coli* (x 10^{10} cfu ml^{-1}) or 20 ml of *S. typhimurium* (x 10^{10} cfu ml^{-1}) or 20 ml of a mixture of *E. coli* and *S. typhimurium* (x 10^{10} cfu ml^{-1}) combined with 20 ml of *E. coli* phage (x 10^{10} pfu ml^{-1}) or 20 ml of *S. typhimurium* phage (x 10^{10} pfu ml^{-1}) or 20 ml of a mixture of *E. coli* phage and *S. typhimurium* phage (x 10^{10} pfu ml^{-1}).

Control treatments consisted of chicken corn-soy diet treated as above, but autoclaved twice at 121°C for 30 minutes immediately prior to use. The flasks were occasionally shaken to ensure uniformity of colonization. After 2 hours, the content of each flask was spread over a sterilized aluminum foil and dried in a laminar flow cabinet for 45 minutes. After drying, the contents were returned back to its original sterilized flask.

In total there were 15 bacteria-phage combinations as described in Table 2.2. The treatments with the broad-spectrum antibiotic oxytetracycline (treatments number 13, 14 and 15) were incorporated into the corn-soy diet at the manufacturer's recommended rate (50g/100Kg). Treatments number 13, 14 and 15 were included as a comparison to all treatments which involved the use of bacteriophages as biological control agents. Each treatment was replicated eight times and the experiment was repeated once.

In all treatments, the flasks were incubated at 37°C for 3 days, one week and three weeks in the dark in order to study the effect of incubation time on the survival of the phages and the bacteria. The flasks were occasionally shaken to ensure uniformity of colonization.

After 3 days, or one week or two weeks of incubation at 37°C in the dark, 10 grams from every treatment were taken in order to determine the population densities of *E. coli* and *S. typhimurium* and also to determine the persistence of the bacteriophages in the chicken diet at the end of the incubation period.

In order to determine the population densities of *E. coli* and *S. typhimurium*, 10 g of the chicken feed from every treatment were dispensed into 100 ml of sterile 1 g l^{-1} agar solution in de-ionized water. The suspension was shaken for 30 minutes at 37°C. Ten-fold dilutions (10^{-2}–10^{-6}) were made in sterile deionized water and 0.2 ml aliquots were spread with a sterile glass rod onto nutrient agar in sterile plastic Petri plates. Ten plates were used per dilution and dried in a laminar flow cabinet for 30 minutes before incubation at 37°C in the dark for 2 days.

Colonies were counted and the population densities of *E. coli* and *S. typhimurium* were expressed as $\log_{10}$ colony forming units (cfu) g^{-1} chicken corn-soy diet as described by Sivasithamparam *et al.* (1979).

In order to determine the concentration of the *E. coli* phage or *S. typhimurium* phage, 10 g of the chicken feed from every treatment were dispensed into Erlenmeyer flasks (250 ml) containing 100 ml of sterile PYCa broth. The flasks were inoculated with 10 ml of *S. typhimurium* or 10 ml of *E. coli*. The flasks were then incubated in a gyratory shaker (Model G76, New Brunswick Scientific-Edison, N.J., USA) at 200

rpm at 37°C for 48 hours. After incubation, the suspensions from each flask were centrifuged for 10 minutes at 4000 rpm and the supernatant were filtered three times through sterile Millipore membrane filters (Millipore Corporation, MA, USA) (0.8 µm, 0.4 µm and 0.2 µm), respectively, and were collected in sterile tubes.

Aliquots (0.3 ml) of *S. typhimurium* or *E. coli* were inoculated separately onto PYCa agar plates. The plates were dried for 30 minutes in a laminar flow (Vickers and Williams, 1987). After drying the plates, 0.2 ml of the bacteriologically filtered sample from every treatment was spotted onto the plates (Bradley *et al.*, 1961). The solution was uniformly spread over the dried plates using sterilized spreaders. The plates were then incubated in the dark for 48 hours at 37°C and examined for lytic zones (plaques) (Williams *et al.*, 1980). The plaques were counted and expressed as $\log_{10}$ plaque forming units (pfu) g^{-1} chicken corn-soy diet as described by Brownell *et al.* (1967).

2.2.16 Statistical analysis

All treatments were arranged in a randomized complete block design for all experiments. Data were subjected to analysis of variance (ANOVA) and Least Significant Differences (LSD) between means were determined using Fisher's Protected LSD Test at $P = 0.05$. Superanova® (Abacus Concepts, Inc., Berkeley, California, USA) was used for all analyses. Values followed by the same letter within a column are not significantly different ($P > 0.05$) according to Fisher's Protected LSD Test.

Table 2.2 Different treatments to study the effect of inoculation with *Escherichia coli* phage or *Salmonella typhimurium* phage or a mixture of *E. coli* phage and *S. typhimurium* on the colony forming units (cfu) of *E. coli* or *S. typhimurium* in chicken corn-soy diet

Treatments
(1) Control (autoclaved corn-soy diet) + killed autoclaved *E. coli* alone
(2) Control (autoclaved corn-soy diet) + killed autoclaved *S. typhimurium* alone
(3) Control (autoclaved corn-soy diet) + killed autoclaved *E. coli* + killed autoclaved *S. typhimurium*
(4) Control (autoclaved corn-soy diet) + *E. coli* phage alone
(5) Control (autoclaved corn-soy diet) + *S. typhimurium* phage alone
(6) Control (autoclaved corn-soy diet) + *E. coli* phage + *S. typhimurium* phage
(7) Autoclaved corn-soy diet + living *E. coli* alone
(8) Autoclaved corn-soy diet + living *S. typhimurium* alone
(9) Autoclaved corn-soy diet + living *E. coli* + living *S. typhimurium*
(10) Autoclaved corn-soy diet + living *E. coli* + *E. coli* phage
(11) Autoclaved corn-soy diet + living *S. typhimurium* + *S. typhimurium* phage
(12) Autoclaved corn-soy diet + living *E. coli* + living *S. typhimurium* + *E. coli* phage + *S. typhimurium* phage
(13) Autoclaved corn-soy diet + living *E. coli* + oxytetracycline
(14) Autoclaved corn-soy diet + living *S. typhimurium* + oxytetracycline
(15) Autoclaved corn-soy diet + living *E. coli* + living *S. typhimurium* + oxytetracycline

CHAPTER III: RESULTS

3.1 Untreated raw waste water samples

The two untreated raw waste water samples obtained from two different sewerage treatment plants in Al Ain, (Al Saad Plant) and (Seih Lehma Plant) gave different results regarding the isolation of the lytic active phages against *E. coli* and *S. typhimurium*.

The untreated raw waste water sample from Al Saad Plant did not contain any active phages against *E. coli* and *S. typhimurium*. However, the two different phages used in the present study were isolated and purified from untreated raw waste water samples obtained from Seih Lehma Plant. Bacteriophage number 1 (O_1) was isolated using *S. typhimurium* as a propagation host and bacteriophage number 2 was isolated using *E. coli* as a propagation host.

3.2 Isolation of *S. typhimurium* phage from waste water sample

Bacteriophage number 1 (O_1) was isolated using *S. typhimurium* as a propagation host. When 0.2 ml of the raw-untreated bacteriologically filtered waste water samples was spotted (as a centrally placed droplet) onto the PYCa agar plates seeded with *S. typhimurium*, a clear lytic zone (big plaque) was produced after 48 hours of incubation at 37°C in the dark (Fig. 3.1)

3.3 Isolation of *E. coli* phage from waste water sample using the easy phage-100 medium kit method

Bacteriophage number 2 (O_2) was isolated from untreated raw waste water samples using *E. coli* as a propagation host. This kit was used only to isolate the bacteriophages only active against *E. coli* as they used pretreated Petri dishes. Blue

plaques were obtained on a red background (lawn) at the end of the incubation period (Fig. 3.2).

3.4 Quality assurance procedures in the easy phage kit method

No *E. coli* phages were obtained when the sterile water was used (negative control). No blue plaques were obtained on a red background (lawn) at the end of the incubation period (Fig. 3.3).

The MS2 pure *E. coli* phage obtained from Scientific methods Inc., Beckley Street, Granger, Indiana, USA, (the positive control) was very effective in attacking the *E. coli* cells as indicated by the formation of the typical blue plaques on a red background (lawn) at the end of the incubation period (Fig. 3.4). The MS2 pure *E. coli* phage gave results very similar to the unknown untreated raw waste water samples using *E. coli* as a propagation host. This was clear from the very similar typical blue plaques on a red background (lawn) at the end of the incubation period (Fig. 3.4).

The reagents employed during the sample run of the easy phage kit were shown to be of high standard as expected.

3.5 Purification of the bacteriophages from the phage isolation plates

After incubation, a single plaque (single individual lysis zone) of bacteriophage number 1 (O_1) on the lawns of the bacterial hosts was obtained using PYCa agar plates previously inoculated with *S. typhimurium* (Fig. 3.5).

After incubation, a single plaque (single individual lysis zone) of bacteriophage number 2 (O_2) on the lawns of the bacterial hosts using PYCa agar plates previously inoculated with *E. coli* was obtained (Fig. 3.6).

3.6 Phage assay

The optimum concentration of bacteriophage number 1 (O_1) active against *S. typhimurium* was found to be 10^{10} pfu ml^{-1} when the serial tenfold dilution technique (up to 10^{12}) prepared in PYCa plates was employed. Similarly, it was also found that, the optimum concentration of bacteriophage number 2 (O_2) active against *E. coli* was found to be 10^{10} pfu ml^{-1}.

3.7 Plaques morphology

Bacteriophage number 1 (O_1) selected to inhibit the growth of the Gram negative bacterium *S. typhimurium* formed small circular clear plaques (0.5 mm) (Fig. 3.5).

However, bacteriophage number 2 (O_2) selected to inhibit the growth of the Gram negative bacterium *E.coli* formed small circular clear plaques (0.9 mm) (Fig. 3.2). In the case of bacteriophage number 2 (O_2), the plaques were typically round, blue areas that lack the reddish color observed on the bacterial lawn. The bacterial lawn produced reddish color and the plaques were blue in color (Fig. 3.2).

3.8 Host range (Activity spectra of the phage)

Bacteriophage number 1 (O_1) isolated using *S. typhimurium* as a propagation host was found to be monovalent and lysed only *S. typhimurium* (Table 3.1). Bacteriophage number 1 (O_1) failed to lyse the Gram positive bacteria *B. subtilis*, *S. aureus*, *S. epidermidis* and *E. faecalis*. Bacteriophage number 1 (O_1) failed also to lyse the Gram negative bacteria *E. coli*, *P. vulgaris*, *P. aeruginosa* and *S. dysenteriae* (Table 3.1).

Bacteriophage number 2 (O_2) isolated using *E. coli* as a propagation host was found to be polyvalent and lysed the Gram positive bacteria *B. subtilis*, *S. aureus*, *S.*

epidermidis and *E. faecalis* (Table 3.1). In addition bacteriophage number 2 (O_2) also was found to be polyvalent against the gram negative bacteria. In addition to its ability to lyse *E. coli*, it was also able to lyse *S. typhimurium* (Table 3.1) and *P. vulgaris*, *P. aeruginosa* and *S. dysenteriae* (Table 3.1).

Table 3.1: Host range of bacteriophages number 1 and 2 isolated from untreated raw waste water samples on different Gram positive and gram negative bacteria.

Bacterial species	bacteriophage number 1	bacteriophage number 2
Bacillus subtilis (DSM 1088)	-	+
Staphylococcus epidermidis (ATCC 12228 D-5)	-	+
Staphylococcus aureus (ATCC 29213)	-	+
Enterococcus faecalis (ATCC 51299)	-	+
Salmonella typhimurium (ATCC 14028)	+ (PH)	+
Pseudomonas aeruginosa (ATCC 27853)	-	+
Escherichia coli (ATCC 25922)	-	+ (PH)
Proteus vulgaris (ATCC 33420)	-	+
Shigella dysenteriae (ATCC 13313)	-	+

(+) = host species susceptible to phage lysis; (-) = host species not susceptible to phage lysis; PH = propagation host.

3.9 Electron microscopy

Negatively stained particles of the bacteriophage number 1 (O_1) active against *S. typhimurium* fitted the *Siphoviridae* (B1) morphotype (Francki *et al.*, 1991) which consisted of icosahedral heads (Fig. 3.7). On the other hand, negatively stained particles of the bacteriophage number 2 (O_2) active against *E. coli* fitted also the *Siphoviridae* (B1) morphotype (Francki *et al.*, 1991) which consisted of icosahedral heads (Fig. 3.8).

3.10 Effects of medium composition on phage propagation and viability

For the bacteriophage number 1 ($Ø_1$) active against *S. typhimurium*, peptone-yeast extract agar was the optimum medium for phage propagation followed by nutrient agar and tryptic soy agar (Table 3.2). There was no significant difference between nutrient agar and tryptic soy agar on phage propagation and viability (Table 3.2).

The incorporation of 0.05% $Ca(NO_3)_2$ into peptone-yeast extract agar, nutrient agar and tryptic soy agar was found to increase the number of plaques on all the three media types compared to the same medium but without the addition of calcium nitrate (Table 3.2). Increased NaCl concentrations reduced the numbers of congruent pfu ml^{-1} on all the three media types (Table 3.2). The incorporation of 0.1 M NaCl to the media used killed the bacteriophage number 1 ($Ø_1$) and yielded no pfu (Table 3.2).

For the bacteriophage number 2 ($Ø_2$) active against *E. coli*, peptone-yeast extract agar was the optimum medium for phage propagation followed by tryptic soy agar and nutrient agar (Table 3.2). Tryptic soy agar was found to be significantly better than nutrient agar as a medium for bacteriophage number 2 (Table 3.2).

The incorporation of 0.05% $Ca(NO_3)_2$ into peptone-yeast extract agar, nutrient agar and tryptic soy agar was found to increase the number of pfu ml^{-1} on all the three media types compared to the same medium but without the addition of calcium nitrate (Table 3.2). Increased NaCl concentrations reduced the numbers of congruent plaques on all the three media types (Table 3.2). The incorporation of 0.1 M NaCl to the media used killed the bacteriophage number 1 ($Ø_1$) and yielded no pfu (Table 3.2).

Table 3.2: Effect of different complex media on the viability and propagation of the two phages.

Medium	Supplement	(log_{10} pfu ml^{-1}) of Phage number 1	(log_{10} pfu ml^{-1}) of Phage number 2
Peptone-yeast extract agar	+ 0.1 M NaCl	0.00 *a*	0.00 *a*
Peptone-yeast extract agar	+ 0.01 M NaCl	3.89 *c*	4.31 *c*
Peptone-yeast extract agar	+ 0.00 M NaCl (W/V)	5.51 *e*	5.85 *f*
Peptone-yeast extract agar	+ 0.00 M NaCl + 0.05% (W/V) $Ca(NO_3)_2$	7.41 *g*	7.91 *g*
Nutrient agar	+ 0.1 M NaCl	0.00 *a*	0.00 *a*
Nutrient agar	+ 0.01 M NaCl	3.36 *b*	2.25 *h*
Nutrient agar	+ 0.00 M NaCl (W/V)	4.61 *d*	2.81 *c*
Nutrient agar	+ 0.00 M NaCl + 0.05% (W/V) $Ca(NO_3)_2$	5.69 *f*	3.13 *k*
Tryptic soy agar	+ 0.1 M NaCl	0.00 *a*	0.00 *a*
Tryptic soy agar	+ 0.01 M NaCl	3.29 *b*	3.71 *b*
Tryptic soy agar	+ 0.00 M NaCl (W/V)	4.55 *d*	4.67 *d*
Tryptic soy agar	+ 0.00 M NaCl + 0.05% (W/V) $Ca(NO_3)_2$	5.71 *f*	5.21 *e*

Values are means of 5 replicates for each treatment and the values with the same letter within a column are not significantly ($P > 0.05$) different according to Fisher's Protected LSD Test.

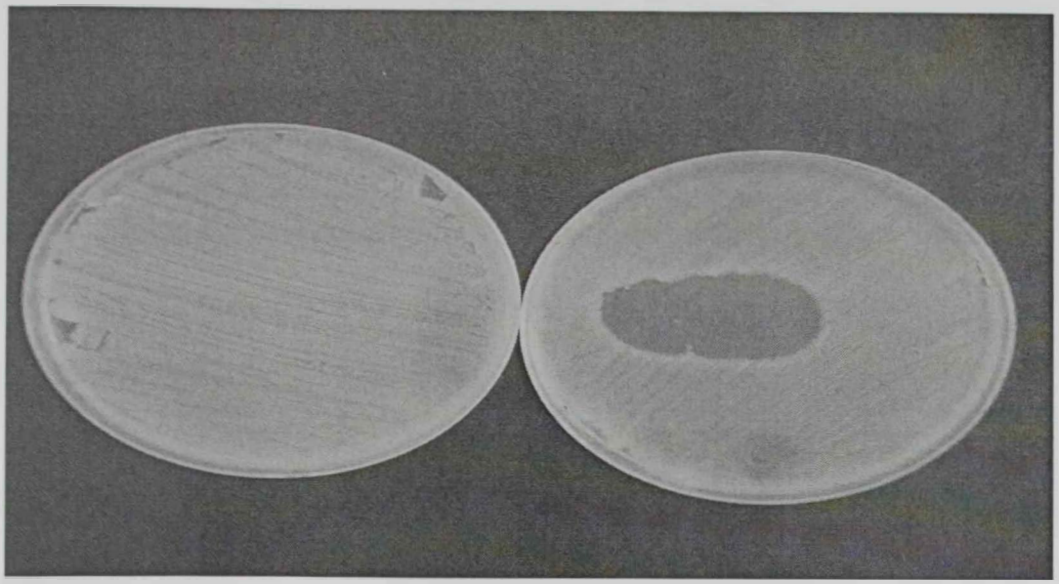


Figure 3.1: Lysis of *Salmonella typhimurium* by bacteriophage number 1. Plate on the left hand, is the control. Note the central lytic zone (plaque) in the middle of the peptone-yeast extract agar amended with calcium nitrate at the end of the incubation period (48 hours) at 37°C.

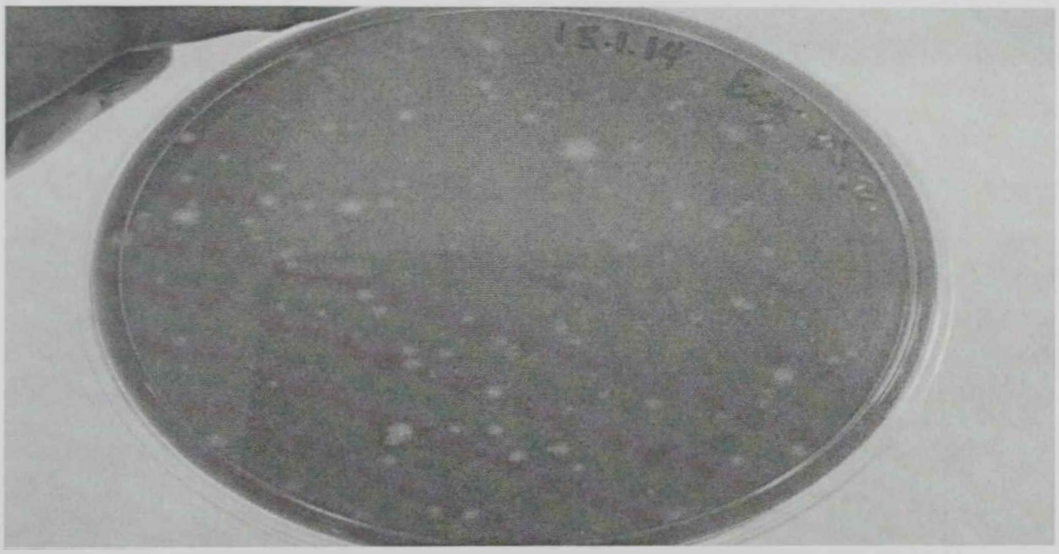


Figure 3.2: Lysis of *Escherichia coli* by bacteriophage number 2. Note the blue plaques on a red background (lawn) at the end of the incubation period (48 hours) at 37°C.

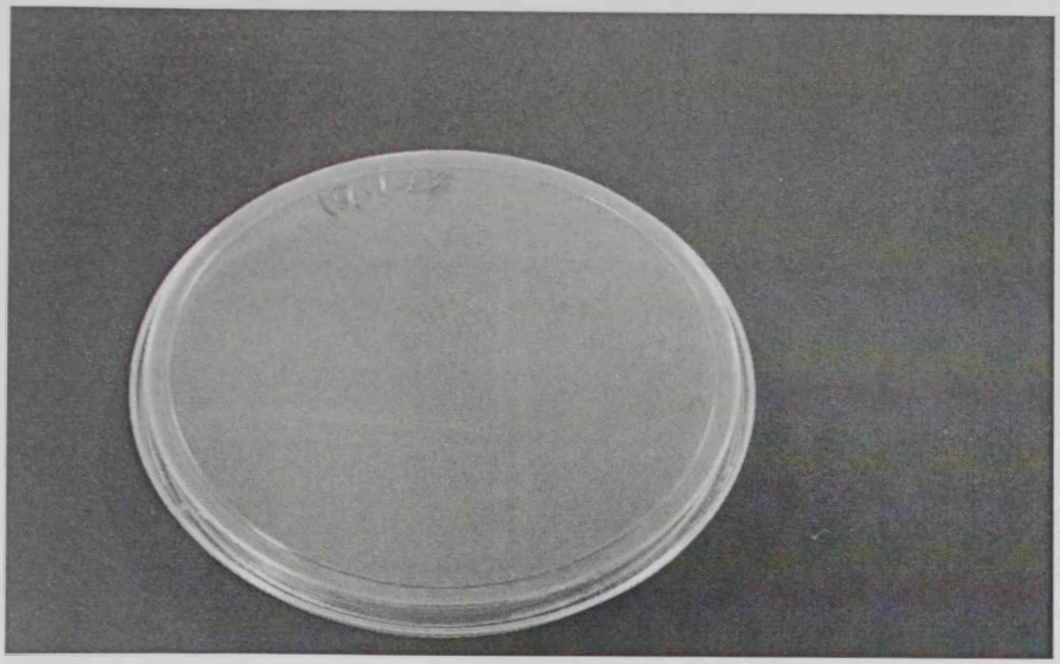


Figure 3.3: Negative control plate without the addition of phage number 2. Note the red background (lawn) at the end of the incubation period (48 hours) at 37°C.

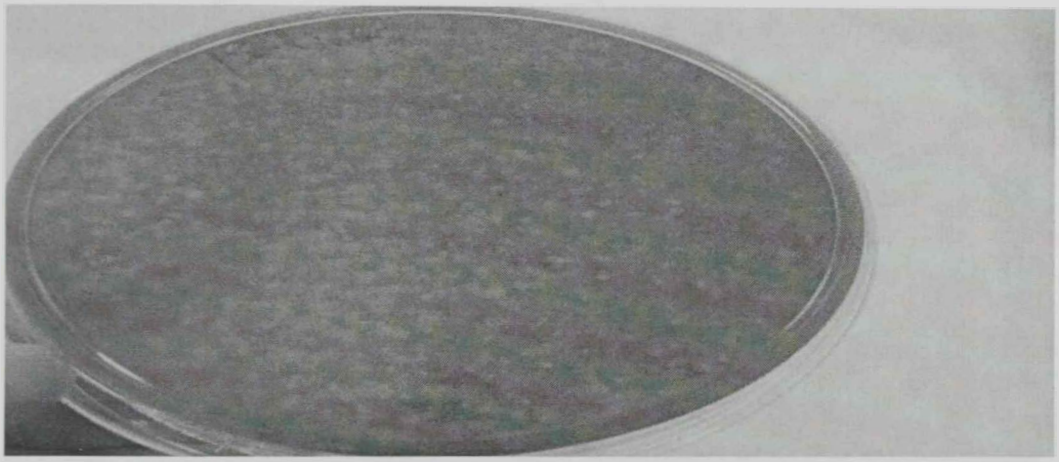


Figure 3.4: Lysis of *Escherichia coli* by MS2 pure *E. coli* phage (positive control). Note the blue plaques on a red background (lawn) at the end of the incubation period (48 hours) at 37°C.

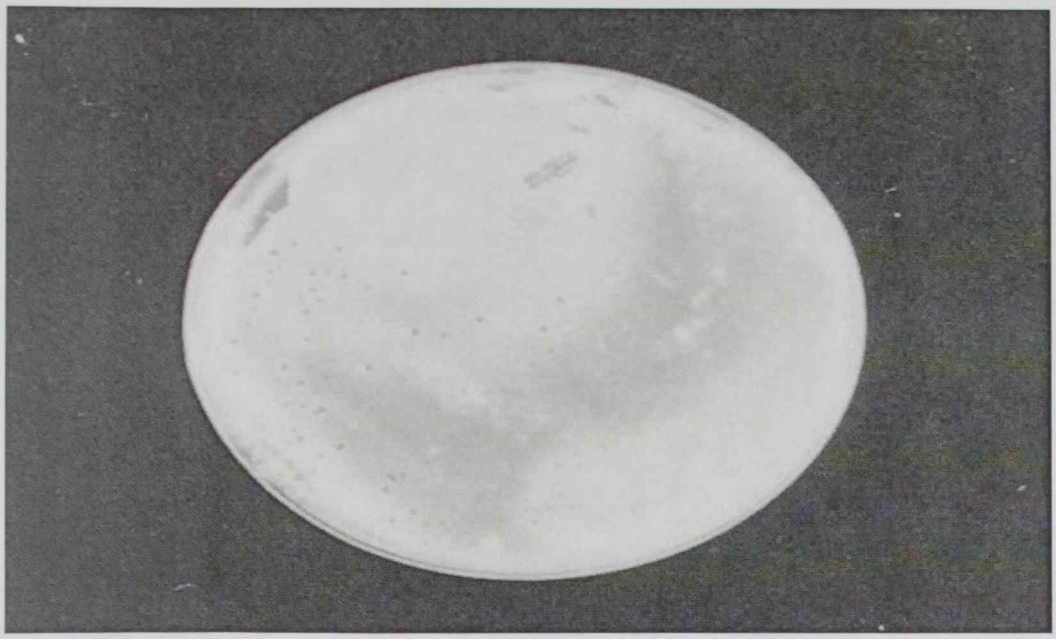


Figure 3.5: Single plaque formation of bacteriophage number 1 active against *Salmonella typhimurium* on peptone-yeast extract agar amended with calcium nitrate after 2 days of incubation at 37°C.

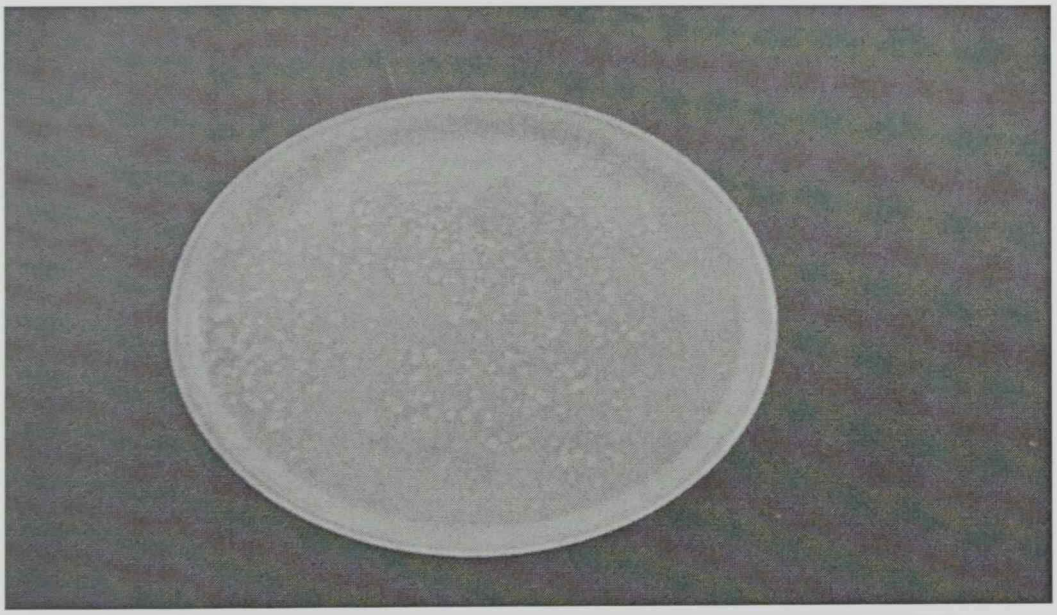


Figure 3.6: Single plaque formation of bacteriophage number 2 active against *Escherichia coli* on peptone-yeast extract agar amended with calcium nitrate after 2 days of incubation at 37°C.

Figure 3.7 Electron microscopy of bacteriophage number 1 active against *Salmonella typhimurium*. Magnification 205,000 X.

Figure 3.8 Electron microscopy of bacteriophage number 2 active against *Escherichia coli*. Magnification 205,000 X.

3.11 Effects of physical agents on phage propagation and viability

Bacteriophage number 1 (O_1) active against *S. typhimurium* was sensitive to the treatments with freezing, refrigeration, heating, boiling, ultrasonic treatment and Ultra Violet radiation (Table 3.3). All these physical agents greatly reduced the numbers of pfu (Table 3.3).

Freezing Bacteriophage number 1 (O_1) for 30 minutes, one hour, two hours showed slight adverse effect on the viability of the virus (Table 3.3). However, freezing for 3 hours, four hours, five hours, six hours, 12 hours, 24 hours, and 48 hours had an adverse effect on Bacteriophage number 1 (O_1) titre.

At 4°C bacteriophage number 1 (O_1) numbers decreased markedly with time (Table 3.3). Heating the bacteriophage number 1 (O_1) at 45°C for 15 minutes and for 30 minutes did not show any adverse effect on the virus viability(Table 3.3). Heating bacteriophage number 1 (O_1) at 55°C for 15 minutes and for 30 minutes also did not show any adverse effect on the virus viability(Table 3.3). However, heating the bacteriophage number 1 (O_1) at 65°C for 15 minutes and for 30 minutes had an adverse effect on phage titre and reduced the numbers of pfu (Table 3.3).

Boiling bacteriophage number 1 (O_1) at 100°C for 5 minutes and for 10 minutes completely; killed the phage as evident with no pfu formation (Table 3.3). Bacteriophage number 1 (O_1) was able to resist the ultrasonic treatment for 60 seconds and 120 seconds but with a reduced rate compared to the control treatment (Table 3.3). Exposing bacteriophage number 1 (O_1) to Ultra Violet radiation for 30 seconds and 60 seconds showed no adverse effect on the page viability. However, the

exposure to Ultra Violet radiation for 120 seconds, 180 seconds and 240 seconds reduced bacteriophage number 1 (O_1) viability and reduced the pfu (Table 3.3).

Bacteriophage number 2 (O_2) active against *E. coli* was sensitive to the treatments with freezing, refrigeration, heating, boiling, ultrasonic treatment and Ultra Violet radiation (Table 3.3). All these physical agents greatly reduced the numbers of pfu (Table 3.3).

Freezing Bacteriophage number 2 (O_2) for 30 minutes, one hour, two hours, 3 hours showed slight adverse effect on the viability of the virus (Table 3.3). However, freezing four hours, five hours, six hours, 12 hours, 24 hours, and 48 hours had an adverse effect on bacteriophage number 2 (O_2) titer.

At 4°C bacteriophage number 2 (O_2) numbers decreased markedly with time (Table 3.3). Heating the bacteriophage number 2 (O_2) at 45°C for 15 minutes and for 30 minutes did not show any adverse effect on the virus viability (Table 3.3). Heating bacteriophage number 2 (O_2) at 55°C for 15 minutes and for 30 minutes also did not show any adverse effect on the virus viability(Table 3.3). However, heating the bacteriophage number 2 (O_2) at 65°C for 15 minutes and for 30 minutes had an adverse effect on phage titer and reduced the numbers of pfu (Table 3.3).

Boiling bacteriophage number 2 (O_2) at 100°C for 5 minutes and for 10 minutes completely; killed the phage as evident with no pfu formation (Table 3.3). Bacteriophage number 2 (O_2) was able to resist the ultrasonic treatment for 60 seconds and 120 seconds but with a reduced rate compared to the control treatment (Table 3.3). Exposing bacteriophage number 2 (O_2) to Ultra Violet radiation for 30 seconds, 60 seconds and 120 seconds, showed no adverse effect on the phage

viability. However, the exposure to Ultra Violet radiation for 180 seconds and 240 seconds reduced bacteriophage number 2 (O_2) viability and reduced the pfu (Table 3.3).

Table 3.3: Effect of physical agents on the viability and propagation of the two phages.

Medium	(log_{10} pfu ml^{-1}) of phage number 1	(log_{10} pfu ml^{-1}) of phage number 2
Untreated control	8.25 *b*	7.82 *b*
Freezing (30 Minutes)	7.45 *c*	7.51 *c*
Freezing (1 hours)	7.11 *d*	7.38 *c*
Freezing (2 hours)	6.55 *e*	7.11 *d*
Freezing (3 hours)	4.52 *c*	7.05 *d*
Freezing (4 hours)	3.88 *k*	6.42 *e*
Freezing (5 hours)	3.21 *l*	5.51 *f*
Freezing (6 hours)	2.75 *m*	4.35 *g*
Freezing (12 hours)	0.00 *a*	0.00 *a*
Freezing (24 hours)	0.00 *a*	0.00 *a*
Freezing (48 hours)	0.00 *a*	0.00 *a*
4°C for 1 day	8.11 *b*	7.45 *c*
4°C for 7 days	6.54 *e*	7.21 *d*
4°C for 14 days	6.11 *f*	6.35 *e*
4°C for 30 days	5.76 *g*	5.23 *f*
4°C for 45 days	2.15 *n*	4.25 *g*
4°C for 60 days	1.25 *o*	1.56 *h*
45°C for 15 minutes	8.20 *b*	7.55 *c*
45°C for 30 minutes	8.18 *b*	7.25 *d*
55°C for 15 minutes	8.05 *b*	6.51 *e*
55°C for 30 minutes	8.00 *b*	6.43 *e*
65°C for 15 minutes	5.25 *h*	3.25 *l*
65°C for 30 minutes	4.75 *c*	2.98 *k*
Boiling 100°C for 5 minutes	0.00 *a*	0.00 *a*
Boiling 100°C for 10 minutes	0.00 *a*	0.00 *a*
Ultrasonic treatment for 60 seconds	7.25 *c*	7.15 *d*
Ultrasonic treatment for 120 seconds	7.05 *d*	6.88 *e*
UV irradiation for 30 seconds	8.20 *b*	7.79 *b*
UV irradiation for 60 seconds	8.03 *b*	7.71 *b*
UV irradiation for 120 seconds	6.53 *e*	7.51 *c*
UV irradiation for 180 seconds	6.21 *f*	5.45 *f*
UV irradiation for 240 seconds	5.75 *g*	5.23 *f*

Values are means of 5 replicates for each treatment and the values with the same letter within a column are not significantly ($P > 0.05$) different according to Fisher's Protected LSD Test.

3.12 Effects of chemical agents on phage propagation and viability

Bacteriophage number 1 (O_1) active against *S. typhimurium* was sensitive to the treatments with chloroform (0.5%, 1% and 3%), thymol (1% and 3%), hydrogen peroxide (1% and 3%), phenol (1% and 3%), ethyl alcohol (70% and 100%), Clorox (1% and 3%), iodine (1% and 3%), merthiolate (1% and 3%) (Table 3.4). All of these chemicals greatly reduced the numbers of pfu (Table 3.4). However, the bacteriophage number 1 (O_1) showed some degree of resistance to these chemicals in these concentrations thymol (0.5%), hydrogen peroxide (0.5%), phenol (0.5%), ethyl alcohol (30%), Clorox (0.5%), iodine (0.5%) and merthiolate (0.5%) (Table 3.4). Chloroform (3%), thymol (3%), hydrogen peroxide (3%), phenol (3%), Clorox (3%), iodine (3%), merthiolate (3%) completely killed bacteriophage number 1 (O_1) (Table 3.4).

Bacteriophage number 2 (O_2) active against *E. coli* was sensitive to the treatments with chloroform (0.5%, 1% and 3%), thymol (3%), hydrogen peroxide (3%), phenol (1% and 3%), ethyl alcohol (70% and 100%), Clorox (3%), iodine (3%), merthiolate (1% and 3%) (Table 3.4). All of these chemicals greatly reduced the numbers of pfu (Table 3.4). However, the bacteriophage number 2 (O_2) showed some degree of resistance to these chemicals in these concentrations thymol (0.5% and 1%), hydrogen peroxide (0.5% and 1%), phenol (0.5%), ethyl alcohol (30%), Clorox (0.5% and 1%), iodine (0.5% and 1%) and merthiolate (0.5%) (Table 3.4).

Chloroform (3%), thymol (3%), hydrogen peroxide (3%), phenol (1% and 3%), ethyl alcohol (70%), Clorox (3%), iodine (3%), merthiolate (3%) completely killed bacteriophage number 2 (O_2) (Table 3.4).

Table 3.4: Effect of chemical agents on the viability and propagation of the two phages.

Medium	(log_{10} pfu ml^{-1}) of phage number 1	(log_{10} pfu ml^{-1}) of phage number 2
Untreated control	8.25 *b*	7.82 *b*
Chloroform (0.5%)	7.55 *c*	4.05 *e*
Chloroform (1%)	3.54 *d*	2.15 *g*
Chloroform (3%)	0.00 *a*	0.00 *a*
Thymol (0.5%)	8.20 *b*	5.42 *d*
Thymol (1%)	7.48 *c*	4.11 *e*
Thymol (3%)	0.00 *a*	0.00 *a*
Hydrogen peroxide (0.5%)	8.15 *b*	5.28 *d*
Hydrogen peroxide (1%)	7.50 *c*	4.22 *e*
Hydrogen peroxide (3%)	0.00 *a*	0.00 *a*
Phenol (0.5%)	8.17 *b*	4.32 *e*
Phenol (1%)	3.44 *d*	0.00 *a*
Phenol (3%)	0.00 *a*	0.00 *a*
Ethyl alcohol (30 %)	8.11 *b*	5.55 *d*
Ethyl alcohol (70 %)	3.38 *d*	0.00 *a*
Ethyl alcohol (100 %)	7.44 *c*	5.31 *d*
Clorox (0.5%)	8.22 *b*	5.39 *d*
Clorox (1%)	3.21 *e*	3.75 *f*
Clorox (3%)	0.00 *a*	0.00 *a*
Iodine (0.5%)	8.08 *b*	6.87 *h*
Iodine (1%)	3.16 *e*	5.31 *d*
Iodine (3%)	0.00 *a*	0.00 *a*
Merthiolate (0.5%)	8.03 *b*	6.71 *h*
Merthiolate (1%)	3.05 *f*	4.22 *e*
Merthiolate (3%)	0.00 *a*	0.00 *a*

Values are means of 5 replicates for each treatment and the values with the same letter within a column are not significantly ($P > 0.05$) different according to Fisher's Protected LSD Test.

3.13 Effects of host age on phage propagation and viability

Fewer bacteriophage number 1 ($Ø_1$) active against *S. typhimurium* were obtained when propagation hosts were incubated for 0-3 h, in comparison to those incubated for 5-10 h (Table 3.5). More pfu where obtained when the propagation hosts were incubated for 10 hours. Meanwhile, fewer bacteriophage number 2 ($Ø_2$) active against

E. coli were obtained when propagation hosts were incubated for 0-3 h, in comparison to those incubated for 5-10 h (Table 3.5).

3.14 Effects of host inoculum size on phage propagation and viability

Greater levels of host inoculum resulted in greater phage titers (Table 3.6) for bacteriophage number 1 (O_1) active against *S. typhimurium* (Table 3.6). Propagation host at 10^7 cfu ml^{-1} yielded the highest pfu ml^{-1} compared to 10^6 cfu ml^{-1} and 10^5 cfu ml^{-1} (Table 3.6).

Also, greater levels of host inoculum resulted in greater phage titers (Table 3.6) for bacteriophage number 2 (O_2) active against *E. coli* (Table 3.6).

Propagation host at 10^7 cfu ml^{-1} yielded the highest pfu ml^{-1} compared to 10^6 cfu ml^{-1} and 10^5 cfu ml^{-1} (Table 3.6).

3.15 Effects of phage inoculum size on phage propagation and viability

Greater phage inoculum size resulted in greater phage output (Table 3.7) for bacteriophage number 1 (O_1) active against *S. typhimurium* (Table 3.7). Phage inoculum size of 10^7 pfu ml^{-1} gave the highest pfu ml^{-1} compared to10^6 pfu ml^{-1} and 10^5 pfu ml^{-1} (Table 3.7).

Also, greater phage inoculum size resulted in greater phage output (Table 3.7) for bacteriophage number 2 (O_2) active against *S. typhimurium* (Table 3.7). Phage inoculum size of 10^7 pfu ml^{-1} gave the highest pfu ml^{-1} compared to10^6 pfu ml^{-1} and 10^5 pfu ml^{-1} (Table 3.7).

3.16 Determination of adsorption rate constant, latent period and burst size

Adsorption rate constant (K) of bacteriophage number 1 (O_1) arising from *S. typhimurium* was 1.22 X 10^{-6} ml min^{-1} (Table 3.8). The Adsorption rate constant of

bacteriophage number 2 (O_2) arising from *E. coli* was 1.41 X 10^{-6} ml min^{-1} (Table 3.8).

Counts from samples taken after 35-40 min suggested that infected cells were lysing and liberating phage. Latent period values obtained for the bacteriophage number 1 (O_1) arising from *S. typhimurium* was 40 minutes (Table 3.8). Latent period values obtained for the bacteriophage number 2 (O_1) arising from *E. coli* was 35 minutes (Table 3.8).

The rise periods of the bacteriophage number 1 (O_1) arising from *S. typhimurium* was 30 minutes (Table 3.8). The rise periods of the bacteriophage number 2 (O_2) arising from *E. coli* was 40 minutes (Table 3.8).

The average burst sizes of bacteriophage number 1 (O_1) arising from *S. typhimurium* was 21.5 virions/cell (Table 3.8). The average burst sizes of bacteriophage number 2 (O_2) arising from *E. coli* was 15.2 virions/cell (Table 3.8). The second burst began at around 70-80 minutes; nearly 30-40 min after the first burst began which confirmed the minimum latent period of 30-40 min for the two phages (Table 3.8).

Table 3.5: Effect of incubation time of host (in hours) prior to inoculation (host age) on the phages productivity and yield of phages.

Incubation time (in hours) at 37°C of host prior to inoculation with the phage	(log_{10} pfu ml^{-1}) of phage number 1	(log_{10} pfu ml^{-1}) of phage number 2
0	6.14 *a*	7.78 *a*
3	6.11 *a*	7.92 *a*
5	6.51 *b*	9.14 *b*
10	8.38 *c*	9.44 *c*

Values are means of 5 replicates and the values with the same letter within a column are not significantly ($P > 0.05$) different according to Fisher's Protected LSD Test.

Table 3.6: Effect of propagation host inoculum size on the phages productivity and yield of phages

Propagation host inoculum size (cfu ml^{-1} of host suspension)	(log_{10} pfu ml^{-1}) of phage number 1	(log_{10} pfu ml^{-1}) of phage number 2
10^7	6.81 *a*	6.41 *a*
10^6	5.90 *b*	5.32 *b*
10^5	5.16 *c*	4.81 *c*
10^4	4.62 *d*	4.35 *d*

Values are means of 5 replicates and the values with the same letter within a column are not significantly ($P > 0.05$) different according to Fisher's Protected LSD Test.

Table 3.7: Effect of phage inoculum size on the phages productivity and yield of phages.

Phage inoculum size (pfu ml^{-1} of phage suspension)	(log_{10} pfu ml^{-1}) of phage number 1	(log_{10} pfu ml^{-1}) of phage number 2
10^7	6.42 *a*	6.19 *a*
10^6	6.11 *b*	5.91 *b*
10^5	5.77 *c*	5.23 *c*
10^4	5.23 *d*	4.89 *d*
10^3	4.76 *e*	4.35 *e*
10^2	3.25 *f*	3.93 *f*
10^1	2.31 *g*	2.21 *g*

Values are means of 5 replicates and the values with the same letter within a column are not significantly ($P > 0.05$) different according to Fisher's Protected LSD Test.

Table 3.8: Biological properties of phage number 1 (O_1) active against *Salmonella typhimurium*, and phage number 2 (O_2) active against *Escherichia coli*.

Phage number	Adsorption rate constant K (mL min)	Latent period (min)	Rise period (min)	Burst size relative increase (virions/cells)
Phage number 1 ($Ø_1$) active against *S. typhimurium*	1.22×10^{-6}	40	30	21.5
Phage number 2 (O_2) active against *E. coli*	1.41×10^{-6}	35	40	15.2

3.17 The effect of incorporation of bacteriophages on the population densities of *S. typhimurium* and *E. coli* in the chicken feed

3.17.1 Effect of mixing the two phages on bacterial growth

When bacteriophage number 1 (O_1) selected to inhibit *S. typhimurium* and bacteriophage 2 (O_2) selected to inhibit *E. coli* were mixed together, there were no inhibitory effects of the two phages on each other's. The two phages in one mixture were still able to inhibit the growth of *S. typhimurium* and *E. coli*.

3.17.2 The effect of incorporation of *S. typhimurium* and *E. coli* bacteriophages on the population densities of *S. typhimurium* and *E. coli* in the chicken feed

In control treatments which contained the killed *E. coli* alone (treatment 1), killed *S. typhimurium* alone (treatment 2) and killed *E. coli* + killed *S. typhimurium* (treatment 3), no bacteria were found at the end of the incubation period after 3 days, or after 7 days or after 21 days (Tables 3.9, 3.10 and 3.11).

In control treatments which contained the *E. coli* phage alone (treatment 4), *S. typhimurium* phage alone (treatment 5) and *E. coli* phage + *S. typhimurium* phage (treatment 6), the bacteriophage number 1 (O_1) active against *S. typhimurium* and bacteriophage number 2 (O_2) active against *E. coli* were found to be at high levels at the end of the incubation period after 3 days, or after 7 days or after 21 days (Tables 3.9, 3.10 and 3.11). The $\log_{10}$ plaque forming units (pfu) g^{-1} chicken corn-soy diet of the two phages were found to be with high titres (Tables 3.9, 3.10 and 3.11). No bacteria were observed in treatments 4, 5 and 6 (Tables 3.9, 3.10 and 3.11).

In the treatments which contained the living *E. coli* alone (treatment 7), living *S. typhimurium* alone (treatment 8) and living *E. coli* + living *S. typhimurium* (treatment

9), the bacteria were found at the end of the incubation period after 3 days, or after 7 days or after 21 days (Tables 3.9, 3.10 and 3.11). The population densities of *E. coli* and *S. typhimurium* expressed as $\log_{10}$ colony forming units (cfu) g^{-1} chicken corn-soy diet was found to be significantly higher compared to other treatments (treatments 10, 11 and 12) which contained the bacteria and the phages together. No bacteriophages were detected in treatments 7, 8 and 9 (Tables 3.9, 3.10 and 3.11).

The application of bacteriophage number 1 (O_1) selected to inhibit *S. typhimurium* in autoclaved corn-soy diet infested with *S. typhimurium* (treatment 11), or the application of bacteriophage number 2 (O_2) selected to inhibit *E. coli* in autoclaved corn-soy diet infested with *E. coli* (treatment 10), or the application of bacteriophage number 1 (O_1) selected to inhibit *S. typhimurium* and bacteriophage number 2 (O_2) selected to inhibit *E. coli* in autoclaved corn-soy diet infested with *S. typhimurium* and *E.coli* (treatment 12) significantly ($P < 0.05$) reduced the population densities of *S. typhimurium* in treatment 11 and the population densities of *E.coli* in treatment 10 and the population densities of *S. typhimurium* and *E.coli* in treatment 12 compared to the treatments which included the application of living *E. coli* alone (treatment 7), living *S. typhimurium* alone (treatment 8) and living *E. coli* + living *S. typhimurium* (treatment 9) (Tables 3.9, 3.10 and 3.11). Treatment 12 (the two bacteriophages together + living *E. coli* + living *S. typhimurium*) in *E. coli* + *S. typhimurium* amended chicken feed gave the best control and significantly ($P < 0.05$) reduced the population densities of *S. typhimurium* and *E.coli* compared with other treatments (Tables 3.9, 3.10 and 3.11).

The treatment which received the mixture of the two phages (treatment 12) or the application of bacteriophage number 1 (O_1) selected to inhibit *S. typhimurium* (treatment 11), or the application of bacteriophage number 2 (O_2) selected to inhibit *E. coli* (treatment 10) was almost as effective as the oxytetracycline applications (treatment 13), or (treatment 14) or (treatment 15) in reducing the incidence of *S. typhimurium* and *E. coli* in the chicken feed (Tables 3.9, 3.10 and 3.11).

There were, however, no significant ($P > 0.05$) differences between the bacterial levels of the phage treated chicken feed (treatments 10, 11 and 12) and the oxytetracycline treated chicken feed (treatments 13, 14 and 15) (Tables 3.9, 3.10 and 3.11).

The same trends of the reduction of the population densities of *S. typhimurium* and *E. coli* were observed after 3 days, or after 7 days or after 21 days of incubation with bacteriophage number 1 (O_1) or bacteriophage number 2 (O_2) or both bacteriophage (O_1) + bacteriophage (O_2) (Tables 3.9, 3.10 and 3.11).

There were more reduction in the population densities of *S. typhimurium* and *E. coli* at 21 days of incubation more than 7 days of incubation and 3 days of incubation.

Table 3.9 Effect of incorporation of bacteriophages on the population densities of *S. typhimurium* and *E. coli* (expressed as $\log_{10}$ cfu g^{-1} chicken corn-soy diet) and on the population densities of *S. typhimurium* and *E. coli* phages (expressed as $\log_{10}$ pfu g^{-1} chicken corn-soy diet) after incubation at 37°C for 3 days.

Treatments	Phage population densities as $\log_{10}$ (pfu) g^{-1} chicken corn-soy diet	Bacteria population densities as $\log_{10}$ (cfu) g^{-1} chicken corn-soy diet
(1) Control (autoclaved corn-soy diet) + killed autoclaved *E. coli* alone	0.00 *a*	0.00 *a*
(2) Control (autoclaved corn-soy diet) + killed autoclaved *S. typhimurium* alone	0.00 *a*	0.00 *a*
(3) Control (autoclaved corn-soy diet) + killed autoclaved *E. coli* + killed autoclaved *S. typhimurium*	0.00 *a*	0.00 *a*
(4) Control (autoclaved corn-soy diet) + *E. coli* phage alone	7.31 *b*	0.00 *a*
(5) Control (autoclaved corn-soy diet) + *S. typhimurium* phage alone	6.88 *c*	0.00 *a*
(6) Control (autoclaved corn-soy diet) + *E. coli* phage + *S. typhimurium* phage	8.51 *d*	0.00 *a*
(7) Control (autoclaved corn-soy diet) + living *E. coli* alone	0.00 *a*	8.42 *b*
(8) Control (autoclaved corn-soy diet) + living *S. typhimurium* alone	0.00 *a*	8.81 *c*
(9) Control (autoclaved corn-soy diet) + living *E. coli* + living *S. typhimurium*	0.00*a*	9.3 *d*
(10) Control (autoclaved corn-soy diet) + living *E. coli* + *E. coli* phage	7.25 *b*	4.21 *g*
(11) Control (autoclaved corn-soy diet) + living *S typhimurium* + *S. typhimurium* phage	6.79 *c*	3.54 *f*
(12) Control (autoclaved corn-soy diet) + living *E. coli* + living *S. typhimurium* + *E. coli* phage + *S. typhimurium* phage	8.41 *d*	3.15 *e*
(13) Control (autoclaved corn-soy diet) + living *E. coli* + oxytetracycline	0.00 *a*	4.05 g
(14) Control (autoclaved corn-soy diet) + living *S. typhimurium* + oxytetracycline	0.00 *a*	3.61 *f*
(15) Control (autoclaved corn-soy diet) + living *E. coli* + living *S. typhimurium* + oxytetracycline	0.00 *a*	3.05 *e*

Values are means of 8 replicates and the values with the same letter within a column are not significantly ($P > 0.05$) different according to Fisher's Protected LSD Test.

Table 3.10 Effect of incorporation of bacteriophages on the population densities of *S. typhimurium* and *E. coli* (expressed as $\log_{10}$ cfu g^{-1} chicken corn-soy diet) and on the population densities of *S. typhimurium* and *E. coli* phages (expressed as $\log_{10}$ pfu g^{-1} chicken corn-soy diet) after incubation at 37°C for 7 days.

Treatments	Phage population densities as $\log_{10}$ (pfu) g^{-1} chicken corn-soy diet	Bacteria population ensities as $\log_{10}$ (cfu) g^{-1} chicken corn-soy diet
(1) Control (autoclaved corn-soy diet) + killed autoclaved *E. coli* alone	0.00 *a*	0.00 *a*
(2) Control (autoclaved corn-soy diet) + killed autoclaved *S. typhimurium* alone	0.00 *a*	0.00 *a*
(3) Control (autoclaved corn-soy diet) + killed autoclaved *E. coli* + killed autoclaved *S. typhimurium*	0.00 *a*	0.00 *a*
(4) Control (autoclaved corn-soy diet) + *E. coli* phage alone	8.51 *b*	0.00 *a*
(5) Control (autoclaved corn-soy diet) + *S. typhimurium* phage alone	6.95 *c*	0.00 *a*
(6) Control (autoclaved corn-soy diet) + *E. coli* phage + *S. typhimurium* phage	8.91 *d*	0.00 *a*
(7) Control (autoclaved corn-soy diet) + living *E. coli* alone	0.00 *a*	8.83 *b*
(8) Control (autoclaved corn-soy diet) + living *S. typhimurium* alone	0.00 *a*	9.15 *c*
(9) Control (autoclaved corn-soy diet) + living *E. coli* + living *S. typhimurium*	0.00*a*	9.61 *d*
(10) Control (autoclaved corn-soy diet) + living *E. coli* + *E. coli* phage	8.75 *b*	3.05 *g*
(11) Control (autoclaved corn-soy diet) + living *S. typhimurium* + *S. typhimurium* phage	7.05 *c*	3.34 *f*
(12) Control (autoclaved corn-soy diet) + living *E. coli* + living *S. typhimurium* + *E. coli* phage + *S. typhimurium* phage	9.05 *d*	2.58 *e*
(13) Control (autoclaved corn-soy diet) + living *E. coli* + oxytetracycline	0.00 *a*	2.95 *g*
(14) Control (autoclaved corn-soy diet) + living *S. typhimurium* + oxytetracycline	0.00 *a*	3.29 *f*
(15) Control (autoclaved corn-soy diet) + living *E. coli* + living *S. typhimurium* + oxytetracycline	0.00 *a*	2.49 *e*

Values are means of 8 replicates and the values with the same letter within a column are not significantly ($P > 0.05$) different according to Fisher's Protected LSD Test.

Table 3.11 Effect of incorporation of bacteriophages on the population densities of *S. typhimurium* and *E. coli* (expressed as $\log_{10}$ cfu g^{-1} chicken corn-soy diet) and on the

population densities of *S. typhimurium* and *E. coli* phages (expressed as $\log_{10}$ pfu g^{-1} chicken corn-soy diet) after incubation at 37°C for 21 days.

Treatments	Phage population densities as $\log_{10}$ (pfu) g^{-1} chicken corn-soy diet	Bacteria population densities as $\log_{10}$ (cfu) g^{-1} chicken corn-soy diet
(1) Control (autoclaved corn-soy diet) + killed autoclaved *E. coli* alone	0.00 *a*	0.00 *a*
(2) Control (autoclaved corn-soy diet) + killed autoclaved *S. typhimurium* alone	0.00 *a*	0.00 *a*
(3) Control (autoclaved corn-soy diet) + killed autoclaved *E. coli* + killed autoclaved *S. typhimurium*	0.00 *a*	0.00 *a*
(4) Control (autoclaved corn-soy diet) + *E. coli* phage alone	8.85 *b*	0.00 *a*
(5) Control (autoclaved corn-soy diet) + *S. typhimurium* phage alone	7.11 *c*	0.00 *a*
(6) Control (autoclaved corn-soy diet) + *E. coli* phage + *S. typhimurium* phage	9.08 *d*	0.00 *a*
(7) Control (autoclaved corn-soy diet) + living *E. coli* alone	0.00 *a*	9.25 *b*
(8) Control (autoclaved corn-soy diet) + living *S. typhimurium* alone	0.00 *a*	9.55 *c*
(9) Control (autoclaved corn-soy diet) + living *E. coli* + living *S. typhimurium*	0.00*a*	9.91 *d*
(10) Control (autoclaved corn-soy diet) + living *E. coli* + *E. coli* phage	8.95 *b*	2.15 *g*
(11) Control (autoclaved corn-soy diet) + living *S. typhimurium* + *S. typhimurium* phage	7.21 *c*	2.41 *f*
(12) Control (autoclaved corn-soy diet) + living *E. coli* + living *S. typhimurium* + *E. coli* phage + *S. typhimurium* phage	9.17 *d*	1.77 *e*
(13) Control (autoclaved corn-soy diet) + living *E. coli* + oxytetracycline	0.00 *a*	2.25 *g*
(14) Control (autoclaved corn-soy diet) + living *S. typhimurium* + oxytetracycline	0.00 *a*	2.35 *f*
(15) Control (autoclaved corn-soy diet) + living *E. coli* + living *S. typhimurium* + oxytetracycline	0.00 *a*	1.85 e

Values are means of 8 replicates and the values with the same letter within a column are not significantly ($P > 0.05$) different according to Fisher's Protected LSD Test.

CHAPTER IV: DISCUSSION

Diaz *et al.* (1989) indicated that the isolation and identification of bacteriophages are of interest for a variety of reasons, which include: (i) the problems they cause to fermentation industries (Chater, 1986), (ii) their value for microbial identification using the bacteriophage typing technique (Korn-Wendish and Schneider, 1992), (iii) their use for the detection and understanding of host controlled restriction-modification systems (Diaz *et al.*, 1989), (iv) their utilization as tools for genetic exchange and analysis in bacteria (Herron and Wellington, 1990), (v) the study of bacteriophage general and molecular biology and ecological characteristics (Lomovskaya *et al.*, 1980; Williams *et al.*, 1987), and (vi) their recent role in bacteriophage therapy to replace the application of antibiotics (Brussow, 2007).

The objectives of the present study were to: (i) isolate super lytic bacteriophage that can kill *Salmonella typhimurium* and *E. coli in vitro*; (ii) test the effectiveness of the isolated bacteriophages in chicken feed; (iii) compare the isolated bacteriophages with the currently in use antibiotics in chicken feed.

In the current study and in an attempt to isolate lytic bacteriophages active against Gram negative bacteria *Salmonella typhimurium* and *Escherichia coli* known to cause food poisoning diseases in chicken feed, two bacteriophages were isolated from untreated raw waste water samples collected from Al-Ain sewage treatment factory. The optimum concentration of the two phages used was found to be 10^{10} pfu ml^{-1}.

Bacteriophage number I (O_1) was isolated using *S. typhimurium* as a propagation host and was found to be monovalnet and only lysed *S. typhimurium*. Bacteriophage

(O_1) failed to lyse the Gram positive bacteria *B. subtilis*, *S. aureus*, *S. epidermidis* and *E. faecalis* and failed also to lyse the Gram negative bacteria *E. coli*, *P. vulgaris*, *P. aeruginosa* and *S. dysenteriae*. On the other hand, bacteriophage number (O_2) isolated using *E. coli* as a propagation host was found to be polyvalent and lysed the Gram positive bacteria (*B. subtilis*, *S. aureus*, *S. epidermidis* and *E. faecalis*), and the Gram negative bacteria (*P. vulgaris*, *P. aeruginosa* and *S. dysenteriae*). Bacteriophages active against bacteria are readily detected in sewage and it appears that bacteriophages are widespread in the sewage environment (Tartera and Jofre, 1987; Ricca and Cooney, 2000).

The inability of bacteriophage number 1(O_1) to kill Gram positive bacteria and all Gram negative bacteria except *S. typhimurium* indicates the importance of specific receptor sites on the bacterial cell wall and also indicates that the structural differences in the cell wall between Gram positive and negative bacteria are critical. These findings in our study are in agreements with those reported by Ricca and Cooney (2000) and Kimura *et al.* (2008).

The increase in the (pfu ml^{-1}) of bacteriophages when $Ca(NO_3)_2$ was added, confirmed the findings of Adams (1959) who concluded that divalent cations such as Ca^{++} are required for adsorption of phage to their receptors. Gold (1959) also noted that addition of divalent cations to the medium increased the size and number of plaques produced by phage. The increased NaCl concentrations used in this study reduced the numbers of congruent plaques on the three media types. These findings also agreed with Walton (1951) who concluded that certain concentrations of alkaline

cations, sodium, potassium and ammonium completely inhibit multiplication of phages that attack bacterial host (Walton, 1951).

The physical and chemical treatments used in the study reported in this investigation had adverse effect on the two phages used, with bacteriophage (O_1) isolated against *S. typhimurium* was the most affected. Bacteriophage (O_1) was sensitive to the treatments with freezing, refrigeration, heating, boiling, ultrasonic treatment and UV radiation. Freezing bacteriophage (O_1) for 30 minutes, one hour, two hours (but not 3 hours, four hours, five hours, six hours, 12 hours, 24 hours, and 48 hours) showed slight adverse effects on the viability of the bacteriophage. At 4°C bacteriophage (O_1) numbers decreased markedly with time. Heating the bacteriophage (O_1) at 45°C, 55°C (but not at 65°C) for 15 minutes and for 30 minutes did not show any adverse effect on the virus viability and the numbers of pfu. Boiling bacteriophage (O_1) at 100°C for 5 minutes and for 10 minutes completely; killed the phage as evident with no pfu formation. Bacteriophage (O_1) was able to resist the ultrasonic treatment for 60 seconds and 120 seconds but with a reduced rate compared to the control treatment. Exposing bacteriophage (O_1) to UV radiation for 30 seconds and 60 seconds (but not 120 seconds, 180 seconds and 240 seconds) showed no adverse effect on the bacteriophage viability. Although most phages are resistant to detergents and other chemical and physical agents, their inactivation by these agents is commonly observed (Goyal, 1987).

Bacteriophage (O_2) was sensitive to the treatments with freezing, refrigeration, heating, boiling, ultrasonic treatment and UV radiation. Freezing bacteriophage (O_2) for 30 minutes, one hour, two hours, 3 hours (but not four hours, five hours, six hours,

12 hours, 24 hours, and 48 hours) showed slight adverse effect on the viability of the bacteriophage. At 4°C bacteriophage (O_2) numbers decreased markedly with time. Heating the bacteriophage (O_2) at 45°C, and 55°C for 15 minutes and for 30 minutes (but not 65°C for 15 minutes and for 30 minutes) did not show any adverse effect on the virus viability.

Boiling bacteriophage (O_2) at 100°C for 5 minutes and for 10 minutes completely; killed the phage as evident with no pfu formation. Bacteriophage (O_2) was able to resist the ultrasonic treatment for 60 seconds and 120 seconds but with a reduced rate compared to the control treatment. Exposing bacteriophage (O_2) to UV radiation for 30 seconds, 60 seconds and 120 seconds (but not 180 seconds and 240 seconds), showed no adverse effect on the page viability.

Many phages have been reported to be resistant to many physical agents as suggested by Goyal (1987). This supports the finding in the present study which demonstrated that bacteriophages (O_1) and (O_2) showed some degrees of resistance to physical agents.

Bacteriophage (O_1) was sensitive to the treatments with chloroform (0.5%, 1% and 3%), thymol (1% and 3%), hydrogen peroxide (1% and 3%), phenol (1% and 3%), ethyl alcohol (70% and 100%), Clorox (1% and 3%), iodine (1% and 3%), merthiolate (1% and 3%). However, the bacteriophage (O_1) showed some degree of resistance to these chemicals in these concentrations thymol (0.5%), hydrogen peroxide (0.5%), phenol (0.5%), ethyl alcohol (30%), Clorox (0.5%), iodine (0.5%) and merthiolate (0.5%). Bacteriophage (O_2) was sensitive to the treatments with chloroform (0.5%, 1% and 3%), thymol (3%), hydrogen peroxide (3%), phenol (1%

and 3%), ethyl alcohol (70% and 100%), Clorox (3%), iodine (3%), merthiolate (1% and 3%). All of these chemicals greatly reduced the numbers of pfu. However, the bacteriophage (O_2) showed some degree of resistance to these chemicals in these concentrations thymol (0.5% and 1%), hydrogen peroxide (0.5% and 1%), phenol (0.5%), ethyl alcohol (30%), Clorox (0.5% and 1%), iodine (0.5% and 1%) and merthiolate (0.5%). Many phages have been reported to be resistant to many chemical agents as suggested by Goyal (1987). This supports the finding in the present study which demonstrated that bacteriophages (O_1) and (O_2) showed some degrees of resistance to chemical agents.

This degree of resistance of the two bacteriophages against physical and chemical agents will strengthen the application of the bacteriophages as a biological product as bacteriophages can now tolerate adverse effects during storage and application of the bacteriophages product in the chicken feed industry.

In the current study, fewer bacteriophage (O_1) were obtained when propagation hosts were incubated for 0-3 h, in comparison to those incubated for 5-10 h. Meanwhile, fewer bacteriophage (O_2) were obtained when propagation hosts were incubated for 0-3 h, in comparison to those incubated for 5-10 h. Greater levels of host inoculum resulted in greater phage titres for bacteriophage (O_1). Also, greater levels of host inoculum resulted in greater phage titres for bacteriophage (O_2).

Greater phage inoculum size resulted in greater phage output for bacteriophage (O_1). Also, greater phage inoculum size resulted in greater phage output (Table 3.7) for bacteriophage (O_2). Adsorption rate constant (K) of bacteriophage (O_1) and (O_2) were 1.22×10^{-6} ml min^{-1} and 1.41×10^{-6} ml min^{-1}, respectively. Latent period

values obtained for bacteriophage (O_1) and (O_2) were 40 minutes and 35 minutes, respectively. The rise periods of the bacteriophage (O_1) and (O_2) were 30 minutes and 40 minutes, respectively. The average burst sizes of bacteriophage (O_1) and (O_2) were 21.5 virions/cell and 15.2 virions/cell, respectively.

The burst sizes obtained for the two phages used in this study were lower than the ones obtained by Dowding (1973) but higher than some of the values obtained by Sykes *et al.* (1981) for phages utilising neutrophilic hosts. However, many factors such as the nature of the host, and host age can influence phage burst size (Bradley *et al.*, 1961). For example, Dowding (1973) indicated that actinomycetes, including streptomycetes, do not germinate synchronously, so that by the time a high enough proportion of spores have germinated, some of them will be large clumps of mycelium. Therefore, the one-step growth experiments in this study agree with Dowding (1973) who suggested that the concept of multiplicity of infection will remain meaningless in host-phage systems until a method is found to cause synchronous and uniform spore germination.

The negatively stained particles of the bacteriophage number 1 (O_1) active against *S. typhimurium* and the bacteriophage number 2 (O_2) active against *E. coli* fitted the *Siphoviridae* (B1) morphotype (Francki *et al.*, 1991) which consisted of icosahedral heads (Fig. 3.8). Many bacteriophages are known to consist only of icosahedral heads with tails and tails fibers. Some bacteriophages also are simple in structure without the formation of the head and tails as suggested by Ackermann and Heldal (2010).

Bacteria which are resistant to most or all currently available antibiotics in the market are causing increasingly serious problems, raising widespread fears of returning to a pre-antibiotic era of untreatable infections and epidemics (Brussow, 2007; Hanlon, 2007; Kimura *et al.*, 2008). Despite intensive work by drug companies, no new classes of antibiotics have been found in the last 30 years (Brussow, 2007; Hanlon, 2007). There are hopes that the new found ability to sequence entire microbial genomes and to determine the molecular bases of pathogenicity will open new avenues for treating infectious diseases, but other approaches are also being sought with increasing fervor (Soothill *et al.*, 2004; Sulakvelidze, 2005; Hanlon, 2007). One result is a renewed interest in the possibilities of bacteriophage therapy which can be defined as "the application of a specific kind of bacterial viruses "phages or bacteriophages" that attack only bacteria to kill pathogenic microorganisms (Barrow and Soothill, 1997; Sulakvelidze *et al.*, 2001; Brussow 2005; Sulakvelidze, 2005).

In the current study, the application of bacteriophage (O_1) in corn-soy diet infested with *S. typhimurium*, or the application of bacteriophage (O_2) in corn-soy diet infested with *E. coli*, or the application of bacteriophage (O_1) and bacteriophage (O_2) in corn-soy diet infested with *S. typhimurium* and *E.coli* significantly ($P < 0.05$) reduced the population densities of *S. typhimurium* and the population densities of *E.coli* compared to the treatments which included the application of living *E. coli* alone, living *S. typhimurium* alone and living *E. coli* + living *S. typhimurium*. The treatment which received the mixture of the two phages or the application of bacteriophage (O_1), or the application of bacteriophage (O_2) was almost as effective

as the oxytetracycline applications in reducing the incidence of *S. typhimurium* and *E. coli* in the chicken feed. There were, however, no significant ($P > 0.05$) differences between the bacterial levels of the phage treated chicken feed and the oxytetracycline treated chicken feed. Results show that there is a potential to use a mixture of these two bacteriophages rather than antibiotics for the prevention of *S. typhimurium* and *E. coli* food poisoning diseases in chicken feed and in poultry production.

Bacteriophages are highly specific in killing certain strains of bacteria, just like antibiotics, but with the added benefit of multiplying as they consume the host, so they spread rapidly. Recent work has shown that bacteriophages are highly effective at clearing pathogens from poultry carcasses and at killing them in the intestines of live birds and in their eggs (Häusler, 2006).

Many studies have shown that the application of bacteriophages in chicken feed reduced the incidence of *Salmonella* spp. in the feed (Miller *et al.*, 2010; Lim *et al.*, 2011; Janez *et al.*, 2013; Wong *et al.*, 2014). Fiorentin (2005) demonstrated that chicken skin samples were experimentally contaminated by immersing whole legs (thighs and drumsticks) in a suspension containing 10^6 cfu ml^{-1} of *Salmonella enteritidis* phage type 4 (SE PT4) at the slaughter day. One day later, samples from one group were immersed in a suspension pool containing 10^9 cfu ml^{-1} of each of three wild *Salmonella* lytic bacteriophages previously isolated from feces of free range chickens (Fiorentin, 2005). *Salmonella* counting was performed at three-day intervals in the chicken legs stored at 5°C and showed a significant reduction

($P<0.05$) of SE PT4 in bacteriophage-treated cuts on days 3, 6 and 9 post-treatment (Fiorentin, 2005). These findings support the work obtained in the present study.

Carvalho *et al.* (2010) tested the efficacy of a phage cocktail composed of three phages for the control of poultry infected with *C. coli* and *C. jejuni*. They evaluated the effectiveness of two routes of phage administration (by oral gavage and in feed) in order to provide additional information regarding their future use in a poultry unit (Carvalho *et al.*, 2010). They reported a reduction in the population densities of the pathogenic bacteria. These findings support the work obtained in the present study.

Phage therapy is the therapeutic use of lytic bacteriophages to treat pathogenic bacterial infections (Chopra *et al.*, 1997). Bacteriophages or "phages" are viruses that invade only bacterial cells and, in the case of lytic phages, cause the bacterium to burst and die, thus releasing more phages (Carlton, 1999). Phage therapy is one of the viable alternatives to antibiotics, being developed for clinical use in the 21st century by many research groups in Europe and in the Unites States of America (USA) (Brussow, 2005). After having been extensively used and developed mainly in former Soviet Union countries for about 90 years ago, phage therapy is now becoming more available in other countries such as USA for a variety of bacterial and poly-microbial biofilm infections (Levin and Bull, 1996; Smith and Huggins, 1987; Sulakvelidze, 2005). Phage therapy has many applications in human medicine as well as dentistry, veterinary science and agriculture (Brussow, 2005). An important benefit of phage therapy is that bacteriophages can be much more specific than more common drugs, so can be chosen to be harmless to not only the host organism (human, animal or plant), but also other beneficial bacteria, such as gut

flora, reducing chance for opportunistic infections (Sulakvelidze, 2005). They also have few if any side effects as opposed to drugs, and do not stress the liver (Sulakvelidze *et al.*, 2001). Because they replicate *in vivo*, a single, small dose is sometimes sufficient. On the other hand this specificity is also a disadvantage, as phage will only kill a bacterium if it is matching to the specific subspecies. Accordingly a mixtures of more than one phage are often applied to improve the chances of success rather than using individual phage in the phage therapy (Sulakvelidze *et al.*, 2001; Brussow, 2005). This supports the findings of the present study which demonstrated that a mixture of two phages was much better than using individual phage alone in reducing the population densities of *S. typhimurium* and *E. coli*.

Phages are currently being used therapeutically to treat bacterial infections that do not respond to conventional antibiotics (Brussow, 2007; Hanlon, 2007). They tend to be more successful where there is a microbial biofilm covered by a polysaccharide capsule layer that antibiotics typically cannot penetrate (Hanlon, 2007). Other microbial biofilms include those on medical instruments and devices, so an enzyme added to a phage can effectively and selectively wipe out even bacteria beneath these films (Brussow, 2007; Hanlon, 2007).

Phages have been explored as means to eliminate pathogens like *Campylobacter* in raw food and *Listeria* in fresh food or to reduce food spoilage bacteria. In agricultural practices, phages were used to fight pathogens like *Campylobacter*, *Escherichia* and *Salmonella* in farm animals, *Lactococcus* and *Vibrio* pathogens in fish from aquaculture and *Erwinia* and *Xanthomonas* in plants of agricultural

importance (Hanlon, 2007). McKenna *et al.* (2001) reported that a highly virulent and polyvalent *Streptomyces* phage isolated from potato field in Australia was able to significantly reduce the incidence of potato scab disease caused by *Streptomyces scabies*. They propagated and cultivated the phage using new novel mini-bioreactors (McKenna *et al.*, 2001).

Phages were used against diarrheal diseases caused by *Escherichia coli*, *Shigella* or *Vibrio* and against wound infections caused by facultative pathogens of the skin like staphylococci and streptococci (Soothill *et al.*, 2004; Brussow, 2007; Hanlon, 2007).

A clear benefit of phage therapy is that it does not have the potentially very severe adverse effects of antibiotics (Hanlon, 2007). It can be fast-acting, once the exact bacteria are identified and the phages can be administered. Another benefit of phage therapy is that although bacteria are able to develop resistance to phages the resistance is much easier to overcome (Sulakvelidze, 2005; Hanlon, 2007). As a consequence phage therapy is devoid of problems similar to antibiotic resistance (Sulakvelidze, 2005; Hanlon, 2007). Bacteriophages are often very specific, targeting only one or a few strains of bacteria. Traditional antibiotics usually have more wide-ranging effect, killing both harmful bacteria and useful bacteria such as those facilitating food digestions. The specificity of bacteriophages reduces the chance that useful bacteria are killed when fighting an infection (Sulakvelidze, 2005; Hanlon, 2007).

Research groups in the West are engineering a broader spectrum phage and also target methicillin resistant *Staphylococcus aureus* (MRSA) treatments in a variety of

forms including: impregnated wound dressings, preventative treatment for burn victims, phage-impregnated sutures (Hanlon, 2007; Kimura *et al.*, 2008).

In the USA, the Food and Drug Administration (FDA) approved spraying meat and poultry products with phages (Brussow, 2007). Phage therapy is used for the treatment of a variety of bacterial infections including: laryngitis, skin infections, dysentery, conjunctivitis, periodontitis, gingivitis, sinusitis, urinary tract infections and intestinal infections, burns, boils, and also poly-microbial biofilms on chronic wounds, ulcers and infected surgical sites (Brussow, 2007; Hanlon, 2007).

Isolation of phages from different substrates and neglected environments will increase our knowledge on the ecology of species, genus and family specific phage as well as provide additional information on phage ecology and phage-host interactions (Williams and Lanning, 1984).

The results obtained in the present investigation will open the door to find a new alternative drug that can be used to kill antibiotic-resistant bacteria for which antibiotics are no longer effective. This will also increase the public awareness about food safety and about the necessity of having our foods free from any antibiotic residues.

In conclusion, the isolated bacteriophages have great potential in the field of bacteriophage therapy and chicken feed industry because they are self-replicating and can be targeted against bacterial receptors that are essential for bacterial pathogenesis. The phages obtained can be used as a safe and alternative technique to eradicate bacterial infections and chronic poly microbial biofilm caused by antibiotic resistant bacteria.

Future work is needed to establish that bacteriophages obtained in the present study can reduce or eliminate *Salmonella* and *E. coli* not only in chicken feed but also in small flocks of live chickens. The future significant aim is to ensure that such birds fed on feed treated with bacteriophages can provide poultry products that are fit for human consumption and have greatly reduced levels of contamination by these two pathogens. If these trials demonstrate significant reductions in bacteria in the birds and their products, the stage will be set for larger trials involving several hundred birds.

The possibility of the emergence of phage-resistant strains, quantity of phages required for administration, route and timing of administration, and destruction of phages by stomach acidity following oral administration will also need to be investigated in future work using live birds.

CONCLUSIONS

1- Two powerful lytic bacteriophages designated bacteriophage number 1 (O_1) and bacteriophage number 2 (O_2) were isolated from untreated raw waste water samples collected from Al-Ain sewage treatment plant.

2- Bacteriophage number 1 (O_1) was shown to be a monovalent lytic phage and only lysed its propagation host *Salmonella typhimurium*.

3- Bacteriophage number 2 (O_2) was shown to be a polyvalent lytic phage and lysed its propagation host *Escherichia coli* and many other Gram positive and Gram negative bacteria.

4- The optimum concentration of the two phages used was found to be 10^{10} plaques forming unit (PFU ml^{-1}).

5- Peptone-yeast extract agar amended with calcium nitrate was the best medium for phage propagation and cultivation.

6- Bacteriophage (O_1) was sensitive to the treatments with freezing, refrigeration, heating, boiling, ultrasonic treatment and Ultra Violet (UV) radiation. However, bacteriophage (O_2) was much more resistant compared to bacteriophage (O_1).

7- The application of bacteriophage (O_1) in corn-soy diet infested with *S. typhimurium*, or the application of bacteriophage (O_2) in corn-soy diet infested with *E. coli*, or the application of bacteriophage (O_1) and bacteriophage (O_2) in corn-soy diet infested with *S. typhimurium* and *E.coli* significantly reduced the population densities of *S. typhimurium* and the population densities of *E.coli* compared to the treatments which included the

application of living *E. coli* alone, living *S. typhimurium* alone and living *E. coli* + living *S. typhimurium*.

8- The treatment which received the mixture of the two phages or the application of bacteriophage (O_1), or the application of bacteriophage (O_2) was almost as effective as the oxytetracycline applications in reducing the incidence of *S. typhimurium* and *E. coli* in the chicken feed.

9- These two bacteriophages obtained in the present study can replace the application of antibiotics in chicken feed industry as they gave almost equivalent reduction in the population densities of *S. typhimurium* and *E. coli* as the broad spectrum antibiotic oxytetracycline.

10- The isolated bacteriophages have great potential in the field of bacteriophage therapy and chicken feed industry because they are self-replicating and can be targeted against bacterial receptors that are essential for bacterial pathogenesis.

11- The phages obtained can be used as a safe and alternative technique to eradicate bacterial infections caused by any antibiotic resistant bacteria.

REFERENCES

Abduladze, T., Li, M., Menetrez, M., Dean, T., Senecal, A., and Sulakvelidze, A. (2008). Bacteriophages reduce experimental contamination of hard surfaces, tomato, spinach, broccoli, and ground beef by *Escherichia coli* O157:H7. *Journal of Applied and Environmental Microbiology*, 74, 6230–6238.

Abdul-Hassan, H. S., El-Tahan, E., Massoud, B., and Gomaa, R. (1990). Bacteriophage therapy of *pseudomonas* burn wound sepsis. *Annual Mediterranean Burn Club*, 3, 262–264.

Ackermann, H.-W., and Heldal, M. (2010). Basic electron microscopy of aquatic viruses. In: S. W. Wilhelm, M. G. Weinbauer, and C. A. Suttle [eds.]: *Manual of Aquatic Viral Ecology*., Taxas, USA, American Society of Limnology and Oceanography.

Adamia, R. S., Matitashvili, E. A., Kvachadze, L. I., Korinteli, V. I., Matoyan, D. A., Kutateladze, M. I., and Chanishvili, T. G. (1990). The virulent bacteriophage IRA of *Salmonella typhimurium*: cloning of phage genes which are potentially lethal for the host cell. *Journal of Basic Microbiology*, 10, 707–716.

Adams, M. (1959). *Bacteriophages*. Wiley InterScience, New York, U.S.A. pp 169-172.

Alisky, J., Iczkowski, K., Rapoport, A., and Troitsky, N. (1998). Bacteriophages show promise as antimicrobial agents. *Journal of Infection*, 36, 5–15.

Arikan, O., Sikora, L., Mulbry, W., Khan, S., and Foster,G. (2007). Composting rapidly reduces levels of extractable Oxytetracycline in manure from therapeutically treated beef calves. *Bioresource Technology*, 98, 169–176.

Armon R., and Kott Y. (1993). A simple, rapid and sensitive presence/absence detection test for bacteriophage in drinking water. *Journal of Applied Bacteriology*, 74, 490- 496.

Bach, S., McAllister, T., Veira, D., Gannon, V., and Holley, R. (2003). Effect of bacteriophage DC22 on *Escherichia coli* O157:H7 in an artificial rumen system (Rusitec) and inoculated sheep. *Animal Research*, 52, 89–101.

Barrow, P. A., and J. S. Soothill. (1997). Bacteriophage therapy and prophylaxis: rediscovery and renewed assessment of potential. *Trends in Microbiology*, 7, 268–271.

Barrow, P., Lovell, M., and Berchieri, A. (1998). Use of Lytic Bacteriophage for Control of Experimental *Escherichia coli* Septicemia. *Clinical and Diagnostic Laboratory Immunology*, 5, 294-298.

Borie, C., Sanchez, M.L., Navarro, C., Ramirez, S., Morales, M.A.., Retamales, J., and Robeson, J. (2009). Aerosol spray treatment with bacteriophages and competitive exclusion reduces *Salmonella enteritidis* infection in chickens. *Avian Disease*, 53, 250–254.

Bradbury, J. (2004). My enemy's enemy is my friend. Using phages to fight bacteria. *Lancet*, 363, 624–625.

Bradley, B. E. (1967). Ultrastructure of bacteriophage and bacteriocins. *Bacteriology Review*, 31, 230--314.

Bradley, S. G., Anderson, D. L., and Jones, L. A. (1961). Phylogeny of actinomycetes as revealed by susceptibility to actinophage. *Developments in Industrial Microbiology*, 2, 223-237.

Brownell, G. H., Adams, J. N., and Bradley, S. G. (1967). Growth and Characterization of nocardiophages for *Nocardia canicruria* and *Nocardia erythropolis* mating types. *Journal of General Microbiology*, 47, 247-256.

Brussow, H. (2005). Phage therapy: the *Escherichia coli* experience. *Microbiology*, 151, 2133-2140.

Brussow, H. (2007). *Phage Therapy: The Western Perspective. Bacteriophage: Genetics and Molecular Biology*. Norfolk, UK, Caister Academic Press.

Burm, R., and Steward, G.F. (2010). Morphological characterization of viruses in the stratified water column of alkaline, hypersaline Mono Lake. *Microbial Ecology*, 60, 636-643.

Butaye, P., Devriese, L., and Freddy, A. (2003). Antimicrobial Growth Promoters Used in Animal Feed: Effects of Less Well Known Antibiotics on Gram-Positive Bacteria. *Clinical Microbiology Reviews*, 16, 175-188.

Capita, R., Álvarez-Astorga, M., Alonso-Calleja, C., Moreno, M., and Garcia-Fernández, M. (2003). Occurrence of *salmonellae* in retail chicken carcasses and their products in Spain. *International Journal of Food Microbiology*, 81, 169-173.

Carlton, R M. (1999). Phage therapy: past history and future prospects. *Archivum Immunologiae et Therapia Experimentalis*, 5, 267–274.

Carrillo, C. L., Atterbury, R. J., and El-Shibiny, A. (2005). Bacteriophage therapy to reduce *Campylobacter jejuni* colonization of broiler chickens. *Journal of Applied and Environmental Microbiology*, 71, 6554–6563.

Carvalho, C., Gannon, B., Halfhide, D., Santos, S., Hayes, C., Roe, J., and Azeredo, J. (2010). The *in vivo* efficacy of two administration routes of a phage cocktail to reduce numbers of *Campylobacter coli* and *Campylobacter jejuni* in chickens. *BMC Microbiology*, 10, 232-250.

Chater, K. F. (1986). *Streptomyces phages and their applications to Streptomyces genetics*. New York, U.S.A, Academic Press.

Chibani, S., Sidoti, J., Bruttin, A., Kutter, E., Sarker, S., and Brussow, H. (2004). *In vitro* and *in vivo* bacteriolytic activities of *Escherichia coli* phages: implications for phage therapy. *Antimicrobial Agents and Chemotherapy*, 48, 2558–2569.

Chopra, I., Hodgson, J., Metcalf, B., and Poste, G. (1997). The search for antimicrobial agents effective against bacteria resistant to multiple antibiotics. *Antimicrobial Agents and Chemotherapy*, 41, 497–503.

Clark, F. E. (1965). *The Concept of Competition in Microbial Ecology. In: Ecology of Soil-borne Plant Pathogens*, Berkeley, U.S.A, University of California Press.

Craig, S., and Zich, D. (2009). *Rosen's Emergency Medicine: Concepts and Clinical Practice*. 7th ed. Philadelphia, USA, ELSIVER.

Diaz, L. A., Hardisson, C., and Rodicio, M. R. (1989). Isolation and characterization of actinophages infecting *Streptomyces* species and their interaction with host restriction-modification systems. *Journal of Genetic Microbiology*, 135, 1847-1856.

Dowding, J. E. (1973). Characterization of bacteriophage virulent for *Streptomyces coelicolor* A3 (2). *Journal of General Microbiology*, 76, 163-176.

Faller, P., and Michael, A. (2006). Flavophospholipol use in animals: Positive implications for antimicrobial resistance based on its microbiologic properties. *Journal of Diagnostic Microbiology and Infectious Disease*, 56, 115– 121.

FAO/WHO, (2001). Health and nutritional properties of probiotics in food including powder milk with live lactic acid bacteria; Report of a Joint FAO/WHO Expert Consultation on Evaluation of Health and Nutritional Properties of Probiotics in Food Including Powder Milk with Live Lactic Acid Bacteria; Amerian Córdoba Park Hotel, Córdoba, Argentina: FAO/WHO.

Fiorentin, L., Vieira, N. D., and Barioni, W. (2005). Use of Lytic Bacteriophages to Reduce *Salmonella enteritidis* in Experimentally Contaminated Chicken Cuts. *Journal of Avian Disease*, 7, 255 – 260.

Flint, S.J., L.W. Enquist, R.M. Krug, V.R. Racaniello M. and Skalka. A. (2000). *Principles of Virology*. First edition, Washington DC, USA, American Society of Microbiology Press.

Francki, R. I. B., Fauquet, C. M., Knudson, D. L., and Brown, F. (1991). Classification and nomenclature of viruses. Fifth report of the International Committee on Taxonomy of Viruses. *Arch Virology Supplement*, 2 , 161-166.

Fuller, R. (1989). Probiotics in man and animals. *Journal of Applied Bacteriology*, 66, 365–378.

Fuller, R. (2001). The chicken gut microflora and probiotic supplements. *Journal of Poultry Science*, 38, 189–196.

Gold, W. (1959). Effects of the medium and its composition on the activities of actinophage for *Streptomyces griseus*. *Annuals of the New York Academy of Science*, 81, 995-1016.

Goyal, S. M. (1987). *Methods in phage ecology*. New York, U.S.A, John Wiley and Sons Incorporation.

Guard-Petter, J., (2001). The chicken, the egg and *Salmonella enteritidis*. *Journal of Applied and Environmental Microbiology*, 3, 421-430.

Guttman, B., Raya, R., and Kutter, E. (2005). *Bacteriophages: Biology and Applications*. Chapter 3, Florida, USA. CRC Press.

Hanlon, G.W. (2007). Bacteriophages: an appraisal of their role in the treatment of bacterial infections. *International Journal of Antimicrobial Agents*, 30, 118-28.

Harper, D. (2012). *Virus's biology, application and control*. First edition. London, UK, Garland Science.

Harper, D. R. (2006). *Biological control by micro-organisms*. In Encyclopedia of Life Sciences. Chichester, UK, John Wiley & Sons.

Harper, D. R., and Kutter, E. (2008). *Therapeutic use of bacteriophages*. In Encyclopedia of Life, Chichester, UK. Sciences. John Wiley & Sons.

Harrison W.A., Griffith C.J., Tennant D., and Peters A.C. (2008). Incidence of *Campylobacter* and *Salmonella* isolated from retail chicken and associated packaging in South Wales. *Letters in Applied Microbiology*, 33, 450 – 454.

Hauer, J., Hacein-Bey-Abina, S., and Lim, A. (2010). Efficacy of gene therapy for X-linked severe combined immunodeficiency. National English *Journal of Medicine*, 363, 355–364.

Häusler, T. (2006). *Viruses vs. Superbugs: A Solution to the Antibiotics Crisis.* London, UK. Macmillan.

Herron, P. R., and Wellington, E. M. H. (1990). New method for extraction of streptomycete spores from soil and application to the study of lysogeny in sterile amended and non sterile soil. *Journal of Applied Environmental Microbiology*, 56, 1406-1412.

http://ec.europa.eu/agriculture/antimicrobials/phagevet.

Huff, W., Huff, G., Rath, J., Balog, R, and Donoghue, M. (2002). Prevention of *Escherichia coli* infection in broiler chickens with a bacteriophage aerosol spray. *Journal of Poultry Science*, 81, 1486–1491.

Huff, W., Huff, G., Rath, J., Balog, R., and Donoghue, M. (2003). Evaluation of aerosol spray and intramuscular injection of bacteriophage to treat an *Escherichia coli* respiratory infection. *Journal of Poultry Science*, 82, 1108–1112.

Huff, W., Huff, G., Rath, J., Balog, R., and Donoghue, M. (2006). Evaluation of the influence of bacteriophage titer on the treatment of colibacillosis in broiler chickens. *Journal of Poultry Science*, 85, 1373–1377.

Janež, N., and Loc-Carrillo, C. (2013). Use of phages to control *Campylobacter spp.* *Journal of Microbiological Methods*, 95, 68-75.

Johnke, J., Yossef, C., Marina, D., Ariel, K., Edouard, J., and Antonis, E. (2014). Multiple micro-predators controlling bacterial communities in the environment. *Current Opinion in Biotechnology*, 27, 185-190.

Kalha, C., Singh, S., Kang, M., Hunjan, V., Gupta, R., and Sharma, S. (2014). Entomopathogenic Viruses and Bacteria for Insect-Pest Control. *Integrated Pest Management*, 12, 225-244.

Kimura, Z., Nakayama, N., and Asakawa, S. (2008). Ecology of viruses in soils: Past, present and future perspectives. *Journal of Soil Science and Plant Nutrition*, 54, 1-32.

Korn-Wendish, F., and Schneider, J. (1992). Phage typing - a useful tool in actinomycete systematics. *Gene*, 115, 243-247.

Kowalski, C., Pomorska, M., Lebkowska, B., and Slawik, T. (2007). Determination of Oxytetracycline in Biological Matrix. *Journal of Liquid Chromatography & Related Technologies*, 64, 277-280.

Krylov, V.N. (2014). Bacteriophages of *Pseudomonas aeruginosa*: Long-Term Prospects for Use in Phage Therapy. *Advances in Virus Research*, 88, 227-278

Lacey, L.A., and Kaya, H.K. (2007). *Field Manual of Techniques in Invertebrate Pathology: Application and Evaluation of Pathogens for Control of Insects and Other Invertebrate Pests*. Netherlands, Springer.

Levin, B., and J. J. Bull (1996). Phage Therapy Revisited: The Population Biology of a Bacterial Infection and its Treatment with Bacteriophage and Antibiotics. *The American Naturalist*, 147, 881-898.

Lim, TH., Kim, MS., Lee, DH., Lee, YN., Park, JK., Youn, HN., Lee, HJ., Yang, SY., Cho, YW., Lee, JB., Park, SY., Choi, IS., and Song, CS. (2011). Efficacy of bacteriophage therapy on horizontal transmission of *Salmonella gallinarum* on commercial layer chickens. *Avian Disease*, 55, 435-438.

Lim, TH., Kim, MS., Lee, DH., Lee, YN., Park, JK., Youn, HN., Lee, HJ., Yang, SY., Cho, YW., Lee, JB., Park, SY., Choi, IS., and Song, CS. (2012). Use of bacteriophage for biological control of *Salmonella enteritidis* infection in chicken. *Avian Disease*, 56, 143-701.

Lomovskaya, N. D., Chater, K. F., and Mkrtumian, N. M. (1980). Genetics and molecular biology of *Streptomyces* bacteriophages. *Journal of Microbiology Review*, 44, 206-229.

Lomovskaya, N. D., Mkrtumian, N. M., Gostimskaya, N. L., and Danilenko, V. N. (1972). Characterisation of temperate actinophage C 31 isolated from *Streptomyces coelicolor* A3 (2). *Journal of Virology*, 9, 258-262.

Mahony, J., McAuliffe, O., Paul Ross, R., and Sinderen, D. (2010). Bacteriophages as biocontrol agents of food pathogens. *Opinion in Biotechnology*, 22, 157-163.

McCarthy, M., Brennan, M., Kelly, A., Ritson, C., de Boer, M., and Thompson, N. (2007). Who is at risk and what do they know? Segmenting a population on their food safety knowledge. *Journal of Food Quality and Preference*, 18, 205-217.

Mckenna F., El-Tarabily, K. A.,Hardy, G. E. St. J., and Dell, B. (2001). Novel *in vivo* use of a polyvalent Streptomyces phage to disinfest *Streptomyces scabies* infected seed potatoes. *Plant Pathology*, 50, 666-675.

Meader, E., Mayer, M., Gasson, M., Steverding, D., Simon, R., and Narbad, C. (2010). Bacteriophage treatment significantly reduces viable *Clostridium difficile* and prevents toxin production in an *in vitro* model system. *Anaerobe*, 16, 549-554.

Miles, RD., and Bootwalla, SM. (1991). *Direct-Fed Microbials in Animal Production.* Iowa, USA, National Food Ingredient Association.

Miller, R. W., Skinner, EJ., Sulakvelidze, A., Mathis, GF., and Hofacre, CL. (2010). Bacteriophage therapy for control of necrotic enteritis of broiler chickens experimentally infected with *Clostridium perfringens*. *Avian Disease*, 54, 33-40.

Modi R., Hirvi Y., Hill A., and Griffiths M.W. (2001). Effect of Phage on Survival of *Salmonella enteritidis* during Manufacture and Storage of Cheddar Cheese Made from Raw and Pasteurized Milk. *Journal of Food Protection*, 64, 927-933.

Monk, C. Rees, Barrow, P. Hagens, S., and Harper, D. (2010). Bacteriophage applications: where are we now? *Letters of Applied Microbiology*, 51, 363–369.

Murphy, T., Parra, R., Radman, R.., Roy, I., Harrop, A., Dixon, K., and Tajalli Keshavarz, T. (2007). Novel application of oligosaccharides as elicitors for the enhancement of bacitracin a production in cultures of *Bacillus licheniformis*. *Enzyme and Microbial Technology*, 40, 1518–1523.

Nigam, A., Gupta, D., and Sharma, A. (2014). Treatment of infectious disease: Beyond antibiotics. *Journal of Microbiological Methods*, 69, 83-88.

O'Flaherty, S., Ross, R.P., and Coffey, A. (2009). Bacteriophage and their lysins for elimination of infectious bacteria. Federation of European Societies *Microbiol Review*, 33, 801-19.

O'Flynn, G., Ross, R., Fitzgerald, A., and Coffey, A. (2004). Evaluation of a cocktail of three bacteriophages for biocontrol of *Escherichia coli* O157:H7. *Journal of Applied and Environmental Microbiology*, 70, 3417–3424.

Oliveira, A., Sereno, R., and Azeredo, J. (2010). *In vivo* efficiency evaluation of a phage cocktail in controlling severe colibacillosis in confined conditions and experimental poultry houses. *Journal of Veterinary Microbiology*, 146, 303–308.

Oliveira, M., Viñas, I., Colàs, O., Anguera, M., Usall, I., and Abadias, M. (2014). Effectiveness of a bacteriophage in reducing *Listeria monocytogenes* on fresh-cut fruits and fruit juices. *Journal of Food Microbiology*, 38, 137-142.

Parker, RB. (1979). Probiotics, the other half of the antibiotics story. *Journal of Animal Nutrition and Health*, 29, 4–8.

Projan, S. (2004). Phage-inspired antibiotics. *National Biotechnology*, 22, 167–168.

Purdy, R.N., Dancer, B.N., Day, M.J., and Stickler, D.J. (1984). A novel technique for the enumeration of bacteriophage from water. *FEMS Microbiology Letters*, 21, 89-92.

Quiberoni, V., Suárez, A.G., Binetti, J.A., and Reinheimer, A. (2011). Bacteriophage Biological Aspects. *Journal of Bacteriophage*, 1, 31-45.

Raya, R., Oot, A., and Moore-Maley, B. (2011). Naturally resident and exogenously applied T4-like and T5-like bacteriophages can reduce *Escherichia coli* O157:H7 levels in sheep. *Journal of Applied and Environmental Microbiology*, 76, 8144-8160.

Raya, R., Varey, R., and Oot, R. (2006). Isolation and characterization of a new T-even bacteriophage, CEV1, and determination of its potential to reduce *Escherichia coli* O157:H7 levels in sheep. *Journal of Applied and Environmental Microbiology*, 72, 6405–6410.

Ricca, D.M., and Cooney J.J., (2000). Screening environmental samples for source-specific bacteriophage hosts using a method for the simultaneous pouring of 12 petri plates. *Journal of Industrial and Microbiological Biotechnology*, 24, 124–126.

Rivas, L., Coffey, B., and McAuliffe, O. (2010). *In vivo* and *ex vivo* evaluations of bacteriophages e11/2 and e4/1c for use in the control of *Escherichia coli* O157:H7. *Journal of Applied and Environmental Microbiology*, 76, 7210–7216.

Rozema, E., Stephens, T., and Bach, S. (2009). Oral and rectal administration of bacteriophages for control of *Escherichia coli* O157:H7 in feedlot cattle. *Journal of Food Protection*, 72, 241–250.

Schiller, L., and Sellin, J. (2010). *Sleisenger & Fordtran's Gastrointestinal and Liver Disease*. 9th ed. Philadelphia, USA, SAUNDERS.

Sheng, H., Knecht, I., Kudva, T., and Hovdem C. (2006). Application Of bacteriophages to control intestinal Escherichia coli O157:H7 levels in ruminants. *Journal of Microbiology*, 72, 5359–5366.

Simon, M., and Taha, M. (2013). *Poultry and products annual: UAE report*. Dubai, GAIN publications.

Sivasithamparam, K., Parker, C.A., and Edwards, C.S. (1979). Rhizosphere microorganisms of seminal and nodal roots of wheat grown in pots. *Journal of Soil Biology and Biochemistry*, 11, 155-160.

Smith, H. W., and Huggins, R. B. (1987). The control of Experimental E. coli Diarrhea in calves by means of Bacteriophage. *Journal of General Microbiology*, 133, 1111-1126.

Sodha, S., Griffin, P., and Hughes, J. (2009). *Principles and Practice of Infectious Diseases*. 7th ed. Philadelphia, USA, Churchill Livingstone.

Soothill, J., Hawkins, C., Anggård, E., and Harper, D. (2004). Therapeutic use of bacteriophages. *Lancet Infectious Disease*, 4, 544-545.

Stanford, K., McAllister, M., and Niu, Y. (2010). Oral delivery systems for encapsulated bacteriophages targeted *Escherichia coli* o157: H7 in feedlot cattle. *Journal of Food Protection*, 73, 1304–1312.

Sulakvelidze, A. (2005). Phage therapy: an attractive option for dealing with antibiotic-resistant bacterial infections. *Drug Discovery Today*, 15, 807-809.

Sulakvelidze, A., Alavidze, Z., and Morris J.G. (2001). Bacteriophage Therapy. Antimicrobial *Agents and Chemotherapy*, 45, 649–659.

Sunderland, J., Lovering, A., Tobin, C., MacGowan, A., Roe, J., and Delsol, A. (2004). Determination of avilamycin A and B in pig feces by solid phase extraction and reverse-phase HPLC assay. *International Journal of Antimicrobial Agents*, 24, 511-514.

Sykes, I. K., Lanning, S., and Williams, S. T. (1981). The effect of pH on soil actinophage. *Journal of General Microbiology*, 122, 271-280.

Tartera, C., and Jofre, J. (1987). Bacteriophages active against *Bacteroides fragilis* in sewage-polluted waters. *Journal of Applied and Environmental Microbiology*, 53, 1632–1637.

Templeton, N. S. (2008). *Gene and Cell Therapy: Therapeutic Mechanisms and Strategies*, 3rd ed. Boca Raton, USA, CRC Press.

Thacker, P.D. (2003). Set a microbe to kill a microbe: drug resistance renews interest in phage therapy. *Journal of American Medical Association*, 290, 3183–3185.

Toro, H., Price, SB., McKee, AS., Hoerr, FJ. Krehling, J., Perdue, M., and Bauermeister, L. (2005). Use of bacteriophages in combination with competitive exclusion to reduce *Salmonella* from infected chickens. *Avian Diseases*, 49, 118–124.

Tsonos, J., Vandenheuvel, D., Briers, Y., De Greve, H., Hernalsteens, J., and Lavigne, R. (2013). Hurdles in bacteriophage therapy: Deconstructing the parameters Veterinary Microbiology. *Science*, 15, 124-150.

Vickers, J. C., and Williams, S. T. (1987). An assessment of plate inoculation procedures for the enumeration and isolation of soil streptomycetes. *Microbiology Letters*, 35, 113-117.

Walton, R. B. (1951). Effects of cations upon multiplication of actinophage for *Streptomyces griseus*. *Antibiotics Chemotherapy*, 1, 518-522.

Wegener, F., and Henrik C. (2003). Antibiotics in animal feed and their role in resistance development. *Current Opinion in Microbiology*, 6, 439–445.

Weld, R.J., Butts, C., and Heinemann, J.A. (2004). Models of phage growth and their applicability to phage therapy. *Journal of Theoretical Biology*, 227, 1–11.

Williams, S. T., and Lanning, S. (1984). *Studies on the ecology of streptomycete phage in soil. In: Biological, Biochemical and Biomedical Aspects of Actinomycetes.* Orlando, USA, Academic Press.

Williams, S. T., Mortimer, A. M., and Manchester, L. (1987). *Ecology of soil bacteriophages. In: Phage Ecology*. New York, U.S.A, John Wiley and Sons Incorporation.

Williams, S. T., Wellington, E. M. H., and Tipler, L. S. (1980). The taxonomic implications of the reactions of representative *Nocardia* strains to actinophage. *Journal of General Microbiology*, 119, 173-178.

Wong, L.C., Sieo, C., Siang Tan, W., Abdullah, N., Hair-Bejo, M., Abu, J., and Yin Wan Ho, (2014). Evaluation of a lytic bacteriophage, Φ st1, for biocontrol of *Salmonella enterica* serovar *typhimurium* in chickens. International *Journal of Food Microbiology*, 172, 92-101.

Wright, A., Hawkins, C.H., Änggård, E. E., and Harper, D.R. (2009). A controlled clinical trial of a therapeutic bacteriophage preparation in chronic otitis due to antibiotic-resistant *Pseudomonas aeruginosa*. *Journal of Clinical Otolaryngology*, 34, 349-357

www.Scientificmethods.com. Protocol for detection of coliphages in water sample using Easy Phage medium-100, Indiana, USA. Scientific methods Incorporation.

Yata, T., Nianiaris, N., Songsivilai, S., and Hajitou, A. (2013). *Bacteriophage: From Bacteria to a Successful Targeted Systemic Gene Delivery for Cancer.* Gene Therapy of Cancer, Third Edition, London, UK, Academic Press.

Appendix 1

1- Peptone-yeast extract calcium nitrate (PYCa) (Bradley *et al.*, 1961)

Peptone	5 g
Yeast extract	3 g
Calcium nitrate	0.5 g
Distilled water	1000 mL
Agar	20.0 g

Appendix 2 Protocol of Easy Phage Kit

Scientific Methods

Protocol for Detection of Coliphage in Water Samples Using Easyphage-100 Medium

Version 3, 4/20/09

1. Reagents and Supplies:

   1.1 Tryptic Soy Broth - Sterile 1X TSB (Not provided, prepare according to manufacturer's instructions.)

   1.2 Bacterial host cells for male specific Coliphages (Provided as a slant or lyophilized form of *E. coli* Famp/CN13 upon request, fee may be applied)
   Log phase *E. coli* Famp host cells should be prepared the day before the assay (see EPA Method 1602 for details). Use an inoculation loop or other sterile transfer instrument to inoculate sufficient TSB with the *E. coli* Famp (e.g. 40 mL will be enough for 10 x 100 mL water samples). Each 100 ml sample to be analyzed will require 3.5 ml of this bacterial culture. After inoculation, incubate for 18-22 hours at 36°C ± 1°C to produce log phase *E. coli* Famp.

   Use *E. coli* CN13 for somatic coliphage.

   1.3 Sterile reagent water for negative control if perform (Not provided)

   1.4 Sterile EasyPhage medium (Provided, 100 mL per bottle)
   Medium may settle, showing two layers during storage at 4°C. Mix the medium well before use (Do not shake vigorously). Warm up the medium to room temperature or 35°C before use. Medium can be used when it is cold but will take longer to solidify.

   1.5 Bacteria Stain, sterile (Provided)
   0.7 mL Bacteria Stain is needed per bottle of 100 mL Easyphage medium

   1.6 Sterile pretreated petri dishes (Provided)
   Only pretreated petri dishes should be used with Easyphage medium (regular Petri dishes will NOT work).

   1.7 MS2 Positive Control (Provided upon request; fee may be applied)
   Male-specific coliphage, MS2, stock solution is provided for spiking the positive control. Use the amount according to the instructions on the label and following the procedure in Section 3-Quality Assurance Procedures

2. Procedures

   2.1 Warm up Easyphage and TSB to room temperature or 35°C.

12441 Beckley Street ▪ Granger, Indiana 46530
574.277.4078 (Ph) ▪ www.scientificmethods.com ▪ 574.968.0269 (Fax)

2.2 Into a bottle of Easyphage, add 100 ml water sample, 0.7 mL of Bacteria Stain, and 3.5 mL log phase *E. coli* Famp or CN13 culture. Mix the solution by swirling the bottle several times (do not vortex to avoid creating bubbles). Pipette 20 mL of the mixture into each pretreated petri dish for a total of 10 dishes.

Note: After dispensing all 10 petri dishes, if there is any medium mixture left in the bottle, dispense 1-2 mL per dish to those 10 petri dishes until all the mixture is finished. On the other hand, sometimes, the last Petri dish may have less than 20mL. It is perfectly Ok if the volume is at least 16 mL.

2.3 Swirl the dish gently several times to settle the bubbles on the edge of the dish. Let the medium mixture solidify on a horizontal bench for 1h. Transfer all the dishes to incubator. Petri dishes should be incubated in the upright position.

Alternative, the petri dishes can be placed directly into an incubator to let the medium solidify, but check that the incubator shelves are level. After dispensing medium mixture to the 10 pretreated petri dishes, immediately stack up the dishes and carefully move them to incubator. Petri dishes should be incubated in upright position.

Note: Do not move or transfer the petri dishes during gel solidification.

2.4 Incubate Petri dishes at 36°C ± 1°C for 16-24 hr.

2.5 Count the blue plaques on the background of red bacterial lawn. Plaques are typically round, blue areas that lack the reddish color observed on the bacterial lawn. They may show up in different sizes and shapes, and all should be counted.

3. Quality Assurance Procedures

3.1 To demonstrate that the reagents and laboratory systems employed during the sample run were performing as expected, analyze a negative and a positive control sample.

3.2 Negative Control: Analyze one 100 mL sample containing no coliphage (i.e. 100 mL sterile reagent water) according to the procedures 2.1-2.5. This sample must produce no plaque to demonstrate that the analytical run is valid. This control result is associated with all samples analyzed during the analytical run.

3.3 Positive Control: Analyze one 100 mL sample containing viable coliphages (i.e. 100 mL water sample spiked with pure coliphage stock solution). Lab strain male-specific coliphage such as MS2, ATCC#15597-B1 can be used to serve as a positive control. Filtered raw sewage also can be used (EPA Method 1602). Add 100 mL of sterile reagent water to a 100 mL Easyphage bottle. Then spike sufficient amount (specified in the positive control label) stock solution of MS2 to the Easyphage medium mixture to produce a countable number of plaques. Add bacterial stain and *E. coli* Famp and process according to procedures 2.1-2.5. Total plaque count for the 10 petri dishes is summed to be in the range of 100 to 1000 pfu.

3.4 This positive control sample must produce plaques to demonstrate that the analytical run is valid. This positive control result is associated with all samples analyzed during the analytical run.

Additional Suggestions:

1. For analyzing field water samples that may have high background bacteria (such as river water, treated waste water etc.), adding Ampicillin and Streptomycin to the Easyphage mixture is strongly recommended for male-specific coliphages. Add nalidixic acid for somatic coliphages.

Stock ampicillin/streptomycin preparation (100X Amp/Strep):

Dissolve 0.15 g of ampicillin sodium salt (Sigma A9518 or equivalent) and 0.15 g of streptomycin sulfate (Sigma S6501 or equivalent) in 100 mL of reagent water. Filter through 0.22-um-pore size membrane filter or syringe filter for sterilization. Dispense in small volumes (depending on how fast you can finish using an aliquot) and store frozen at -20 °C for up to one year. For one bottle of Easyphage plus water sample with final volume approximately 200 mL, add 2 mL of 100X Amp/Strep during sample processing.

Stock nalidixic acid (Sigma N4382, or equivalent)—***Please note: Nalidixic acid is considered toxic. Wear suitable protective clothing, gloves, and eye/face protection anduse in a chemical fume hood.***

Dissolve 1 g of nalidixic acid sodium salt in 100 mL reagent water. Filter through a sterile, 0.22-µm-pore-size membrane filter assembly. Dispense 5 mL per 5-mL freezer vial, date vial, and store frozen at -20°C for up to one year. Thaw at room temperature or rapidly in a 36°C ± 1.0°C water bath. Mix well prior to use.

2. After incubation, there may be some condensate on the lid of the dishes. Dry the lids with Kim wipe tissue paper and start counting.

3. It is suggested that MS2 or equivalent male specific Coliphage should be used to get familiar with the procedures and used as positive control with water field samples.

4. A light source from the top of the plate may be used in addition to a light box or a colony counter. The blue color of plaques will be more intense when the light shines from the top of the plate. Sometimes, tiny blue plaques may not show up well if this tiny plaque is observed from the bottom of the dish but it will be very visible on the surface of the gel.

5. This Easyphage-100 medium detection system is capable of producing quantitative results and quality control schemes for investigating the precision and accuracy of the microbiological assays, such as initial and ongoing coliphage precision and recovery experiments.

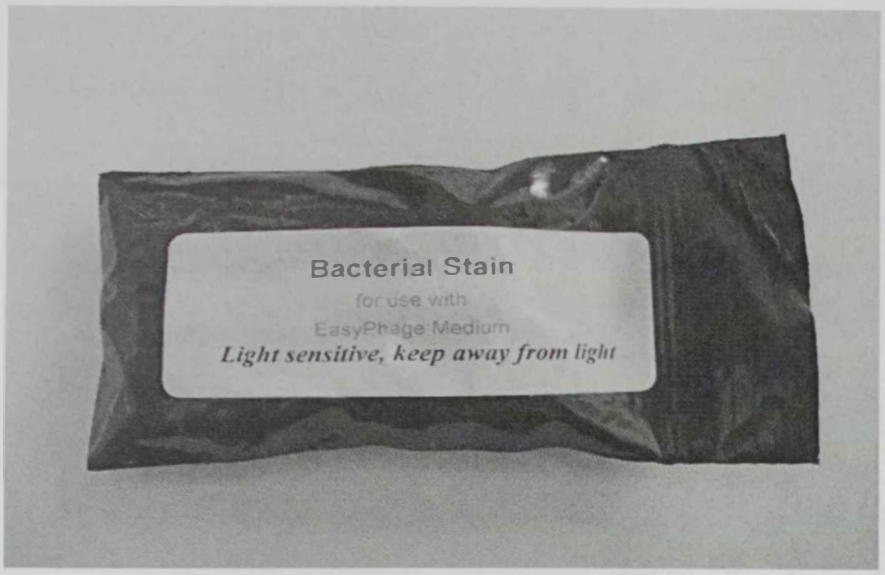


Figure 6.1: Bacterial Stain of the Easy Phage kit.

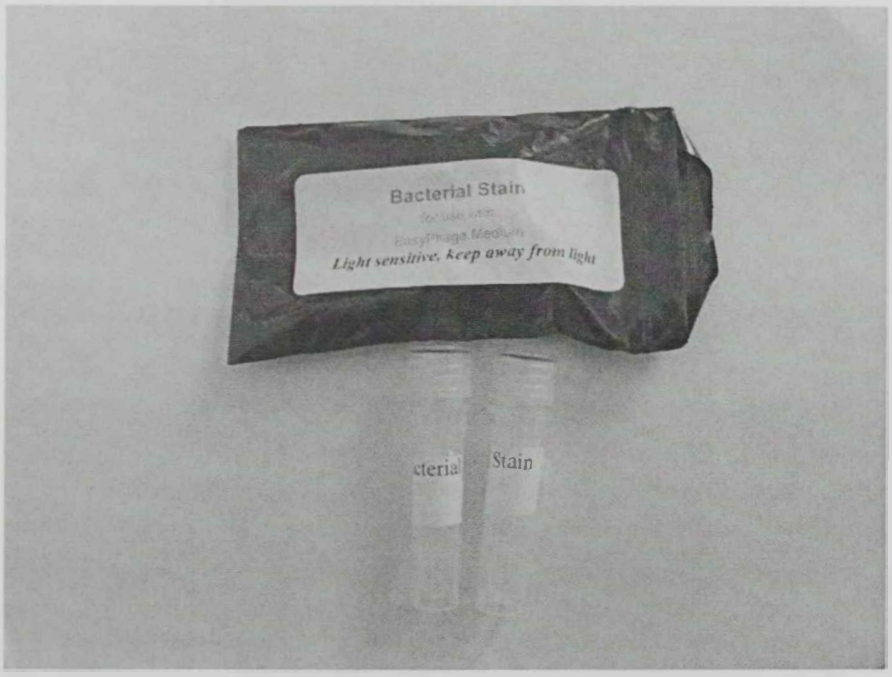


Figure 6.2: Bacterial Stain of the Easy Phage kit.

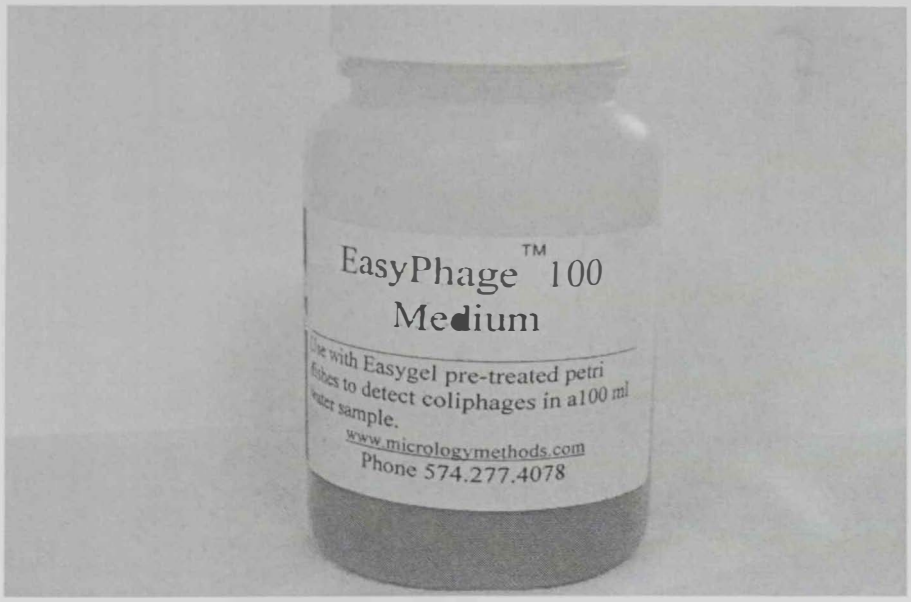


Figure 6.3: Easy Phage Medium-100.

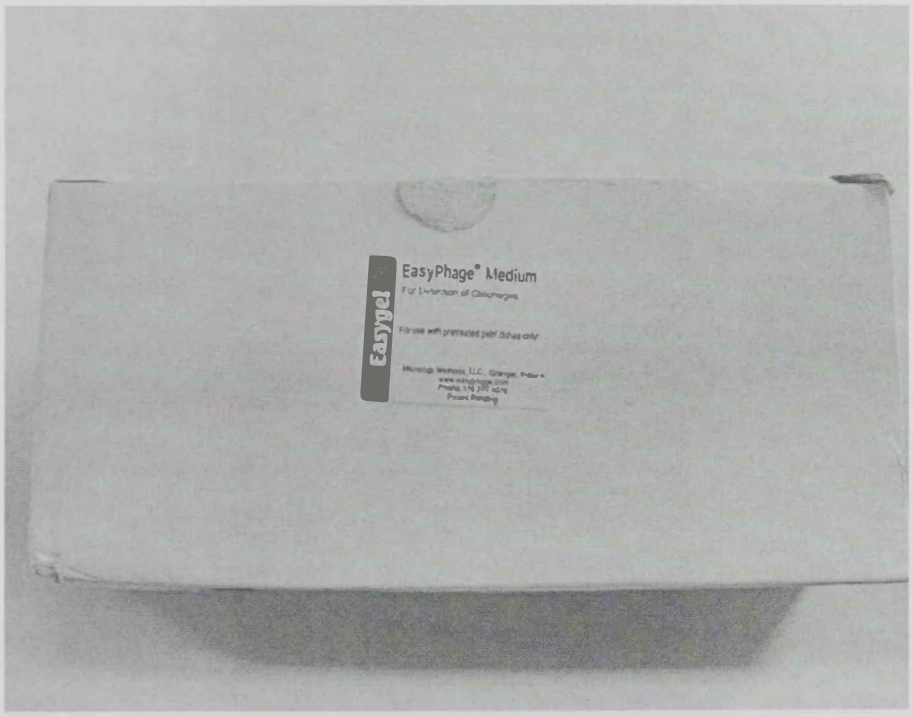


Figure 6.4: The Easy Phage Kit.

Appendix 3

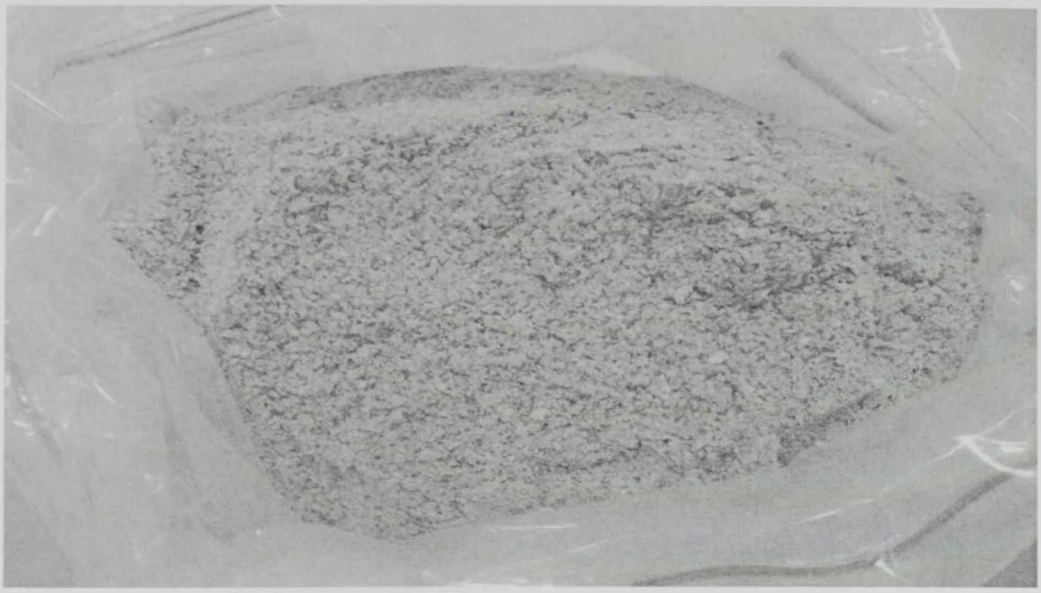


Figure 6.5: A sample of chicken feed.

Figure 6.6: The growth of *Escherichia coli* on EMB agar medium.

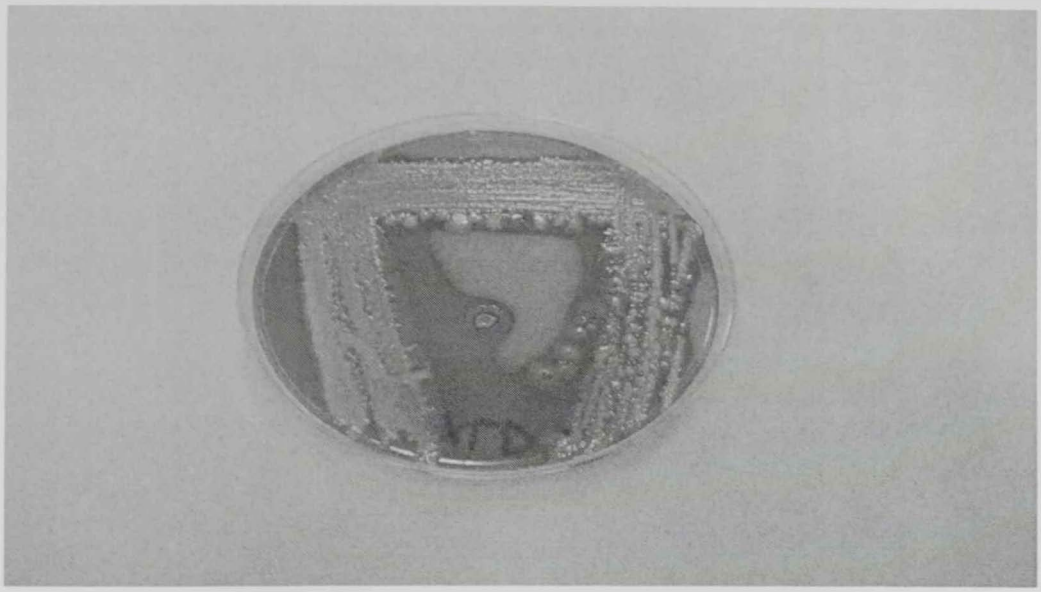


Figure 6.7: The growth of *Salmonella typhimurium* on XLD agar medium.

و عند دراسة العوامل الاخرى المؤثرة في نمو الفيروس وجد انه عند حضانة البكتيريا لمدة 0-3 ساعات و اضافتها للفيروس رقم 1 فان عدد وحدات الفيروس تكون اقل بالمقارنة مع اضافة بكتيريا ذات فترة حضانة اطول اي من 5-10 ساعات. و اتت النتائج مشابهة للفيروس رقم 2. و ايضا وجد ان زيادة مقدار البكتيريا المضافة للفيروس رقم 1 و الفيروس رقم 2 ادت الى تركيز اكبر للفيروسات اي زادت عدد وحدات الفيروس. و ايضا عند زيادة مقدار الفيروس المراد مضاعفته يزيد من عدد وحدات الفيروس الناتجة.

اجريت تجارب لاختبار مدى قدرة الفيروس رقم 1 ضد السالمونيلا و الفيروس رقم 2 ضد الايكولايفي محاربة البكتيريا في اعلاف الدواجن , حيث تم اضافة كل فيروس على حدة و ايضا تمت اضافتها كخليط معا , و كانت النتيجة ان الفيروسات قد قللت من تركيز بكتيريا السالمونيلا و الايشريشياكولاي بشكل كبير و ملحوظ (ب < 0.05) عند مقارنتها بنسبة تركيز البكتيريا المضافة لأعلاف الدواجن كعامل ضبط. عند المقارنة وجد ان اضافة خليط من الفيروس رقم 1 و الفيروس رقم 2 اعطى نتائج افضل في التقليل من تركيز البكتيريا من نتائج اضافة الفيروس كل على حدة.

و كانت النتائج الدالة على التقليل من نسبة تركيز البكتيريا في اعلاف الدواجن تقريبا مشابهة لنتائج اضافة المضاد الحيوي الاوكسيتتراسايكلين في انقاص و تقليل اعداد البكتيريا.

في الختام , فان هذه الفيروسات المعروفة ايضا بالبكتيريوفاج لديها فرصة كبيرة باقتحام مجال العلاج بالبكتيريوفاج و صناعة اعلاف الدواجن لانها ذاتية التكاثر او الانقسام و يمكنها استهداف المستقبلات الموجودة على البكتيريا المسببة للأمراض. و في المحصلة يمكننا استخدام البكتيريوفاج كبديل آمن للقضاء على الامراض و العدوى البكتيرية و البكتيرية المتعددة المزمنة الناتجة عن البكتيريا المقاومة للمضادات الحيوية.

الملخص

في هذه الدراسة تم عزل نوعين من الفيروسات أكلات البكتيريا "لاقمات البكتيريا" من مياه الصرف الصحي الغير معالجة من مدينة العين بدولة الإمارات العربية المتحدة. تم عزل الفيروس النوع الاول باستخدام البكتيريا سالمونيلا تايفيميوريومو تمت الاشارة اليه بالفيروس رقم 1 و تم عزل الفيروس النوع الثانيباستخدام البكتيريا اشريشياكولاي المعروفة بالتسبب بالتسمم الغذائي في اعلاف الدواجن و تمت الاشارة اليه بالفيروس رقم 2.

وجد إن التركيز الأمثل للفيروسات المستخدمة في هذه الدراسة 10^{10} وحدة فيروس لكل ملليلتر. وجد ان الفيروس رقم 1 ذو ميول احادي حيث قام بتحليل بكتيريا السالمونيلا فقط و هي المستخدمة في عزله في حين ان الفيروس رقم 2 كان ذا ميول تعددي حيث قام بتحليل العديد من البكتيريا موجبة صبغة الجرام مثل باسيلسسبتيليس و ستافلوكوكس اوري و ستافلوكوكسابيديرمديس و انتروكوكسفيكالس ،بالاضافة الى البكتيريا سالبة صبغة الجرام مثل بروتيسفالجاريس و سيدوموناساريجنوزا و شيجلاديزينتري. نتج عن الفيروس رقم 1 دوائر صغيرة خالية من البكتيريا (0.5 مليمتر) في حين نتج عن الفيروس رقم 2 مناطق صغيرة دائرية خالية من الصبغة الحمراء الموجودة على خلفية البكتيريا (0.9 مليمتر). باستخدام تقنية الصبغة السالبة للفيروس رقم 1 و الفيروس رقم 2 في المجهر الالكتروني, وجد ان كلا الفيروسين متطابق مع الشكل الخارجي المعروف لعائلة الفيروسات المسماة بالسايفوفريدي و التي يتكون فيها الفيروس من راس و سطح قاعدي.

تم اختبار اضافة 0.05 % من كربونات الكالسيوم للبيئة التي تحوي الفيروس رقم و الفيروس رقم 2, سواء كانت بيئة البيتون او البيئة المغذية او بيئة الصويا, و وجد ان هذه الاضافة زادت من عدد وحدات الفيروس في البيئة مقارنة بالبيئة الغير مضاف اليها كربونات الكالسيوم. و جاء تأثير اضافة الملح او كلورا يد الصوديوم بنتائج معاكسة حيث لوحظ ان عدد وحدات الفيروس سواء للفيروس رقم 1 او الفيروس 2 اقل عن عددها في البيئة الغير مضاف لها كلورا يد الصوديوم.

بدراسة بعض العوامل الفيزيائية و المواد الكيميائية على نمو الفيروسات وجد إن الفيروس رقم 1 كان أكثر حساسية للتجميد و للحرارة و التبريد و الأشعة فوق البنفسجية و الموجات فوق الصوتية و لمواد كيميائية مثل : الكلوروفورمو ثايمول و هيدروجين بيروكسايد و فينول و ايثيل الكحول و كلوروكس و اليود و ميرثيوليتمقارنة بالفيروس رقم 2.

جامعة الإمارات العربية المتحدة

كلية العلوم

قسم علوم الحياة

إستخدام الفيروسات أكلات البكتيريا كبديل لاضافة المضادات الحيوية في أعلاف الدجاج بدولة الامارات العربية المتحدة

رسالة مقدمة من /

وفاء عوض الشامسي

مقدمة إلى /

جامعة الإمارات العربية المتحدة

استكمالا لمتطلبات الحصول على درجة الماجستير في علوم البيئة

تحت اشراف الدكتور خالد الطرابيلي و الدكتور عبد المجيد الخاجة

يونيو 2014

Digitized By
جامعة الإمارات العربية المتحدة
United Arab Emirates University
UAEU Libraries Deanship
S.M.Wolieed
Shrieen
2016.11.20
09:25:53
+04'00'