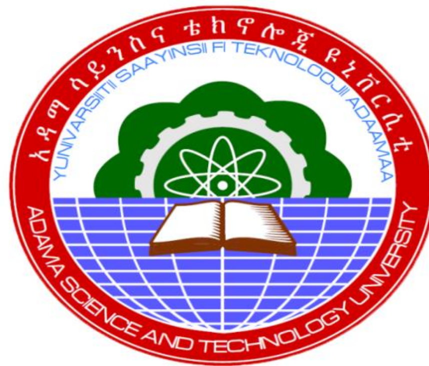
Isolation of Bacteriophages from sewage and their molecular characterization and *in-vitro* antimicrobial activities on contaminated food items collected from Bishoftu town, Oromia Regional State, Ethiopia

By: Kemila Ebrahim



A Thesis Submitted to Department of Applied Biology

School of Applied Natural Science

Presented in Partial Fulfilment for the Requirements of Degree of Masters of Science in Applied Biology (Specialisation in Biotechnology)

Office of Graduate Studies

Adama Science and Technology University

December, 2018

Adama, Ethiopia

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SCHOOL OF APPLIED NATURAL SCIENCES

APPLIED BIOLOGY DEPARTMENT

APPROVAL OF BOARD OF EXAMINERS

We, the under signed, members of the Board of Examiners of the final open defence by Kemila Ebrahim have read and evaluated her thesis entitled "**Isolation of Bacteriophages from sewage and their molecular characterization and *in-vitro* antimicrobial activities on contaminated food items collected from Bishoftu town, Oromia Regional State, Ethiopia**" and examined the candidate. This is, therefore, to certify that the thesis has been accepted in partial fulfilment for the requirement of the Degree of MSc in Applied Biology (Specialisation in Biotechnology).

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CANDIDATE DECLARATION

I hereby declare that the work which is being presented in this research entitled as **Isolation of Bacteriophages from sewage and their molecular characterization and *in-vitro* antimicrobial activities on contaminated food items collected from Bishoftu town, Oromia Regional State, Ethiopia** in partial fulfillment for the requirement of degree of MSc in Applied Biology (Specialisation in Biotechnology) is authentic record of my original work carried out in Bishoftu NVI under the advisors of Dr. Hunduma Dinka and Dr. Esayas Gelaye. The matter embodied in this has not been submitted by other person or me for the award of any other degree. All relevant resources of information used in this body have been duly acknowledged.

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Candidate	Signature	Date

This MSc Thesis has been submitted for examination with my approval as thesis advisor.

Name: ____

Signature: ____

Date of submission: …………..

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LIST OF ACRONYMS AND ABBREVATIONS

API	Analytical Profile Index
ANOVA	Analysis of Variance
BSA	Bovine Serum Albumin
CDC	Centers for Disease Control and Prevention
CFU	Colony Forming Unit
ESKAPE	*Enterococcus faecium, Staphylococcus aureus, Klebsiella pneumoniae, Acinetobacter baumanni, Pseudomonas aeruginosa and Enterobacter* species
FDA	Food and Drug Administration
IBM	International Business Machines
NIAID	National Institute of Allergy and Infectious Diseases
NCR	National Census Report
NVI	National Veterinary Institute
PFU	Plaque Forming Unit
SPSS	Statistical Package for Social Science
TSA	Tryptic Soya Agar
TSB	Tryptic Soya Broth
XLD	Xylose-Lysine-Desoxycholate agar

ABSTRACT

Foodborne pathogens such as Salmonella and Escherichia coli have become big problem to food industries and human health. Emergence of antibiotic resistant bacteria has limited the opportunities of controlling pathogenic bacteria in food commodities and treating foodborne infections. Bacteriophages are viruses of bacteria that infect and kill bacteria. This study is targeted to isolation, purification, quantification, in-vitro evaluation and molecular characterization of phages. Accordingly, four lytic phages, PS1, PS2, PE1 and PE2, were isolated and characterized. In the result, all the isolated phages have very nice lytic ability phenotypically in spot assay with PS1 and PE2 exhibiting more activity and having higher plaque concentration than PS2 and PE1. Bacteriophages titer was determined within range of 10^5 *to* 10^{12}*PFU/mL. In phage efficacy test, Phage of PS1 and PE2 was found to be effective in reducing number of bacteria in two different foods (meat and milk) contaminated with Salmonella Enterica (S1) and E. coli O157:H7 (E2), respectively. Which were spiked with* 1×10^3 *CFU of Salmonella Enterica and* 1×10^5 *CFU E. coli 0157:H7 strain and treated with* 1×10^7*PFU of each PS1 and PE2 phage, respectively at 4ºC and 25ºC. In the result of the present study, the inhibitory effect of both phages was better at 25ºC than at 4ºC when the two storage temperature is compared. A significant reduction in bacterial number was observed in both tested foods having* $p< 0.05$ *in phage treated food samples when compared with its non-phage treated control compartment. Both Phages incubated at 4ºC in each food samples are relatively stable than the one which is at 25ºC. Molecular analysis of all the four phages using gel-electrophoresis revealed clear band patterns all having greater than 12Kb genome sizes, whereas restriction digestion of phage DNA with MseI has shown no digestion but with HinP1I digestion, low concentrated and cleaved band was observed for PE2. Based on the finding of this study, using bacteriophage as antimicrobial agent to prevent pathogenic bacteria is realistic. Bacteriophages ability of infecting and killing food spoiling microorganisms and capacity of extending shelf life of food makes them potential alternative antimicrobial agent to alleviate the risk of transmitting pathogenic bacteria via food commodities. Accordingly more characterization, in-vivo evaluation and safety investigation study should have to be done to take phage therapy to higher and problem solving stage.*

Keywords: Drug resistance, Foodborne disease, Molecular analysis, Phage therapy, *Plaque*

1. INTRODUCTION

1.1. Background of the study

Foodborne disease is a disease of an infectious or toxic nature caused by consumption of contaminated food. Every day many foods are contaminated with spoilage and pathogenic bacteria that cause high losses in food industry and contribute for deteriorating consumer health which demands the use of efficient technology in food industry for consumer safety (Bassett, 2007). Despite advances in modern technologies like antibiotics; the food industry is continuously challenged with the threat of microbial contamination (Patel *et al.,* 2015). The emergence of genetically modified bacterial resistance to antibiotics following widespread clinical, veterinary, and animal or agricultural usage has made antibiotics lesser effective due to development of antibiotics resistant gene in pathogenic bacteria and inherent feature of bacteria to develop resistance (Fischetti, 2008). Recently many genetically modified organisms in various food products and in animal feeds that may contain the antibiotic resistant gene marker have been reported (Lisha *et al*., 2017).

Pathogenic bacteria are involved in public health problems, including risking human health. They are known to cause high mortality in the world. However, there has been little success for the development of new drugs against multidrug-resistant pathogens (Rasmussen and Casey, 2001). Data on the world wide incidence of foodborne disease are fragmented, mainly focusing on specific pathogens or their rate of occurrence in particular countries (Carla *et al.,* 2012), For example, in the United States of America it is estimated that foodborne diseases results in approximately 76 million illnesses; 325,000 hospitalizations and 5,000 deaths each year (Sirsa *et al.,* 2009). As a consequence of this, the true measure of foodborne illness on a global scale is unattainable (Lorrainet *et al.,* 2014). Generally, foodborne diseases are associated with acute, mild and self-limiting gastroenteritis with symptoms such as nausea, vomiting and diarrhea as a consequence of consumption of microbial contaminated food. Besides that, a number of chronic sequels may result from foodborne infections involving diseases that affect the cardiovascular, musculoskeletal, respiratory system and immune systems (Kuchenmuller *et al*., 2013).

The bacterial species in question for foodborne infection have previously been referred to by the acronym ESKAPE, which includes Gram-positive *Enterococcus faecium* and *Staphylococcus aureus,* as well as Gram-negative *Klebsiella pneumoniae*, *Actinobacter baumanni*, *Pseudomonas aeruginosa* and *Enterobacter species* (Rice, 2008). It has been reported that among *Enterobacter* species *Escherichia coli* and *Salmonella* are considered major foodborne pathogens that can infect human mostly through contaminated food. They are of a very important contamination index of water and food and also reported with drug resistance issue (Reid *et al.*, 2001). Contamination of food with such antibiotic resistant bacteria presents a significant risk of illness to human and animal health. The overuse of antibiotics has further escalated this problem, resulting in the increasing emergence of antibiotic-resistant foodborne pathogens (Buncic and Sofos, 2012).

Rapid increase of antibiotic resistance in the bacterial community is an ecological phenomenon, but is also one of the greatest hazards for human and veterinary medicine worldwide. Circulation of antibiotic-resistant bacteria occurs mainly between four ecosystems (humans, animals, soils and aquatic environments) interconnected by water (Koskella and Meaden, 2013). Different physical, chemical and biological methods have been used to control the presence of pathogenic bacteria in food processing chain, but their efficiency was quite low in most cases. New methodology like bacteria and their products was used to kill or reduce the numbers of pathogenic bacteria. For instance pro-biotic bacteria like bacteriocin producing bacteria produce bacteriocins to kill or inhibit the pathogenic bacteria (Grinter *et al*., 2012) and its purified Nisin added to food products to kill such pathogens, although these and other antibacterial alternatives have no adverse effect on human health or to keep quality of the food product (Loretz *et al*., 2010). Therefore, it is necessary to use strict control measures against pathogens and spoilage bacteria. The efforts to develop new methods for controlling microbial contamination and to decrease the risk of foodborne illness in food and food processing environment are extremely important. Accordingly, bacteriophages have emerged as novel, viable, and safe options for the prevention, treatment, and eradication of these contaminants in a range of foods and food processing environments (Ayman, 2016).

Bacteriophage is the virus that infects and kills the bacteria and can be used as an alternative antimicrobial therapy. Bacteriophages are most abundant biological entity on our planet and they are highly specific; infecting only one or a few types of bacteria and non-infectious to human, animals and plants (Nobrega *et al*., 2015). Soon after their discovery in the early 20th century, researchers began using phages for the treatment of infectious disease and their administration as pharmaceutical agents was a common practice in the pre-antibiotic era. However, this approach was abandoned in west following the discovery and use of antibiotics in the 1940s and scientists are concentrated on escalated production of newer and newer antibiotics of commercial importance (Carlton, 1999). Eventually, there have been constantly increasing number of antibiotic-resistant bacterial infections and a deficit in the development of new chemotherapeutics to counteract bacteria, so that, there has been a re-emergence of interest in phages as potential therapeutic agents (Matsuzaki *et al*., 2005). After a century of study, however, there have been no reports of phage particles causing diseases in humans, animals, and plants, despite being ubiquitous in food, intestinal micro-biomes, and the environment (Patel *et al*., 2015). This would imply that phages are safe and generally well tolerated by mammals. Phage applications for bio-control have been approved by Food and Drug Administration's (FDA) as a safe food additive on ready-to-eat products (Bren, 2007).

Phage therapy encompasses several modalities, but common to all is the use of a virus that can present immunological complications for the host. Whole phages, modified phages, and their derivatives have potential as antimicrobials in the areas such as primary production, postharvest processing, bio-sanitation, and bio-detection. The use of lytic bacteriophages as antimicrobial agents for controlling pathogenic bacteria has appeared as a promising new alternative strategy in the face of growing antibiotic resistance in many fields including medicine, veterinary medicine and aquaculture (Weinbauer, 2004). Replication of the several particles and release of the progeny of phage generally leads to death of the host cell and it has been estimated to kill 20-40% of marine bacteria every day (Suttle, 2005). Hence, the use of bacteriophages to remove microbial pathogens from food has recently become an option for the food industry as a novel method for bio-control of bacteria without interfering with the non-targeted microflora, as well as enhancing the safety of food products (Hagens and Loessner, 2010). Moreover, the relative simplicity and economy of phage therapy makes it an

affordable preposition that showed the importance of phage therapy as potential tool for fighting against the drug resistant bacteria (Duckworth and Gulig, 2002).

1.2. Statement of the problem

Despite the effective technologies and the good manufacturing practices, food safety is constantly threatened by the factors related to changes in lifestyle, consumer eating habits, food and agriculture manufacturing processes and also the increased international trade (Newell *et al.,* 2010). Moreover, development of antibiotics resistant pathogenic bacteria in food and food processing environment following widespread clinical, veterinary, and animal or agricultural usage further escalate the problem (Parisien *et al*., 2008). According to Center for Disease Control and Prevention (CDC), foodborne illness is known to be a ubiquitous, costly, yet preventable health problem all over the world (CDC, 2014). Additionally antibiotic resistant species resulted in significant increase in hospitalization and the risks of invasive infections and death (Travers and Barza, 2002).

Nowadays new antibiotics are not progressing and other alternative like bacteriophage to fight against pathogenic bacteria is not developed to the extent of controlling this problem. As a result, foodborne pathogens have becoming a constant threat to consumer and food industry with scarcity of the new drug pipeline to combat these emerging threats. According to World Health Organization (WHO) the world is on the brink of losing antibiotics miracle in which many common infections will no longer have a cure and that my kill unabated if the urgent corrective and protective actions is not taken (Freire *et al.,* 2011). So that, foodborne illnesses of microbial origin and safety of genetically modified organism are one of the biggest threats to health and food security in the universe. These indicate the urgent need for development of novel non-antibiotic approach to fight against the increased incidence of multi-drug resistant pathogen due to the shortage of new antibiotics in developmental pipeline.

1.3. Objectives of the study

1.3.1. General objective

- ❖ To isolate lytic bacteriophage from sewage, evaluate their anti-bacterial activity on contaminated foods and characterize bacteriophages using molecular tools.

1.3.2. Specific objectives

The specific objectives of this study were:

- ❖ To isolate lytic phages against foodborne bacterial pathogens collected from sewage, Bishoftu.
- ❖ To characterize isolated phages in controlling or reducing foodborne bacterial pathogens phenotypically.
- ❖ To isolate and select bacteriophages having strong killing potential against tested host strains with high titer concentration (high PFU/ml).
- ❖ To examine for their (phages) stability on food sample at refrigeration (4ºC) and room (25ºC) temperature and
- ❖ To analyze isolated bacteriophages by molecular tools.

1.4. Significance of the study

Reduced efficacy of antibiotics with emergence of resistant bacteria has limited the opportunities for controlling pathogenic bacteria in food commodities and treating foodborne infections (Patel *et al.*, 2015). Thus, it seems unrealistic to depend entirely on antibiotics against bacterial threats, and even the most effective antibiotics have also detrimental effects on endogenous gut micro biome which plays a vital role in human digestion and nutrition (Premarathne *et al*., 2017). Most importantly, new antibiotics are not progressing at the same rate as the emergence of resistance. So due to this and steadily declined discovery of new class of antibiotics, there is an urgent need to work on novel food preservation strategies to delay spoilage, extend shelf life, and to prevent foodborne illness associated with toxigenic and pathogenic species of bacteria.

When more number of bacteria is becoming resistant to antibiotics, the development of new disease-fighting agents has become essential with increasing today's emphasis and consumers demand on the availability of fresh food products which are free from foodborne pathogens and synthetic chemicals (Tan *et al*., 2014). Accordingly, one possible replacement for antibiotics is the use of bacteriophages that parasitize and kill the bacteria against which it is targeted (Theil, 2004). However, there is a lack of research on the application and utilization of bacteriophages as controlling agent of spoilage bacteria in food commodities, especially in raw animal materials destined for the rendering process in food industries. Therefore, this study will help to narrow the gap via investigating the efficacy of phage treatment in controlling growth of major food spoilage and foodborne pathogenic bacteria using bacteriophages at post-harvest fresh and processed foods. The finding will help to transfer this technology to the field for commercial environment as natural tool for the control of microbial contaminant as a promising and chemical free approach technology. It will also help as a base line for further studies since there is no any reported study on bacteriophage in Ethiopia.

1.5. Research questions

1. What is the level of *in-vitro* efficiency of phages in controlling or killing foodborne bacterial pathogens?
2. How is the efficiency/stability of phage in keeping the safety of food sample at the given temperature (4 ºC and 25 ºC) for the given time interval?
3. What are the characteristics of isolated phages?

1.6. Delimitation of the study

This study was targeted to: Isolation and identification of bacteria (*Salmonella* and *E. coli*) and phage isolation, purification, *in-vitro* analysis, efficacy examination against pathogenic foodborne *Salmonella* and *E. coli* bacterial strains and Phage *lytic* capacity investigation and stability detection at 4 ºC and 25 ºC on food samples. Even though molecular characterization based on PCR is very crucial to identify and characterize bacteriophages, due to the absence of PCR Primer and sequencing machine, this study was limited to characterizing bacteriophages by nucleic acid identification, Gel electrophoresis and Restriction enzyme digestion of isolated and purified phages.

2. LITRATURE RIVIEW

2.1. History of phage discovery and phage therapy

Long before the discovery of antibiotics, bacterial viruses called bacteriophages were discovered independently by a British microbiologist Frederick Twort in 1915 and Felix de Herelle (Felix d'Herelle, 1949). In 1917s, that French Canadian scientist Felix D'Herelle characterized the nature of these viruses and named them 'bacteriophages'. He has also been attributed with coining the word '*plaque'*, to describe the zone of clearing caused by phages on a bacterial lawn and developed protocols for *plaque* assays and burst size studies, and immediately recognized the potential of using phages as an antibacterial. Phages were used to treat and prevent infectious disease in humans and animals (Golkar *et al*., 2014).

In the early years, phage therapies resulted in mixed success, in large part due to a poor understanding of the viruses themselves, as well how they infect and kill bacteria. The extensive work on phage therapy was carried out from 1920 to 1930 in USA to treat infection caused by *Streptococcus* and *Bacilli*. Due to their remarkable antibacterial potency, bacteriophages were implemented in the treatment of human diseases almost instantly after their discovery. Bacteriophages were appeared as the front line therapeutics against infectious disease before the discovery of the broad spectrum antibiotic and were used in various countries until the Second World War and phage therapy saw modest success (Smith and Huggins, 1987). At the same time, antibiotics were discovered and widely used that causes rejection of bacteriophages as therapeutic agent in many countries including Europe and USA even though it have showed benefits in therapy of animal models (Qadir, 2015). Interestingly, in recent years, bacteriophage therapy was "rediscovered" with the work done by Smith and Huggins in 1980s due to increase antibiotics resistance in bacteria with the threat of multiple drug resistance (MDR) infections. There has been a refocusing on the therapeutic use of phages in several countries namely Poland, United States, Europe and Russia to provide a new solution to eradicate unwanted bacteria, such as those associated with infectious diseases and gastrointestinal microbe dysbiosis (Kutateladze and Adamia, 2010).

2.2. General overview on nature of bacteriophage

Bacteriophages are bacterial viruses and they are most abundant organisms on the Earth. They are ubiquitous, obligate intracellular parasites that attack the bacteria cell, hijack the host machinery and finally destroy it. Each kind of bacterium has its own bacteriophage, including Archaea and Cyanobacteria which are also attacked by a group of viruses often called as Cyanophages (Paul and Sullivan, 2005; Clokie *et al*., 2011). Bacteriophage widely occurs in sewage, soil, marine water and other natural environment where their specific hosts proliferate. They have become a focus of interest to control bacterial contamination of food products, having several characteristics that make them attractive as therapeutic or agents of bio-control. Phage is significant due to their effectiveness, specificity, natural residence in the environment, self-replicating and self-limiting. Beside this the most important feature of phages is their narrow host range (Theil, 2004). They kill only specific pathogenic bacteria strain without affecting the balance of beneficial bacterial micro flora and this feature makes them a potential antimicrobial agents and advantageous unlike broad-spectrum antibiotics (Koskella and Meaden, 2013). It is important to note that bacteriophages can significantly modify bacterial communities, which is dependent on the potential for bacteriophage propagation within those communities. Thus, bacteriophages have a potential for propagation, in turn, is a function of the interaction of the *biotic* and *abiotic* components of an environment (Boyd and Brussow, 2002).

2.3. Morphology and taxonomy of bacteriophage

Bacteriophages are classified into single order, 13 families based on their nature of nucleic acid and virion morphology traits and 31 genera (Ackermann, 2005). Structurally, most of bacteriophages consist of three parts which are head, tail and tail fiber. Head of phages is attached to a tail through a connector that functions as adaptor between the two structures of the phage (Figure1). The tail is a hollow tube which acts as a passage way for genetic materials to pass from capsid to host bacteria (Lurz *et al*., 2001). Tail fibers and base plate which are located at the end structure of the phage are involved in the binding process of the phage to the bacterial outer membrane (Sao-Jose *et al*., 2006). The head stores genetic materials (either DNA or RNA but not both) and form a part of the overall feature of a bacteriophage. It encloses nucleic acid which encapsulated with a protein or lipoprotein capsid

connected with a tail that interacts with various bacterial surface receptors via the tip of the tail fibers. The capsid is icosahedral in shape and has the main function to protect the genetic material from the environment (Orlova, 2012).

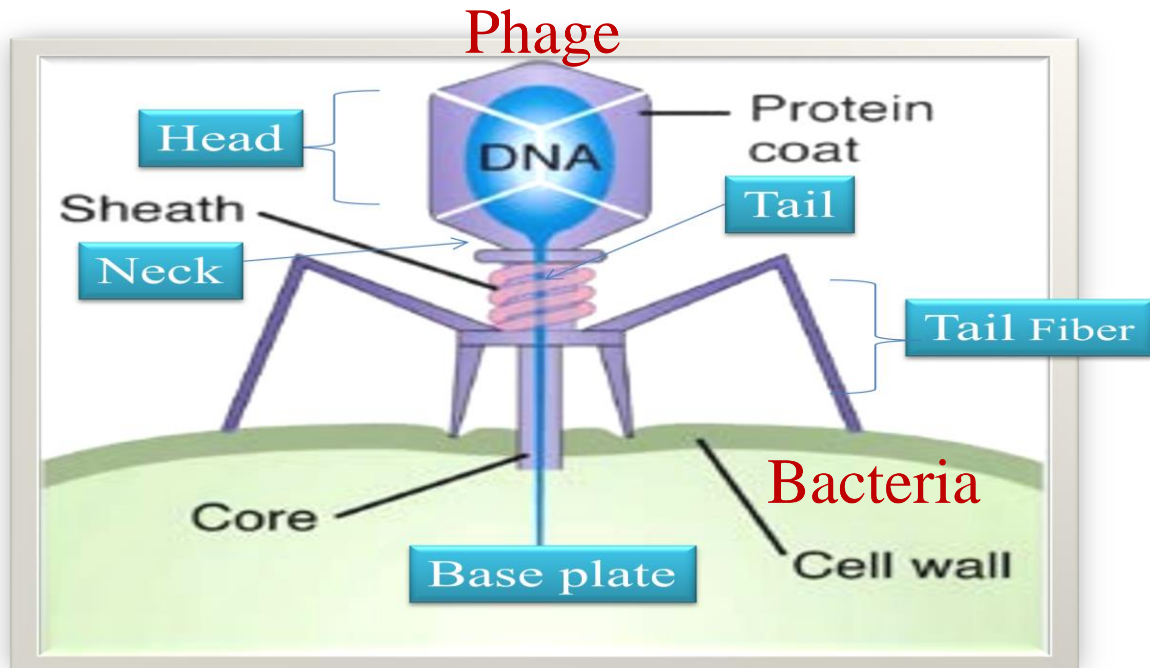


Figure 1: Diagram of a bacteriophage, Adapted from Orlova (2012).

The size of most bacteriophages in general ranges from 22-200nm in length, while the largest bacteriophage known is T4 which is about 200nm long and 80-100nm wide (Clokie *et al*., 2011). Most of the phages are tailed bacteriophage, which accounts for 96% of all phages present on earth, belonging to the order *Caudovirales* (Ackermann, 2009) and they are the predominant group in existence among the phages isolated and characterized (Orlova, 2012). The three families within this order are *Myoviridae* phages, which possess sheathed, contractile tails; *Siphoviridae* possess long non-contractile tails and *Podoviridae*, which possess short tails. The former two families are the most abundant group. The morphology of these viruses is quite diverse; they can be cubic, helical, pleomorphic or tailed, as depicted in Figure 2 (Ackermann, 2006).

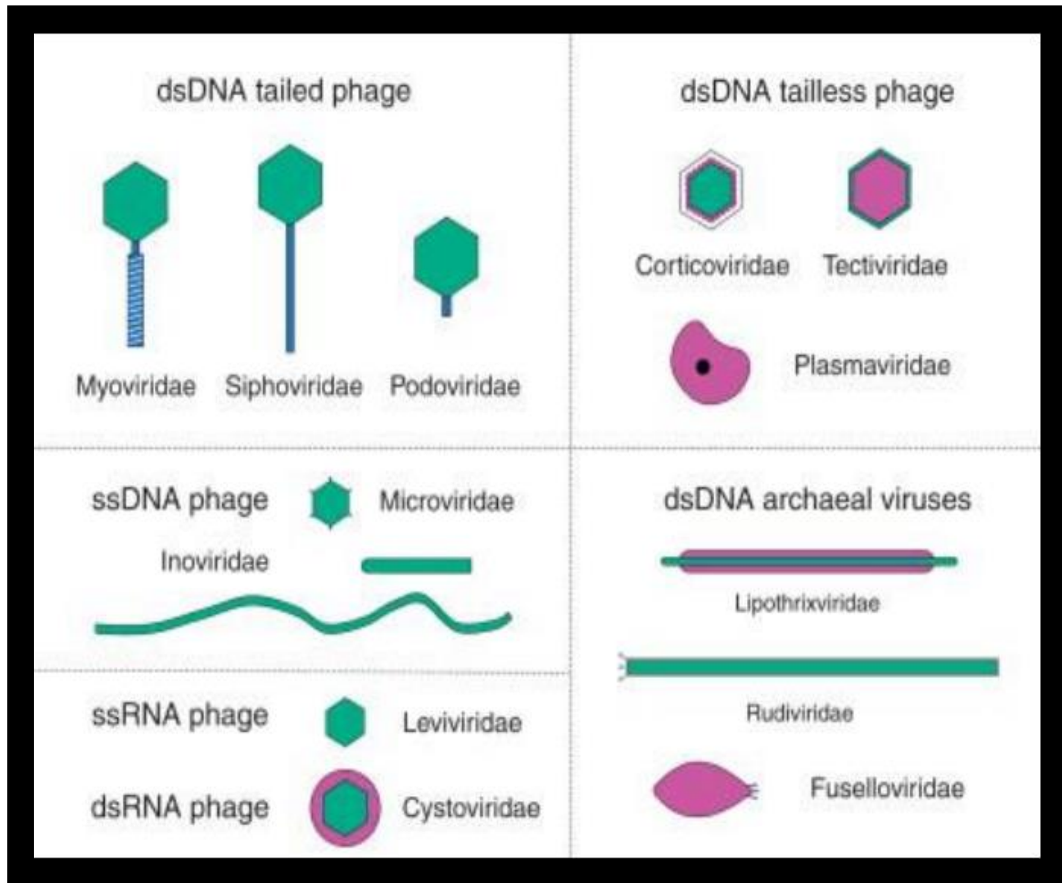


Figure 2: Different phage morphologies, Adapted from Hyman and Abedon (2009).

2.4. Life cycle and habitat of bacteriophage

Bacteriophages are obligate intracellular parasites that infect bacteria and reproduce by hijacking their host's biosynthetic pathway (Michael and Rotem, 2005). As the natural parasites of bacteria, bacteriophages start the infection in bacterial host with adsorption to the suitable host cell reversibly with specific cell-surface proteins and followed by injecting their genetic material in to the cytoplasm. Typically, different phages display different life cycles with the bacterial host after surface adsorption and introduction of viral genetic material. Phages are classified as either *lytic* or *lysogenic* based up on their replication strategy (Figure 3).

In *lytic* cycle, bacteriophage attach on bacteria through various cell surface receptors such as lipopolysaccharids (LPS), teichoic acids and various structural proteins (Omp A, C and F) on bacterial cell wall and injects its nucleic acid into the cytosol then hijack the host cell's protein

machinery and redirects the bacterial synthesis machinery to reproduction of the new and mature phage particles which results indirect damage (*lysis*) to the bacterial host. In this process approximately about 1000 viral particles may be released per cycle (Clokie *et al.,* 2011). To do so, it involves a series of events that occur between attachment of phage particle to a bacterial cell and release of daughter phage particles. There are four phases in the *lytic* cycle which takes about 20 min to 2 hours for adsorption of phage to host cell by binding to specific host, penetration of phage nucleic acid, intracellular development, destruction of the cell wall and finally releasing the newly assembled phages in to the environment (Rao and Lalitha, 2015). After binding and injection of phage genome into the host cell, the virulent bacteriophages will control the host cell's protein synthesis machinery via the expression of specific enzyme encoded by phage genome. The production of phage's enzyme in the later stages such as lysines and holins induced destruction of the cell membrane allowing the newly formed phages burst out from the lysed host cell into the environment (Young, 1992).

A *lysogenic* phage, on the other hand, embeds itself into the genome of its bacterial host, establishing a stable relationship with the bacteria that it has infected and it involves the replication of phage nucleic acid along with host genes for several generations without major destruction to the host cell. The phage genome remains in a repressed state in the host genome and is replicated as part of the bacterial chromosome until *lytic* cycle is induced. This stable relationship is maintained until some stressor, such as high radiation, change in metabolism and DNA damage, disrupts it (John and little, 2005). Hence, temperate phages are not suitable for direct therapeutic use as it may mediate transduction by transferring genetic material of one bacterium to another. This process may lead to the development of antibiotic resistance or even increased virulence of the host by acquiring genes from the pro-phage. Only the *lytic* bacteriophages, which replicate exponentially and eradicate the bacteria rapidly regardless of their antibiotic resistance profile, are more suitable for the biotherapy purposes and they are possibly one of the most harmless antibacterial approaches available (Sillankorva *et al*., 2012). Meanwhile, rather than relying on the killing ability, the unique characteristic of temperate phage is to deliver genetic material into the host genome. This has been exploited and demonstrated in a system that restores antibiotic efficiency by reversing the resistance of the bacterial host, making them susceptible to antibiotics again due to their capability of

transferring genes for toxin production or pathogenicity factors between bacterial populations (Edgar *et al*., 2012).

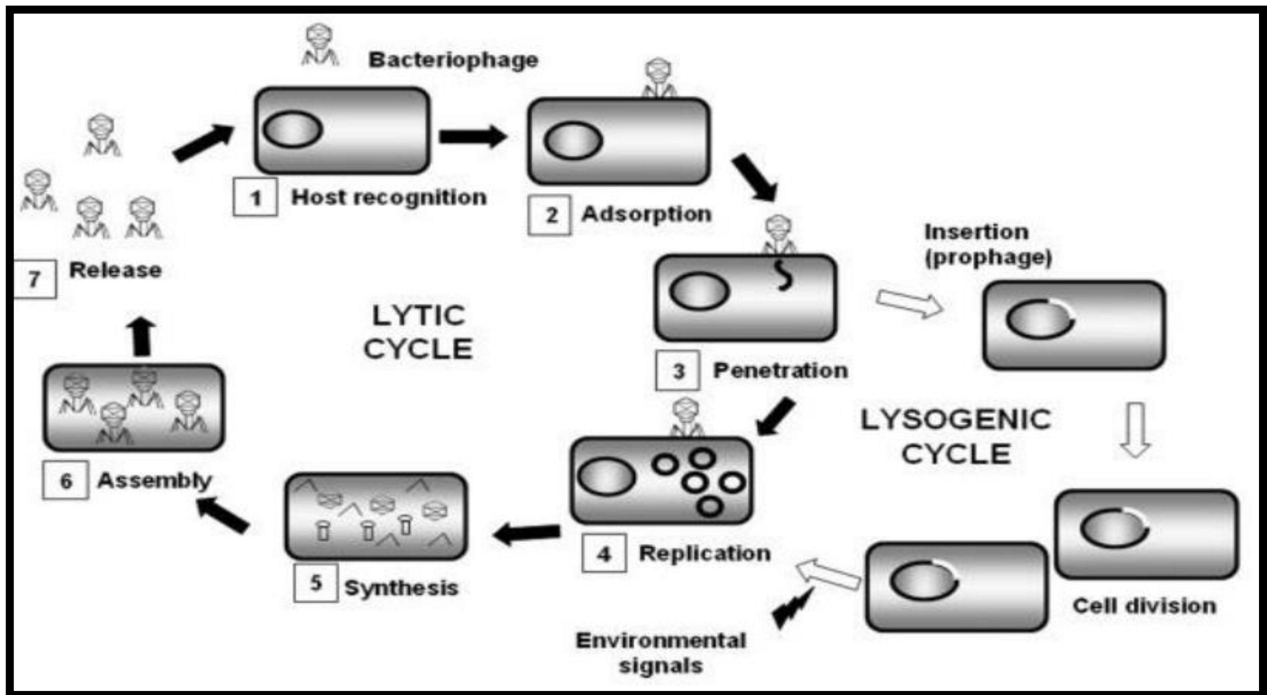


Figure 3: Replication cycles of *lytic* and *lysogenic* bacteriophages, Adapted from Garcia (2010).

2.5. Major Foodborne bacterial pathogens and their origin

Salmonella, Campylobacter and *E. coli O157:H7* is the common contaminants of livestock's and they are carried in the intestinal tract of the animals asymptomatically. *Salmonella spp.* and *Campylobacter spp.* are the top two world's most prominent foodborne pathogens which cause *Salmonellosis* and *Campylobacteriosis,* respectively, in humans. They are usually transmitted between livestock's and food supply through the shedding in fecal materials of the carrier (Tan *et al*., 2014).

1. *Escherichia coli*: *E. coli* is gram-negative bacterium which is a well-known food poisoning pathogen and attributed a third of cases of childhood diarrhea in developing countries. Furthermore, it is also known to be the prominent cause of traveler's diarrhea and associated with diarrhea in domestic and pet animals. Particularly *E. coli O157:H7* causes a wide spectrum of diseases ranging from mild diarrhea to *haemorrhagic colitis*,

haemolytic uremic syndrome and *thrombotic thrombocytopenic purpura* (Karmali *et al.,* 1985). The main reservoirs of *E. coli O157:H7* are comprised of ruminants such as cattle and sheep, as it does not induce significant clinical symptoms and survive well in the intestinal conditions of the ruminants. The main route of transmission to human is via uncooked contaminated meats particularly when proper care is not taken during the slaughtering process leading to contamination of the meat with intestinal contents, fecal materials or dirt on the hide of ruminants. This microorganism is highly virulent and a public health threat because ingestion of a concentration as low as 10 cells is able to cause infection (CDC, 2011).

2. *Salmonella species:* *Salmonella* is a genus of gram-negative facultative intracellular bacteria which has caused an estimated 93.8 million illness worldwide and approximately 155,000 deaths annually. *Salmonella* species is the common pathogen that found in contaminated poultry and it is the top world's most prominent food borne pathogens which cause Salmonellosis in humans and it is considered to be one of the principal causes of zoonotic diseases reported worldwide (Majowicz *et al*., 2010).

Salmonella enteric serovars enteritidis and *typhimurium* are still the most prevalent *Salmonella* responsible for majority of the outbreaks which are often associated with consumption of contaminated eggs, poultry, cattle and swine meats. *Salmonellosis* results in diarrhoea, fever, vomiting and abdominal cramps in humans. Beside infection in human, *Salmonellosis* can occur in swine and is known to be the top 10 most common disease in pig farms (Haley *et al*., 2012). The genus *Salmonella* contains two species which are *S. Bongori* and *S. Enterica*. *Salmonella Enterica* has six subspecies, 99% of the human infections are caused by only one of the subspecies *S. Enterica*. *Salmonella* can colonize the intestinal tract of humans and farm animals. The disease is mainly transmitted with contaminated foods (Majowicz *et al.,* 2010).

3. *Campylobacter species*: *Campylobacter* is a genus of gram-negative spiral, motile, and microaerophilic bacteria which grow optimally at 41ºC. *Campylobacter jejuni* and *Campylobacter coli* are both the *Campylobacter* species that are responsible for majority cases of bacterial gastroenteritis in humans. It has been suggested that *Campylobacter* spp. are the most common enteric pathogen with 2.4 million cases of *C. jejuni* and other species were found annually in United States. This widespread infection is explained because

ingestion of low doses (400-500 cells) can cause *Campylobacteriosis* typically characterized by fever, bloody diarrhea, and acute abdominal pain (Raya *et al*., 2006). *Campylobacter* is capable of colonizing the intestine of poultry and cattle, and thus infection is mostly acquired by fecal-oral contact, ingestion of contaminated foods (raw meat and milk), and water borne through contaminated drinking water (Coffey *et al*., 2011). Therefore, the release of the intestinal contents from the poultry carcasses during slaughtering and processing resulted in contamination of the meat for human consumption is inevitable (Connerton *et al*., 2011). Oral infection with *Campylobacter* has become the most common cause of foodborne disease in industrialized nations (Reich *et al*., 2008).

4. *Listeria species*: *Listeria monocytogenes* is a gram-positive, motile, and facultative intracellular bacterium. Out of the six species of the genus *Listeria* (*L. monocytogenes*, *L. ivanovii*, *L. innocua*, *L. welshimeri*, *L. seeligeri* and *L. grayi*), only two are considered pathogenic: *L. monocytogenes* which is able to infect animals and humans, and *L. ivanovii* infecting animals only. This human pathogenic microorganism is a foodborne pathogen and causes *listeriosis*. It is widely distributed in the environment and foods as it is well adapted to different environmental conditions of food matrices including tolerance to high salt levels, low pH (<6), low oxygen as well as low temperature. The disease infects particularly pregnant woman and elderly people over 65 years (Dworkin *et al*., 2006). Despite its low incidence, the high mortality rate of 15-40% of *listeriosis* is still a great concern, making it a prominent pathogen. *Listeriosis* results in several diseases such as septicaemia, meningitis, encephalitis and even miscarriage in pregnant women. *L. monocytogenes* is commonly isolated from ready to eat food such as vegetables, dairy products and cold-cut poultry or from cross contaminated foods being stored in refrigerator temperature (Vazquez *et al*., 2001). The infection can have the following additional symptoms: gastroenteritis, chorioamnionitis, and neonatal infections. Prominently in immune-compromised patients, the mortality rates are relatively high (Dworkin *et al*., 2006).

2.6. Phage therapy application in control of foodborne bacterial pathogen

The concept of combating pathogens by means of phages is obvious, and was proposed shortly after the discovery of phages approximately 90 years ago. Unfortunately, the discovery of antibiotics basically eliminated research on phage therapy. Today, the increasing problem of

antibiotic resistance rekindled the interest in phage therapy, but only very recently was the concept extended to the field of food safety. According to Bruttin and Brussow (2005) oral administration of *E. coli* specific phages indicated that phages are safe for human. Several current strategies to combat livestock associated pathogens such as toxinogenic *E. coli*, *Campylobacter,* and *Salmonella* are direct extensions of classical phage therapy approaches, in so far as they focus on targeting bacteria in the animals before slaughter (Wagenaar *et al*., 2005). On the other hand, food contamination with *L. monocytogenes*, is more likely to occur during food processing, which consequently is the most reasonable time point for phage bio-control of this pathogen (Fiorentin *et al*., 2005). Phages have recently emerged as a novel approach in the food industry to control bacterial contamination in food in a process called "bio-control" (Hagens and Loessner, 2010). Lots of scientific literature demonstrates the possibility of using phage therapy to effectively reduce the presence of foodborne pathogens in food producing animals and in fresh and processed foods. Some of phages such as ListShield™, EcoShield™ and SalmoFresh™ are already commercially available and approved by FDA (Goodridge and Bisha, 2011). As mentioned above, *lytic* phages have the ability to attach to bacteria and integrate into their cellular machinery, while utilizing the host resources to reproduce. The release of new phages leads to destruction of the bacterial cell (Greer, 2005). Hence, virulent (strictly *lytic*) phages are choice for food safety applications (Mahony *et al*, 2011).

2.6.1. Pre-harvest control of bacteria

Phages are used for treatment of food products, for agricultural and food production systems to control foodborne pathogens. This may include application of phage lysate for production or waste management equipment, introduction of the phages to live food animals to reduce the colonization of pathogens, or application of the phage to consumable implements within the production line to prevent contamination and outgrowth of the target (Atterbury *et al.,* 2007).

According to Carillo et al. (2005) on the study conducted to reduce the colonization of *Campylobacter* in broiler chickens using phage therapy found that, compared to the control, feeding phage particles to young broilers reduced the density of *Campylobacter* in the intestine by 0.5-5 log compared to chickens which had not been fed phage. One potential

limitation to this technology is the potential for phage resistant mutants to develop *in-vivo.* Another application of phage in production settings is the treatment of waste and consumables. As an example, phage may be applied to drainage equipment or fertilizer to reduce or eliminate cross-contamination of food products. A cocktail of phages are applied to compost to control for several *Salmonella Enteric* strains (Erickson, 2012).

2.6.2. Post-harvest control of bacteria

Phage-based technologies in the control of foodborne pathogens in post-harvest foods appeared to be more successful than those phage therapies in pre-harvest foods. The post-harvest intervention is to improve the food safety by applying phages on the surface of foods, hence eliminate or reduce the contamination of foods with foodborne bacterial pathogens, making the foods safe to consume. Phages have been successfully used to control spoilage bacteria and human pathogens during the post-harvest storage of foods under a variety of environmental condition to extend the storage life and/or improve the safety of fruits, dairy products, chicken, and red meats (Tan *et al*., 2014).

3. MATERIALS AND METHODS

3.1. Description of the study area

The study was conducted at Bishoftu town, located at a distance of 40Km southeast of Addis Ababa in Oromia Regional State, Ethiopia (Figure 4). The town has an elevation of 1,920m (6,300ft) above sea level with temperature condition from 13ºC-25ºC (average annual temperature of 18.7ºC). Bishoftu town has a total of 171, 115 population according to the statistical record made by NCR in 2012 (https://en.m.wikipidia.org/wiki/Bishoftu, 20.10.2018). In Bishoftu waste management system is well organized for both liquid and solid municipal wastes which are released from household and animal husbandry. In this town, food sources especially fishes are easily available at local market due to the presence of number of lakes which are the main contributors for fish sources even though not much fishing is done.

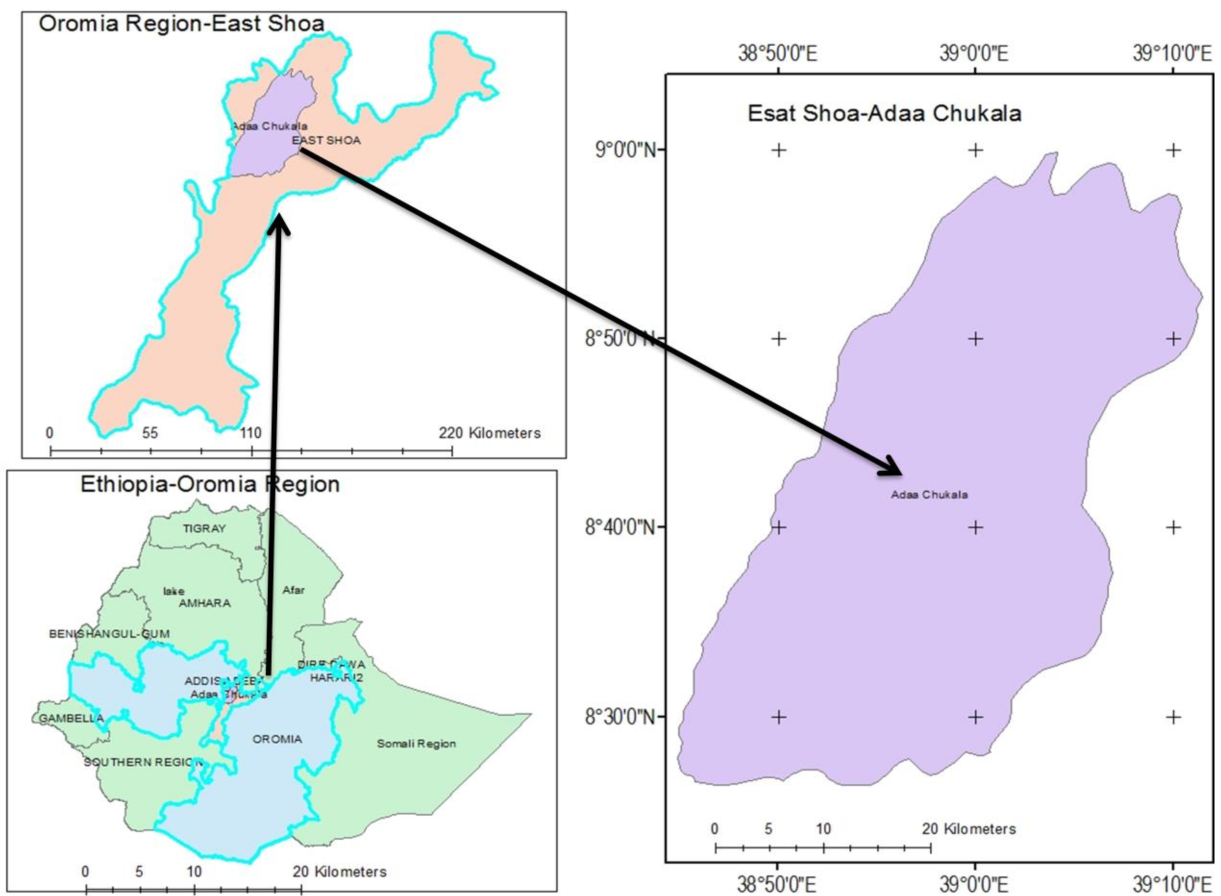


Figure 4: Map of the study area

3.2. Sample collection sites

Samples used during this study were collected both from sewage and foods. Samples for phage isolation was collected from Bishoftu waste water accumulated sites (sewage) (Figure 5).

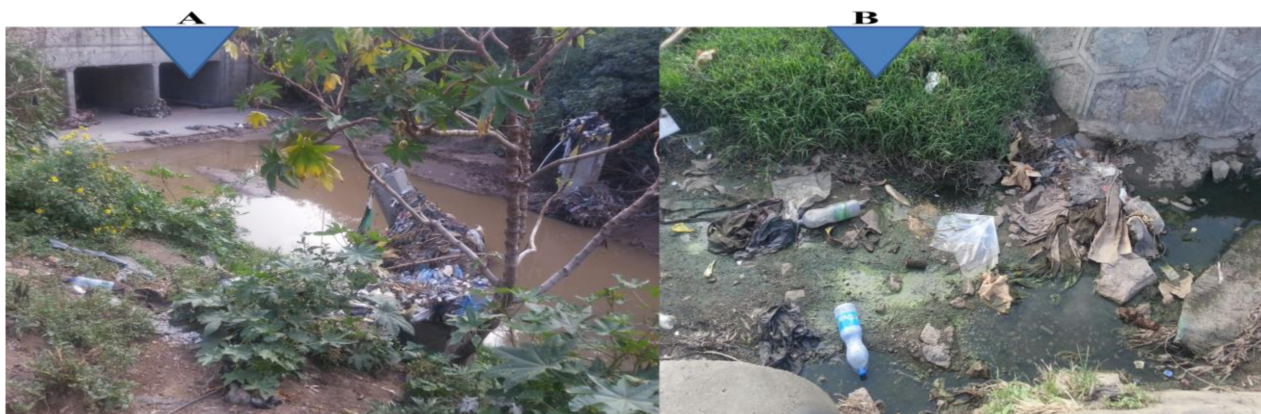


Figure 5: Sample collection sites in Bishoftu town. Where (**A**) is Waste River near the town and (**B**) is drain sewage. Both sampling site was containing calm and stagnant waste water and site **B** is covered with large flouting algae on the surface of the water.

3.3. Sample collection techniques

Bacterial strains (n=2) used in this study were isolated and characterized as part of the study from different food samples and others (n=2) are taken from the National Veterinary Institute (NVI) pathogen bank which are previously isolated and characterized to the strain level. Samples (red meat and fish) and (red meat and milk) were used for host bacterial isolation and used for phage lytic capacity test on food samples respectively. These samples were aseptically collected from market. Sewage sample was used for phage isolation. It was collected using 250 ml flask and immediately kept under refrigerator at 4 ºC after collection until enriched for bacteriophages isolation.

3.4. Host bacterial strain isolation and preparation

The host bacteria (*Salmonella Enterica* and *E. coli1)* used for phage isolation and enumeration was isolated and characterized in NVI bacteriology Lab, Bishoftu. Isolation and identification of those bacterial pathogens from food sample (fish and red meat) was carried out based on growth pattern and colony characteristics on selective and differential medias (MacCkonkey, Xylose-Lysine-Desoxycholate agar (XLD) and Tryptic Soya Agar (TSA)). The identification of bacterial typical colonies was first verified morphologically by Gram staining and later confirmed by microbiological techniques and biochemical assays using Analytical profile Index (API 20E) kit for *Enterobacteriaciae.*

3.4.1. Gram staining

Gram staining was carried out to identify bacteria according to their gram character (Gram positive or Gram negative). Briefly, the overnight cultured bacteria were placed on glass slide aseptically. Cristal violet was added and allowed to stay for about a minute, then the slide was washed and followed with Gram`s iodine for a minute and it was drained off with alcohol and washed with tap water, and then safranin was added and after one minute it was washed off with tap water. Finally the slide was drained and the colony was observed under the Microscope (400×).

3.4.2. Biochemical test for *E. coli* and *Salmonella*

API 20E biochemical kit was used to characterize both isolated bacteria following the instruction provided by the manufacturers. For inoculation of the strip, the overnight cultured bacteria was prepared by inoculating on 5 ml of TSB, and then for CIT, VP and GEL test both the tube and cupule filled with bacterial suspension, but for the other tests (ONPG, ADH, LDC, ODC, H_2S, URE, TDA, IND, GLU, MAN, INO, SOR, RHA, SAC, MEL, AMY and ARA) only the tube (but not cupule) was filled with bacterial suspension. Anaerobiosis was created in the tests of ADH, LDC, ODC, H2S and URE by overlaying with two drops of mineral oil and then the incubation box was closed and incubated at 37 °C for 24 hours. After the incubation period one drop of TDA reagent was added to TDA test, JAMES reagent to IND test and VP1 and VP2 reagent to VP test, respectively. Finally the strip was observed using API 20E Reading catalogue, (Appendex 3).

After identification, the isolated bacteria were inoculated in nutrient broth (Tryptic Soya Broth) and later it was preserved at +4ºC for further activities.

3.5. Isolation of bacteriophage

3.5.1. Processing of sewage samples for isolation and enrichment of bacteriophages

Isolation and enrichment of bacteriophages specific for the target bacterium from sewage sample was carried out according to the standard protocol of Twest and Kropinski (2009) with slight modification in some utilized devises and activities. From previously collected enough sewage samples, volume of 20 ml were centrifuged at 4500 × g for 20 min to remove particulate material. Then, the supernatant was filter sterilized by passing it through 0.22 µm low protein binding membrane filter and volume of 10 ml of the sample was mixed with equal volume of sterile nutrient broth (TSB) in a test tube and then it was seeded with 1ml of log-phase refreshed (for 2-4hour) indicator strain from overnight cultured bacteria and the mixture was incubated overnight at 37 ºC with shaking (agitator) at 150 rpm. Next day, the mixture was centrifuged at 4500 ×g for 25 min and then the supernatant containing phage was passed through a 0.22 µm pore membrane filter under aseptic conditions and the pellet left at the bottom was discarded.

3.5.2. Amplification of bacteriophage

To amplify and propagate the filtrate for phage activity test, the filtered supernatant from enriched phage was again mixed with 10 ml of TSB and inoculated with 1 ml of indicator strain. Then the mixture was incubated at 37 ºC in a shaking incubator at 150 rpm overnight and the supernatant considered to contain more enriched phages was filter sterilized through a 0.22 µm pore membrane filter under aseptic condition after centrifuged at 4500 ×*g* for 25 min. Then, four enrichment phage of *Salmonella Enterica, Salmonella Thypimurium*, phage of *E. coli* 1 and phage of *E. coli* O157:H7 was prepared and named as PS1, PS2, PE1and PE2, respectively.

3.6. Characterization of bacteriophage

To study bacteriophage activity (storage stability of phage at various temperature and their capacity in reducing bacterial pathogens from food) in food sample, *Salmonella Enterica* (S1) and *E. coli* O157: H7 (E2) was selected with their respective phages based on their capacity of forming high PFU in higher titer and based on their ability of forming very clear *plaque* in spot plate.

3.6.1. *In-vitro* evaluation of phage efficiency in controlling foodborne pathogenic bacteria

Isolated phage *in-vitro* evaluation was done by using both *spot assay* to test bacteriophage activity against the host strain and *double agar overlay assay* for the quantitative analysis of bacteriophage using the standard protocol.

3.6.1.1. *Spot assay* for bacteriophage activity detection

Bacteriophage activity against the host strain was checked using *spot assay* as described by (Cerveny *et al.,* 2002) with slight modification in concentration of the soft agar and utilized media. Briefly, sterile nutritive soft agar (0.75 %) was prepared and maintained in molten state at 45°C in a water bath. 0.1 ml of indicator cells (bacteria) which were isolated from food samples and grown over night in the TSB was added to molten agar, mixed quickly and poured on previously prepared nutrient agar plate (TSA).Volume of 10 µl of the amplified filtrate of bacterial free filtrate was spotted at two to five different places on the plate, allowed to dry at room temperature in the safety cabinet for about 10 minutes to avoid floating on the plate surface and incubated overnight at 37 °C, then the plates were examined for clearance at the spotted area on the next day.

3.6.1.2. Quantitative analysis for bacteriophage (*Double agar overlay* assay)

The *double agar overlay assay* was carried out for the quantitative analysis of bacteriophage using the standard protocol of Adams (1959). Serial dilution of the bacteriophage sample was carried out in sterile TSB. Volume of 0.1 ml phage suspension from selected dilution with 0.1 ml of indicator cells (bacteria) was added to 5 ml of molten soft agar which was kept at 45 ºC. The mixture was mixed thoroughly and quickly poured over previously moisture dried nutrient agar plate (TSA) without producing air bubbles and the plate were gently swirled to ensure

even distribution of mixture. The overlays was allowed upright for 10min to solidify at room temperature and then incubated overnight in inverted position (upside down) at 37ºC, expecting that each phage particle infects a cell, multiplies and releases mature phages that proceed to a second round of infecting neighboring cells and leaving a clear area on the lawn called *plaque*. Then on the next day *plaque* morphology was observed and plates with 30–300 *plaques* was selected and counted. Original phage count (titer) was determined by using the following formula to calculate the *plaque* number in particular titer plate.

$$\left[\frac{\text{PFU}}{\text{ml}}\right] = \text{Number of plaques counted/Volume of lysate infected with} \times \text{Dilution}$$

Where, **PFU/ml** is *Plaque* Forming Unit per Milliliter. It is the number of *plaques* per milliliter of phage lysate which bacteria have been infected with and formed in the bacterial lawn due to each phage particle infects a cell and leave zone of clearance.

3.6.2. Evaluation of bacteriophage activity on food sample

3.6.2.1. Bacterial infected food treatment with phage

Pasteurized milk sample and red meat was purchased from local market and abattoir respectively. Preliminarily the food was analyzed to check for the possible pre-contamination by *Salmonella* and *E. coli* according to the standard procedures. Meat sample was sliced aseptically and placed into Petri dishes aside 5ml of Pasteurized milk sample was poured in a bio-safety cabinet to detect activity of bacteria with phage on food samples. Host bacteria (*Salmonella Enterica* (S1) *and E. coli O157:H7* (E2)) were grown individually in TSB at 37 ºC overnight. PS1, and PE2 phages were selected from previously isolated and examined four phages based on their lytic capacity and PFU to higher titer level (having high concentration) and used individually to challenge the respective hosts which food samples (red meat and milk respectively) were infected with. Experiment was conducted at variant temperature (4 ºC and 25 ºC) to represent refrigerator and room temperature. For each sample, meat and milk samples were infected with 100µL of individual host strain and allowed to attach for 10 min at room temperature in a bio-safety cabinet. Then after, the same volume (100µL) of enriched phage was added to each sample. Bacteria were monitored by viability counting on selective media plates after 0, 3, 6, and 9 hours of phage treatment. For the control the same bacteria

infected food without phage treatment was used after balancing the concentration of phage free control bacteria with that of phage treated bacteria. To quantify *Salmonella* on meat it was homogenized in 5 ml of TSB while, *E. coli* was detected directly in milk samples since it was liquid. *Salmonella* and *E. coli* were counted on 90mm plates by pouring 10µL of diluted with TSB and homogenate samples in Xylose Lysine Deoxycholate agar (XLD) and MacCkonkey plate, respectively.

3.6.2.2. Phage stability test

The same procedure was used with section 3.6.1 to detect phage stability on food sample at 4 ºC and 25 ºC for continuous 3 days or 72 hours. Concentration of phage was determined throughout the monitored time interval using *double agar overlay* technique to count *plague* forming unit per milliliter or PFU/ml of diluted phage. Twenty-five micro liters of diluted phages were inoculated directly onto the surface of the meat and in milk sample which were experimentally contaminated by their host bacteria first. The samples (bacteria infected and then phage treated) were then incubated at 4 ºC and 25 ºC. Volume of 100 µL of 7-fold serial diluted samples was mixed with 5mL of molten soft agar (0.75%) to determine phage concentration at each detection time. Then the phage titers were determined after 0, 5, 24, 48 and 72 hours of phage treatment, separately (Appendix 2).

3.7. Molecular analysis of bacteriophages

3.7.1. *Plaque* selection and purification

Plaques from phage of *E. coli O15:H7*(PE2), phage of *E. coli 1*(PE1), phage of *Salmonella Thypimurium* (PS2) and phage of *Salmonella Enterica* (PS1) was selected and purified by taking *plaques* based on size and clarity using small sized pastor pipette. To take the *plaque* tip of the pipette was inserted carefully into the center of the *plaque* and plug of soft agar containing bacteriophage was taken out and inoculated into tube containing TSB. Phage suspension was centrifuged at 14,000×g at 4ºC for 20 min and filtered using 0.22 µm to remove any bacterial debris and the supernatant was transferred to sterile micro centrifuge tube for DNA extraction.

3.7.2. Phage DNA extraction

Previously purified phage particles (10^{9-11} PFU/ml) were used for phage DNA extraction using QIAGEN™ extraction kit. The centrifuged and filtered (to separate phage particles from co-precipitated bacterial debris) pure supernatant was treated with lysing buffer. Phage capsids were disintegrated using *Proteinase K* enzyme. Then the phages DNA were precipitated by standard 70% ethanol and washed repeatedly using illusion buffers. The flow-through and collection tube was discarded, as extraction control 200ml of *RNase* free-water was used, the spin column with DNA of phage was transferred to new micro-centrifuge tube, then to collect the phage DNA 200µl of Buffer AE was added to the center of the spin column membrane and incubated at room temperature for three minutes and then the eluted sample was stored for further usage.

3.7.3. Gel Electrophoresis

For Gel electrophoresis 15µL of phage DNA was mixed with 3 µL of Blue 6× loading Dye and loaded into well of 1.5% agarose gel in 1×Tris-Borate- EDTA (TBE). The DNA molecular marker (12Kb, Promega) was used to estimate molecular weight of phage DNA and then the gel were viewed under UV trans-illuminator to visualize the presence of DNA bands.

3.7.4. Restriction enzyme digestion

Phage DNA was digested using two restriction enzymes (*MseI and HinP1I*) following the instruction provided by the manufacturers (Promega). *MseI* have 5`….T*TAA....3` forward and 3`….AAT*T….5` reverse recognition site, where as the *HinP1I* have 5`….G*CGC….3` forward and 3`….CGC*G…. 5` reverse recognition site. Mixture for restriction digestion was prepared as indicated on Appendix 1, based on the protocol adopted from (Soo, 2013) with triplication on phage DNA concentration (volume) to increase band visibility. Then the mixture was agitated by mini-spine and incubated in water bath at 46ºC for 1hour. Enzyme digested DNA were separated on 1.5% agarose gel electrophoresis at 140 V for 2:50 hours and visualized by trans-illumination with UV light.

3.8. Statistical Data Analysis:

Concentration (CPU/ml) of bacteria was determined both for treatment group and control group. Data were analyzed using IBM SPSS 20.0 Software and Excel Microsoft Word. One-way analysis of variance (ANOVA) was used to compare phage-treated sample with its control counterpart and the results were presented using tables and graphs. Significant differences were discriminated with significance set at $p<0.05$, where p-value of less than 0.05 was considered statistically significant at 95% level of confidence.

4. RESULTS AND DISCUSSION

4.1. *E. coli* and *Salmonella* isolation from food samples

Salmonella Enterica (S1) and *E. coli* (E1) strains typical colony was observed under microscope and the result show that both *E. coli* and *Salmonella* is Gram negative. Because they have a thin cell wall with only 1-2 layers of peptidoglycan (10% of cell envelope) so they do not retain the purple stain color caused by crystal violet rather stained pink by safranin (Figure 6). The result for standard biochemical test using Analytical Profile index for confirmation of the *Salmonella* and *E. coli* isolates were presented (Figure 7).

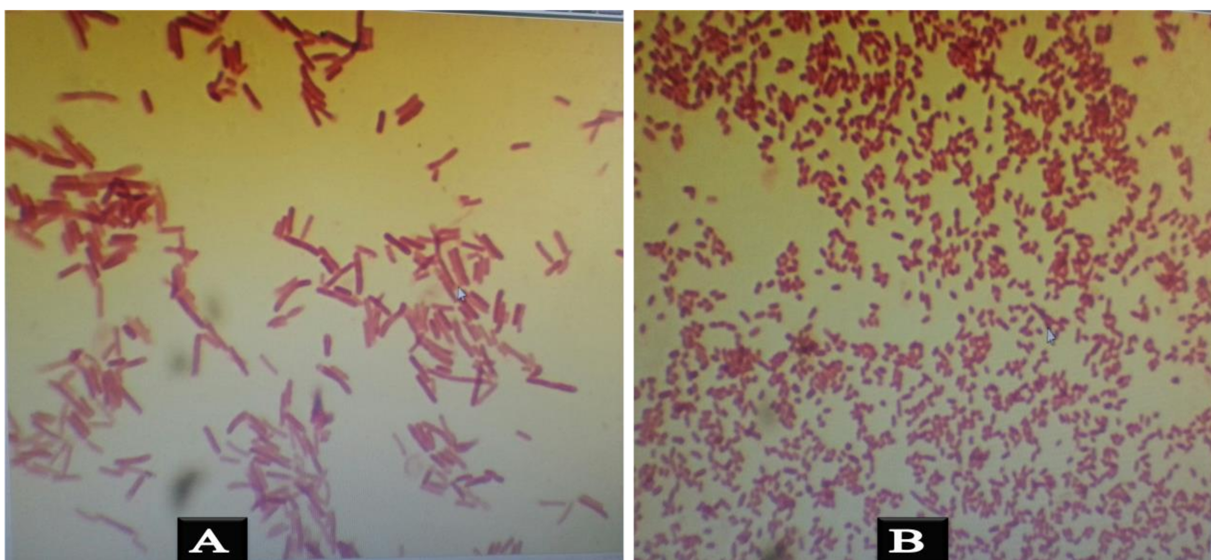
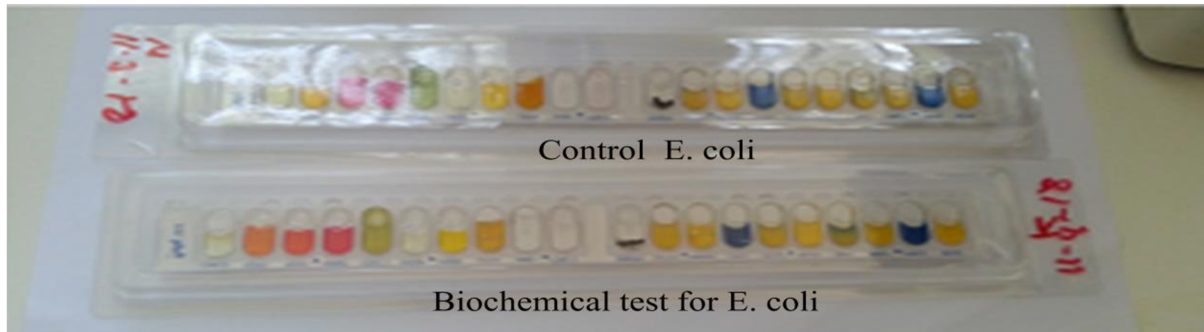


Figure 6: Gram staining for *E. coli* (**A**) and *Salmonella* (**B**).



ABIS online - Results

AUTO MODE [N]
USER: KEMILA
DATABASE VERSION: Enterobacteriaceae 2.6.6-052018
STRAIN CODE: Expect E. coli

RESULTS

1

1. Escherichia coli ~ similarity 93%
No opposing tests Probability: 94.57% Integrity: 100%

ABIS online - Results http://www.tgw1916.net/bacteria_r

AUTO MODE [N]
USER: KEMILA
DATABASE VERSION: Enterobacteriaceae 2.6.6-052018
STRAIN CODE: Salmonella En.

RESULTS

2

1. Salmonella spp. (possibility of S. enterica subsp. enterica) ~ similarity 82.7%
Expected tests: Catalase+ Gelatin hydrolysis– Lactose– Probability: 96.01% Integrity: 100%

Figure 7: API 20E Biochemical test for *E. coli* and *Salmonella*. (**1**), Indicate that E2 is 93.0% *Escherichia coli*. (**2**), Shows that S1 is 82.7% *Salmonella Enterica* subspecies *Enterica.* Where, API 20E kit contains twenty different inoculation tubes (strip) of biochemical test for *enterobacteriaceae* identification, Apendix3.

4.2. Characterization of bacteriophage *plaque*

Enriched phages of two *Salmonella* and two *E. coli* strain were isolated from sewage and designated as PS1, PS2, PE1 and PE2 (phage of *Salmonella Enterica*, phage of *Salmonella Thypimurium*, phage of *E. coli* 1 and phage of *E. coli O157:H7*, respectively) based on their capacity of propagating on their own target host strain and their *plaque* morphology. According to Akhtar *et al*. (2014) sewage samples have the most diverse bacteriophages with respect to their wide host range.

4.2.1. Bacteriophage activity detection by *spot assay*

To detect *lytic* activity of isolated phage following overnight incubation at 37°C, zones of inhibition where the enriched phage spotted was observed (Figure 8) forming clearance zone on plate of S1, S2, E1 and E2 which indicated the presence of *lytic* bacteriophages (successful isolation of *lytic* phage) and also it shows the isolated and enriched phages are appropriate for phage activity detection.

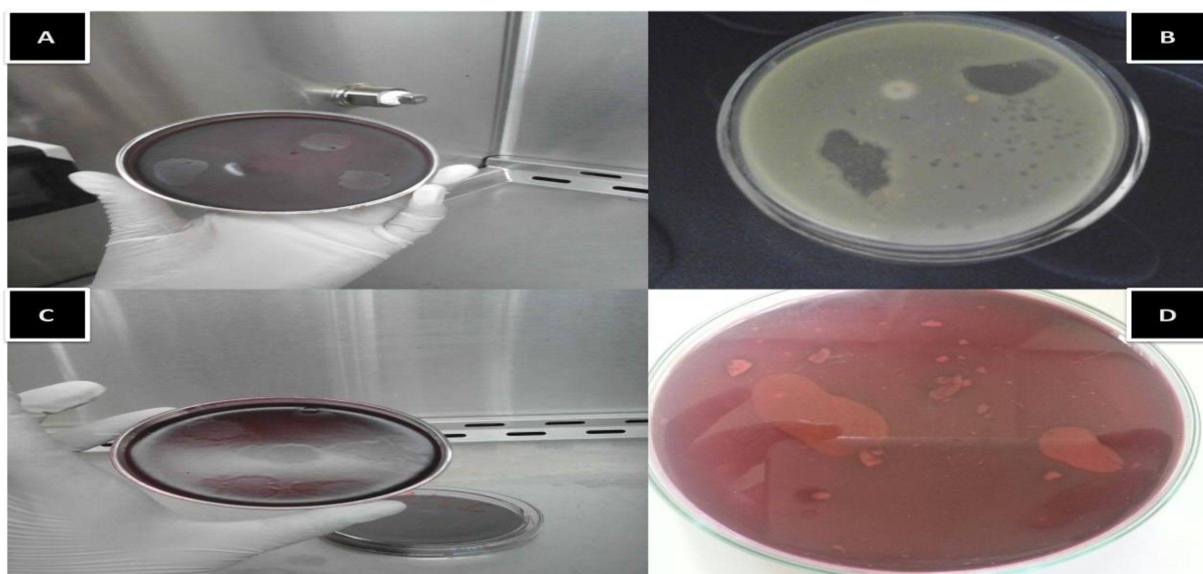


Figure 8: Zone of inhibition on bacterial plate spotted with their phage. Inhibition zone of S1 and S2 on EMB media by PS1 and PS2 (**A** and **C**) are shown respectively, while inhibition zone of E1 by PE1 is shown on TSA (**B**) and inhibition zone of E2 by PE2 on EMB media (**D**).

In the result on *spot* plate test, *plaque* morphology was recorded with all four phage producing clear *plaques* and no any turbid *plaques* were formed. According to Yoon *et al*. (2007), enriched sample applied to a lawn of host bacteria on agar medium and phages in the sample was absorbed to the host bacterium and start lysis to form visible clear zone on bacterial lawn plate which is called *plaque*, which confirms with the finding of this study and also with that of Kutter (2005). Additionally, formation of this clear *lytic* zone indicates that the isolated bacteriophages are not *lysogenic* (temperate phage) rather they were lytic phage because temperate bacteriophages have nature of leading to *lysogenic* infection in which they transfer their genomic material into the host bacterial genome and phage that enter in to the *lysogenic* growth cycle produces turbid *plaque* on bacterial lawn (Ai *et al.*, 2000).

4.2.2. Quantitative assay for bacteriophage *in-vitro* analysis

Throughout the plates, the cloudy areas were seen where the bacteria have grown, except for small clear spots called *plaques,* formed due to that each phage particle infect bacteria and form zone of clearance on the plate. According to the result, all the isolated phages are strictly *lytic* forming round, clear and transparent zones (*plaque*) on their own hosts. The result for phage concentration test revealed that, PS1 and PE2 exhibit more multiplicity of infection or better *plaque* forming unit per milliliter of lysate used at higher dilution factor. The isolated phages have high ability in inhibiting their host bacteria, while PS1 (**A**) and PE2 (**E**) exhibit more *lytic* activity than PS2 (**B**) and PE1 (**C**). The highest bacteriophage titer at 10^{12} PFU/mL was detected for E2 bacteriophage (PE2). The initial phage count was found to be 4.7×10^{6} PFU/ml for PS1 and 6.2×10^{12} PFU/ml for PE2, and no significant reduction in the counts was recorded during two month storage at -21°C. The p*laque* sizes were varying in diameter and have well defined edge (Figure 9). *Plaque* formation is due to the expansion of the bacteriophage population as mediated by multiple rounds of bacteriophage adsorption, infection, and *lysis* of individual bacteria, including bacteriophage diffusion. This is in line with the work of Sulakvelidze (2012), that *lytic* bacteriophage infect bacteria, which in turn leads to *plaque* formation by increasing bacteriophage numbers unlike that of *lysogenic* bacteriophage.

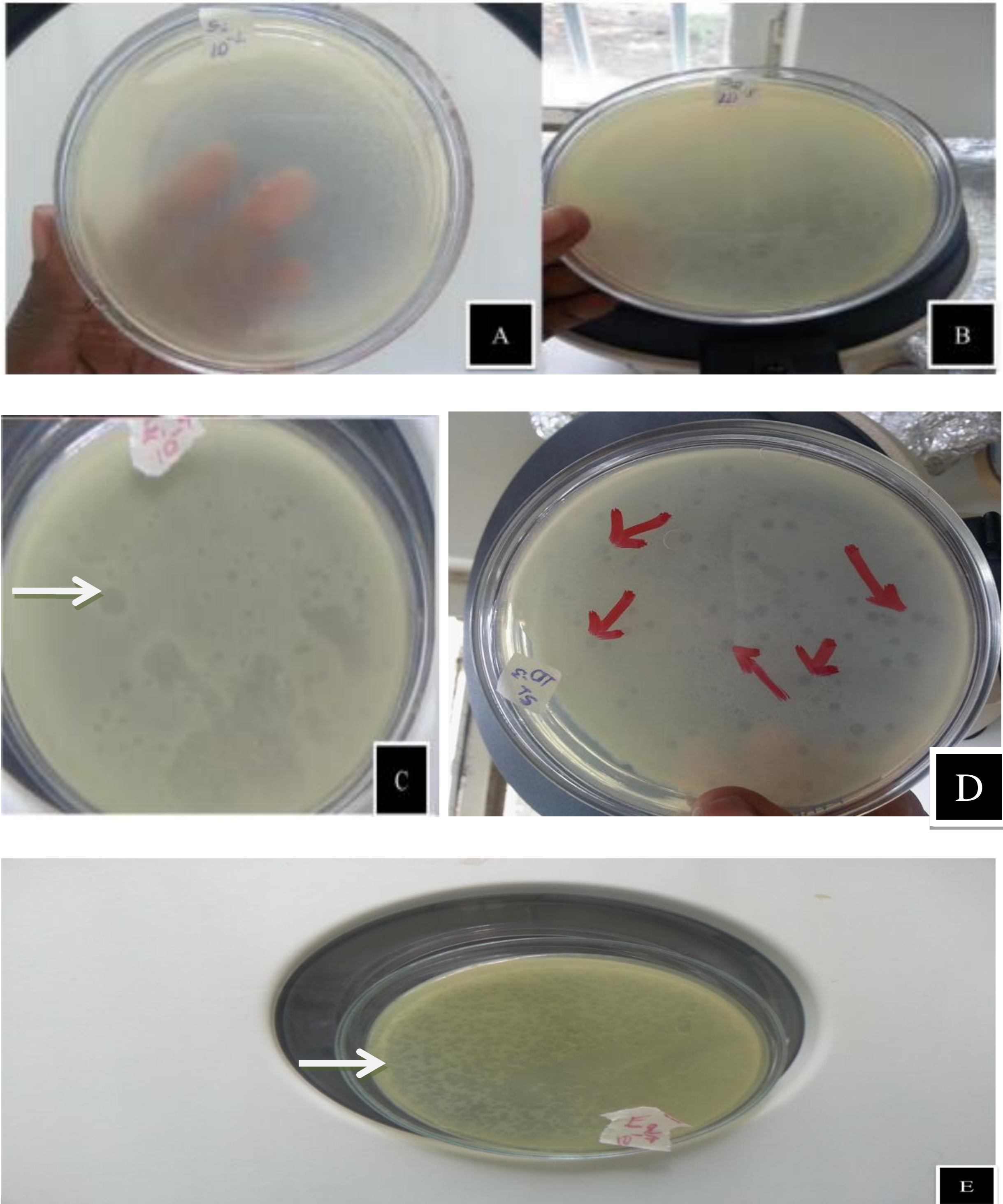


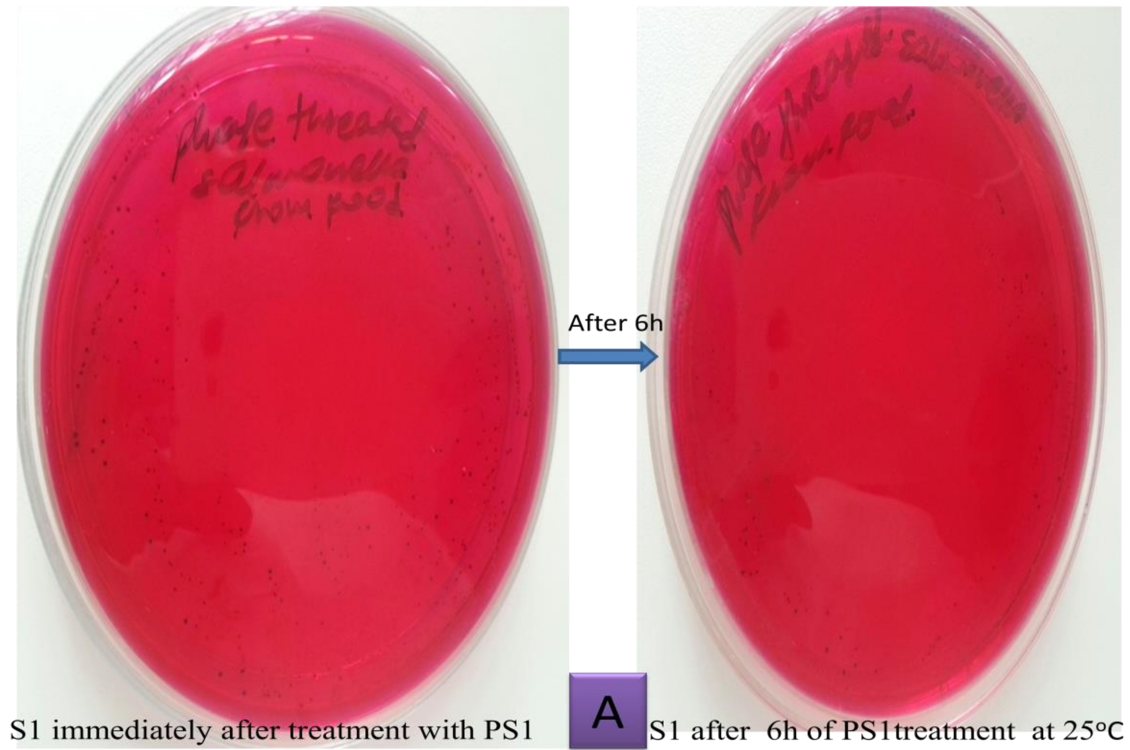
Figure 9: *Plaque* morphology of each isolated phages, *plaque* size and titer concentration on bacterial plate for *Salmonella Thypimurium* (**B**), *Salmonella Enterica* (**A** and **D**), *E. coli 1* (**C**) and *E. coli* 0157:H7 (**E**).

In this study during propagation, phages proved capable of replication and achieved high concentrations of up to 10^{12} PFU/mL for *E. coli* O157:H7 and 10^{6} PFU/mL for *Salmonella Enterica*. This is in close similarity with the report of Hungaro *et al*. (2013) who observed that bacteriophages in high concentration (near to 10^{9} PFU/mL) are necessary for optimum efficacy of phage, however, concentrations equal or less than 10^{6} PFU/mL were not able to reduce growth of *Salmonella Enteritidis* in chicken carcasses. Accordingly it is convincing that PE2 having high concentration (10^{12} PFU/ml) is more effective phage in reducing growth of foodborne *E. coli* O157:H7 (E2) in case of this study too. According to Cormier and Janes (2014) the size and appearance of a *plaque* can be associated with the volume and density of agar, concentration and stage of growth of the host bacterium and constancy of the top agar. This is in agreement with the finding of the current study. It was tried to use same volume and density of bottom agar, the same volume and thickness having soft agar (top agar), almost similar concentration of host bacterium at related growth stage in this study, although different *plaque* size and morphology is observed over each bacterial lawn. Thus, this diverse morphology of *plaque* may indicate that how much phages are sensitive and there lysing capacity are dependent on the environment to infect the neighboring cell more actively and rapidly to form different sized *plaques* which is directly proportional to *plaque* size formation. Therefore this result is also similar with the work of Premarathne *et al.* (2017), which has isolated different sized (1.0-3.0mm) *lytic* plaques in double agar overlay assay.

4.3. Characterization of phage activity on food samples

4.3.1. Efficacy of phage on controlling *Salmonella* and *E. coli* contaminated food samples

The effects of phage (PS1 and PE2) on *Salmonella Enterica* and *E. coli O157:H7* contaminated food sample (Meat and Milk) at 4 ºC and 25 ºC and the concentration of bacteria on these food products at 4 ºC and 25 ºC was detected. For *Salmonella Enterica* (S1) treated with phage (PS1) and stored at 4 ºC and 25 ºC, number of bacteria have been decreasing each sampling time, when compared to the initial inoculums and its control sample without phage treatment (Figure 10A). For *E. coli* O157:H7 treated with phage at both temperatures, number of bacteria has been significantly decreased (Figure 10B).



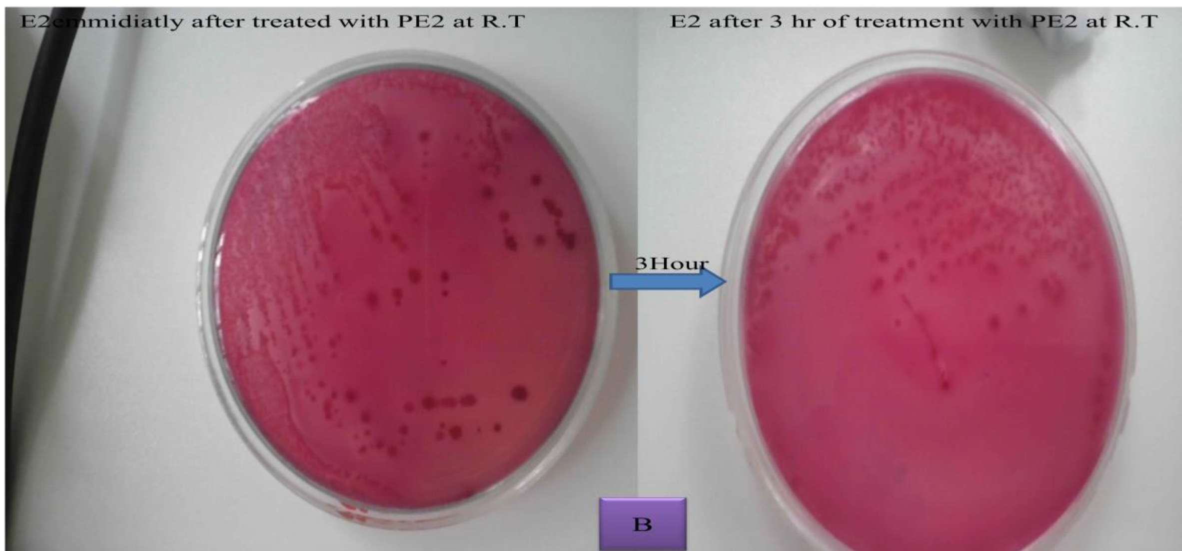


Figure 10: Bacteriophage effect (PS1) on growth of host bacteria (S1) after 6 hour of treatment (**A**) and E2 after 3 hour of treatment with PE2 (**B**) at room temperature (25 ºC).

The number of viable bacteria (CFU/ml) was reduced much more significantly on phage treated ample, when compared to that of control one. Raw data recorded for bacterial (CFU/ml) in phage treated and control group was demonstrated on Table 1.

Table 1. Effect of bacteriophage on phage treated food sample and CFU/ml of bacteria on non treated control group.

Temperature					
4 ºC				**25 ºC**	
Microbial	Time	CFU/ml of sample	CFU/ml of control	CFU/ml of sample	CFU/ml of control
E2	0hr	2.48×10^{8}	2.82×10^{8}	2.1×10^{8}	2.82×10^{8}
	3hr	1.51×10^{8}	2.89×10^{8}	0.98×10^{8}	3.05×10^{8}
	6hr	1.03×10^{8}	2.67×10^{8}	0.63×10^{8}	3.38×10^{8}
	9hr	0.76×10^{8}	2.73×10^{8}	0.27×10^{8}	3.17×10^{8}
S1	0hr	3.5×10^{5}	4.3×10^{5}	2.8×10^{5}	4.3×10^{5}
	3hr	2.7×10^{5}	4.9×10^{5}	1.6×10^{5}	5.7×10^{5}
	6hr	2.0×10^{5}	5.1×10^{5}	0.9×10^{5}	6.2×10^{5}
	9hr	1.7×10^{5}	3.7×10^{5}	0.32×10^{5}	6.5×10^{5}

When the two storage temperatures for bacterial reduction test was compared, storage at room temperature shows much more reduction of bacteria both in case of *E. coli* and *Salmonella* than that of storage at refrigerator temperature (4 ºC), and this shows that, bacteriophages are more active bacterial inhabitants at room temperature than at refrigerator temperature according to the finding of this study. So this indicates that, successful phage bio-control depends on the temperature and this is in line with the previous study that the phage activity is sensitive to the physiological state of the host, which can be affected by growth conditions such as temperature, nutrient availability and oxygen tension (Hongduo *et al*., 2015). The effect of phage PE2 on *E. coli O157:H7* (E2) contaminated food sample (pasteurized milk) at 25 ºC and 4 ºC is demonstrated in Figure 11, while the effect of phage PS1on *Salmonella Enterica* (S1) contaminated meat at 25 ºC and 4 ºC in Figure 12.

The statistical analysis for deference between treatment and control group for *E. coli* and *Salmonella* at various temperatures was demonstrated on Table 2 and 3, having average significant difference expressed in P-value which is 0.046 and 0.045 respectively.

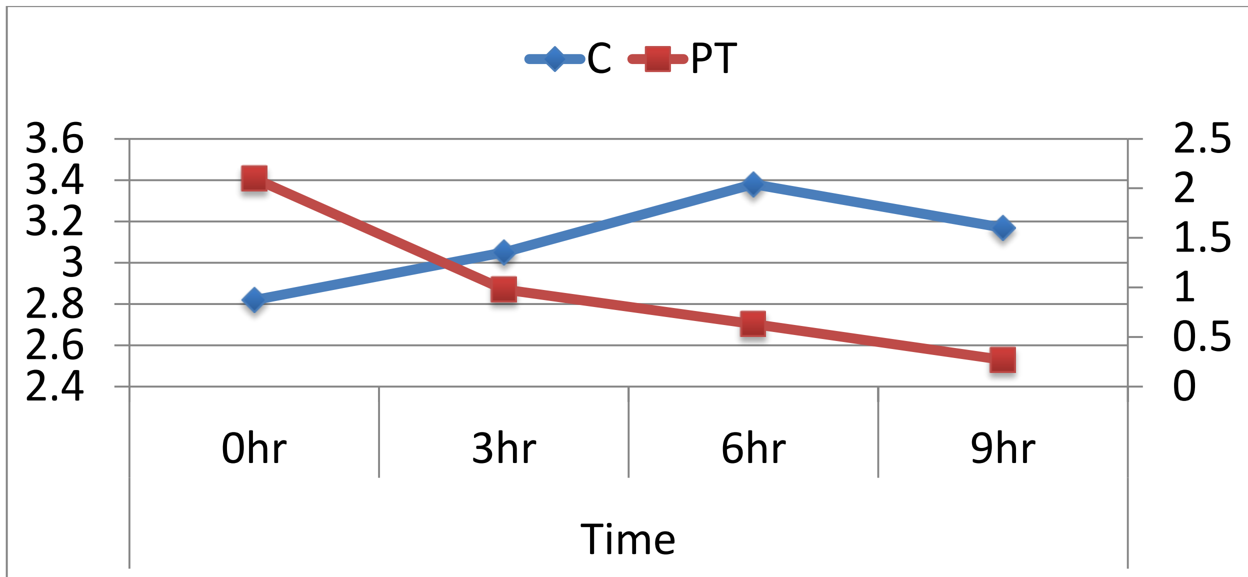
Table 2: Statistical analysis for deference between treatment and control group for *E. coli* in milk sample.

		Sum of Squares	Df	Mean Square	F	Sig.
Time 0	Between Groups	.281	1	.281	7.781	0.108
	Within Groups	.072	2	.036		
	Total	.353	3			
Time 3	Between Groups	2.976	1	2.976	38.834	0.025
	Within Groups	.153	2	.077		
	Total	3.129	3			
Time 6	Between Groups	4.818	1	4.818	29.020	0.033
	Within Groups	.332	2	.166		
	Total	5.150	3			
Time 9	Between Groups	5.929	1	5.929	54.685	0.018
	Within Groups	.217	2	.108		
	Total	6.146	3			

Table 3: Statistical analysis for deference between treatment and control group for *Salmonella* on meat sample.

		Sum of Squares	Df	Mean Square	F	Sig.
Time0	Between Groups	.281	1	.281	7.781	0.108
	Within Groups	.072	2	.036		
	Total	.353	3			
Time3	Between Groups	2.976	1	2.976	38.834	0.025
	Within Groups	.153	2	.077		
	Total	3.129	3			
Time6	Between Groups	4.818	1	4.818	29.020	0.033
	Within Groups	.332	2	.166		
	Total	5.150	3			
Time9	Between Groups	5.688	1	5.688	65.817	0.015
	Within Groups	.173	2	.086		
	Total	5.861	3			

E2 on Milk at 4 ºC



E2 on Milk at 25 ºC

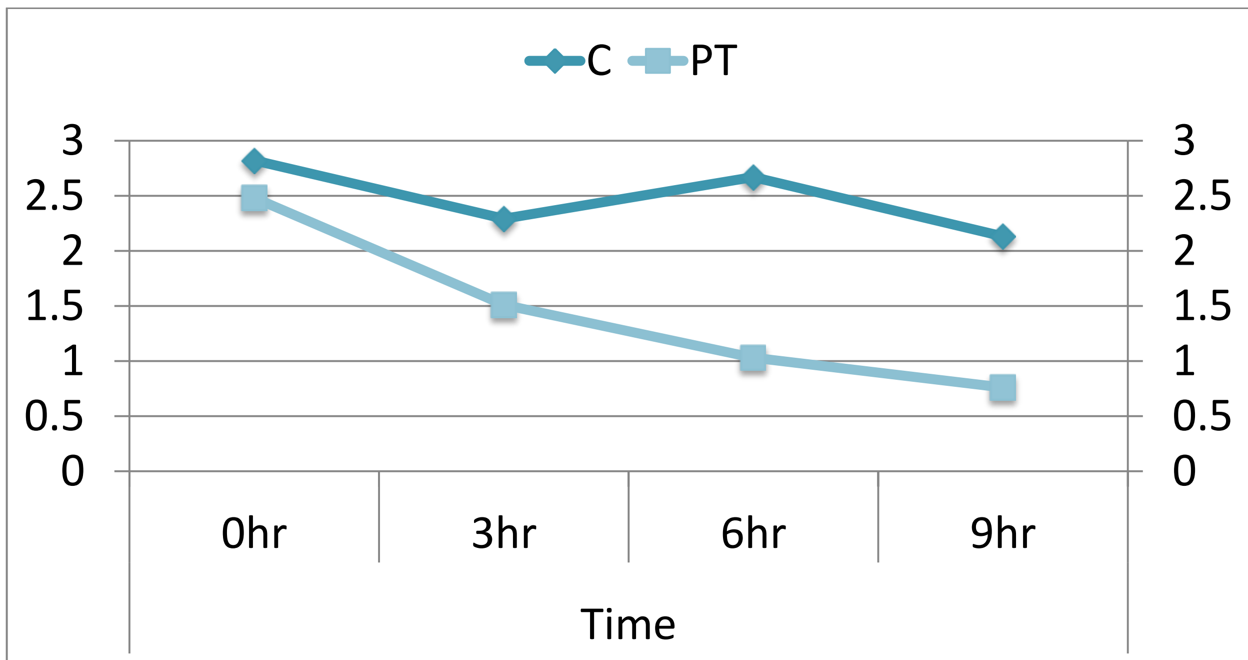
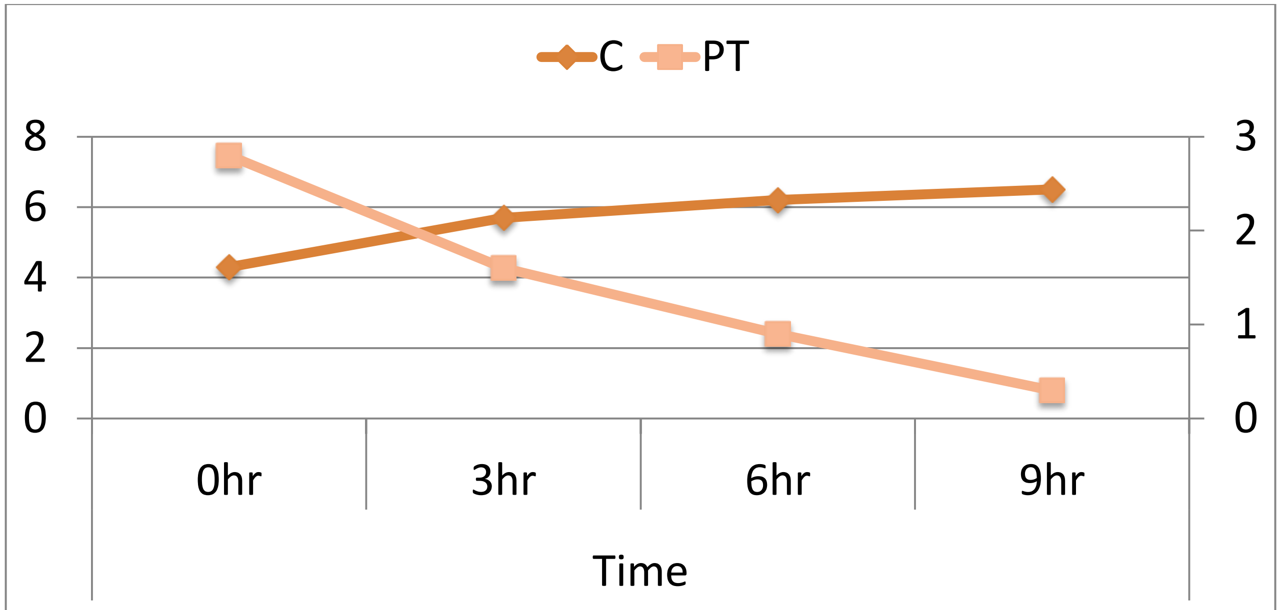


Figure 11: Effect of phage (PE2) on growth of *E. coli* 0157:H7 (E2) in milk sample at 4ºC and 25ºC. Milk sample was inoculated with E2 and later treated with PE2. Where, C represent control sample without phage treatment and PT is phage treated test sample. There is significant difference between treatment and control group with ($p < 0.05$). Statistical significance between control group and phage treated group is demonstrated in Table 2.



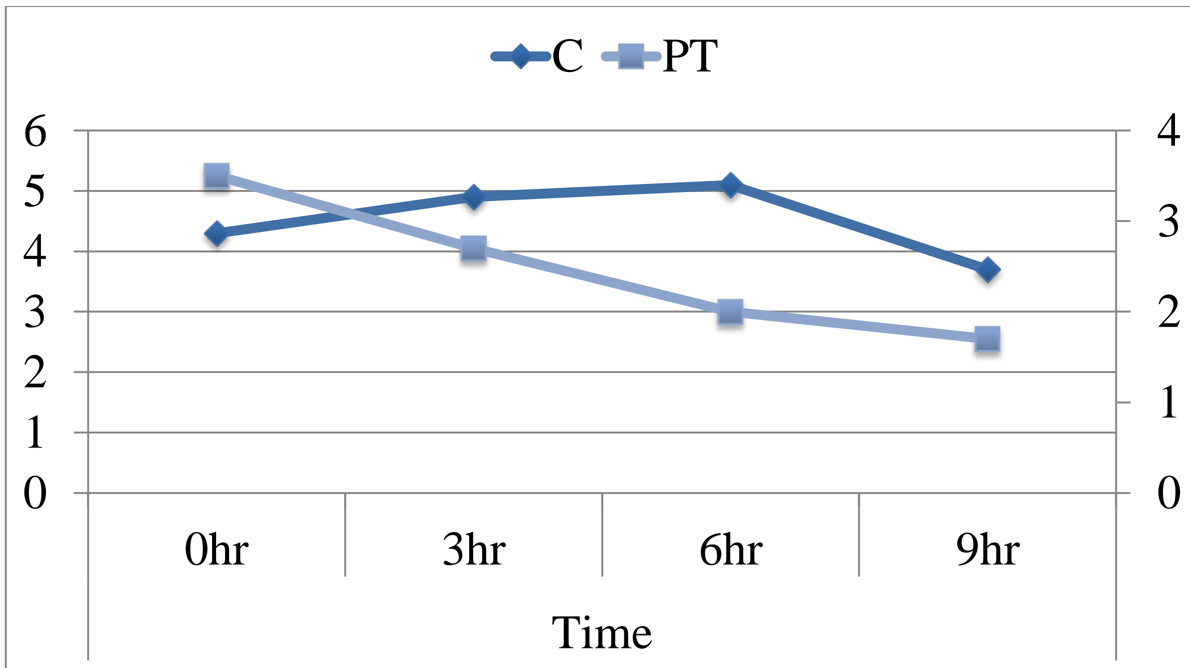


Figure 12: Effect of phage (PS1) on growth of *Salmonella* (S1) on meat sample at 4 ºC and 25 ºC. Meat sample was inoculated with S1 and later treated with PS1.Where C represent CFU for control sample without phage treatment and PT PFU for phage treated test sample having significant difference ($p < 0.05$) under both temperatures, Table 3.

Generally, these results indicated that, the application of phages as a therapeutic agent to treat foodborne bacterial pathogens is effective in reducing the numbers of *Salmonella* and *E. coli* contaminated red meat and milk sample respectively. This finding is in agreement with previous work done by Ayman (2016) that the application of phages was very effective in reducing the numbers of *Salmonella* and *E. coli* contaminated surface of cucumber and eggs to below the limit of detection in his study.

4.3.2. Stability of phage on phage treated food sample at various temperatures

The *plaque* forming unit of phage was investigated at refrigeration (4 ºC) and room (25 ºC) temperature to know the concentration of phage PS1 and phage PE2 added to the food samples over the targeted time of study. Stability and concentration of phage PS1 and phage PE2 added to the food samples were monitored over 72 hours (Table 4).

Table 4. Stability of phage (PFU/ml) on food sample at varies Temperatures.

Temperature			
		4 ºC	**25 ºC**
Microbial	Time	PFU/ml	PFU/ml
S1	At-0	2.77×10^{10}	2.77×10^{10}
	5hr	2.71×10^{10}	3.11×10^{10}
	24hr	2.62×10^{10}	3.04×10^{10}
	48hr	2.23×10^{10}	2.40×10^{10}
	72hr	1.73×10^{10}	1.07×10^{10}
E2	At-0	3.0×10^{10}	3.0×10^{10}
	5hr	3.14×10^{10}	3.26×10^{10}
	24hr	3.07×10^{10}	2.89×10^{10}
	48hr	2.91×10^{10}	2.17×10^{10}
	72hr	2.75×10^{10}	1.64×10^{10}

In meat sample, for the first 24 hours titer of phage PS1 at refrigerator temperature was stable, but PS1 at room was stable only for the first 5 hours. While, there was significant loss on phage titer of PS1 at 25 ºC after 48 hour, PS1 at 4 ºC was still stable up to 48 hours with slight loss, but with increasing time of incubation, much decrease in phage PFU/mL sample was observed. Phage PS1 in meat sample after 48hour at 25 ºC and after 72 hour at 4 ºC decreased much more significantly. For the phage PE2 added to milk sample, the phage stock was observed being relatively stable throughout the first 48 hour of incubation at 4 ºC and slight loss of phage was observed after 24 hour of incubation at 25 ºC. Phage PE2 in milk sample after 72 hour at both room and refrigerator temperature shows much more reduction in *plaque* number.

According to the reports of some researchers (Jepson and March, 2004; Ly-Chatain, 2014) bacteriophages are stable in different chemical and physical conditions and those factors could play important role in their replication dynamics. Reduction in phage numbers could be due to phage decay. When phage stock reduction of PS1 and PE2 is generally compared with each other in terms of storage temperature, much more phage reduction is observed at early time in both PS1 and PE2 at room temperature (25°C) than at refrigerator temperature (4°C), and these results indicated that the isolated phages were stable at refrigeration temperature (4 °C) for long time period (Figure 13A and 13B) and these results are consistent with the reports of Hooton *et al*. (2011) and Ayman (2016), which suggested, bacteriophages remained stable for a long period of time at low temperature.

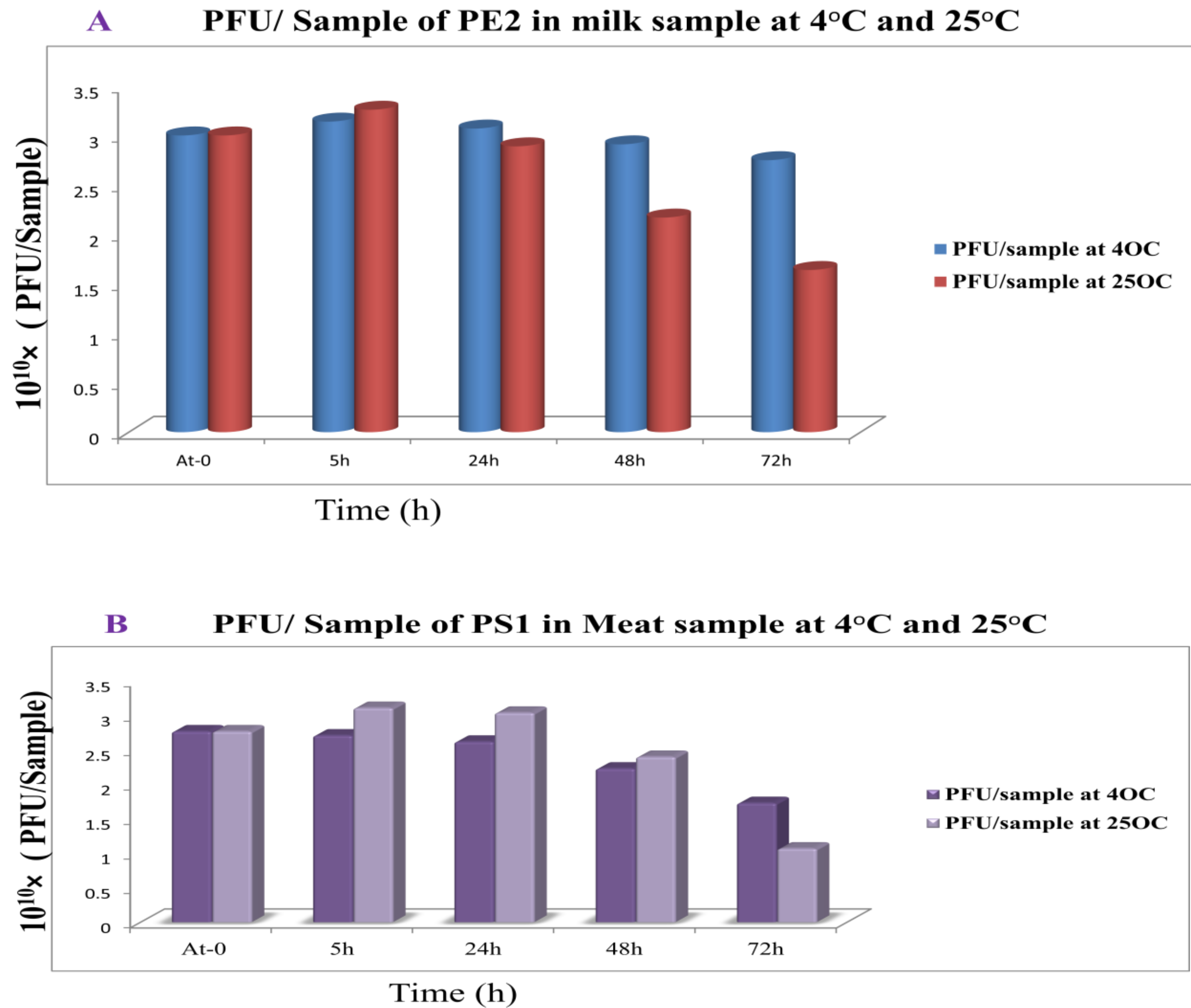


Figure 13: Stability of phage PS1 and PE2 over 72hour of incubation on bacterial infected milk and meat samples. Where (**A**), is PE2 on meat and (**B**), is S1 in milk sample at variable temperature.

Phages where increasing in PFU/ mL at 25ºC for the first incubation times than at 4ºC, this could be due to that, phages are more active in bacterial infection and rapid replication at higher temperature (25ºC), so that they can increase in number and become stable, even though they are decreasing at longer incubation period most probably due to starvation. This is in line with the reports of Ayman (2016) in which phages were quite stable until the end of his experiments (10 days) even though no increment in PFU, which suggested due to starvation condition of bacteria indicating that phage replication can only happen if bacterial host cells are continuously growing.

4.4. Molecular analysis of bacteriophage

4.4.1. Gel-electrophoresis and genome size estimation

DNA of all isolated phage of *Salmonella* and *E. coli* were isolated and electrophoresed along with 12Kb molecular markers (Invitrogen). The banding pattern of PE1 and PE2 was clear showing high concentration of phage DNA extracted while that of PS1 and PS2 shows low concentration of phage DNA (figure 14). By visualizing the band pattern, it was seen that the concentration of the extracted PE2 DNA is high and suitable to proceed for restriction digestion analysis than the rest ones.

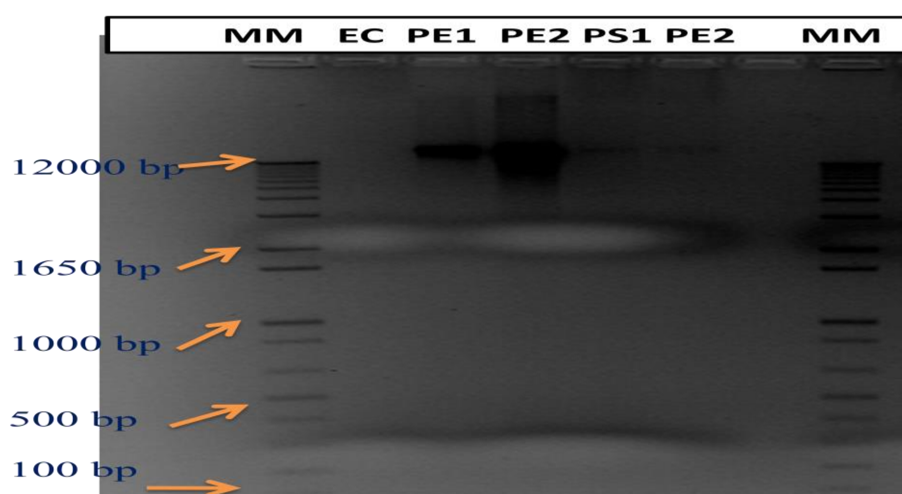


Figure 14: Agarose gel electrophoresis showing of DNA isolated from two *E. coli* and two *Salmonella* Phages. Where, lane 1 is 12Kb MM; lane 2 is extraction control; lane 3 is phage of *E. coli* 1; lane 4 is phage of *E. coli* 2; lane 5 is phage of *Salmonella* 1 and lane 6 is phage of *Salmonella* 2.

Phages are the most abundant life form on earth and estimated to be in the range of 10^{30} to 10^{32} (Brussow and Kutter, 2005). In this study it is observed that the genome size of all isolated bacteriophages were about or above 12Kb when compared with 12Kb molecular marker and all are found to be DNA phage. According to Ackermann (2005) majority of the phages contain dsDNA. While, small proportion of phage groups has ssDNA, ssRNA or dsRNA. According to the report of Abedon (2011) bacteriophages are differentiated into four genome size categories: very small, with genome size that range in 3.5 to 4Kb; small, which

have genome range from 4.5Kb- 9Kb; Medium-sized having genomes that range in size from approximately 9 to 15Kb and large sized with greater than 16Kb genome size. Even though further molecular and morphological characterizations are required, with the reference of the above findings of Ackermann (2005) and Abedon (2011), all the isolated phages (n=4) are DNA phage and have related molecular sizes and they are more likely to be medium to large sized bacteriophage.

4.4.2. Restriction digestion of phage DNA

The results from digestion with two restriction enzymes (*MseI* and *HinP1I*) revealed that all the DNA treated with *MseI* restriction enzyme were not sensitive to the applied enzyme, so there is no digestion of the band with utilized enzyme, which could be probably due to absence of recognition site on phage genetic material for *MseI* enzyme (figure 15A)**.** This is in line with the work of Krylov (2006), that, inability to digest phage DNA by restriction enzymes could be due to the absence of target sites for the restriction enzyme utilized. For DNA digested with *HinP1I* enzyme phage of *E. coli* 2 (PE2 or lane 3) is sensitive to *HinP1I* which exhibited banding patterns which ranges between 2Kb to 0.4Kb. But for the rest of the three phages (PE1, PS1and PS2), neither digested nor undigested band was seen on agarose gel (figure 15B). This might be due to the very low concentration of DNA as it is shown in non-enzyme treated band above to be detected by forming visible bands. In addition to genome (DNA or RNA) identification and genome size estimation, restriction enzyme patterns will help to establish the taxonomic affiliation between isolated phages. However, it is not clear band to observe the exact position of cut and compared the band patterns of all four phages.

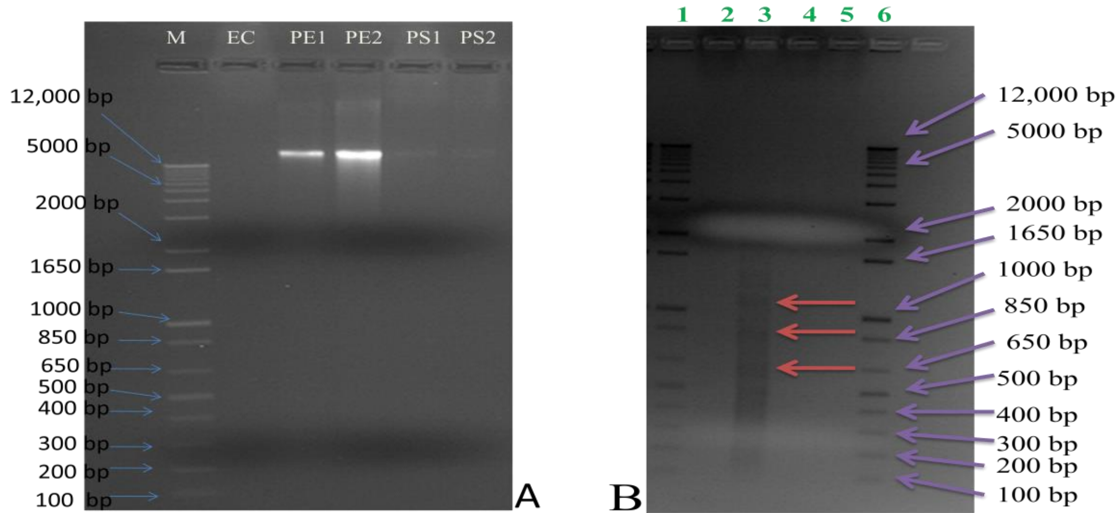


Figure 15: Restriction endonuclease digestion patterns of DNA isolated from two *Salmonella* and two *E. coli* phages. (**A**), *MesI* treated phage DNA (Lane M is ladder, Lane EC is extraction control, Lane PE1 is phage of *E, coli* 1, Lane PE2 is phage of *E, coli* 2, Lane PS1 is phage of *Salmonella* 1 and Lane PS2 is phage of *Salmonella* 2). (**B**), *HinP1I* digested phage DNA (Lane 1 is ladder, Lane 2 is phage of *E, coli* 1, Lane 3 is phage of *E, coli* 2, Lane 4 is phage of *Salmonella* 1, Lane 5 is phage of *Salmonella* 2 and lane 6 is ladder).

5. CONCLUSION AND RECOMMENDATIONS

Four lytic phages for two *Salmonella* and two *E. coli* were isolated from municipal sewage, Bishoftu. The high titers and lytic ability exhibited by these phages makes them promising and potential candidates to be used as effective bio-control agents against pathogenic bacteria. Especially, PE2 and PS1 exhibit a big potential of spoilage bacteria reduction both in food and *in-vitro* analysis along with high concentration (capacity of propagation) achieved. This study has shown that bacteriophages can significantly reduce or eliminate the most pathogenic *E. coli* and *Salmonell*a inoculated with 10^3CFU of both bacteria *in vitro* and in food samples. Thus, these results demonstrated that bacteriophage treatment has the potential to be used as antibacterial agent and to be developed as an alternative strategy to treat very harmful and deadly foodborne bacterial pathogens (*Salmonella* and *E. coli*) infection in processed and fresh food commodities.

Based on the above conclusion the following recommendations are forwarded:

- ✓ More identification and characterization tests should be done to confirm this finding and also to take phage therapy to higher and problem solving stage.
- ✓ Further understanding of the interaction between bacteria and phage is essential to be used as any therapeutic agents.
- ✓ *In vivo* studies with appropriate animal models and complete genome analysis to assess the safety of that specific phage and also pharmacokinetics response of model organism to the phage therapy should be studied.
- ✓ When phage is isolated from waste or frowzy sample like this study, before utilization of phage as food additives to control foodborne bacteria and/or as any phage therapy applications, many questions should have to be addressed by producers and researchers and more work needs to be done to determine whether the specifically isolated phage (from sewage) can be used to disinfect food products without any side effects or not.

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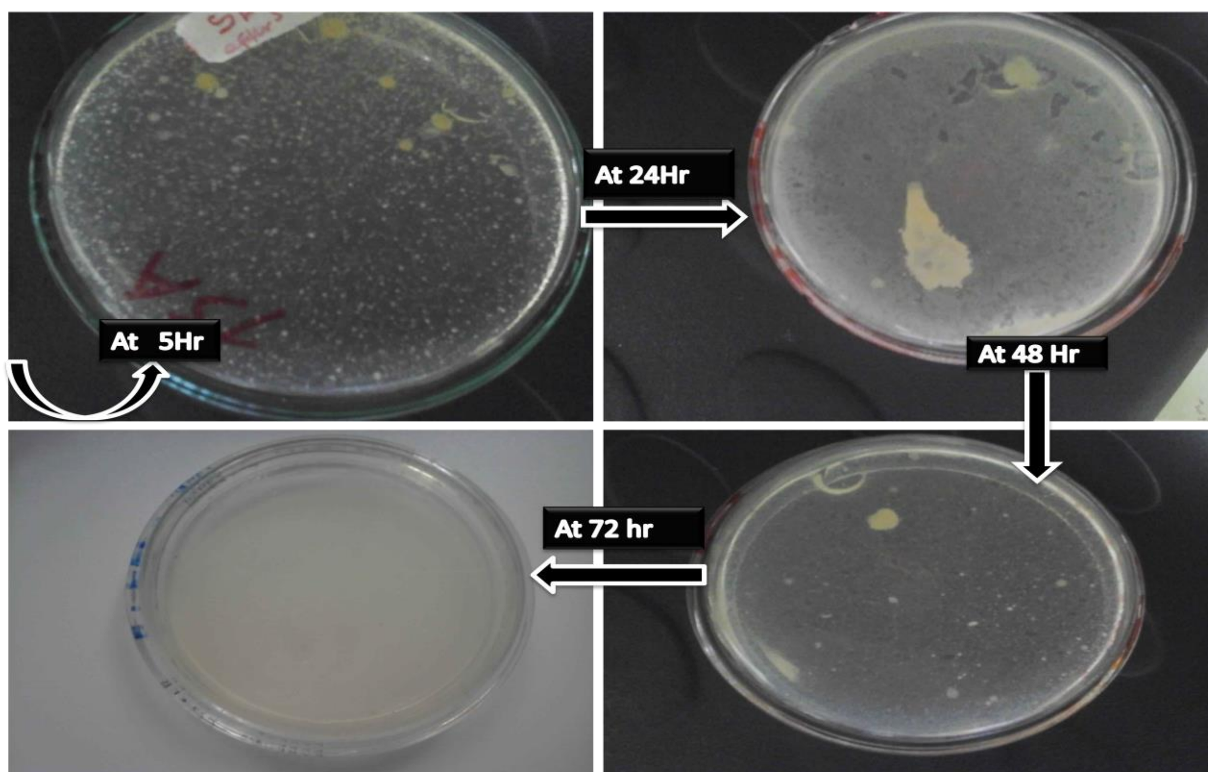
7. APPENDICES

Appendix 1

Supplementary table on restriction enzyme digestion for phage DNA

Components	**Per-reaction**
Phage DNA	15µL
Restriction enzyme	2.5 µL
Restriction enzyme buffer	1.5µL
BSA	1µL
PCR water	3µL
Total	**23µL**

Appendix 2



Supplementary figure, on stability of phage PS1on host bacteria (S2) in meat sample over incubated period of time at 25ºC.

Appendix 3

eTopic 28.1 Reading the API 20E.

Tests	Substrate	Reaction tested	Negative results	Positive results
ONPG	ONPG[a]	Beta-galactosidase	Colorless	Yellow
ADH	Arginine	Arginine dihydrolase	Yellow	Red/orange
LDC	Lysine	Lysine decarboxylase	Yellow	Red/orange
ODC	Ornithine	Ornithine decarboxylase	Yellow	Red/orange
CIT	Citrate	Citrate utilization	Pale green/yellow	Blue-green/blue
H_2S	Na thiosulfate	H_2S production	Colorless/gray	Black deposit
URE	Urea	Urea hydrolysis	Yellow	Red/orange
TDA	Tryptophan	Deaminase	Yellow	Brown-red
IND	Tryptophan	Indole production	Yellow	Red (2 min)
VPNa pyruvate	Acetoin production	Colorless	Pink/red (10 min)	
GEL	Charcoal gelatin	Gelatinase	No diffusion of black	Black diffuse
GLU	Glucose	Fermentation/oxidation	Blue/blue-green	Yellow
MAN	Mannitol	Fermentation/oxidation	Blue/blue-green	Yellow
INO	Inositol	Fermentation/oxidation	Blue/blue-green	Yellow
SOR	Sorbitol	Fermentation/oxidation	Blue/blue-green	Yellow
RHA	Rhamnose	Fermentation/oxidation	Blue/blue-green	Yellow
SAC	Sucrose	Fermentation/oxidation	Blue/blue-green	Yellow
MEL	Melibiose	Fermentation/oxidation	Blue/blue-green	Yellow
AMY	Amygdalin	Fermentation/oxidation	Blue/blue-green	Yellow
ARA	Arabinose	Fermentation/oxidation	Blue/blue-green	Yellow

[a]ONPG = orthonitrophenyl beta-D-galactoside.

Supplementary figure, API 20E Reading Catalogue.