Synergistic Effect of Phage-Antibiotic Combination against *Pseudomonas Aeruginosa* and Microencapsulation of Bacteriophages to Improve their Stability in Gastronomic Conditions

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2023

Synergistic Effect of Phage-Antibiotic Combination against *Pseudomonas Aeruginosa* and Microencapsulation of Bacteriophages to Improve their Stability in Gastronomic Conditions

A thesis submitted in partial fulfillment of the requirements for the Degree of

Masters of Philosophy

In

Microbiology

By

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Islamabad
2023

بسم الله الرحمن الرحيم

"IN THE NAME OF ALLAH THE MOST BENEFICENT, THE MOST MERCIFUL"

Read! And the Lord is Most Honorable and Most Benevolent, Who taught (to write) by pen, He taught man that which he knew not.

(Surah Al-Alaq 30: 3-5)
Al-Quran

Dedication

I Dedicate My Humble Effort to My Loving Parents and amazing Husband for their Immense Support and Unconditional Encouragement in Every Path of Life.

Declaration

The material and information contained in this thesis is my original work. I have not previously presented any part of this work elsewhere for any other degree.

Alina Majid

Certificate

This thesis by **Alina Majid** is accepted in its present form by the Department of Microbiology, Quaid-i-Azam University, Islamabad, for the requirement of the degree of Master of Philosophy in Microbiology.

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Dated: 01-02-2024

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List of Acronyms

BS	Bile Salts
SIF	Simulated Intestinal Fluid
°C	Centigrade
min	Minute
hr	Hour
%	Percentage
mm	Millimeter
mg	Milligram
pH	Power of hydrogen ions
L	Liter
SGF	Simulated Gastric fluid
NaCl	Sodium Chloride
mM	Milli Molar
Fig	Figure
µg	Microgram
µl	Microliter
NA	Nutrient Agar
M	Molar
Sp.	Species
Ag	Alginate
A-Ag	Alginate-agarose
MOI	Multiplicity of Infection
A-G	Alginate Gelatin
PAS	Phage Antibiotic Synergy
PEG	Poly Ethylene Glycol
CFU	Colony Forming Unit
PFU	Plaque Forming Unit
ml	Milliliter

Acknowledgments

All the praise and appreciation is for Allah, the Lord of the Alamine (mankind, jinn's and all that exists), In the name of Allah, the Most Gracious and the Most Merciful. Alhamdulillah, all praises to Allah for the strengths and His blessing in completing this thesis. May Allah shower the choicest of His Blessings on all those who have contributed in ailing humanity! The deepest respect and love for the Holy Prophet, Hazrat Muhammad (P.B.U.H), who enlightened our lives with Islam, saved us from the dark and helped us to recognize our only and true Creator. I am extremely grateful to my parents, who facilitated and encouraged me at every single step of my academic career.

First and foremost I am extremely grateful to my supervisor, Dr. Samiullah Khan, for his invaluable advice, continuous support, and patience during my MPhil research study. His immense knowledge and plentiful experience have encouraged me in all this time of my academic research and daily life. His invaluable help, constructive comments, suggestions and inspiration helped me a lot throughout the experimental process and thesis.

I would like to extend my gratitude to Prof. Dr. Naeem Ali, Chairman Department of Microbiology, Quaid-i-Azam University Islamabad, for his friendly attitude and for facilitating my research in the department. I owe my deep gratitude to all the faculty members especially, Dr. Malik Badshah, Dr. Asif Jamal, Dr. Fariha Hassan, Dr. Safia Ahmed, Dr. Javed Dasti, Dr. Muhammad Ishtiaq Ali and *Dr. Rabaab Zahra,* for their extremely cooperative attitude and humble guidance throughout my stay in the department.

I would like to thank my esteemed senior and co-supervisor Ms. Aneela Nawaz for her treasured support which was really influential in shaping my experimental ways and critiquing my results. She imparted unconditional physical and moral support throughout the tenure and her precious help in passing through the thick and thins of this study and to cope with the setbacks of our research.

I would like to express my appreciation and thanks to all my lab seniors and fellows, Ubaid, Nasir, Muqaddas, Memoona, Ismail and others for their moral support and advices, who willingly shared their expertise and were always a source of guidance, reassurance and pleasant company.

I appreciate all my friends especially Warda, Ayesha, Alviya, Eman, Rumaisa and Munira who have always been a major source of support when things would get a bit discouraging, for talking and laughing with me about nothing and always being a wonderful distraction.

I am also thankful to all the attendants and technicians for their help and support during my laboratory work.

Last but not the least, special thanks to my parents, husband, daughter and siblings for their prayers, love and endless encouragement without which I would not have been able to achieve this milestone.

Finally, I again thank Allah the Almighty, the constant Guider, and the source of all good, Who listened to my prayers and destined the accomplishment of this task.

Alina Majid

Abstract

Multidrug-resistant bacterial infections are propagating and escalating in frequency across the globe, jeopardizing practically every aspect of modern medicine and posing a serious challenge to public health. The excessive consumption of existing medications and a scarcity of newer medications due to restrictive legal constraints and diminished business incentives have both been blamed for the antibiotic resistance dilemma. In trials, progressive alternative therapies like phage treatment demonstrated encouraging results, alluding to its potential for use as preventive or adjuvant therapy in the future. The aim of the study was to determine antimicrobial effectiveness of combining sub-inhibitory concentrations of gentamicin, cefepime and meropenem with *P. aeruginosa*-specific bacteriophages belonging to *Siphoviridae* family. Phages showed remarkable stability with sub MIC doses of antibiotics with no significant change in plaque size and morphology. Further, the optimal MOI of phages was determined to be employed synergistically with antibiotics. While observing PAS, it was determined that phages PAA, PAM and PAR exhibited excellent synergy with antibiotics at sub MIC values. Moreover the combinatorial effect of these phages as a cocktail with antibiotics displayed remarkable synergy depicting complete bacterial removal. Furthermore, the synergistic effect of phages and antibiotics was tested on *P.aeruginosa* biofilms. It was observed that the PAS phenomenon proved to be highly effective in eradicating biofilm at sub MIC doses of antibiotics. The successful application of phages as antimicrobials was determined by testing the stability and activity of free phages in contrast to phages encapsulated in combinatorial bio-composite hydrogels of Sodium Alginate with other polymeric substances in the gastrointestinal environment. It was observed that free phages lost their viability significantly at gastric pH, however the encapsulated phages retained stability to further continue their passage to intestinal environment. The intestinal environment was determined to be highly favourable for phages as maximal phage release occurred in simulated intestinal fluid, to efficiently treat the intestinal ailments. Thus, the encapsulation strategy proved to enhance the effectiveness of phage therapy as an alternative to antibiotics.

Introduction

Antimicrobial resistance (AMR) has evolved as one of the key health concerns of the twenty-first century, posing an imminent danger to the effective prevention and treatment of an array of infections caused by bacteria, viruses, parasites and fungi that have developed resistance and are no longer curable with the standard drugs. Bacteria that cause both serious and mild illnesses have gradually, over many years, become resistant to every novel antibiotic that joins into circulation, and the extent of this resistance varies (Luong et al., 2020). In consideration of this reality, it is imperative to take action to halt this mounting global health care catastrophe. Antimicrobial resistance (AMR) is a plausible basis for the dynamic "resistome" and a threat to global health brought on by elevated usage of antibiotics in livestock breeding and healthcare facilities, a population explosion, shoddy sanitation, inadequate sewerage and waste disposal arrangements, and selection stress. Antibiotics, which once revolutionized medical practices, are currently in peril due to antibiotic-resistant bacteria (Davies et al., 2010).

The discovery of antibiotics was a pivotal point in human history that preserved countless of lives; these miracle bolts were accompanied by newly arising pathogenic-resistant bacteria. A recent review of the bacterial genome identified 20,000 genes that could be resistant, underscoring the serious danger of returning to the pre-antibiotic era. For the first time, the contemporary era revealed the antibiotic resistance pattern, particularly for intestinal infectious agents including *E. coli, Shigella,* and *Pseudomonas Aeruginosa* (Lauman and Dennis, 2021). In the industrialized world, where it was thought to be a minor health issue, but rather in impoverished states, these resistant infections caused tremendous clinical and monetary losses.

Since the range of viable treatments for some infections has gradually been reduced as resistance characteristics to various classes of antibiotics have accumulated, giving rise to strains with multidrug-resistant (MDR) phenotypes. Although resistance can be linked to a decline in fitness or virulence, some MDR strains nevertheless exhibit an impressive capacity for infection and propagation in the clinical context and have the ability to quickly develop epidemics (Moradali et al., 2017). Hence, the search for alternative options has become inevitable. The hunt for newer approaches has led to

the resurgence of phage utilization termed as ―Phage Therapy" as the appreciated recommendation in the quest for novel strategies of bacterial infection control and prophylaxis. Phage therapy proponents highlight numerous significant advantages that phages have over antibiotics, including host-specificity, self-amplification, biofilm destruction, and low human toxicity (Potron et al., 2015). The exploration of these minuscule living entities has only recently been possible due to the advent of analytical technologies like next-generation sequencing and electron microscopy. As seen by an influx of recent human clinical trials and animal studies, these technological breakthroughs have sparked a renewed interest in phage therapy research.

Bacteriophages therapies against the multi-drug resistant pathogens can be employed as alternative therapies. Several advantages are associated with the utilization of bacteriophages compared to traditional antibiotics, as, phages are very specific towards their host and avoid the damage of natural microbiota. Bacteriophages are self-replicating and increase their number at the site of infection and are able to kill the drug resistant bacteria. The concept of the utilization of bacteriophages alternative to antibiotics is not new, as initially they were utilized a century ago for therapeutic purpose. With the discovery of antibiotics and the lack of knowledge about the replication of bacteriophages, bacteriophage based therapies were abandoned in western world. Details of the replication process of bacteriophages are clarified now which facilitates the utilization of bacteriophages in therapy (Umadevi et al., 2011).

Phages are targeted bacterial viruses with several antibacterial effector properties that assist to minimize multi-drug resistant infections in a human body. They can target bacteria that are enclosed in biofilms and are typically unaffected by antibiotic resistance. Phages offer an infinite source of antibacterial drugs against all human bacterial infections due to their immense abundance, considerable diversity, and relatively simple isolation (Thacharodi and Lamont, 2022).

Due to the lengthy product development and FDA clearance processes, clinical applications of phage treatments are still uncommon. This has resulted in an array of nonclinical applications with a focus on food safety, agriculture, and clinical diagnostics, as well as limits in the phage treatments that are now accessible for clinical therapy. The FDA has licensed a number of phage cocktail products to treat

agricultural pathogens or pathogens found in processed foods. *Escherichia coli* and *Salmonella enterica* are only two pathogens that are currently the target of phage therapy. Clinical treatments are presently being evaluated for approval, however they are frequently still only being attempted on animals. Several pathogens, including *Pseudomonas aeruginosa, Staphylococcus aureus, vancomycin-resistant Enterococcus faecium, Clostridium difficile, and Klebsiella pneumoniae*, have been successfully treated with phage treatments in animals, according to reported studies (Khan et al., 2021).

Sub-lethal quantities of some antibiotics have been discovered to stimulate the development of some aggressive phage by host bacterial cells, a phenomenon known as phage-antibiotic synergy (PAS), which aids in the prevention and treatment of infections. According to reports, combining phage therapy with low doses of specific antibiotics may offer a treatment option for bacterial infections that are challenging to treat, including infections that are resistant to medicines. Antibiotics now in use are quickly losing their effectiveness against newly emerging antibiotic-resistant bacteria, which presents a practical challenge in the fight against antibiotic resistance (Karimi et al., 2021). Phage usage in conjunction with current antibiotics may resolve this problem. Antibiotics have been demonstrated to synergistically increase the lytic activity of phages. Antibiotic-resistant P. aeruginosa is an area of study about which very little has been researched and about which this phenomena is still not fully understood.

Phage treatments could be used as a solution to the dilemma of antibiotic resistance and the treatment of bacterial illnesses. It has been demonstrated that lytic phages can preferentially infect and lyse bacteria that are resistant to many drugs, resulting in an efficient antibacterial response both in vitro and in vivo. Modified controlled delivery techniques for phage therapeutics have a number of advantages over free phage distribution, including stability, localised availability, and protection from enzyme degradation, adhesion, and active site delivery. When phage therapies are developed and standardised, biomaterial encapsulation of bacteriophages must be created in order to increase storage stability, do dispense with the requirement for a cold supply chain, and enable phages to withstand production stresses and stay functional (Thiyagarajan et al., 2017)..

A recent innovation with a broad range of possible applications is microencapsulation technology. This method encloses the core material in a semipermeable or sealed capsule membrane utilizing natural or synthetic polymers as the encapsulation material. The outer component and the core material are the substances that are enclosed within each other. Polymers are primarily utilized in microencapsulation technology to encapsulate functional, active substances and create an extensive rigid barrier around the core material to lessen its reactivity with the surrounding environment (Mushegian, 2020). Functional compounds can have their physical characteristics and stability improved through microencapsulation, which can also prevent or mitigate deactivation and enhance storage time.

Conclusively, phage treatments, either alone or in conjunction with currently available antibiotics, will be used increasingly to treat infections as they are proven to be safe and effective. Phage therapy is a method for lowering antibiotic resistance to frequently used treatment regimens, enabling the on-going use of antibiotics that are currently on the market (Kutter et al., 2010). When bacteriophage genomes are modified, target ranges and the amount of genetic payload delivered to bacterial cultures are expanded while maintaining host target specificity. This specificity will target prospective infections in vivo while having less of an influence on natural microbiome cultures and healthy host systems. Emphasising on materials that are hydrophilic, able to be synthesised under harmless environments, non-hazardous and environmentally friendly, engineering of encapsulation or alteration of phage to enhance stability and storage capabilities will keep permitting larger, more manageable, and foreseeable volumes of phage to be introduced into host systems for therapy (Abedon et al., 2011).

Aim and Objectives

Aim:

To cope the arising dilemma of antibiotic resistance against *Pseudomonas Aeruginosa* by determining the phage-antibiotic synergy and Microencapsulation of phages for improved storage, oral administration and stability in gastrointestinal tract with the following objectives,

Objectives:

- Evaluation of phage antibiotic synergy between the previously characterized phages and commercially available antibiotics (Gentamicin, Cefepime and Meropenem).
- Assessing the anti-biofilm potential of phage antibiotic synergy phenomenon to treat resistant bacterial infections..
- Development of effective delivery route (Encapsulation) of oral phages into the gastrointestinal tract of humans and animals to treat resistant ailments.
- Discerning the stability of encapsulated phages at various gastronomic conditions for efficient therapeutic effects.

Literature Review

Antimicrobial resistance (AMR) has emerged as one of the major public health issues of the twenty-first century, posing a serious threat to the effective prevention and treatment of a wide range of infections caused by bacteria, viruses, parasites, and fungi that have developed resistance and are no longer treatable with the conventional medications. Over many years, bacteria that cause both severe and minor illnesses have steadily developed varying degrees of resistance to every new antibiotic that enters the market. Given this fact, it is crucial to act in order to stop the escalating global health care catastrophe (Dion et al., 2020). Antimicrobial resistance (AMR) is a plausible basis for the dynamic "resistome" and a threat to global health brought on by elevated usage of antibiotics in livestock breeding and healthcare facilities, a population explosion, shoddy sanitation, inadequate sewerage and waste disposal arrangements, and selection stress. Antibiotics, which once revolutionized medical practices, are currently in peril due to antibiotic-resistant bacteria (Huh et al., 2019).

The discovery of antibiotics was a pivotal point in human history that preserved countless of lives; these miracle bolts were accompanied by newly arising pathogenic-resistant bacteria. A recent review of the bacterial genome identified 20,000 genes that could be resistant, underscoring the serious danger of returning to the pre-antibiotic era. For the first time, the contemporary era revealed the antibiotic resistance pattern, particularly for intestinal infectious agents including *E. coli, Shigella,* and *Pseudomonas Aeruginosa*. In the industrialized world, where it was thought to be a minor health issue, but rather in impoverished states, these resistant infections caused tremendous clinical and monetary losses (Coleman et al., 2012).

2.1. Global crisis caused by AMR

Antibiotic-resistant infections persist to raise concerns across the globe about accurate evaluation of their socioeconomic impact. Quantification of the patterns of diffusion connected to antibiotic resistance is a crucial prerequisite in this regard. AMR costs the world economy an extensive amount of resources. Hospital acquired infections with multiple drug resistance are responsible for 99,000 fatalities per year in the USA. According to the Infectious Diseases Society of America (2011), sepsis and pneumonia, two prevalent HAIs, resulted in over 50,000 deaths in the USA in 2006

and cost an estimated $8 billion (Loc-Carrillo and Abedon, 2011). The annual total of infections and fatalities brought on by the most prevalent multidrug-resistant pathogens (*Staphylococcus aureus, Klebsiella pneumonia, Enterococcus faecium, Streptococcus pneumoniae, Escherichia coli and Pseudomonas aeruginosa*) was forecast to reach 400,000 and 25,000 across Europe per year in 2007. The treatment of frequent infections in neonatal critical care is getting more challenging and in certain cases is now impossible. Sixty to seventy percent of infections are caused by eight Staphylococcal species, most often *S. epidermidis* and *S. aureus*. In addition, these facilities have seen multiple outbreaks of *methicillin-resistant S. aureus* (MRSA) (Abedon et al., 2011).

In response to the growing resistance of pathogens to antibiotics, WHO identified three groups of pathogens for which there is an urgent need for new antibiotic developments and research. The critical most categories includes those multi-drug resistant bacteria that pose a specific threat to life or quality of life in nursing homes, hospitals, or in patients using ventilators or blood catheters. Included in this group of pathogens are the ESKAPE (*Enterococcus faecium, Staphylococcus aureus, Klebsiella pneumonia, Acinetobacter baumannii, Pseudomonas aeruginosa, and Enterobacter species*). Among ESKAPE, *P. aeruginosa* has probably been the first pathogen to acquire MDR and XDR attributes with the advent of strains resistant to all anti-pseudomonal drugs except polymyxins (Edwards-Jones, 2013). The MDR and XDR strains of *P. aeruginosa* have been identified as high-risk clones associated with global clonal lineages. These strains have accumulated a variety of resistance indicators through mutation, including those that upregulate AmpC beta lactamase, or that affect the regions of topoisomerases that determine quinolone resistance, or by horizontal acquisition of resistance genes, as aminoglycoside-modifying enzymes, ESBLs, or carbapenemases. According to a report by the U.S. Centres for Disease Control and Prevention, *P. aeruginosa* infections concerning healthcare are anticipated to impact 51,000 individuals annually. Of these infections, 13% are multidrug-resistant (MDR), and these infections account for about 400 deaths annually (Shokri et al., 2017).

The range of viable treatments for some infections has gradually been reduced as resistance characteristics to various classes of antibiotics have accumulated, giving

rise to strains with multidrug-resistant (MDR) phenotypes. Although resistance can be linked to a decline in fitness or virulence, some MDR strains nevertheless exhibit an impressive capacity for infection and propagation in the clinical context and have the ability to quickly develop epidemics. Hence, the search for alternative options has become inevitable. The hunt for newer approaches has led to the resurgence of phage utilization termed as ―Phage Therapy" as the appreciated recommendation in the quest for novel strategies of bacterial infection control and prophylaxis (Mapes et al., 2016). Phage therapy proponents highlight numerous significant advantages that phages have over antibiotics, including host-specificity, self-amplification, biofilm destruction, and low human toxicity. The exploration of these minuscule living entities has only recently been possible due to the advent of analytical technologies like next-generation sequencing and electron microscopy. As seen by an influx of recent human clinical trials and animal studies, these technological breakthroughs have sparked a renewed interest in phage therapy research.

2.2. Phage Therapy

The recognition of bacteriophages and their relevance to infectious diseases first introduced in the 20th century, paved the way for the employment of phage therapy to reduce the prevalence of various infectious diseases. The efficacy of Phage therapy was subjected to intense discussion from the time it was initially successfully used to treat children with Shiga dysentery in 1921 at the Hospital in Paris, France, through the time it was applied to humans all over the world. Phage products were removed from the market in 1945 as the golden period of antibiotics came to an end. However, penicillin resistance rapidly evolved into a significant clinical issue. Thus, in consequence, the inevitable extinction of all currently used antibiotics has rekindled the remarkable phage treatment research (Chegini et al., 2020). .

Frederick Twort initially reported the distinctive zone of lysis associated with phage infection in 1915. Soon after, Felix d'Herelle was acknowledged for determining the origin of this event, attributing the plaques to bacterial viruses, and coining the term "bacteriophage". In addition, d'Herelle is recognized with developing the concept of using phages therapeutically and initiating the first known clinical application of phage in 1919 at the Paris hospital Hôpital des Enfants-Malades. Despite a number of

successful trials, d'Herelle's early studies were controversial for being inadequately monitored, and the scientific community vehemently contested his work. Despite this, d'Herelle proceeded to advance phage therapy in the early 20th century by developing a number of phage therapy facilities and industrial phage manufacturing plants across Europe and India for the treatment of cholera, bubonic plague, and the dysentery. 1931 marks the application of phage to treat cholera in Punjab, India, which was another notable success. 73 experimental subjects who got phage therapy and 119 participants who served as controls were included in this study (Ling et al., 2022). D'herelle noted that only five people perished in the experimental group while there were 74 fatalities in the control group, with roughly 90% of the experimental group exhibiting lower mortality. Other pioneers in an effort to commercialize phage therapy, yielded several cocktail of phages against major disease-causing pathogens including *streptococcus, staphylococcus, and E. coli*, but the conflicting outcomes of these treatments led the world to abandon phage therapy and embrace antibiotic therapies until recently (Raz et al., 2019).

2.2.1. Bacteriophages; The active agent

Phages are targeted bacterial viruses with several antibacterial effector properties that assist to minimize multi-drug resistant infections in a human body. They can target bacteria that are enclosed in biofilms and are typically unaffected by antibiotic resistance. It is believed that there are $>10^{31}$ phages in the biosphere, and they are readily differentiated from soil, faeces, water and hospital/domestic sewage. Although phages may exhibit a wide range of morphologic shapes and genome types, nearly most of the documented ones have double-stranded DNA genomes and an un-enveloped icosahedral head and unique tail structure. Phage tails, which are exclusive to them are either contractile, short non-contractile, or long non-contractile, enabling for the distinction of different types of viruses into several classification groups as siphoviruses, myoviruses and podoviruses Phage genomes are incredibly diverse genetically, with up to 80% of estimated genes having no recognized biological use. Additionally, even among phage variants that infect the same bacterial host, phages show significant gene variety. Phages offer an infinite source of antibacterial drugs against all human bacterial infections due to their immense abundance, considerable diversity, and relatively simple isolation (Watanabe et al., 2006).

2.2.2. How phages cause infection?

In vitro infection of susceptible host bacteria by pathogenic double-stranded DNA phages results in a gradual increase in the quantity of phages in the culture medium that typically takes 30 to 40 minutes. During this time, phage particles have to inject their genome into the host organism's cell, take over the host's metabolism, and express their viral genes, form phage particles, and release offspring into the environment. Viral endolysins break down peptidoglycan linkages in the cell wall, causing the cell wall to become unstable and an osmotic breach due to the high internal pressure. This results in cell lysis. Cell lysis is a constant result of a virulent phage infection that is successful. In contrast, lysogeny may result from infection with a temperate phage. In a nutshell, during bacterial cell division, temperate phages multiply alongside the bacterial chromosome after infecting bacteria as a commensal prophage. The prophage initiates its lytic cycle to produce phage particles when it is subjected to environmental triggers or stresses. Prophages' innate ability to facilitate the exchange of genes across bacteria through specialised transduction is a mechanism that could boost bacterial virulence or encourage antibiotic resistance. Temperate phages are typically ineligible for therapeutic use (Fukuda et al., 2012). However, by eliminating so-called lysogeny genes from their genomes, developments in synthetic biology have widened up new possibilities for the therapeutic application of temperate phages as a remedy against bacterial diseases.

The majority of phages can only infect bacteria that have their corresponding receptor, which effectively limits the spectrum of hosts that they can infect. Phages differ in their host specificity; some are strain-specific, while others have shown the potential to infect a variety of bacterial strains and even species. Bacteria have developed a variety of defenses against lytic phage infection, while phages have developed an equally astonishing range of defenses to overcome this resistance. When it comes to bacteria, this can involve changing or losing receptors as well as integrating phage DNA into the clustered regularly interspaced palindromic repeats associated system (CRISPR/Cas) system. When it comes to phage, this can involve recognizing new or modified receptors as well as anti-CRISPR genes (McVay et al., 2007).

The eradication of bacteria by phages via a parasitic and enzymatic approach differs from all other therapies' antibacterial modes of action and enables phages to combat the majority of MDR bacteria. Additionally, self-replication in the presence of bacteria that is sensitive, offers density-associated dosing at the area of infection. Lowering daily doses from extended-release medications can lower expenses, patient failure to adhere, disease recurrence and side effects of drug therapy. The relationship between the frequency of phage dosages and conditions relapse has yet to be properly assessed. The prospect for low-dose phage therapies that rely on self-replication to increase the phage content at the area of infection represents one of the special features of phage therapy. Target host cell densities must exceed a replication threshold of 10^4 CFU/mL for self-dosing to take place (Watanabe et al., 2006).

Once the viral tail fibers have formed an irreversible bond with particular receptors on the bacterial cell wall, such as LPS (endotoxin), polysaccharide, teichoic acid, outer membrane proteins, pili and efflux pumps, only then phages are enabled to cause the infection. Phages become more particular when attacking bacterial targets as a result of this strict compatibility, which also prevents mammalian cell tropism. However, phages have a quick translocation capacity and can even enter areas of the body that were once thought to be sterile. There is, however, little knowledge of how phages interact with human cells, despite the rise in clinical uses (Duplessis et al., 2018).

2.2.3. Clinical data and therapeutic studies on phage therapy

The shift from the observable and subjective to the repeatable and quantified is a crucial step for any scientific endeavor. Numerous recent clinical trials have shown that bacteriophage therapy still seems to be approaching a turning point in its transition from qualitative to quantitative treatment. In March 2016 at the University of California, San Diego, a bacteriophage preparation was used to treat an acute infection caused by the multi-antibiotic resistant bacteria *Acinetobacter baumannii*, establishing the first known successful clinical application of intravenous bacteriophage therapy in the United States. This instance rekindled interest in bacteriophage therapy across the world, inspiring formal public lectures, a book, and numerous more initiatives to raise awareness of the therapeutic potential of phages (Soothill, 1994).

2.2.4. Effectiveness of various isolated phages against *Pseudomonas Aeruginosa*

Pseudomonas aeruginosa; a multi-drug resistant (MDR) bacteria is regarded as a prominent bacterial pathogen that is responsible for acute infections and a high rate of fatalities because of its resistance to a broad spectrum of antibiotics. The formation of its biofilm leads to recurring infections that are resistant to standard antibiotics. This bacterium produces significant tissue damage with various levels of virulence. In light of the fact that *P. aeruginosa* biofilm cannot be eradicated with antibiotics, researchers have considered alternative approaches. One of these revolutionary treatments is phage therapy. Bacteriophages can be employed for eliminating *P. aeruginosa* biofilm because they impair the extracellular matrix, enhance the diffusion of antibiotics into the biofilm's inner layer, and inhibit quorum-sensing activities. Numerous studies have demonstrated the effectiveness of abundant phages against *Pseudomonas aeruginosa* infections. Bacteriophages were identified against 96 clinical strains of *P. aeruginosa* in a 2017 study by Shokri D et al. According to the standards established by the European Centre for Disease Prevention and Control, 94 of these isolates were identified to be MDR, 63 were XDR, 1 was PDR, and 2 were non-MDR (Shokri et.al, 2017). These phages were combined to form cocktail that was highly effective against MDR, XDR, and PDR bacterial isolates. This study demonstrated the significant advantages of using phage therapy as a prospective therapeutic strategy. Mapes AC et al. produced a cocktail of four phages (E2005, Paer4, PA2, and KMV) and broadened their host range, by introducing 16 clinical strains of *Pseudomonas aeruginosa* into a 96-well micro-titer plate along with a mixture of phage cocktails of sequential dilution. The ensuing wells showed two different results, with some showing complete lysis of the bacterium while others showed clear bacterial lysis. The host range of the phage clones was then further re-characterized, and the phages were added to concoctions with predicted host range capabilities. The phage mixtures or combinations had a dosage response, with a statistically noteworthy decrease occurring with increasing phage titer/dose.

2.2.5. Animal trials of phage therapy against clinically significant pathogens and *Pseudomonas Aeruginosa*

Phage therapy against a variety of pathogens that are therapeutically significant has recently been investigated using animal models. A single dose of phage given concurrently with the administration of *Clostridium difficile* was sufficient to prevent infection in a hamster model of *Clostridium difficile*-induced ileocecitis; subsequent phage treatments prevented infection in 11 out of 12 mice, whereas control animals given *Clostridium difficile* and clindamycin died within 96 hours. Using a hamster model, phage combinations also drastically reduced *Clostridium difficile* multiplication in vitro and constrained proliferation in vivo. In another study, Oral phage delivery prevented 66.7% mice from dying when they were exposed to gut-derived sepsis caused by *P. aeruginosa* as compared to the control group, which experienced 0% mortality (Miró et al., 2013) .

A temperate phage identified as Pan70 was investigated by Holgun AV, Rangel G, and colleagues for its effects in vivo against a mouse burn model. Phage treatment for the mouse burn model demonstrated a substantially greater probability of survival (80%–100%) compared to the control group (0%). Phage treatment was administered to mice after *P. aeruginosa* P4 strain infection at 0 min, 45 min, 24 h, and 48 h. The findings demonstrated that temperate phage Pan70 was successful in enhancing the health and survival of mice when exposed to high CFU of bacteria at 24 and 48 hours after infection (Jault et al., 2019).

2.2.6. Human trials of phage therapy against clinically significant pathogens and *Pseudomonas Aeruginosa*

At various institutions in Eastern Europe, phage therapy studies on humans have been conducted for almost a century. In both experimental and clinical settings, the Eliava Institute has employed phage intensively to treat a variety of common bacterial infections, including *S. aureus, E. coli, Streptococcus spp., P. aeruginosa, Proteus spp., S. dysenteriae, Salmonella spp., and Enterococcus spp.* Successful applications include both therapeutic and preventative surgical and gastroenterological procedures. A superficial application of a *S. aureus*-specific phage was all that was required for healing in a six patient clinical study of diabetic foot ulcers that were resistant to

antibiotics. 219 patients diagnosed with bacterial dysentery in a 1938 trial were treated with a phage cocktail constituting a variety of phages targeting *Shigella spp., E. coli, Proteus spp., P. aeruginosa, Salmonella spp, Staphylococcus spp., Streptococcus spp. and Enterococcus spp*. administered both orally and rectally. Blood in the stools was eased in 28% of patients within 24 hours, and another 27% showed improvement within 2-3 days. In total, 74% of the 219 patients experienced reduced symptoms or betterment overall. A cohort of 18577 kids was also engaged in a preventative intervention trial employing typhoid phages during a 1974 typhoid epidemic. Typhoid incidence was reduced five times less after phage therapy in comparison with control group (Chang et al., 2019).

Holgun A. et al. investigated the impact of the newly discovered phage PA709 on human skin cells both in vitro and ex vivo. Following 4 hours of incubation, the in vitro phage caused the 5 log reduction in the concentration of *P. aeruginosa*, from 10^7 CFU/ml to 10^2 CFU/ml in the control group, even though the uninfected bacteria simultaneously reproduced swiftly and achieved exactly the same numbers of CFU as of the control group. In the scenario of in vivo testing, fresh human skin was removed during surgery and cleaned up before the administration of the control and phage treatments. Within 4 hours of incubation, the bacterial CFU was 10^6 CFU/cm^2; however in the group receiving the phage therapy, the CFU was lowered to 10^2 CFU/cm^2. An infected burn site on the skin was treated with a phage cocktail made up of 12 lytic phages (PP1131) in the first stage of a randomized clinical study. 26 subjects with burn wounds were taken into consideration; 13 received phage therapy, 12 had 1% sulfadiazine silver emulsion cream as normal care, and the final participant had no infection at all. The effects of the phage cocktail were inferior to those of the standard procedure, according to the results. This indicated that two segments or more of the average bacterial load in the most afflicted lesion had been successfully reduced in at least half of the participants. Since phages are frequently effective against the intended pathogen when coupled with antibiotics, a therapeutic choice that is yet to be explored in human beings, the prospect for phage therapy yet remains a mystery to be unveiled (Zhang et al., 2013).

2.3. Anti-Biofilm potential of Bacteriophages

The integration of several phage components allows phage-based therapy to attack biofilm through a variety of ways. Utilizing phage against bacterial activity is a prime instance of microorganism-mediated bio control, which also includes using the entire organism or only its byproducts as the bacterial antagonist. Phages penetrate biofilm before adhering to the host cell by employing depolymerases that are stored at the tail structure of the virion to increase its affinity towards the target bacteria. The receptor-binding protein on the long tail fibre then specifically binds to the receptors on the surface of the host cell, activating the initial connection between phages and bacteria hosts that result in viral infection. The tail contraction, tail tube permeation, genome injection, and eventually cell lysis happens subsequent to the irreversible attachment phase. Unique to virulent phages, which cause the escape of progeny phages from the infected cells at the last stage of the lytic cycle, is the intra- to extracellular destruction of the host cell. Additionally, holins and endolysins, two phage proteins that initiate cell lysis, are activated concurrently with this release of lytic progeny virions (Zhang et al., 2013). The cytoplasmic membrane of the host is breached by holins, which also allow endolysin ingress to and degradation of bacterial peptidoglycan, an essential aspect of the bacterial cell wall. *Pseudomonas aeruginosa* can adhere to a variety of surfaces and form biofilms, which increase resistance to antibiotics, cleaning agents, various radiological treatments, environmental variables, the body's immune response and cause persistent infection. Zhang et al. investigated the impact of biofilm formation and the removal of existing biofilm using phage and chlorine treatment. Phage therapy alone decreased the production of biofilm by 45 and 73 percent with 4x10*7 pfu per ml, respectively. When phage concentrations of 6x10*7 pfu per ml were used, pre formed biofilm was eliminated by 45 and 75%. Chlorine at 210 mg/L had no effect on extant biofilm, but it did lower the production of new biofilm by 86 percent. The effectiveness of two bacteriophages, MAG1 and MAG4, to inhibit carbapenem-resistant *P. aeruginosa* in planktonic and biofilm models was examined by Kwiatek et al. Each phage was demonstrated to affect roughly 50% of P. aeruginosa strains when applied alone, but when they were combined, the anti-biofilm capability improved to 72.9%. While MAG4 quickly decreased biofilm with effectiveness, MAG1 had an impact on biofilm after a longer

period of time. According to this study, bacteriophages can destroy biofilms employing three different methods: destruction of the bacteria that form biofilms through typical phage infection, synthesis of EPS depolymerase, and "lysis from without" that does not require phage gene expression after consumption. On the basis of these researches, it can be speculated that discovering new phages can be a wonderful antimicrobial agent substitute for treating MDR *P. aeruginosa* biofilm and may even be able to completely remove infections spurred on by MDR *P. aeruginosa* in combination with bacteria in vitro. The outcomes of in vitro research can aid in expanding the use of phages against nosocomial MDR *P. aeruginosa* infections. The application of bacteriophage mixtures can improve anti-biofilm effectiveness and reduce bacteriophage resistance (Sarhan and Azzazy, 2015).

Researchers are searching for an alternative to antibiotic treatment, such as phage therapy (PT), to treat various bacterial illnesses in both animals and humans due to the increasing level of bacterial resistance to antibiotics. Despite the fact that this therapeutic approach has been known for more than a century, there are currently very few controlled clinical studies that, by today's criteria, could demonstrate the effectiveness of using bacterial viruses to treat bacterial diseases. Nevertheless, the outcomes of numerous published case studies are encouraging.

Bacteriophages are specialized in killing bacteria; however, there are still several possibilities for bacteria to develop an anti-phage system, as,

- Altering microbial surface receptors to hinder phage DNA entrance,
- Modifying foreign (phage) DNA with (CRISPR) and associated genes,
- Developing a restriction-modification mechanism through which enzymes within the host bacteria comprehend and cut the foreign phage DNA to halt the invasion, thereby terminating the infection by inducing "self-destruction mechanism in bacterial hosts and hindering phage assembly.

Scientists have expanded mono-phage therapy to incorporate a combination therapy that combines phages and antibiotics as a preventative measure against the potential emergence of resistance to individual antibacterial drugs. It has been effectively demonstrated that phage-antibiotic therapy reduces the development of phage-resistant and antibiotic-resistant strains. The combination therapy employing phages

and antibiotics has recently received extensive research as an intriguing treatment for bacterial infection. This is due in particular to its enhanced efficacy in reducing phage and antibiotic resistance in addition to the synergistic antibacterial effects produced by enhancement in phage replication when coupled with antibiotics (Hall et al., 2012).

2.4. Phage-Antibiotic Synergy (PAS)

Some antibiotics induce elevated production of phages and larger plaques in their presence. Additionally, some antibiotics at sublethal doses may promote the release of phage progeny from bacterial cells. The presence of PAS could decrease the quantity of antibiotics required for treatment and eventually limit the development of bacterial antibiotic resistance. Researchers hypothesised that phages combined with antibiotics may be more efficient at controlling bacteria than either agent alone as the known mechanisms through which antibiotics and phages eradicate bacteria varies. According to research, using phages and antibiotics to treat Gram-positive bacteria, such as *methicillin-resistant Staphylococcus aureus* (MRSA) and multidrug-resistant *Enterococcus* strains, is specifically encouraged (Alemayehu et al., 2012).

2.4.1. Historical beginnings to current scenarios of Phage-Antibiotic Synergy (PAS)

Comeau et al. conducted the first study on PAS, reporting T4-like phages against common *E. coli* strains, T3 and T7 phages against *E. coli* isolate AS19, and T4-type Yersinia phage PST against *Yersinia pseudotuberculosis* all resulted in substantial plaque size expansion when combined with sublethal -lactams like aztreonam, ampicillin, nalidixic acid and ampicillin Enhanced phage generation during plaque formation, which leads to more bacteria being inhibited from growing or being destroyed, is effectively implied by the plaque size expansion. In addition to the phages unique to *E. coli* and *Y. pseudotuberculosis*, increase in phage plaque size was also observed in the phages specific to *P. aeruginosa*, *Bacillus cereus* and *Burkholderia cepacia* (Forti et al., 2018).

According to Oechslin et al., ciprofloxacin and meropenem at subinhibitory dosages entirely prevent the development of phage resistant mutants. In experiments by Kebriaei et al. against MRSA strains, PAS was also noted. In these research, *S. aureus* phage Sb-1-daptomycin/vancomycin combos outperformed antibiotics alone in

preventing phage resistance development. Other in vitro investigations revealed that when PAS was present, there were less phage resistant cells. Li et al. defined PAS, a promising mechanism, as a combination of phages and antibiotics that lowers the dose of drugs and prevents the emergence of antibiotic resistance. In an in vitro experiment using the antibiotic colistin and the phage Phab24 against the *Acinetobacter baumannii* Myoviridae, Wang et al. observed that phage-resistant bacteria that proliferated in the absence of antibiotics showed greater sensitivity to colistin despite maintaining their original antibiotic resistance mechanism. The phage-resistance strategy is the direct reason for this increase in antibiotic sensitivity. The use of temperate phages is an intentional administration to re-sensitize bacteria to drugs (Oliveira et al., 2018).

A reduction in the MIC of antibiotics may potentially contribute to the synergy between phages and antibiotics. Another study found that the *S. aureus* Siphoviridae phage Henu2 and sub-lethal antibiotic doses had an in vitro synergistic effect on the reduction of *S. aureus* by over 3 logs in just 12 hours. The growth of *S. aureus* was only moderately inhibited by phage Henu 2. According to the study, phage Henu2 and antibiotics together boosted phage production.

2.4.2. Phage-Antibiotic combination to treat *Pseudomonas Aeruginosa* infections

Several researches during the past 20 years have discussed the use of PAS and phage-antibiotic combination to combat *Pseudomonas aeruginosa*. In vitro sensitivity to a number of antibiotics significantly increased in two *P. aeruginosa* strains infected with the phages Pf3 and Pf1, according to a study by Hagens et al. According to Knezevic et al., P. aeruginosa's growth was inhibited when phages and subinhibitory levels of ceftriaxone were administered together. According to the research done by Torres-Barceló et al., employing phages and antibiotics synergistically can significantly improve the bacterial control as compared to employing either one individually.

2.4.3. Mechanisms of Phage-Antibiotic synergy

Several mechanisms have been reported in literature through the phage antibiotic combination inhibits the bacterial growth. Below discussed are some of the well-established mechanisms of this process.

- **Cell Filamentation**

Cell filamentation is seen when the PAS phenomenon occurs, according to Comeau et al. In *Escherichia coli* and *Yersinia enterocolitica*, beta-lactams and quinolones both produced filamentation and PAS, whereas gentamicin and tetracycline could not. Given that filamentation promotes changes in the peptidoglycan layer and that these changes likely result in increased sensitivity to the action of the enzymes encoded by phage lysis genes, the authors hypothesize that some antibiotics may hasten cell lysis. This behavior may lead to quicker lysis and a higher rate of phage generation. Some antibiotics are known to prevent protein synthesis or DNA gyrase activity, which can disrupt phage propagation. Phage-host and phage-antibiotic combinations both influence the extent to which a phage and antibiotic can function synergistically. According to the present studies, cell elongation/filamentation is one of the mechanisms leading to PAS, although there are additional components included. Antibiotics that do not promote cell filamentation do, in fact, exhibit synergy (Nathan, 2014).

- **Antibiotic induced phage production and amplification**

The impact of the antibiotics may increase plaque size, hasten phage amplification, and expand the burst size, representing another mechanism of PAS. MR-5, a Myoviridae phage of *S.aureus* displayed an increase in plaque size by three times in response to sublethal dosages of certain antibiotics as ketolide, tetracycline and linezolid in contrast to beta-lactam or quinolones. . Similarly with antibiotics as clarithromycin, cefotaxime, and ciprofloxacin, the generation of phage progeny was significantly enhanced, in accordance to an in vitro investigation.

- **Sensitizing pathogens to potential antibiotics**

A phage cocktail of *P. aeruginosa* and antibiotics have been administered in an in vitro investigation to monitor the development of bacterial resistance to antibiotics. In the presence of the *P. aeruginosa* phage cocktail PAM2H, treatment with antibiotics as meropenem, ciprofloxacin and gentamicin raised the *P. aeruginosa* sensitivity to antibiotics by 56%, 81% and 31% respectively. The application of temperate phages to re-sensitize bacteria to drugs is deliberate. The other mechanisms allude to the possibility that antibiotics may make natural lysogens

more vulnerable because of how they alter the lytic cycle of phages. Al-Anany et al. revealed evidence of the interaction between antibiotics and temperate phages. In this research, ciprofloxacin and the temperate phage *E. coli* HK97 were utilised together to eliminate E. coli in culture. The temperate phage-antibiotic synergy contrasts from the lytic phage-antibiotic synergy in its mechanism as the antibiotic acts through the RecA protein, a component of the bacterial Distress response, rather than merely encouraging the formation of phages. Lysogen deficiency is the factor that triggers this occurrence (Wiggins, 1985).

- **Decrease in MIC of antibiotics**

A reduction in the MIC of antibiotics may potentially contribute to the synergy between phages and antibiotics. Another study found that the *S. aureus* Siphoviridae phage Henu2 and sub-lethal doses of antibiotics functioned synergistically to reduce the amount of the bacteria by over three logs in just 12 hours. The growth of *S. aureus* was only moderately inhibited by phage Henu 2 alone. According to the study, phage Henu2 and antibiotics together boosted the phage replication.

- **Targeting Bacterial sites**

By focusing on several bacterial receptors, Manohar et al. and Torres-Barceló et al. demonstrated that phage-antibiotic combination can limit bacterial growth and resistance to antibiotics. They provided an explanation for this by bringing out that various invasion routes render it more challenging for pathogens to build resistance. This mechanism is comparable to that of phage cocktails, which prevent the simultaneous attack on several bacterial receptors from leading to the establishment of multi-drug resistance (Whitman, 2003).

- **Phage-Induced Antibiotic Penetration into Biofilm**

In the Necel et al. 2022 study, an *E. coli* biofilm was completely eliminated by a phage cocktail employed along with the antibiotics ciprofloxacin and rifampicin. The effectiveness of mixed phage-antibiotic therapy against biofilm was described by Usiak-Szelachowska et al. in 2020 by demonstrating that PAS speeds up the breakdown of the framework of biofilm by phage enzymes, thus rendering it easier for antibiotics to penetrate.

- **Phage Depolymerases**

The function of phage depolymerases is connected to the occurrence of PAS. Phage-encoded polysaccharide depolymerases have the ability to specifically break down exopolysaccharide complexes (EPS) in bacterial biofilms as well as structural or capsular polysaccharides. These exo-polysaccharides are crucial for preserving the viability of bacteria and their pathogenicity. Depolymerases that break down EPS allow phages to infect bacteria that reside within biofilms and penetrate those biofilms. Additionally, depolymerases may contribute to the PAS event by improving antibiotic dispersion and enhancing cell penetration. For instance, alginate lyase from *Pseudomonas* phages can aid in the dispersion of aminoglycosides to prevent *P. aeruginosa* proliferation or successfully remove *P. aeruginosa* biofilms (Coleman, 2012).

2.4.4. Effect of phages and antibiotics on bacterial biofilms

The combination of phages and antibiotics to combat Gram-negative bacteria's biofilms has been studied, and the results are encouraging. A drop in the minimum biofilm eradication concentration (MBEC) value after the incorporation of cefotaxime and the T4 phage, particularly at a high titre to an *E. coli* biofilm suggests that PAS was involved in the total eradication of E. coli biofilms in vitro. Furthermore, when utilizing bacteriophages and antibiotics separately to eliminate biofilms, a considerable number of phage- and antibiotic-resistant cells frequently develop. Tobramycin and the T4 phage were used to break up *E. coli* biofilms, which led to a drop in the number of cells that were resistant to the antibiotics and the phage, respectively, of more than 99% and 39%.

In another study, a combination therapy reduced the number of cells resistant to antibiotics and the PB-1 phage by 60% and 99%, respectively, in *P. aeruginosa* biofilms. The elimination of *Pseudomonas* from biofilms on cultivated epithelial cells was also improved by combined therapy. Phages may halt the increase of antibiotic resistance in minority groups. Because their respective methods of action complement one another, combining phages and antibiotics to cure biofilms is more successful. According to research on *Proteus mirabilis*, decrease in both planktonic and biofilm-forming bacteria is most effective when ampicillin and phage vB_PmiS-TH are used

together. When contemplating combined therapies, the concentration of phages and antibiotics as well as the timing of antibiotic administration are crucial considerations. This type of treatment may successfully remove biofilms produced by both Gram-positive and Gram-negative bacteria, according to studies using phages and certain antibiotics.

2.4.5. Phage Antibiotic synergy in animal models

A wide range of antibiotics were used to examine the phage-antibiotic synergy in several animal models, either in vivo or ex vivo. Mice were used to conduct in vivo research on the interaction between the *P. aeruginosa* Inoviridae phage Pf1 and gentamicin. Gentamicin, the *P. aeruginosa* phage Pf1, or a combination of the antibiotic and the phage were given intraperitoneally to mice before administering *P. aeruginosa* K-PAK. Within 24 hours of the bacterial challenge, the control group and the group receiving only the antibiotic died. Mice treated with the phage alone died within 48 hours, while more than 70% of the mice were saved by the combined therapy.

In a hindpaw infection brought on by the *methicillin-resistant S. aureus* in diabetic mice, the synergistic relationship between the *S. aureus* phage MR-10 and linezolid was examined. Linezolid and the *S. aureus* Myoviridae phage MR-10 were tested solely and in conjunction. The oedema levels were dramatically reduced and tissue recovery was accelerated in the combination therapy group. The combined therapy was the most effective at lowering the bacterial load, according to a comparison of the bacterial loads obtained from each group (mono- or combined therapy); this shows that the phage and antibiotic work in concert.

Intranasal administration of the *S. aureus* phage MR-10 and mupirocin was tested to confirm the efficacy of this combined treatment for a mouse *S. aureus* nasal infection (MRSA). The *S. aureus* phage MR-10 and mutirocin were administered intravenously the following day. The combined therapy was able to totally eliminate pathogens by day 5 following infection, according to ex vivo testing on murine nasal epithelial cells (NEC) that investigated the nasal bacterial load in tissue. Contrarily, monotherapies required 7 days to considerably lower the bacterial burden, and by day 10 after treatment, there were no longer any bacteria in the samples. Furthermore, studies have

shown that a combination approach reduced the incidence of spontaneous mupirocin-resistant mutants emerging to minimal amounts.

2.4.6. Phage Antibiotic Synergy in human therapy

Research interests include investigating how phages and antibiotics work together to combat various bacterial diseases in humans. In the 1980s, studies on individuals with suppurative bacterial infections utilised phages or phage combinations in addition to antibiotics. Phages were given orally or topically, and patients who used solely phages experienced better results (approximately 96% of positive cases), compared to those who used phages and antibiotics together (about 85% of positive instances). The outcomes gained imply that phages and antibiotics are antagonistic to one another. In 87 patients with chronic urinary tract infections, Zilistenau et al. also evaluated the effectiveness of phage therapy or a combination of phage and antibiotic treatment. Phages were administered orally five days in a row at a dose of 20 ml. Up to 10 days antibiotic treatment was provided. In 92.8% of positive instances, phage treatment produced the best performance, although favorable results were seen in 64.4% of patients who received phages and antibiotics. The therapeutic outcomes for both patient groups point to antagonism between phages and antibiotics.

Human orthopedic infections have also been successfully treated with phages and antibiotics. Using a blend of the phages *A. baumannii* and *K. pneumoniae* given via IV for 5 days, and then 1 week afterwards for 6 days with meropenem and colistin iv concurrently, a 42-year-old patient with a trauma-related left tibial infection caused by drug-resistant *A. baumannii* and *K. pneumoniae* had a favorable clinical response with swift tissue healing and positive culture elimination. AB-PA01 *P. aeruginosa* phage combination was administered intravenously for 8 weeks to a 26-year-old cystic fibrosis (CF) patient who suffered from MDR *P. aeruginosa* pneumonia and had been awaiting lung transplantation. Ciprofloxacin and piperacillin-tazobactam were also administered systemically for 3 weeks at this time. Later, doripenem was introduced and ciprofloxacin was removed. Within 100 days of phage therapy, no recurrence of pneumonia or CF aggravation was noticed.

In the past few years, the administration of phages and antibiotics, particularly in the treatment of recurring bacterial infections in people, has yielded encouraging results.

In investigations from 2018 to 2022, combining treatments in severe cases of orthopaedic, respiratory, and urinary tract infections had positive clinical outcomes. Combining therapies in human medicine has been found to improve the efficacy of phages and antibiotics while decreasing the development of phage resistance, which may be crucial in the current phase of antibiotic resistance. Phage-antibiotic interactions should be researched in vitro to avoid antagonistic effects and to establish synergy for the purpose to better understand the effects of therapies in the absence of randomized controlled trials.

2.5. Drawbacks in administration of phages

With great success, phages have been employed in the prevention and treatment of bacterial infections in animals. However, there are several drawbacks concerning employing phages in medicine that are intrinsic to their nature. For instance, administering phages requires identifying the bacterial strain and might necessitate phage hunting or phagograms prior to the therapy due to their great specificity. Particularly in cases of acute illnesses, this could take a while and be challenging to accomplish. Phage cocktails, which combine several different phages to broaden their host range or forestall resistance, have been suggested as more effective therapy options. The problem at present is that phages do not currently have adequate dosage forms, despite the fact that they can be supplied in a variety of methods, including parenterally, topically, orally, inhaledly, by lavage, and through ocular and nasal drops. Additionally, when exposed to certain severe circumstances including organic solvents, pH, temperature, and salinity, phages are susceptible to protein misfolding, aggregation, or denaturation, which results in a loss of stability and infectivity, leading to a depletion of antibacterial action. Moreover, in certain situations, phage particles have a limited tissue diffusion and penetration rate, which renders it challenging for them to penetrate the infection sites.

All of these shortcomings highlight the need for new phage-based formulations to be developed. Combining phage therapy with nanotechnology, or various kinds of nanoparticles (NPs) or microparticles (MPs), provides a rational way to increase the clinical outcomes of phage therapy. This includes developing platforms that increase the bioavailability of phages, inhibit their inactivation, provide protection from the

effects of neutralizing antibodies (Abs), and reduce their swift elimination by the reticulum.

2.5.1. Microencapsulation of phages

A recent innovation with a broad range of possible applications is microencapsulation technology. This method encloses the core material in a semipermeable or sealed capsule membrane utilizing natural or synthetic polymers as the encapsulation material. The outer component and the core material are the substances that are enclosed within each other. Polymers are primarily utilized in microencapsulation technology to encapsulate functional, active substances and create an extensive rigid barrier around the core material to lessen its reactivity with the surrounding environment. Functional compounds can have their physical characteristics and stability improved through microencapsulation, which can also prevent or mitigate deactivation and enhance storage time (Huh, 2019).

2.5.2. Purpose of phage encapsulation

On average, phage preparations have a variety of applications, including

- **Protection.** The phages receives protection against enzymatic attack, hydrolysis, and deactivation by immune system components through encapsulation utilizing, for instance, liposomes.
- **Constancy.** Phages are biological organisms that become inactive when their proteins or nucleic acids break down. This is crucial for their storage in particular.
- **Delivery on an active site.** When liposomes or detergent-lipid particles are used, the encapsulating cargo can enter the tissue, which is frequently impossible when utilizing free chemicals.
- **Accessibility.** Phages can be embedded in a three-dimensional network using fibres and hydrogels, enabling a continuous release of phages to the site of action.
- **Adhesion.** Positively charged substances, like cationic hydrogels or liposomes, in particular, permit greater muco-adhesiveness and extended residence and emission at the active site.

2.5.3. Encapsulation

Phage therapy can be made more successful as a delivery and defence mechanism by encapsulating therapeutic phages. In general, liposomes and both natural and artificial polymers are used in the majority of delivery systems appropriate for phage therapy. Each of them has unique qualities and employs various synthesis techniques. The needed application and administration style determine the appropriate system and manufacturing process to use. Other elements that differ between encapsulation techniques and affect the effectiveness of the final formulation include the size, shape, stability, and titer of phages that have been encapsulated. Since the variety of phages and potential hosts is limitless, it is impossible to build a "all-encompassing" platform for phage encapsulation. Instead, individualized techniques are required. More intriguingly, encapsulation can improved the mucus layer's permeability, permitting phages to enter some bacterial pathogens' homes. Encapsulation also contributes to the phage's regulated release and provides defense against neutralizing antibodies. Following describes few of the materials suitable for phage encapsulation.

- ✓ **Natural biopolymers**

Natural macromolecules are capable of producing polymers called biopolymers. They often have exceptional biocompatibility and biodegradability due to their natural origin, making them ideal for a variety of biological applications, including phage encapsulation. The primary building blocks of natural polymers are polysaccharides, peptides, amino acids, lipids and proteins. These polymers are cross linkable and can be utilized to create unique Nano particles topologies. Their strong resistance to enzymatic breakdown or pH fluctuations is one advantage. More significantly, two polysaccharides—chitosan and alginate—stabilized by Ca2+ were shown to improve the probability of survival of encapsulated phages across the digestive system in broilers infected with *Salmonella* spp., particularly over the long term.

- ✓ **Synthetic polymers**

Synthetic polymers are well known for their potential for controlling a variety of properties, such as size and shape, as well as for their ease of manufacture. Solvent evaporation, nano-precipitation, ionic coagulation, and microfluidics are some of the

diverse synthesis methods. The crucial characteristics of NPs, such as loading effectiveness and release kinetics, which can be precisely adjusted by alterations in composition, responsiveness, stability, or surface charge. Regarding structure, nano- or microcapsules and nano- or microspheres are the two most frequent options. As an illustration, *Salmonella* sp. was treated in farms using phage FGS011 encased in synthetic polymer microparticles. In another study, freeze-dried PLGA MPs were successfully used to encapsulate phages that target *S. aureus* and *P. aeruginosa*. Their shape, make-up, and pattern of release made them seem like an intriguing lung infection treatment. Phages placed on the surface of porous PLGA MPs for nebulized delivery was suggested as a further option for the treatment of P. aeruginosa in lung infections. The transport of phage-loaded MPs across the lungs was demonstrated by in vivo delivery in a mouse model, with no safety concerns noted.

- ✓ **Liposomes**

For the treatment of respiratory, gastrointestinal, and intracellular diseases, phages have been widely researched when encapsulated in lipid-based formulations. Liposomes are one of the lipid-base systems that attracts the most attention. Liposomes are spherical nanoparticles made of an aqueous cavity surrounded by a phospholipid lipid bilayer. Due to the liposome's twofold nature, hydrophobic compounds can be contained in the lipid membrane while hydrophilic and amphiphilic molecules can be enclosed in the polar core. Liposomes' key strength is their composition, which gives them a high level of biocompatibility and makes them simple to produce. This was proven following the liposomal encapsulation of the PEV2 and PEV40 *Pseudomonas* spp. phages. The mean size of the produced liposomes for PEV2 (135 to 218 nm) was slightly smaller than for PEV40 (261 to 448 nm) while employing the identical synthesis conditions; this difference corresponds with phage particle dimensions.

- ✓ **Hydrogels**

As polymer scaffolds, fillers, or carriers for biomolecules, hydrogels are among the most frequently utilised materials in tissue engineering. By encasing phages in a polymer or immobilising them on solid supports, phage distribution via hydrogels can be accomplished. Encapsulating phage in hydrogel has been extensively researched

and offers a number of benefits. As an illustration, Staphylococcal phage K was studied, which demonstrated significant antibacterial activity in an alginate encapsulation and demonstrated superior protection from the stomach's acidic pH to free phage.

- ✓ **Chitosan-alginate bead**

Chitosan-alginate bead encapsulation reduced phage deterioration during preservation and allowed the phage titer of Salmonella enterica and E. coli to remain high in a gastrointestinal in vitro model, supporting its application in the therapy or prophylaxis of intestinal pathogens in livestock.

Hence, microencapsulation is an innovative technology that can facilitate the propagartion of bacteriophages through the gastrointestinal tract.

Material and Methodology

3.1. Study area

This research work was conducted in the Applied, Environmental and Geomicrobiology laboratory (AEG), Department of Microbiology, Faculty of Biological Sciences, Quaid-i-Azam University, Islamabad Pakistan during July 2022 to August 2023. The main objective of this research was to exploit the synergistic potential of phages and antibiotics to combat the MDR *Pseudomonas Aeruginosa* and phage encapsulation for their efficient delivery to human gastrointestinal tract (GIT).

3.2. Chemicals and Reagents

All the chemicals/reagents utilized in this research work were from Sigma Aldrich limited (USA), Merck (Germany) and some obtained from local industry.

3.3. Bacterial Strain

Already isolated and characterized multi-drug resistant strain of *Pseudomonas aeruginosa* was collected from Medical Microbiology laboratory of Quaid-i-Azam University, Islamabad. The resistance pattern of this strain was further tested against 30 antibiotics that were purchased from Oxoid (Thermo Fisher Scientific). The biofilm potential of this strain was tested by micro-titer dish biofilm formation assay as described by Kamali et al (Kamali et al., 2020).

3.4. Isolation, Purification, and Preservation of Bacteriophages

Phage PAA, PAM and PAR were isolated from domestic sewage waste water sample. Domestic sewage waste water sample was collected from Punjab Province of Pakistan. 50 ml of sewage waste water was centrifuged and filtered through the filter paper of 10 ml of 24 hours old culture of *Pseudomonas aeruginosa* was mixed with it and incubation of 24 hours at 35°C was given. After 24 hours 1 ml of sample was drawn, centrifuged at 10 000 rpm for 10 minutes and supernatant was filtered through syringe filter of ―Filter-bio Company" with 0.22µm pore size. Filtrate was tested for the presence of bacteriophages by double layer plaque assay described by Montso et al., 2019 (Montso et al., 2019), and plaques with different morphologies were observed. Isolated plaque was streaked on the nutrient agar seeded with host bacterium and incubation of 24 hours was given. After 24 hours, the layer of soft agar

containing pure bacteriophages was scrapped and suspended in 10 ml of SM buffer. Further it was vortexed for 1 minute and left for an hour in shaking incubator at 35°C (Xiao et al., 2022). After incubation, the filtrate was centrifuged for 10 minutes at 10,000 rpm, supernatant was filtered and pelleted soft agar was discarded. 0.5 ml of this supernatant was mixed with 0.5 ml of phage preservation buffer and freeze at -80°C.

In order to determine the phage titer, 2.5 ml of SM buffer containing pure phage was combined with a host bacterium culture that had been grown for four hours. A 1 ml sample was centrifuged at 10,000 rpm for 10 minutes, filtered using a 0.22 m syringe filter, and serial dilution to 10^{-9} was carried out (Türe et al., 2022). Double layer agar assays of odd number dilutions were carried out by combining 10 ml of each dilution with 100 ml of host bacterium culture that had been in existence for 24 hours, together with 3 ml of 0.7% soft agar, and then pouring the mixture onto newly prepared nutrient agar plates. The entire sample was centrifuged and filtered once PFU was determined, and the filtrate was then chilled at 4°C for further testing.

The formulae for the calculation of PFU/ml of stock is: nx10xd

Where n is the number of plaques and d is the dilution number.

3.5. Antibiotic stock solution preparation

The antibiotics used in this research (Table 1) were obtained from Sigma Chemicals in United States. Gentamicin, cefipime, and meropenem were dissolved at a concentration of 1,024 mg/ml to create the stock solutions in autoclaved distil water respectively.

3.6. Antibiotic Sensitivity Testing:

The antibiotic sensitivity test was performed by disk diffusion method. For this purpose, plates of MHA media were prepared. For bacterial suspension, saline was used as a diluent for a small amount of *Pseudomonas aeruginosa* culture as 10 µl bacterial culture diluted in 9900 µl normal saline. Further the bacterial suspension was swabbed on agar plates, starting at the top and carefully wiping down to the bottom of the MHA plate. To equally cover the whole MHA plate with swabbed inoculum, this process was repeated three times after each rotation of the plate, typically at 60

degrees. 13 antibiotic disks were placed on these plates using sterile forceps to place each disc evenly apart from the others. To prevent disc displacement during incubation, each disc needs to be held up against the agar's surface. The plates were then incubated ambient temperature of 37°C for about 20 to 24 hour. On completion of incubation, the zone of inhibition formed around each antibiotic disk was measured via an accurate scale/caliper. The results of the inhibition zones are termed as "susceptible", "resistant" or ―intermediate" depending on the selected cut-off range for zone diameter in the nearest millimetre and microgram per millilitre, respectively. This is in accordance to the guidelines on breakpoints as developed by the Clinical Laboratory Standards Institute (CLSI).

3.7. Determination of Minimum Inhibitory Concentration of Antibiotics:

The MIC of antibiotics (Gentamicin, Cefipime and Meropenem) was determined by broth micro dilution method on a micro-titre plate. For this purpose, antibiotic stock solutions were prepared in falcons in accordance to the potency in µg/ml as recommended by the CLSI. For stock solutions, the calculated amount of each antibiotic per 10 ml was dissolved in 10 ml of normal saline to prepare a final solution of 10 ml volume (range 1–512 mg/ml). Further the stock solutions were serially diluted up to 9th dilution (i.e. 256, 128, 64, 32, 16, 8, 4, 2, and 1) in eppendorf to create different concentrations of antibiotics. The concentrated *Pseudomonas aeruginosa* broth culture was also 1/1000 diluted as 10µl of *Pseudomonas* broth culture dissolved in 9900µl broth. 100 µl of each concentration of all antibiotics (Gentamicin, Cefipime and Meropenem) were suspended in the wells of the micro-titre plate along with the 100 µl of diluted bacterial culture. The final volume maintained in each well was 200 µl. The micro-titre plate was then incubated at 37°C in static conditions for 24 hours to determine the minimum inhibitory concentration.

3.8. Stability of phages with sub-inhibitory concentrations of antibiotics:

The stability of phages with sub-inhibitory concentrations of antibiotics was examined using a double layer agar assay. 500 µl of phage from 10^6 dilution of phage filtrate was mixed with 500 µl of three sub-inhibitory concentrations (1/2,1/4 and 1/6) of each antibiotic in eppendorf and incubated for an hour. After hour incubation, 100 µl of pure *Pseudomonas Aeruginosa* culture (1.14×10^{12} cfu/ml), 3 ml soft agar and 4 µl

of phage-antibiotic mixture was poured on prepared MHA plates and incubated for 24 hours at 37°C in static conditions. The control group included 100 µl of culture and 5µl of pure phage filtrate mixed in soft agar and poured on MHA plate, spots of phage-antibiotic mixture on prepared DLA plates, and spots of sub-inhibitory concentration of each antibiotic on prepared DLA plates. After 24 hours incubation, the plaque formation was determined to check the stability and the effect of sub-inhibitory concentrations of antibiotic on phage morphology.

3.9. Effect of different MOI of PAA, PAM and PAR alone and in combination on turbidity reduction and cfu reduction of host bacterium:

Phages PAA, PAM and PAR were tested against the specific *Pseudomonas* isolate at different MOIs, (MOI = Plaque forming units (PFU) of virus used for infection/number of cells). Suspension of *P. aeruginosa* isolate (1.6×10^{13} cfu/ml), was prepared in nutrient broth. One hundred microliters of the suspension was added to 100 µl of different dilutions ($10^{3,}$ $10^{6,}$ $10^{9,}$ 10^{12}) of phage lysate adjusted at different MOIs (10, 1, 0.1, 0.01) in the wells of a microtiter plate. A change in OD 600 over a 24-hour incubation period enabled to observe how the phages have affected the bacterial growth. Following a 24-hour incubation period at 37°C, 2 µl of the plates' contents were withdrawn, serially diluted (1/1000) and then plated on Mackonkey agar plates. The number of colony-forming units (CFU/mL) was calculated and contrasted with the control. Data from trials conducted at various MOI's suggested that phage activity inhibits bacterial growth.

For phage cocktail, the optimal MOI of all phages at concentration 10^6 PFU/ml to give PAA MOI 0.1, PAR MOI 0.1 and PAM MOI 1 were mixed in equal proportions and then 100 µl of cocktail was suspended in the well of a microtiter plate with 100 µl of bacterial suspension of 1.6×10^{13} cfu/ml. The control group of the experiment included 200 µl broth in a well and 200 µl of bacterial suspension to assess sterility. Plates were then incubated for 24 h at 37°C, and then examined for clearance of the wells at OD 600 nm. Following the incubation period at 37°C, 2 µl of the plates' contents were withdrawn, serially diluted (1/1000) and then plated on Mackonkey agar plates. The number of colony-forming units (CFU/mL) was calculated and contrasted with the control.

3.10. Synergistic effect of phages and their cocktail with antibiotics on host bacterium

To explore the synergistic effect, he phages were employed at a concentration of 10^6 PFU/ml in the micro-titre plates, and the antibiotics (Gentamicin, Meropenem and Cefipime) were administered at sub-inhibitory concentrations (1/2, 1/4, and 1/6 of MIC). Then, 100 µl of 1.14×10^{12} cfu/ml *P. aeruginosa* suspension was added to plate wells. Plates were then incubated for 24 h at 37°C, and then examined for clearance of the wells at OD 600 nm.

Following the incubation period at 37°C, 2 µl of the plates' contents was withdrawn, serially diluted (up to 10^3) and plated on Mackonkey agar plates. The number of colonies per millilitre (CFU/mL) was calculated and contrasted with the control (a phage and antibiotic alone). All the data is displayed as the mean and standard deviation (SD) of at least three separate studies.

For effect of phage cocktails with antibiotics, 1 ml of 10^6 PFU/ml of PAA, PAM and PAR were firstly incubated to form a cocktail and then applied with the sub-inhibitory concentrations of each antibiotic as per the above mentioned method.

3.11. Synergistic effect of phages and their cocktail with antibiotics on biofilm formation inhibition

Sub-inhibitory concentrations of gentamicin, cefipime, and meropenem were used for the anti-biofilm tests. In brief, biofilms were produced using the bacterial cells in the fresh culture multiplying exponentially. *Pseudomonas Aeruginosa* isolate from a fresh culture was used to produce a bacterial suspension in nutrient broth that was subsequently diluted to 1/100 with fresh medium. 100 µl of the diluted culture, 50 µl of bacteriophages at MOI 0.1 and 1 respectively, and 50 µl of sub-inhibitory doses of each antibiotic were added to the wells of sterile 96 well flat bottom tissue culture plates. The plates were then incubated for 24 hours at 37°C. After incubation, the liquid inside the wells was aspirated, and the wells were rinsed three times with PBS. The adherent biofilm was washed, stained, and its OD was measured at 570 nm using a micro-titre plate reader. The control group of the experiment included 200 µl of broth, 200 µl bacterial culture and phages and antibiotics alone to assess sterility.

For effect of phage cocktails with antibiotics, 1 ml of MOI 0.1 of PAA, PAR and MOI 1 of PAM were firstly incubated to form a cocktail and then applied with the sub-inhibitory concentrations of each antibiotic as per the above mentioned method.

3.12. Synergistic effect of phages and their cocktail with antibiotics on pre-formed biofilm

Each well in 96 well flat bottom plates was filled with 100 µl of diluted *Pseudomonas Aeruginosa* culture in nutrient broth (10^{12} CFU/mL), which was then incubated at 37°C for 72 hours to form a strong biofilm. After incubation, the liquid medium was aspirated, and any planktonic cells were washed out of the wells through three PBS washes. Then, 50 µl of bacteriophage lysate with MOI 0.1 and 1 and sub-inhibitory quantities (1/2, 1/4 and 1/6 of MIC) of each antibiotic were added, and the wells were incubated for 24 hours. After incubation, the phage lysate and antibiotic mixture was removed. The wells were then cleaned, dyed, and the remaining biofilm's OD was measured at 570 nm. Wells containing nutrient both inoculated only with tested bacterial isolate was used as a control of the experiment.

For effect of phage cocktails with antibiotics, 1 ml of MOI 0.01 of PAA, PAR and MOI 1 of PAM were firstly incubated to form a cocktail and then applied with the sub-inhibitory concentrations of each antibiotic as per the above mentioned method.

3.13. Synergistic effect of phages and their cocktail on pre-formed biofilm observed on glass slides

The phage cocktail was tested to evaluate the anti-biofilm activity against *Pseudomonas Aeruginosa. Pseudomonas* isolate was cultivated in 10 ml of nutrient broth at 37°C for 24 h. Bacterial samples were then diluted 1:100 in nutrient broth, and 100 µl of each was suspended on glass slides for phage alone and their cocktail treatment. The slides were incubated for 72 hrs at 37°C. Following the completion of incubation, the content on the slides was washed with pre-warmed (37°C) PBS. Then 50 µl of phages (MOI 0.1 and 1) and phage cocktail was suspended on the slides and incubated for 24 hrs at 37°C. Following incubation, phage lysate was washed and the slides were then cleaned, dyed, and visually observed. Further optical microscopy of the slides was performed to determine the reduction in pre-formed biofilm. On a

control slide with bacterial biofilm yet no exposure to PAA, PAM and PAR, the same process was used.

Encapsulation of bacteriophages in polymeric micro-particles

3.14. Synthesis of microcapsules by the combinations of different polymeric substances

The microcapsules were synthesized of biocompatible polymeric substance sodium alginate and in combination with agarose, gelatin and polyethylene glycol (PEG) used as enteric coating agents in accordance to the Ma's description with some modifications.

- **Sodium Alginate Beads**

2 g amount of sodium alginate was mixed and stirred with 50 ml distilled water in order for the sodium alginate to completely absorb water and swell for usage. The mixture was microwaved at short intervals to obtain a definite solution. Then, sodium alginate was combined with 3 ml of each phage (PAA, PAM, and PAR). Further, using a 5 ml syringe and a magnetic stirrer, tiny droplets of the sodium alginate and phage mixture were pushed into a beaker containing 50 ml of 0.7M chilled $CaCl_2$ solution. To generate spherical microcapsules, the droplets were finally allowed to harden in the $CaCl_2$ solution. These capsules were repeatedly cleaned and filtered with distilled water until there was no trace of $CaCl_2$ left on the surface. The resulting microcapsules were preserved in sealed tubes and kept at 4°C.

- **Alginate-Agarose Beads**

These microcapsules were formed by adding sodium alginate and agarose in 2:1 quantity in 50 ml distilled water. The mixture was microwaved at short intervals to obtain a definite solution. Then, 3 ml of each phage was dispensed (PAA, PAM, and PAR) in the alginate-agarose solution. Further, using a 5 ml syringe and a magnetic stirrer, tiny droplets of the sodium alginate and phage mixture were pushed into a beaker containing 50 ml of 0.7M chilled $CaCl_2$ solution. To generate spherical microcapsules, the droplets were finally allowed to harden in the $CaCl_2$ solution. These capsules were repeatedly cleaned and filtered with distilled water until there

was no trace of $CaCl_2$ left on the surface. The resulting microcapsules were preserved in sealed tubes and kept at 4°C.

- **Alginate-Gelatin Beads**

2:1 amount of sodium alginate and gelatin were mixed in 50 ml distilled water. The mixture was microwaved at short intervals to obtain a definite solution. Then, 3 ml of each phage was dispensed (PAA, PAM, and PAR) in the alginate-gelatin solution. Further, using a 5 ml syringe and a magnetic stirrer, tiny droplets of the sodium alginate and phage mixture were pushed into a beaker containing 50 ml of 0.7M chilled $CaCl_2$ solution. To generate spherical microcapsules, the droplets were finally allowed to harden in the $CaCl_2$ solution. These capsules were repeatedly cleaned and filtered with distilled water until there was no trace of $CaCl_2$ left on the surface. The resulting microcapsules were preserved in sealed tubes and kept at 4°C.

- **Alginate-PEG Beads**

Sodium alginate and polyethylene glycol were dissolved in 2:1 quantity in autoclaved distilled water. The mixture was microwaved at short intervals to obtain a definite solution. Then, 3 ml of each phage was dispensed (PAA, PAM, and PAR) in the alginate-gelatin solution. Further, using a 5 ml syringe and a magnetic stirrer, tiny droplets of the sodium alginate and phage mixture were pushed into a beaker containing 50 ml of 0.7M chilled $CaCl_2$ solution. To generate spherical microcapsules, the droplets were finally allowed to harden in the $CaCl_2$ solution. These capsules were repeatedly cleaned and filtered with distilled water until there was no trace of $CaCl_2$ left on the surface. The resulting microcapsules were preserved in sealed tubes and kept at 4°C.

3.15. Entrapment and release of phages in different polymeric enteric coating agents

To determine the efficient entrapment of phages within the different polymeric microcapsules, the $CaCl_2$ solution in which the capsules were formed was centrifuged for 10 mins. The supernatant was retrieved and the free phages in it were determined by double layer agar plaque assay. The deduction of free phage titre from the total phage PFU used for capsule formation provided the amount of phages entrapped within the capsules. Further 2 capsules were suspended in microcapsules breaking

solution (MBS) composed of 0.2M sodium bicarbonate, 50mM sodium citrate and 50mM Tris-HCl. The microcapsules were subsequently broken down by shaking for 30 minutes at room temperature. Double-layer agar was subsequently employed for determining the phage titre in the solution after 100 µl of samples were taken and ten-fold diluted.

3.16. Phage entrapment efficiency (EE %)

Phage microcapsules loaded with phage were suspended in microcapsules breaking solution. The microcapsules were subsequently broken down by shaking for 30 minutes at room temperature. Further the broken capsules within the MBS were centrifuged for 15 mins to determine the phage titre for calculating the entrapment efficiency. The supernatant was then filtered (pore size 0.22 m) and the double-layer agar was subsequently employed for determining the phage titre in accordance to the following equation,

$$EE\% = (100 - \text{pure phage in } CaCl_2 / \text{total PFU}) \times 100$$

Each testing had two replicates, and the data is presented as the mean encapsulation efficiency standard deviation (SD).

3.17. Characterization of Dried Capsules

The microcapsules were air dried to determine their morphology and to perform dry weight assessment.

3.17.1 Analysis of bead morphology

Optical microscopy was performed at 4X to analyze the microcapsules' size and surface appearance. The dried microspheres were suspended on glass slides and mounted on metal grids using double-sided tape. An eyeglass micrometre was used to gauge the diameter of the phage microspheres.

3.17.2. Dry weight determination

The dry weight of the microcapsules was accurately measured on weighing balance. The readings were recorded in triplicate and reported as mean value.

3.18. Stability of free phages at gastronomic conditions

In 10 ml of a 0.5% NaCl solution that had been pH-adjusted to different pH values (2, 5.7, 6, 6.7, and 7.4) by the addition of 1 M HCl solutions, the stability of free phages at the gastrointestinal pH ranges was assessed. 9 ml of various pH solutions were incubated with 1 ml of phage filtrate for 60 min at 37°C. Following incubation, a 100µl sample was taken, diluted ten times, and used in double-layer agar plaque tests to measure the phage titre.

3.19. Stability of encapsulated phages at gastronomic conditions; Entrapment and Release

In 10 ml of a 0.5% NaCl solution that had been pH-adjusted to different pH values (2, 5.7, 6, 6.7, and 7.4) by the addition of 1 M HCl solutions, the stability of encapsulated phages at the gastrointestinal pH ranges was assessed. 2 capsules were suspended in 2 ml of various pH solutions and incubated for 60 min at 37°C. Following incubation, the dried capsules were removed and the supernatant was centrifuged at 120 rpm for 15 mins. A 100 µl sample was retrieved, diluted ten times, and employed in double-layer agar plaque tests to measure the phage titre. This presented the amount of phages hereby stable and entrapped within the microcapsules.

To determine the phage release from these enteric coating agents at various gastrointestinal conditions, the capsules removed from supernatant were dispensed in MBS. The microcapsules were subsequently broken down by shaking for 30 minutes at room temperature. Double-layer agar assay was subsequently employed for determining the phage titre in the solution after 100 µl of samples were taken and ten-fold diluted. This presented the release of phages from microcapsules at various gastrointestinal pH ranges.

3.20. Stability of free phages in gastric fluid (pepsin) and simulated intestinal fluid (pancreatin)

Artificial SGF was prepared containing 0.2% (wt/vol) NaCl and 3.2 mg/mL pepsin adjusted to pH 2.0 to 2.4. For the free phage, 9 ml of pre-warmed SGF (pH 2.0 or 2.4) and 1 ml of phage filtrate were combined, and the mixture was incubated for 60 mins at 37°C with 100 rpm of shaking. After the incubation period was over, a 100µl sample was taken, diluted ten times, and the phage titre was calculated using double-layer agar.

Artificial SIF was prepared containing 10 mg/mL of pancreatin in 50mM KH2PO4 adjusted at pH 6.8. For the free phage, 9 mL of pre-warmed SIF (pH 6.8) and 1 ml of phage filtrate were combined, and the mixture was incubated for 60 min at 37 °C with 100 rpm of shaking. After the incubation period was over, a 100 µl sample was taken, diluted ten times, and the phage titre was calculated using double-layer agar.

3.21. Stability of encapsulated phages in gastric fluid (pepsin) and simulated intestinal fluid (pancreatin); Entrapment and Release

Two phage capsules were introduced to 1 ml of pre-warmed SGF (pH 2.0 or 2.4) pre-warmed SIF for the microencapsulated phages, which were then incubated at 37°C with 100 rpm of shaking. The capsules were then removed after the 60 minutes incubation, and the supernatant was centrifuged at 120 rpm for 15 minutes. For double-layer agar plaque assays to determine the phage titre, a 100 µl sample was obtained, diluted ten times, and utilized. This showed how many phages were contained inside the microcapsules and retained stability. Further, the microcapsules were removed from supernatant and dispensed in MBS. The microcapsules were subsequently broken down by shaking for 30 minutes at room temperature. Double-layer agar assay was subsequently employed for determining the phage titre in the solution after 100 µl of samples were taken and ten-fold diluted. This presented the release and stability of phages from microcapsules in gastric and simulated intestinal fluid.

3.22. Statistical Analysis

Statistical analysis was performed in triplicate. Standard deviation and mean value was calculated for each data set. One-way ANOVA were employed as statistical analysis tests in this investigation. $P < 0.05$ was the significance threshold.

Results

4.1. Bacterial strain:

The bacterial strain *pseudomonas aeruginosa* under study was obtained from the Quaid-i-Azam University, Islamabad ―Laboratory of Genomics and Epidemiology", was tested against variety of antibiotics of different classes. This strain is capable of producing a strong and resistant biofilm. Figure 1 represents the TEM analysis of *Pseudomonas aeruginosa* with a diameter of 1.06mm ±0.0084 and a length of 2.694mm ±0.49.

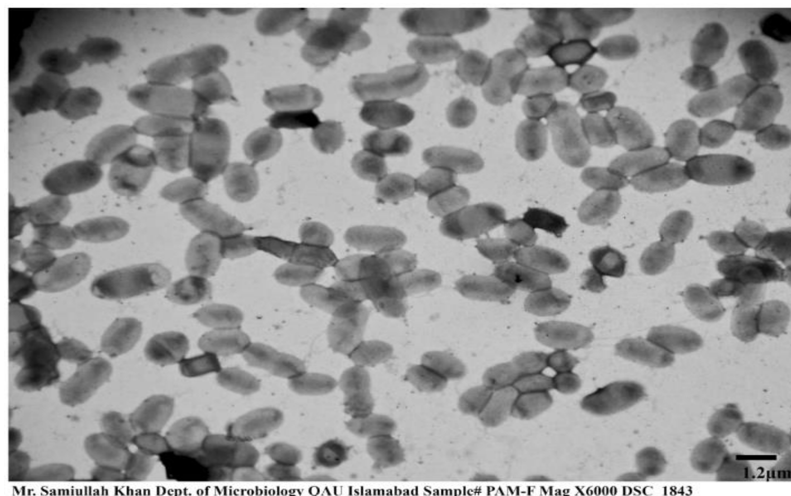

Fig.4.1. TEM analysis of host bacterium *Pseudomonas Aeruginosa*

4.2. Isolation, Purification, and Preservation of Phages

Phages against the MDR strain of *pseudomonas aeruginosa* were successfully isolated from samples of sewage waste via double layer agar spot assay, which produced well-isolated plaques against the bacterial host. The newly isolated phages were termed as PAA, PAM and PAR. Single plaque of each phage was quadrant streaked and further sub-streaked for purification. After three months at -80°C, these phages showed great stability with no significant impact on PFU.

Figure 4.3 represents the TEM analysis of phages PAM (right) and PAR (left). PAM phage belongs to *Siphoviridae* family and has icosahedral symmetry, with a capsid diameter of 55.55 nm and a flexible, long, non-contractile tail with the length of approximately 160.356 nm. Moreover, PAR also belongs to *Siphoviridae* family and

has icosahedral symmetry, with a capsid diameter of 88.756 nm and a flexible, long, non-contractile tail with the length of approximately 370 nm.

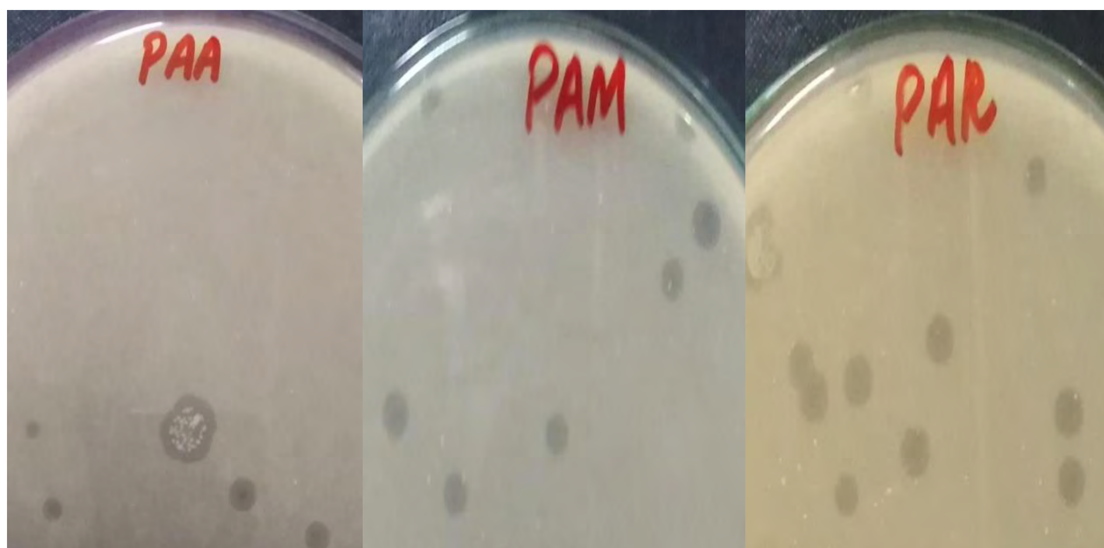

Fig.4. 2. Isolated plaques of PAA, PAM and PAR phages obtained by DLA

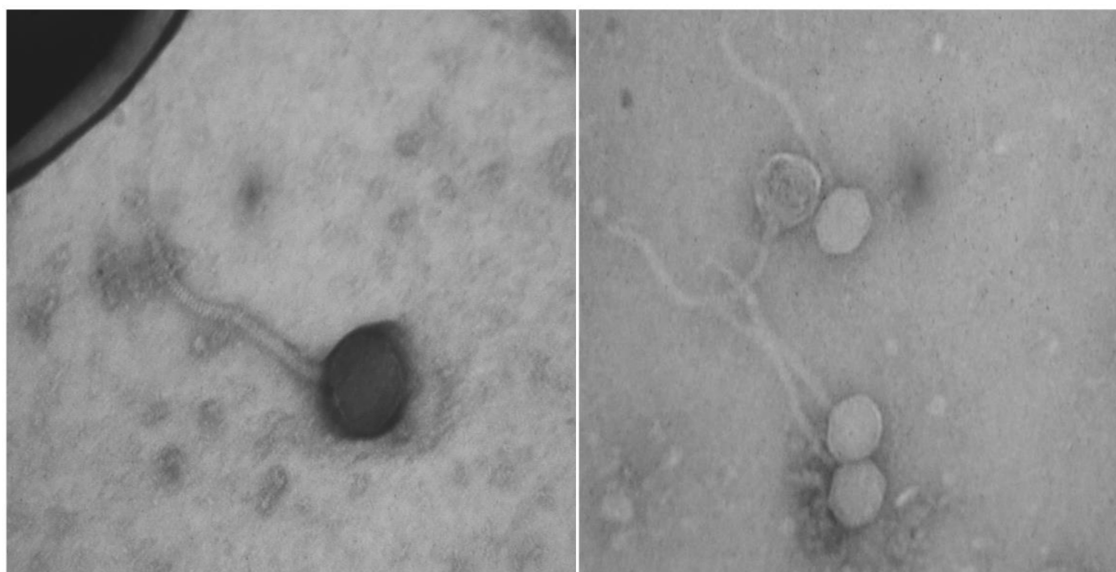

Fig.4. 3. TEM analysis of PAM (right) and PAR (left) phages

4.3. Antibiotic Sensitivity Profile of *Pseudomonas Aeruginosa*:

The MIC values of various antibiotic classes (Table 1) were determined against *P. aeruginosa* to ascertain the bacteria's susceptibility to different antibiotics. Based on the sensitivity and resistance MIC breakpoints, the tested isolate was classified as sensitive (S), intermediate (I), and resistant (R). *P. aeruginosa* exhibited sensitivity

only to chloramphenicol and meropenem, while the majority of the tested antibiotics were highly resistant to it. Meropenem MIC value was assessed to be greater than 4µg/ml. *Pseudomonas aeruginosa* was classified as extended antibiotic-resistant (XDR) based on the MIC data.

Antibiotics	Concentration µg/disc	Zone of inhibition in mm	S/R/I
Amoxicillin	10 µg	0	Resistant
Piperacillin	100 µg	0	Resistant
Ceftazidime	30 µg	0	Resistant
Cefepime	30 µg	13	Resistant
Cefazolin	30 µg	0	Resistant
Cefixime	5 µg	0	Resistant
Gentamicin	10 µg	16	Resistant
Doxycycline	30 µg	13	Intermediate
Chloramphenicol	30 µg	29	Sensitive
Sulfamethoxazole trimethoprim	25 µg	0	Resistant
Meropenem	10 µg	34	Sensitive
Fosfomycin	200 µg	0	Resistant
Nitrofurantoin	300 µg	11	Resistant

Table 1. Antibiotic Susceptibility profile of *Pseudomonas Aeruginosa*

4.4. MIC Determination

Gentamicin, Cefipime and Meropenem were further selected to carry out this study. MIC of the three antibiotics was determined and reported as per CLSI as Gentamicin ≥16 µg/ml (8 µg), Cefepime ≥16 µg/ml (16 µg) and Meropenem ≥4 µg/ml (2 µg). The

selected antibiotic sub-inhibitory concentrations for the PAS study were 1/2, 1/4, 1/6 and 1/8 of MIC.

Antibiotics	**Antibiotics MIC**	**Selected conc for PAS(µg/ml)**			
		1/2	**1/4**	**1/6**	**1/8**
Gentamicin ≥16 µg/ml	8 µg	4 µg	2 µg	1 µg	0.5 µg
Cefepime ≥16 µg/ml	16 µg	8 µg	4 µg	2 µg	1 µg
*Meropenem ≥4 µg/ml	2 µg	1 µg	0.5 µg	0.25 µg	0.125 µg

Table 2. MIC determination of Selected Antibiotics

4.5. Stability of phages with sub-inhibitory concentrations of antibiotics:

Double layer plaque assay was applied to assess the stability of phages with antibiotics in phage-antibiotic combinations. Both the phage infection mechanism and the phage itself exhibited no harmful effects. The configuration and size of the phage plaque didn't vary (Fig 4.4). Additionally, as the amount of phage particles was unaffected by the presence of antibiotics (Gentamicin, Cefipime, Meropenem), no effect on the phage's infectivity could be noted. As the MIC for each of the antibiotics under investigation did not rise in the presence of the phage, further inverse studies also disproved any adverse effects of the phage on the antibiotic compounds.

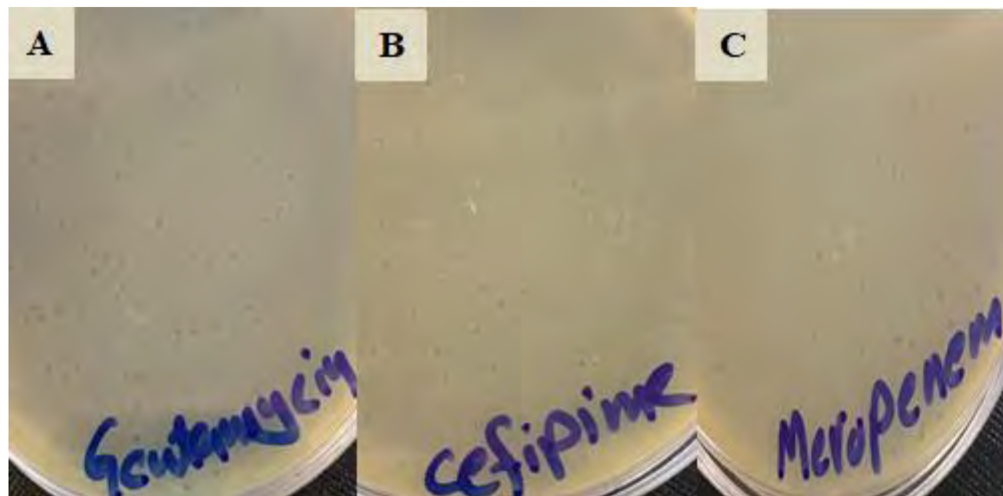

Fig.4.4. PAA, PAM and PAR phages plaque formation in the presence of selected antibiotics

4.6. Effect of different MOI of PAA, PAM and PAR alone and in combination on turbidity reduction and cfu reduction of host bacterium

The inhibitory effect of phages with varying MOI on bacterial host turbidity reduction was evaluated. It was observed, that at higher MOI 10 of each phage, no viable bacterial cells were determined i.e. 0 cells as compared to control 1.6×10^{13} cfu/ml. PAM at MOI 1, reduced the amount of bacteria to 1.23×10^{6} cfu/ml whereas complete reduction was observed in the case of PAA and PAR at MOI 1. Decreasing the multiplicity of infection to 0.1, PAA reduced the bacterial count to 1.2×10^{6} cfu/ml (7 log reduction), PAM reduced the bacterial count to 1.96×10^{6} cfu/ml (7 log reductions) and 1.02×10^{6} cfu/ml (7 log reductions) of host bacterium was observed at MOI 0.1 of PAR phage. Further lowering the phages titer to MOI 0.01, significant viable bacterial cells were obtained. PAA reduced the bacterial cells to 1.6×10^{9} cfu/ml at MOI 0.01, bacterial reduction at MOI 0.01 of PAM was 2.34×10^{9} cfu/ml and PAR at MOI 0.01 reduced the bacterial turbidity to 2.7×10^{8} cfu/ml as compared to the control. Conclusively, the optimal MOI of each phage to obtain viable bacterial cells was deduced from this data as MOI 0.1 for PAA and PAR and MOI 1 for PAM, each representing 7 log reductions in viable bacterial count.

Optimal MOI of each phage was further evaluated to observe the decrease in bacterial count in comparison to the phage cocktail. PAA at optimal MOI 0.1 caused 6 log reduction (1.58×10^{6} cfu/ml) in bacterial count as compared to the control (1.46×10^{12}

cfu/ml) indicating significant reduction. Similarly, PAM was observed at MOI 1, which led to a 6 log reduction (1.41×10^{6} cfu/ml) in bacterial turbidity. 6 log reduction (1.28×10^{6} cfu/ml) was also determined at MOI 0.1 of PAR in contrast to the inoculum count in the control group. A significant reduction of 8 logs was observed when the phage cocktail (constituting PAA MOI 0.1, PAR MOI 0.1 and PAM MOI 1) was administered to determine the effect on the viability of bacterial cells.

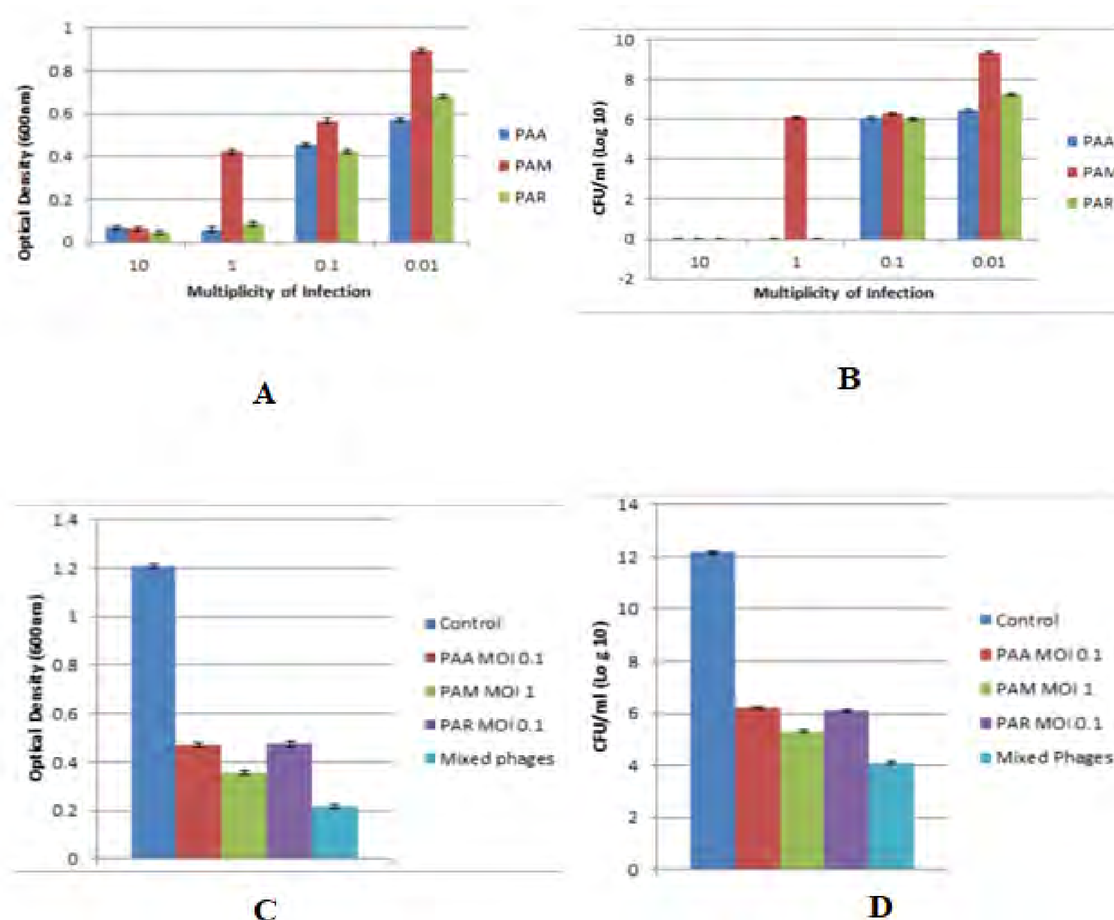

Fig.4.5. (A). Represents the effect of different MOI of PAA, PAM and PAR phages on host bacterium turbidity determined at OD 600 nm. (B). Represents the effect of different MOI of phages on host bacterium turbidity determined by CFU count. (C). Represents the effect of Optimal MOI of phages on host bacterium turbidity determined at OD 600 nm. (D). Represents the effect of Optimal MOI of phages on host bacterium turbidity determined by CFU count.

4.7. Synergistic effect of phages and their cocktail with antibiotics on host bacterium

To configure the synergistic effects of phages in combination with antibiotics, the contribution of each individual component was assessed. Synergistic effects were

observed for all sub-inhibitory concentrations of antibiotics with the optimal MOI of phages. The combination of gentamicin (8 µg/mL) at 1/2, 1/4 and 1/6 of the MIC together with the PAA and PAR phage at MOI 0.1 resulted in a seven-log reduction compared to control (1.14×10^{12} cfu/ml), antibiotic alone which resulted in four-log reduction at 1/2 MIC and a six-log reduction when compared to the phage alone. In the absence of phages, gentamicin alone at 1/4 and 1/6 MIC resulted in only a reduction of three-logs. At lower concentration of gentamicin that is 1/8 of the MIC, six-log reduction by PAA and PAR in combination with antibiotic was observed as compared to the gentamicin alone which led to the two-log reduction at the said MIC. Similarly in the case of gentamicin-PAM phage treatment eight-log reduction was observed at 1/2 and 1/4 sub-inhibitory concentrations of the antibiotic. Furthermore seven-log reduction in bacterial viability was seen at 1/6 and 1/8 of MIC when combined with PAM phage, hence displaying a higher degree of synergistic activity. Higher synergism was reported in the combination of antibiotic and phage cocktail which resulted in nine-log reduction of bacterial cells at 1/2, 1/4, 1/6 and 1/8 of the MIC of gentamicin.

In the case of cefepime–phage treatment (8 µg/ml and 4 µg/ml, 0.5 × MIC, 0.25× MIC and MOI 0.1 phage PAA and PAR), complete elimination of bacterial cells was noted as compared to the antibiotic alone (led to four-log reduction) and phage alone (causing six-log reduction), respectively. However in the case of PAM phage and cefipime combination complete bacterial eradication was observed at 1/2 of MIC and nine-log reduction at 1/4 of the Cefepime's MIC. Eight-log reduction occurred at 1/6 of the MIC and seven-log reduction at 1/8 of the MIC of cefepime in combination with the three phages PAA (MOI 0.1), PAM (MOI 1) and PAR (MOI 0.1). Strong synergistic effect was observed in the case of phage cocktail which led to complete killing of bacteria in combination with 1/2, 1/4 and 1/6 of the MIC of cefepime in comparison to the antibiotic alone which cause three-log reduction at 2 µg and 1 µg concentration of cefepime. Bacterial viability was only observed in 1/8 of MIC of cefepime with phage cocktail which led to eight-log reduction in comparison to the antibiotic and phages alone, hence presenting strong synergism.

Interestingly, Meropenem with PAA, PAM and PAR phage (1 µg/ml and 0.5 µg/ml with MOI 0.1 and 1 respectively) had a synergistic activity resulting in absolute

extermination of bacterial cells in contrast to the control which displayed five and four log reduction in bacterial cfu at the said MIC. Decreasing the sub-lethal dose of meropenem in combination to PAA and PAR, bacterial viability was observed at 0.25µg/ml and 0.125 µg/ml dose of antibiotic resulting in nine and eight-log reduction in cfu/ml respectively. However, PAM displayed eight to seven-log reduction in bacterial turbidity at 1/6 and 1/8 of the MIC of antibiotic in contrast to the control that caused four to three-log reduction at the said MIC. Remarkable synergism was observed in phage cocktail and sub-inhibitory concentrations of meropenem (1/2, 1/4 and 1/6 of MIC) resulting in complete clearance of bacterial colonies. Bacterial turbidity was only observed at 1/8 of the MIC which demonstrates nine-log reduction in bacterial count, hence indicating significant synergy.

Significant results indicating phage antibiotic synergy were obtained in combination with meropenem, followed by cefipime and gentamicin.

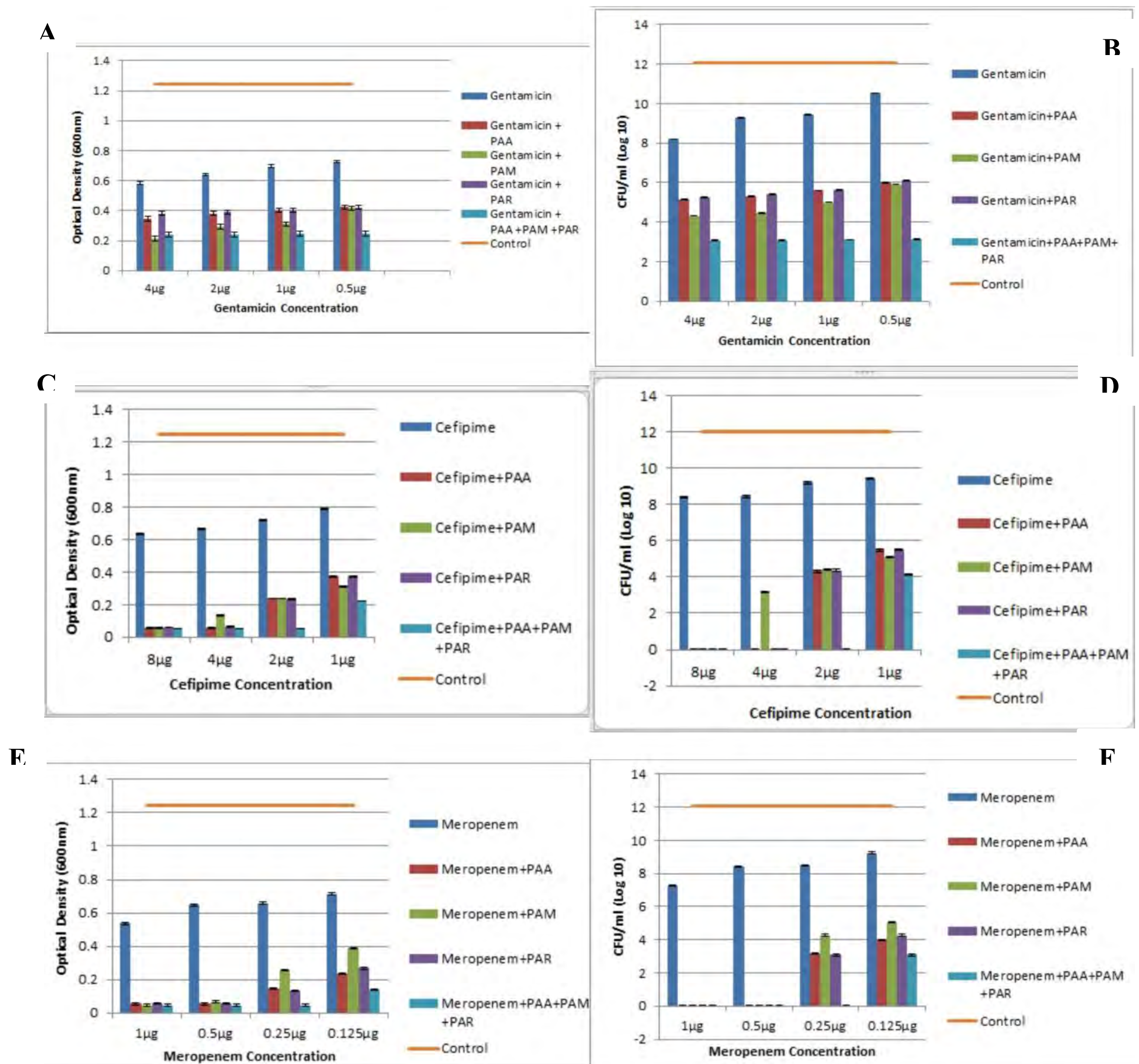

Fig.4.6. (A). Synergistic Effect of gentamicin and PAA, PAM and PAR phages on bacterial turbidity reduction, displaying maximum synergy achieved with phage cocktail, determined at OD 600 nm. (B). Synergistic Effect of gentamicin and phages on bacterial turbidity reduction, displaying maximum synergy achieved with phage cocktail, determined by CFU count. (C). Synergistic Effect of cefepime and phages on bacterial turbidity reduction, displaying maximum synergy achieved with phage cocktail, determined at OD 600 nm. (D). Synergistic Effect of cefepime and phages on bacterial turbidity reduction, displaying maximum synergy achieved with phage cocktail, determined by CFU count. (E). Synergistic Effect of Meropenem and phages on bacterial turbidity reduction, displaying maximum synergy achieved with phage cocktail and significant results, determined at OD 600 nm. (F). Synergistic Effect of gentamicin and phages on

bacterial turbidity reduction, displaying maximum synergy achieved with phage cocktail and significant results, determined by CFU count.

4.8. Synergistic effect of phages and their cocktail with antibiotics on biofilm formation inhibition

Depending on whether a particular bacterial strain is planktonic or lodged in a biofilm, phages and antibiotics frequently affect that strain of bacteria in various ways. Consequently, the potential impacts of phage-antibiotic combinations on bacterial cells buried in biofilms were identified. The results of this study's demonstrate that, for all phage-antibiotic combinations, the biofilm is diminished at high antibiotic concentrations to varied degrees and depending on the antibiotic. In the treatment of the *pseudomonal* biofilm with gentamicin alone, even the highest sub lethal antibiotic concentration of 4 µg/ml did not show any significant anti-biofilm effect. However, when combined with phages the degree of biofilm removal was improved, with maximum biofilm being eradicated when the gentamicin was combined with phage cocktail (PAA, PAR at MOI 0.1 and PAM at MOI 1). When phages were introduced, gentamicin exhibited stronger anti-biofilm effect than when used alone. MIC results showed that the *P. aeruginosa* was sensitive to cefepime. Correlating with this, it was observed that complete biofilm eradication occurred at the higher concentrations of antibiotic in combination with phage cocktail as compared to when antibiotic was used alone, which had no significant inhibitory effect. Cefepime when employed with PAA, displayed maximum removal in comparison to its combination with PAR and PAM. Similarly, meropenem, which had no obvious effect on biofilms on its own, did only reduce the biofilm when phages were present. Clear synergy was observed at concentration of antibiotic higher than 0.25 µg/ml. Remarkable synergistic effect was observed when meropenem was combined with phage cocktail (PAA, PAR at MOI 0.1 and PAM at MOI 1), that displayed maximum removal of the biofilm. Individually PAA in combination with meropenem was able to eradicate biofilm maximally.

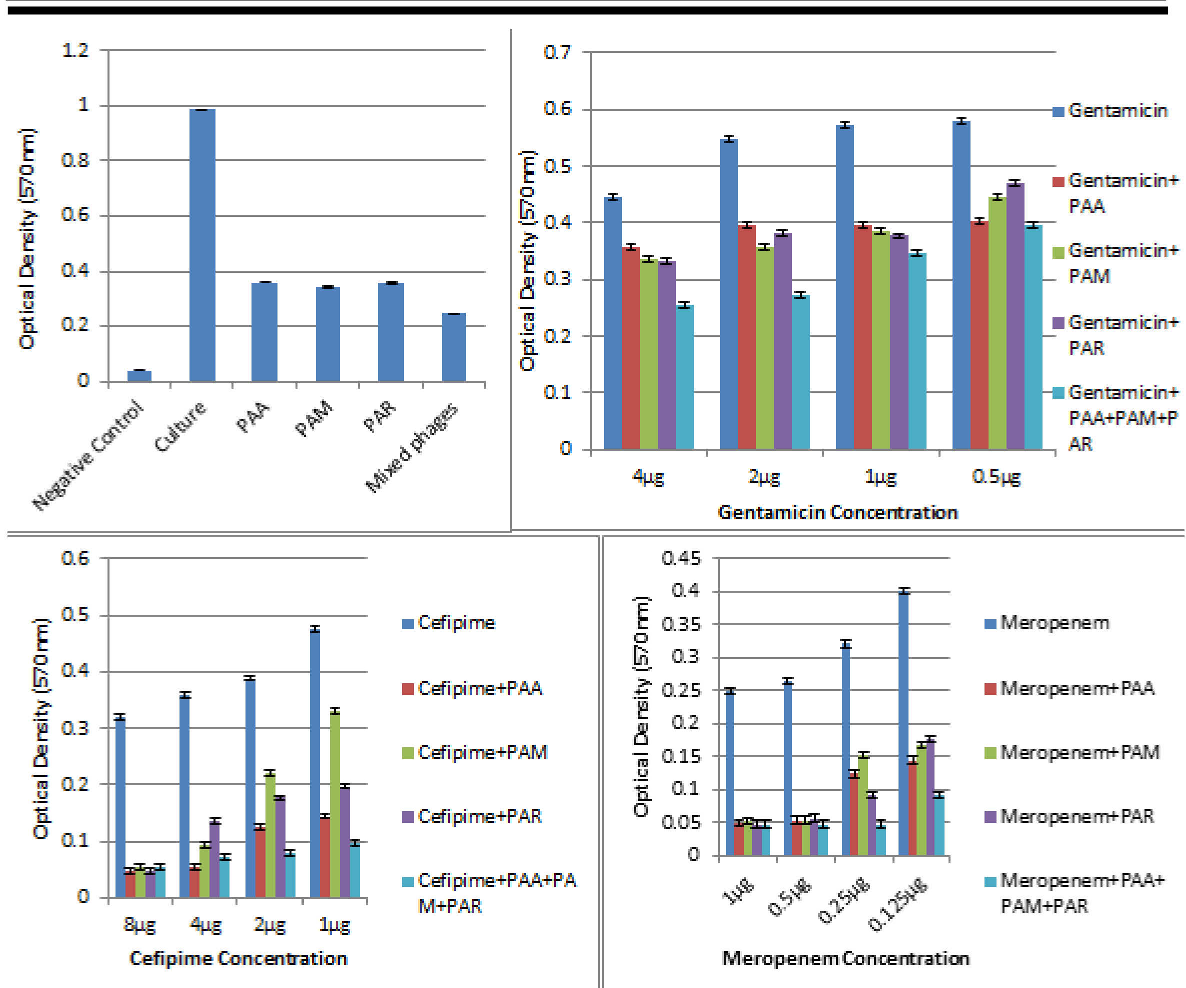

Fig.4.7. (A). Effect of phages and their cocktail on *Pseudomonas* biofilm. (B). Synergistic effect of gentamicin with phages on biofilm inhibition. (C). Synergistic effect of Cefepime with phages on biofilm inhibition. (D). Synergistic effect of meropenem with phages on biofilm inhibition.

4.9. Synergistic effect of phages and their cocktail with antibiotics on pre-formed biofilm

After producing biofilm-embedded cells in microtiter plates for 72 hours, various antibiotic concentrations and phage quantities were applied. It was observed that no significant reduction occurred in biofilm when gentamicin, cefepime and meropenem were applied alone at sub-lethal concentrations. Similarly, slight biofilm removal was observed in their combination with each of the phage. However, mixed phages demonstrated improved results when combined with sub-inhibitory concentrations of the antibiotic, as significant removal of biofilm was observed.

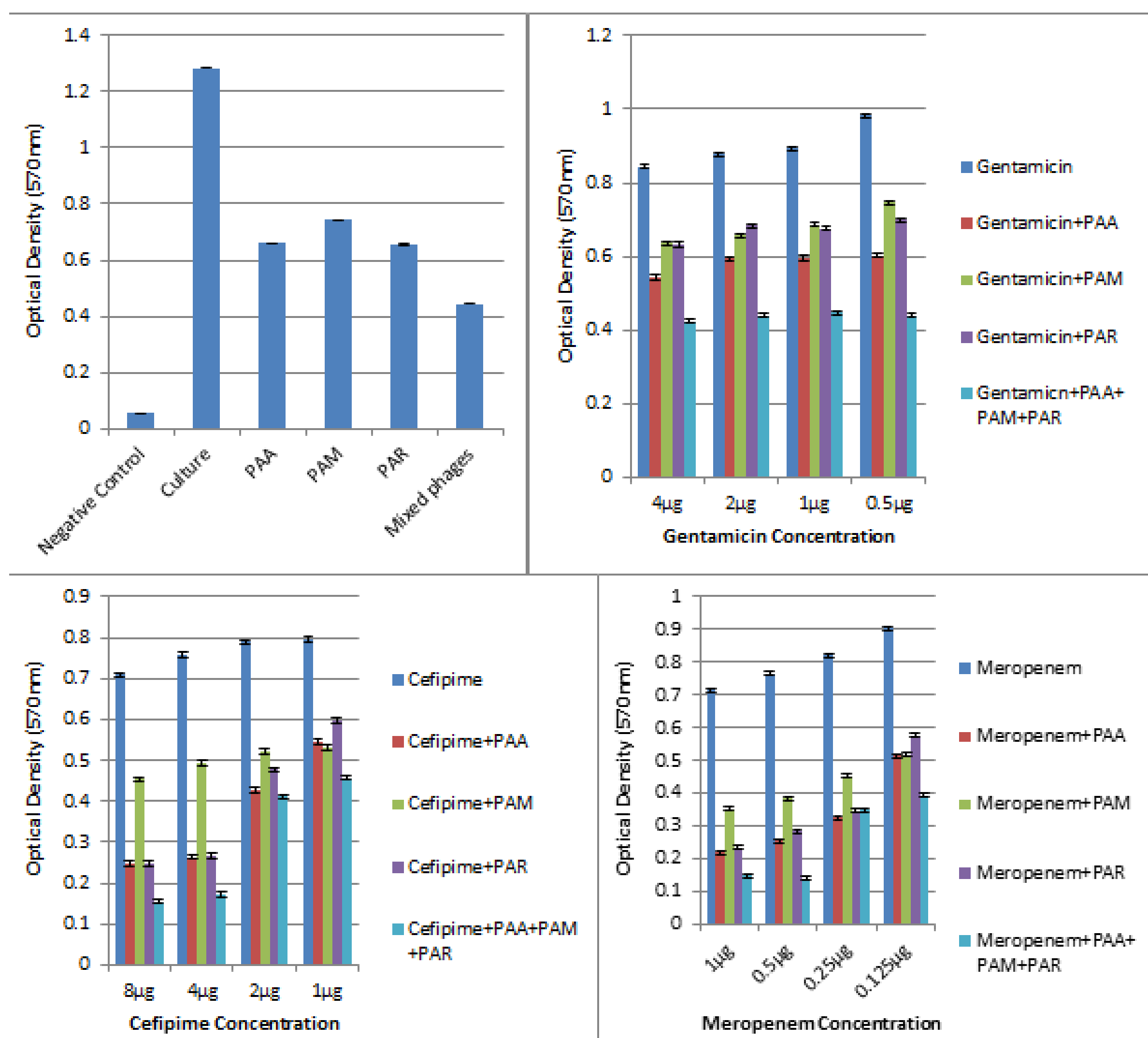

Fig.4.8. (A). Effect of phages and their cocktail on pre-formed biofilm of *Pseudomonas*. (B). Synergistic effect of gentamicin with phages on pre-formed biofilm determined at OD 570 nm. (C). Synergistic effect of Cefepime with phages on pre-formed biofilm determined at OD 570 nm. (D). Synergistic effect of meropenem with phages on pre-formed biofilm determined at OD 570 nm.

4.10. Effect of phages and their cocktail on pre-formed biofilm observed on glass slides

The effect of phages and their cocktail on the treatment of pre-formed biofilm was also observed by producing biofilm embedded cells on glass slides for 72 hours. The qualitative impact of PAA, PAM, and PAR on biofilm reduction was demonstrated by the crystal violet stain of phage treated and untreated biofilms on glass slides. When dyed with crystal violet, the treatment of phages and their cocktail on pre-formed

biofilm on glass slides, revealed a significant difference between the treated and untreated biofilms as depicted by the figure.

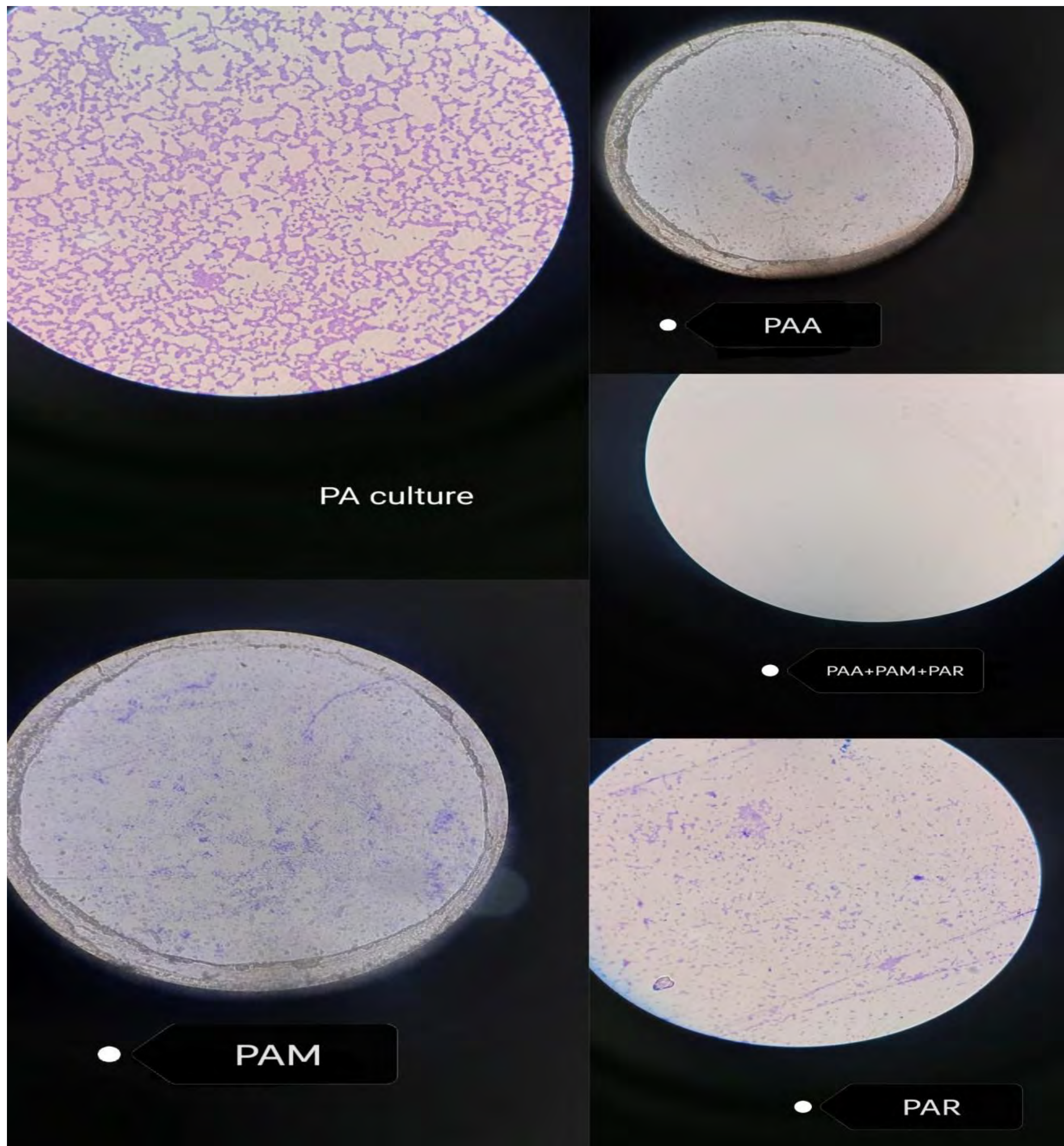

Fig.4.9. Effect of individual phages (PAA, PAM and PAR) and their cocktail on pre-formed biofilm observed on glass slides

Encapsulation of bacteriophages by polymeric micro-particles

4.11. Optimum parameters to synthesize microcapsules

The optimum parameters set to use were determined as, the concentration of sodium alginate was 2%, the ratio of phage to sodium alginate was 3:1, the ratio of other biocompatible polymers to sodium alginate was 2:1, the needle diameter of the 5ml syringe used for extrusion dropping was 0.8 mm and the concentration of CaCl2 was 0.7M.

4.12. Phage entrapment efficiency (EE %)

Each biopolymer formulation's capacity to encapsulate phages is displayed. The integration of phages without biological inactivation was made possible by the mild gelling of alginate and other biopolymeric compositions. Compared to other biopolymeic-based formulations, Alginate-Agarose-based beads displayed significantly greater encapsulation efficiencies (99% entrapment of PAA, PAM and PAR phage). Due to a more efficient and quick crosslinking in the presence of calcium ions, the outstanding encapsulation capacities of alginate-agarose may indicate highest phage lodging in the gel matrix during gelation.

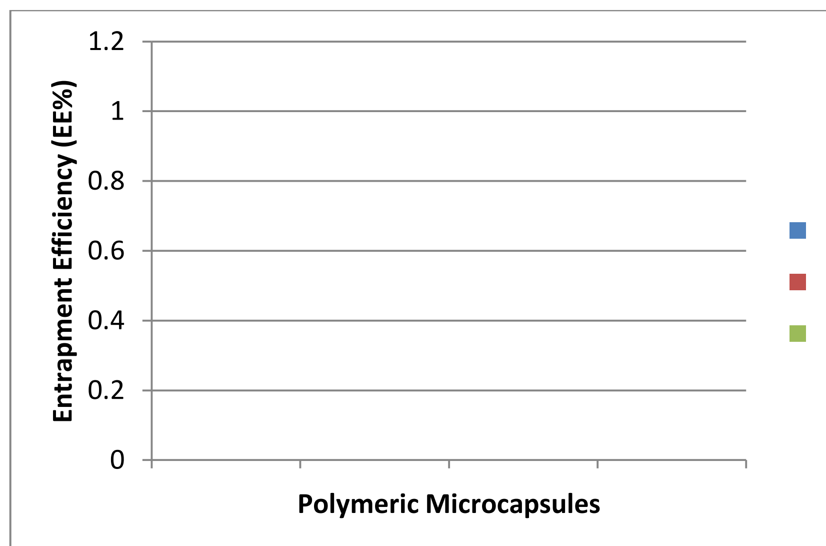

Fig.4.10. Represents the entrapment efficiency of different polymeric combinations

4.13. Characterization of Dried Capsules

4.13.1. Appearance of Microcapsules

The microencapsulated phages had a consistent look and a shape that resembled a sphere. The microcapsule's surface was practically wrinkle-free when viewed under a microscope.

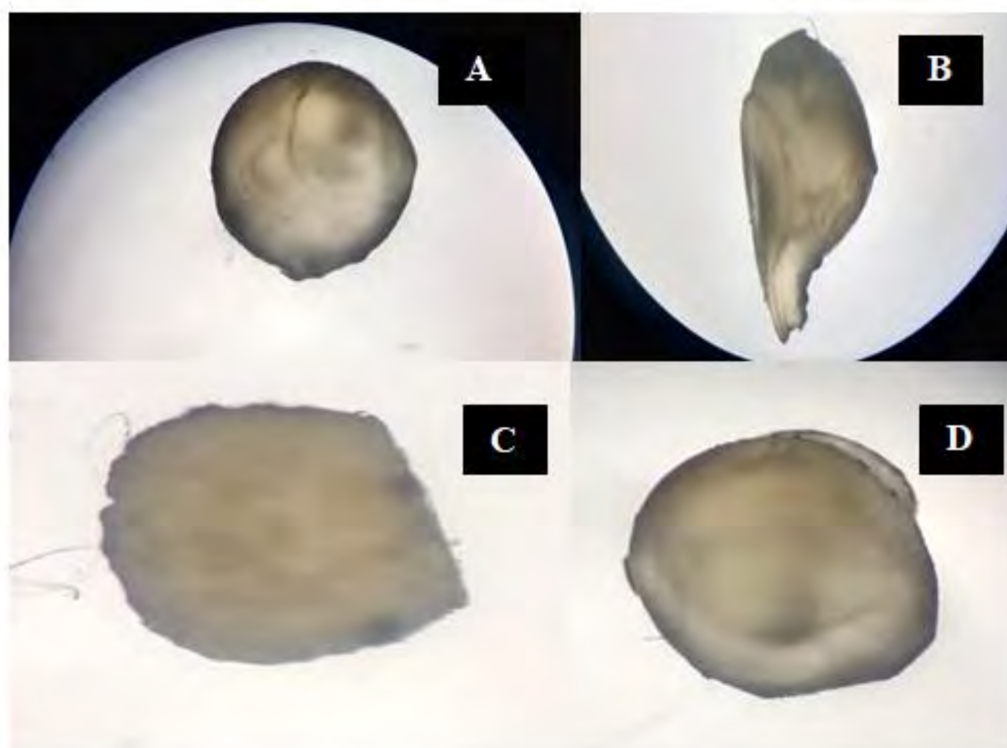

Fig.4.11. (A). Optical microscopy of Alginate Capsule. (B). Optical microscopy of Alginate-Agarose Capsule. (C). Optical microscopy of Alginate-Gelatin Capsule. (D). Optical microscopy of Alginate-PEG Capsule

4.13.2. Dry weight Determination

As per dry weight determination, the weight of the capsules was observed to be 0.0001 and 0.0002 g for each phage (PAA, PAM and PAR).

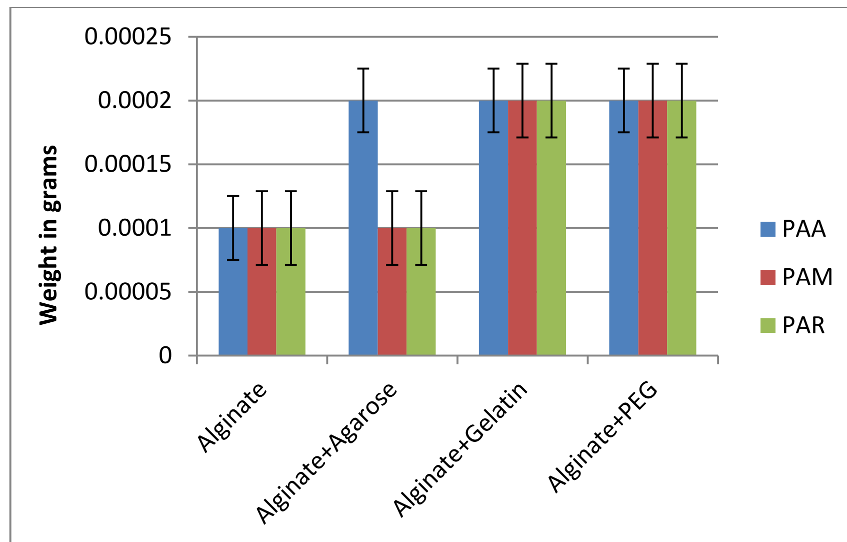

Fig.4.12. Represents the dry weight of each polymeric microcapsule

4.14. Stability of free phages at gastronomic conditions

The stability of the free bacteriophages (PAA, PAM and PAR) were evaluated at a range of pH values of gastrointestinal tract, pH 2, 5.7, 6, 6.7 and 7.4 over 1 h at 37 °C. The viability of non-encapsulated PAA phages at pH 2 was measured after 60mins, and the titers were observed to decrease by 4 log 2 PFU/ ml after in comparison to the control that was 9 log 10 PFU/ml, indicating that the gastric acid efficiently denatures the proteinaceous capsid of phages, ultimately destroying them. The viability of free PAA phages was then observed at pH 5.7 and the titers here were also reduced to 6 log 8 PFU/ml in comparison to the control. However, the phage titers at pH 6, 6.7 and 7.4 remained unchanged signifying the free phage stability at intestinal pH ranges. Similarly, PAM displayed a reduction of 1.4 log 3 PFU/ml and 2.3 log10 PFU/ml at pH 2 and 5.7 respectively in contrast to the phage titer of experimental control of 3 log 13 PFU/ml. However in the case of PAR phage, reduction in phage titer was only observed at acidic pH 2, that is, 4 log 2 PFU/ml and at pH 5.7 that is 3.6 log 9 PFU/ml in comparison to the 5 log11 PFU/ml phage titer of the control. The viability of phage at the remaining pH changes remained unchanged, signifying stability.

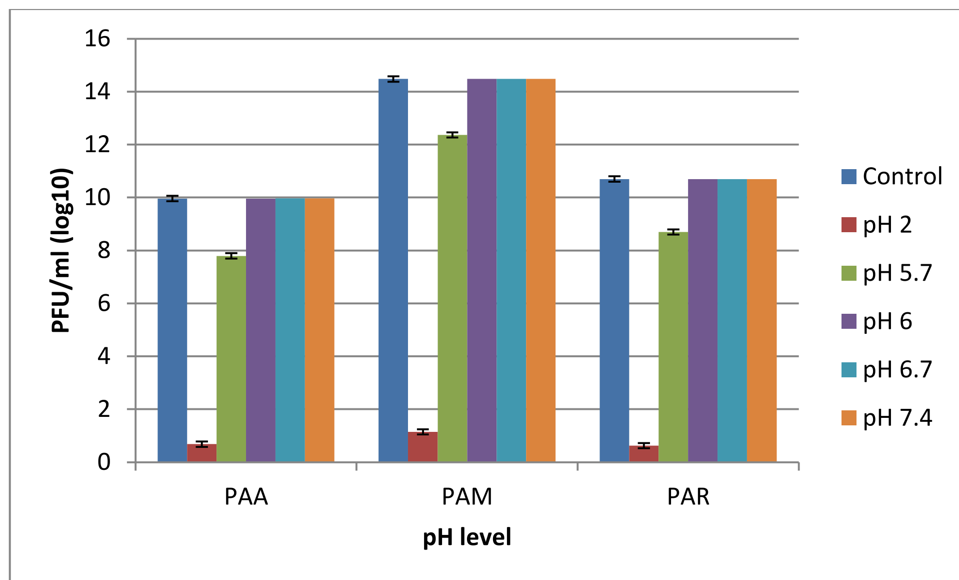

Fig.4.13. Represents the stability of free phages at gastronomic pH

4.15. Stability of Entrapped phages at gastronomic conditions; Entrapment and Release

The stability of the bead-encapsulated bacteriophages was evaluated at acidic pH values, pH 2, 5.7,6, 6.7 and 7.4 over 1 h at 37°C. The viability of encapsulated bacteriophages at pH 2 was measured where their titers were observed to be significantly high, entrapped within the micro-particles indicating stability. Phages remained entrapped within the microcapsules at pH 5.7 and 6, however, the titres of the entrapped phages steadily decreased when increasing the pH to 7.4, indicating their efficient release in the intestines. The release of phages from the polymeric capsules was also observed at the defined pH ranges. No phage was released at pH 2 indicating their instability in the gastric environment. The release of phages was steadily improved with the increase in pH and was observed to be maximum at pH 7.4. Remarkable results indicating entrapment were obtained in the alginate-agarose based capsules signifying their efficiency in protecting the phages at gastric pH and excellent release of phages was observed from the alginate-gelatin capsule in intestinal environment to treat gastrointestinal ailments.

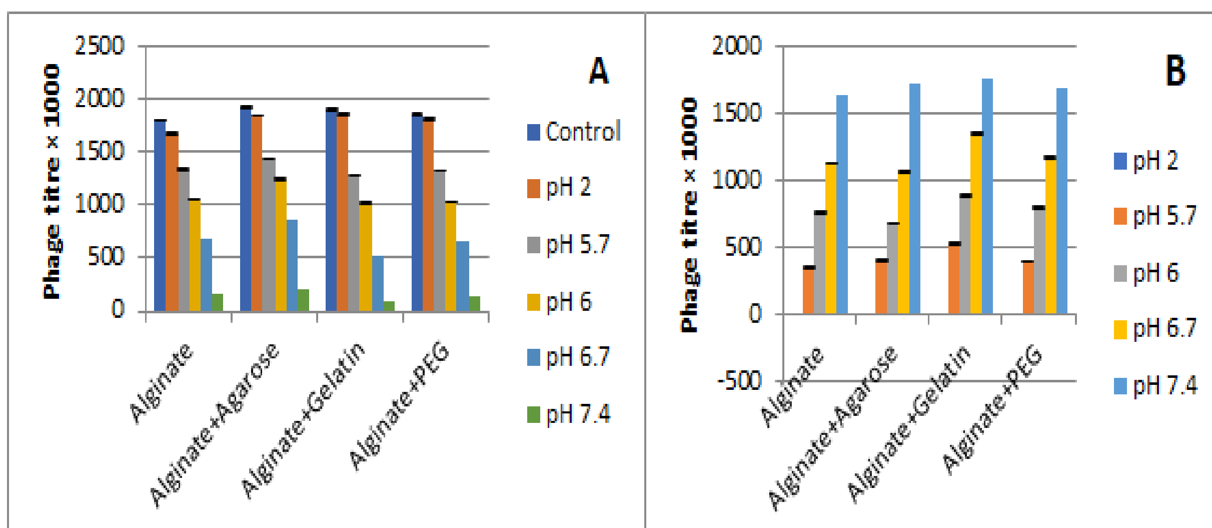

Fig.4.14. (A). Entrapped PAA phages at gastronomic conditions. (B). PAA phages released at gastronomic conditions

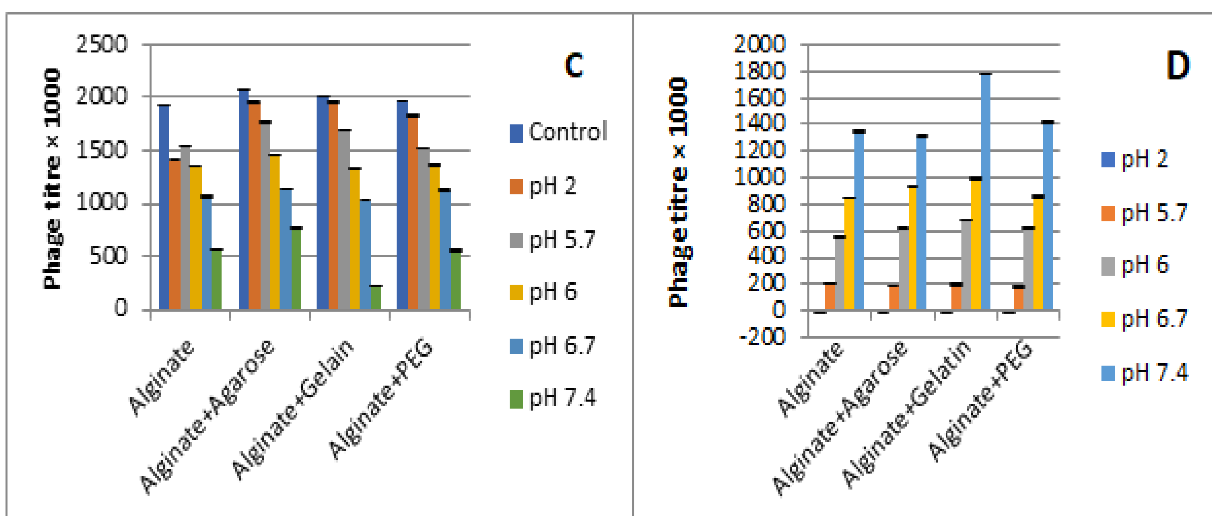

Fig.4.14. (C) Entrapped PAM phages at gastronomic conditions. (D)- PAM phages released at gastronomic conditions

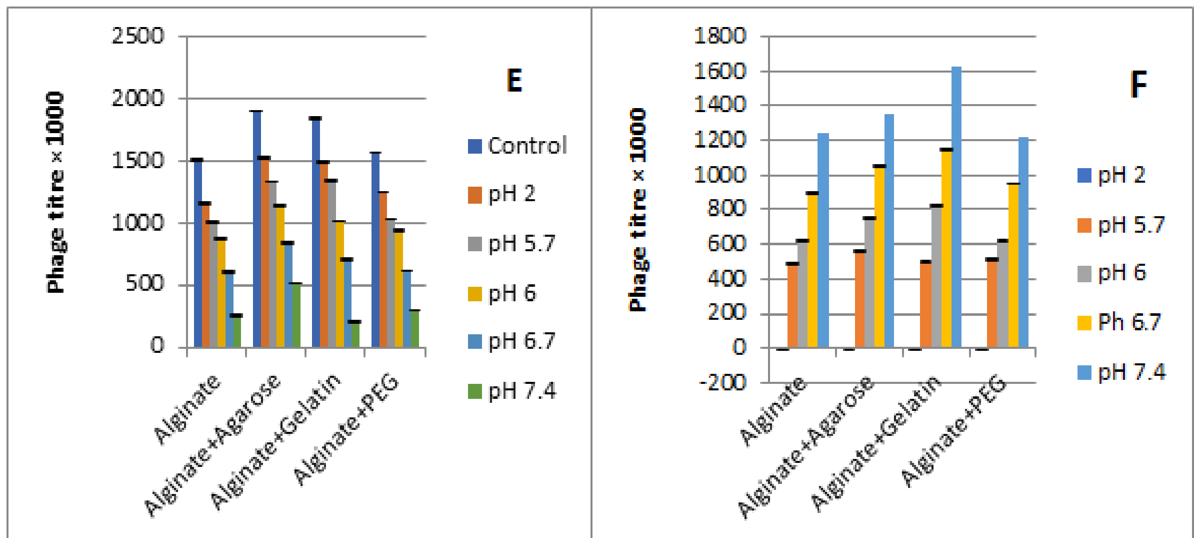

Fig.4.14. (E). Entrapped PAR phages at gastronomic conditions. (F). PAR phages released at gastronomic conditions

4.16. Stability of free in gastric fluid (pepsin) and simulated intestinal fluid (pancreatin)

As hypothesized, microencapsulated phages are deemed to be more stable in SGF since the activity of free phages is rapidly eliminated by stomach acid. To evaluate the effect, both free and microencapsulated phages were exposed to SGF. According to the findings, the free phages PAA, PAM and PAR were susceptible to this acidic environment and were mostly rendered inactive after 15 or 30 minutes of incubation in SGF at pH 2 to 2.4. The viability of non-encapsulated PAA phages at pepsin were observed to decrease by 1.8 log 2 PFU/ ml after in comparison to the control that was 9.2 log9 PFU/ml, indicating that the gastric acid efficiently denatures the proteinaceous capsid of phages, ultimately destroying them. The viability of free PAA phages was then observed at pancreatin and the titers here were slightly reduced to 7.21 log 9 PFU/ml in comparison to the control. Similarly, PAM displayed a reduction of 1.4 log 5 PFU/ml and 1.76 log 10 PFU/ml at pepsin and pancreatin respectively in contrast to the phage titer of experimental control of 2.40 log 12 PFU/ml. However in the case of PAR phage, reduction in phage titer was observed to be 1.4 log 2 at pepsin and 2.87 log 8 PFU/ml at pancreatin in comparison to the 3.8 log 9 PFU/ml phage titer of the control.

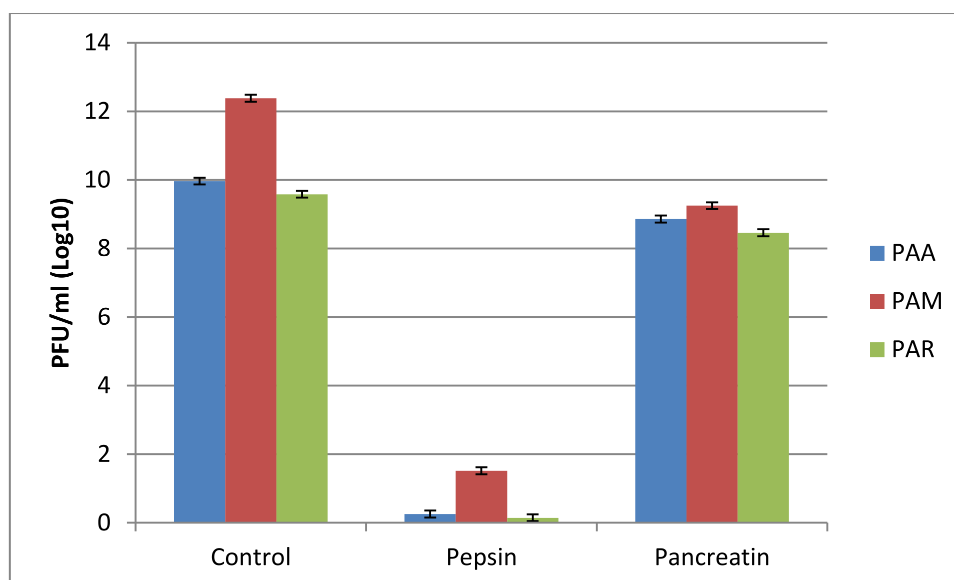

Fig.4.15. Represents the stability of free phages in pepsin and pancreatin

4.17. Stability of Entrapped phages at gastronomic conditions; Entrapment and Release

The stability of the bead-encapsulated bacteriophages was evaluated at pepsin and pancreatin. The viability of encapsulated bacteriophages at pepsin were observed to be significantly high, entrapped within the micro-particles indicating stability, however, the titres of the entrapped phages were slightly reduced in pancreatin. The release of phages from the polymeric capsules was also observed. No phage was released at pH 2 indicating their instability in the gastric environment. The release of phages was steadily improved and was observed to be maximum at pancreatin. The findings suggested that the titre of the liberated phages remained steady in SIF without experiencing any substantial loss. The liberated phages were therefore well tolerant to SIF. The microspheres started to swell and break apart when the microencapsulated phages were added to SIF, and the release rate stabilised following the burst effect. The considerable high titre of the entrapped phages suggests that these were stable in SIF before being released. As a result, the outcome showed that all microencapsulated phages were likely to release in SIF.

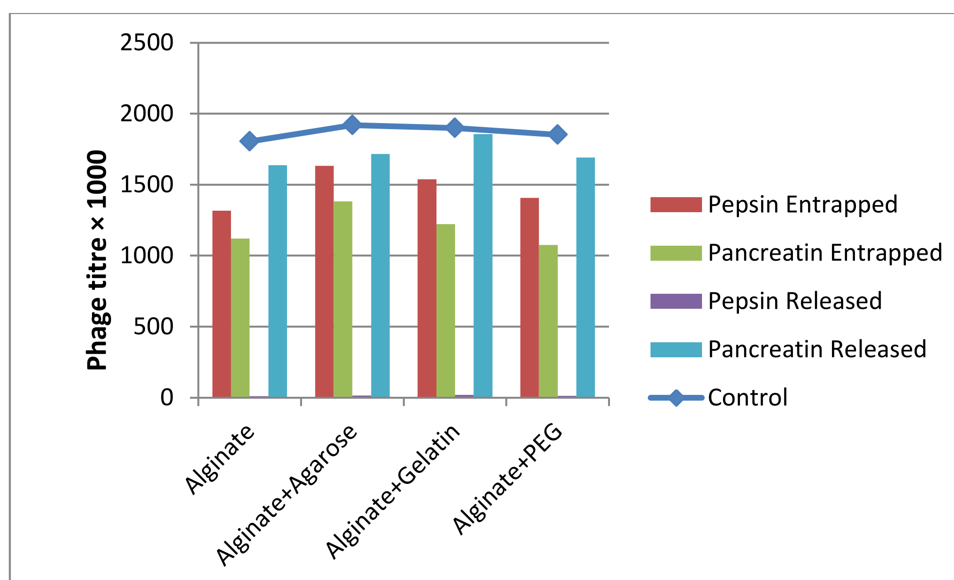

Fig.4.16. Represents the entrapment and release of PAA phages in pepsin and pancreatin

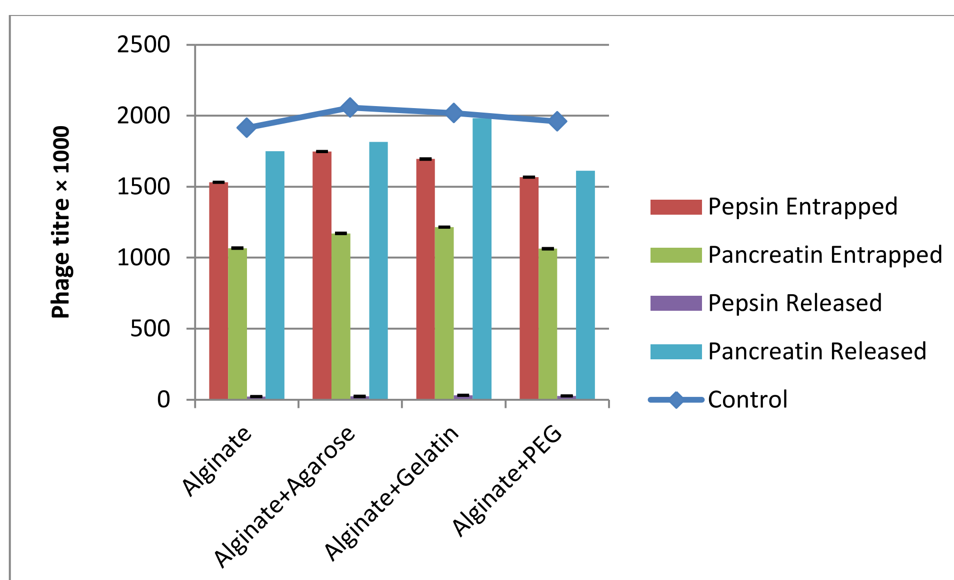

Fig.4.17. Represents the entrapment and release of PAM phages in pepsin and pancreatin

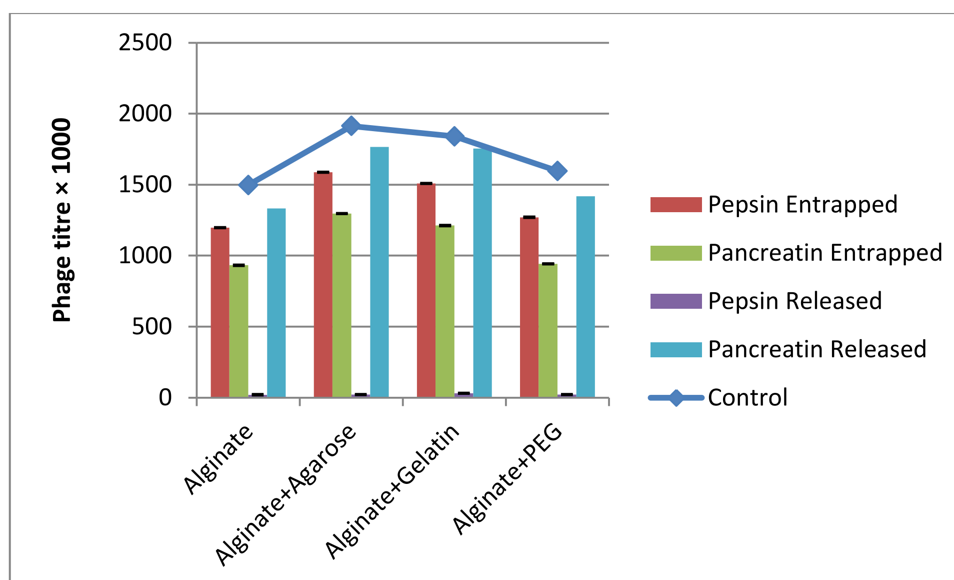

Fig.4.18. Represents the entrapment and release of PAR phages in pepsin and pancreatin

The Alginate-agarose based capsules displayed efficient entrapment and remarkable stability of PAA, PAM and PAR phages in SGF and SIF, respectively, rendering them to be the most desired polymeric combination to synthesize microcapsules. However, remarkable release of phages was observed from the alginate-gelatin capsule in intestinal environment to treat gastrointestinal ailments.

Discussion

Multi-Drug Resistant (MDR) *Pseudomonas aeruginosa* causes disease with an elevated mortality rate as a result of resilience to many antibacterial drugs. With varying degrees of virulence, this bacterium causes significant organ damage, and the development of its biofilm causes recurrent infections that are antibiotic-resistant. One of the unique therapeutic approaches that have been suggested as a result of antibiotics' incapability to remove *P. aeruginosa* biofilm is phage therapy. Importantly, research has shown that using antibiotics in addition to this complementary therapy increases its effectiveness. Utilization of phage therapy is beneficial as phages appear to be equally efficient against bacteria that are susceptible to antibiotics and resistant to them, have low intrinsic toxicities, and can effectively dislodge bacterial biofilms. They also tend to cause minimum disruption of normal flora (Taati Moghadam et al., 2020).

This research aimed to study the synergistic effect of isolated bacteriophages with commercially available antibiotics and the efficient oral delivery of phages to gastrointestinal tract for therapeutic effects. From the sample of sewage waste, potent bacteriophages known as PAA, PAM and PAR were isolated. In a previous study, vB_Pae_AM.P2 phage, from wastewater in Kerala, India was isolated against several MDR clinical *pseudomonas aeruginosa* isolates. Samples of sewage contain a sizable amount of organic materials and microbiological life. Globally popular rivers are thought to be hotspots for phage reservoir and gene transfer events that result in antibiotic resistance. Electron miocrographs have revealed that these phages belong to the *Siphoviridae* family of order caudovirales. Nouran Rezk in 2022 reported that 58% of the examined clinical isolates of the antibiotic-resistant *P. aeruginosa* were susceptible to infection by ZCPA1 phage, a member of the Siphoviridae family (Taati Moghadam et al., 2020).

Sub-lethal quantities of some antibiotics have been discovered to stimulate the development of some aggressive phage by host bacterial cells, a phenomenon known as phage-antibiotic synergy (PAS), which aids in the prevention and treatment of infections. The property of antibiotics, which prevent bacterial cell division and activate the SOS system, which is the driver of DNA repair, increased mutagenesis, and prophase induction, is the basis for the most plausible mode of action. As a result,

bacterial cells may have a greater capacity for biomass and biosynthesis, which pathogenic phages could employ to enhance their own production. Additionally, the existence and impact of these medications can hasten the killing of infected bacteria, enabling the phages to propagate more rapidly (Menon et al., 2021).

In the present study, we assessed the various classes of antibiotics (13 antibiotics) against *P. aeruginosa* to ascertain the bacteria's susceptibility to different antibiotics. Our study revealed that *pseudomonas aeruginosa* displayed sensitivity only against three antibiotics, gentamicin (protein synthesis inhibitor), cefepime and meropenem (cell wall synthesis inhibitors). The mentioned three antibiotics were selected for further testing their inhibitory effect on host bacterium individually and in combination with phages (PAA, PAM and PAR).

In our study, as per the 2014 CLSI breakpoints, the planktonic isolate of pseudomonas aeruginosa and its biofilm were sensitive to the antibiotics gentamicin 16 µg/ml (8µg), cefepime 16 µg/ml (16 µg), and meropenem 4 µg/ml (2 µg) in our study. This is a reflection of the widespread overuse of antibiotics that has resulted in such a high degree of resistance. Such susceptibility was reported by Menon et al while evaluating the PAS effect against *Citrobacter amalonaticus* (Menon et al., 2021).

Further we determined the stability of phages with the selected antibiotics to understand the PAS phenomenon. No harmful effect or change in phage plaque size was determined. Contrary to our findings Ryan et al reported that synergy between cefotaxime and *E.coli* T4 phage resulted in significant changes in plaque size and burst time of the phage. Furthermore, Kokab et al stated in their findings that antibiotics could be majorly responsible for enhancing the phage burst size ultimately resulting in increased lytic activity of the phages against the host bacterium (Kokab et al., 2022).

The inhibitory effect of phages with varying MOI on bacterial host turbidity reduction was evaluated. It was observed, that at higher MOI 10 of each phage, no viable bacterial cells were determined i.e. 0 cells as compared to control. The optimal MOI was for each phage was deduced as MOI 0.01 of PAA, PAR and MOI 1 of PAM. Similar findings were reported by Ling et al that the shorter latency period and inhibited host growth demonstrate that the phage load at MOIs of 0.01 was more

successful against the host bacterium *Aeromonas salmonicida* than those at higher MOIs (Ling et al,. 2022).

However, it is widely recognised that bacteria may swiftly adapt and develop new survival strategies, but the rise of phage-resistant phenotypes is unavoidable regardless the intriguing potential of phages as antimicrobial agents. As a result, combining phage and antibiotic therapy could turn out more advantageous than utilising phages or antibiotics individually.

The results of the present study demonstrated that the association of phages at MOI 1 and 0.1 with the sub-MIC values of gentamicin on *P. aeruginosa* strains, in their planktonic state, was statistically significant. However the combination of phage cocktail at sub-inhibitory concentrations of gentamicin displayed remarkable synergy resulting in elimination of myriad of bacterial cells. In the case of cefepime–phage treatment complete elimination of bacterial cells was noted as compared to the antibiotic and phages alone. Strong synergistic effect was observed in the case of phage cocktail which led to complete killing of bacteria in combination with 1/2, 1/4 and 1/6 of the MIC of cefepime. Bacterial viability was only observed in 1/8 of MIC of cefepime with phage cocktail which hence presenting strong synergism. Interestingly, Meropenem with PAA, PAM and PAR phage had a synergistic activity resulting in absolute extermination of bacterial cells in contrast to the control. Hagens et al. observed results that were similar to ours upon investigating the effects of phage infection along with low concentrations of antibiotics on *P. aeruginosa* strains. They discovered that this combination was capable of killing or inhibiting the growth of *P. aeruginosa* and the phage infection significantly decreased tetracycline resistance (Hagens et al,. 2021).

These results can be attributed to the fact that the outer cell membrane, which in *P. aeruginosa* is strengthened by the active efflux system, serves as the primary barrier to antibiotics in Gram-negative bacteria. This obstacle to the penetration of antibiotics in the bacterial cell, however, might be less effective during filamentous phage progeny expulsion. The second is the filamentation of cells in the presence of sub-inhibitory doses of beta lactams. This was clarified through the fact that, despite having various modes of action, these antibiotics ultimately prevent bacterial cell division. Phage antibiotic synergism, according to Comeau et al., is caused by a

modification in morphology that enables quicker assembly of phages through modified or bigger pools of progenitors crucial to phage maturation and enhances the commencement of cell lysis (Comeau et al., 2016). To interpret the results, it is also necessary to take into account how antibiotics affect bacterial cells. Although peptidoglycan synthesis is not required for phage multiplication, Podoviridae and Siphoviridae families require functioning bacterial ribosomes, integrity of cell membrane and DNA gyrase in order to multiply successfully. We can infer that the criteria for synergy include cell elongation and a specific mechanism of action of antibiotics.

The present study analysed the potential impacts of phage-antibiotic combinations on bacterial cells buried in biofilms were identified. The results of this study demonstrate that, for all phage-antibiotic combinations, the biofilm is diminished at high antibiotic concentrations to varied degrees and depending on the antibiotic. In the treatment of the *pseudomonal* biofilm with gentamicin, cefepime and meropenem in combination with phages displayed remarkable eradication. Maximal synergy was observed in the combinatorial treatment of phage cocktail with antibiotics. A number of reasons, including changed charges on the surface of phage resistant phenotypes and degradation of the biofilm matrix caused by certain of the phages, which can promote antibiotic permeation, can be used to explain the efficiency of the combination treatment of phages and antibiotics on *P. aeruginosa* biofilm. According to Bowler et al., meropenem administration significantly increased the amount of beta-lactamase synthesis in developed biofilm and reduced it in all planktonic bacteria after 24 hours (Bowler et al., 2006).

A stronger penetration of both antibiotics and phages into the biofilm has been proposed as the explanation for the antibacterial synergy between phages and antibiotics. According to reports, phages may employ depolymerases to break down the biofilm matrix, which facilitates their entry into the biofilm's deeper layers. Additionally, it has been suggested that phages can enter the biofilm's lowermost regions by moving via its vacuum spaces. Phages begin multiplying in the deeper layer of the biofilm as a result of this occurrence, attaining large titres and disrupting the biofilm matrix. Following this interruption in the administration of antibiotics

leads to an improved bacterial reduction as a result of these drugs' more profound absorption (Ling et al., 2022).

In the presence of acid, heat, chemical solvents, and the lack of hydration, viruses (including phages) rapidly lose their ability to function. The most popular techniques for phage protection against harmful circumstances are encapsulation technologies. The frequently used procedures and methods for protecting microorganisms include extrusion dropping techniques; spray drying, emulsion and polymerization processes. The extrusion dropping method has been employed in this investigation since it is a relatively simple way which facilitates the synthesis of microcapsules. Additionally, gentle preparation conditions were implemented and the thus produced microcapsules were all precisely the same size. The most frequently utilized biomaterials for phage encapsulation include cellulose, liposomes, alginate, whey protein, gelatin, agarose and polyethylene glycol and have been utilized via various approaches (Ling et al,. 2022).

In the presently conducted research, the viability of free phages was tested at various gastronomic pH. It was observed that the phages became highly inactive at pH 2 (gastric pH) and the viability increased and phages became stable with the increase in pH. These results were in accordance to the study conducted by Yin et al, 2021, where the phages against *E. coli* O157:H7 rendered instability at gastric pH (pH 2) and the stability subsequently increased with the increase in pH. Acidic pH is known to denature the proteinaceous coat (capsid) surrounding phages, ultimately rendering phages to be inactive at low pH ranges (Yin et al, 2021).

In the present study, alginate was used as the principle material for manufacturing the microcapsules in combination to various other polymeric substances, including agarose, gelatin and PEG. The mild gel approach adopted by sodium alginate produces an excellent microencapsulation material with excellent biocompatibility and low toxicology. Furthermore, sodium alginate has a defined pH sensitivity that renders it optimal for surrounding oral phages since its molecular chain contracts at low pH and expands at high pH. Ca2+ and sodium alginate may interact to produce a gel. The identified "egg-box" structure is formed by the interaction of four coordination bonds between Ca2+ and two GG fragments on the sodium alginate molecular chain. This structure causes the neighbouring sodium alginate molecular

chain to change from arbitrarily lined groups into curved band structures that are cross-linked by Ca2+ and intertwine into an intricate three-dimensional gel system. The shape, rigidity, and complexity of generating microencapsulated phages are all influenced by the sodium alginate content in addition to the encapsulation efficiency of the microencapsulated phages. The formation of a thin microcapsule membrane with a soft texture and an irregular shape that is readily damaged occurs when the concentration of sodium alginate is too low for it to fully react with CaCl2. This results in low encapsulation efficiency. The efficiency of encapsulation improves as sodium alginate concentration rises (Yin et al., 2021). The solution's viscosity increases as the sodium alginate concentration exceeds 3%, making it difficult to dispense the solution through the syringe's holes and producing irregularly shaped microcapsules. This is in accordance to the work performed by Abdelsattar et al, on *E. coli* phage ZCEC5 encapsulated in chitosan–alginate beads with 1.5 to 2% concentration of sodium alginate (Abdelsattar et al., 2021)

The entrapment efficiency of each bio-polymeric microcapsule was assessed. It was observed that Alginate-Agarose based capsules were a highly efficient medium for entrapping the phages. The combination was beneficial as alginate gel is porous and allows the diffusion of acid through the gel hence resulting in complete loss of phages activity. Therefore if blended with another polymer, the efficiency of the gel can be increased resulting in stability of phages in harsh acidic environment. The mechanical properties of a bio-composite hydrogel made of alginate and agarose are significantly superior than those of pure agarose or alginate individually. Comparative results from a different investigation additionally demonstrate that alginate and agarose constituents in the hydrogel structure contribute to the development of improved 3D structures with increased uniformity in permeability and interconnectivity, as well as the production of observable pores. The mechanical properties of a bio-composite hydrogel made of alginate and agarose are significantly superior than those of pure agarose or alginate individually. Xiang et al. 2022 previously investigated the potential of alginate-agarose hydrogels as an encapsulating material (Xiang et al, 2022).

Furthermore, the potential stability and activity of encapsulated phages was assessed at the various gastronomic conditions in the current study. It was observed that the

encapsulated phages were exceptionally stable and showed remarkable activity at gastric pH 2. With the elevation in pH ranges, the phage titre within the encapsulated coating kept on decreasing signifying the efficient release of phages at the target site. This is due to the fact that alginate-agarose as a carrier has the potential to prevent the phage from being enzymatically degraded, comparatively to the simple solution of free phage that is degraded in vitro at pH 1-3. The pattern of release was discerned to be most likely caused by the agarose matrix swelling, which encourages the protonation of their amino groups under the acidic state, causing the phage to disintegrate and gradually diffuse from the micro-polymeric network. These outcomes were in conformity with the analysis performed by Kaikabo et al and Rahimzadeh et al (kaikabo et al., 2021) (Rahimzadeh et al, 2021).

As aforementioned, gastric pH substantially reduces the viability of free phages. Hence the effect of pepsin was determined on free and entrapped phages. As per our results, free phages were completely destroyed by the action of proteolytic enzyme, however the encapsulated phages remained entrapped and stable within the enteric coating. Similar results were reported by Lin et al in their investigation to assess the stability of free and encapsulated phages in pepsin. Zhang et al also demonstrated the remarkable viability of encapsulated phages while performing the oral treatment of salmonella infection in chicks (Zhang et al., 2020). The alginate-agarose gel responds to changes in pH. On the surface of the microspheres, the formed alginate gel that is water-insoluble and known as the alginate shell. While the embedded particles are protected from gastric acid and digestive enzymes when the pH is acidic, the gel structure swells and releases its contents when the pH is neutral in the colonic environment. Solutions and tiny pellets (less than 0.02g in size) drain from the stomach relatively quickly and are not significantly impacted by individual digestive conditions, according to a published study. In vivo, the half-emptying period for liquid and pellet systems is typically between 0.5 and 1.5 hours. According to another study, the passage time from a healthy person's small intestine is 3–4 hours on average and up to 6 hours at the most. Since the microencapsulated phages in the current study possessed a dry weight of less than 0.02 g, they could be promptly emptied from the stomach. Additionally, sodium alginate can contract in the stomach to shield the phages from destruction triggered by gastric acid and digestive enzymes,

thereby ensuring the activity of the microencapsulated phages was not impeded at pH 2 or 2.4.

Liver produces bile acids, which accumulate in the gallbladder until it is sufficient for them to be released into the duodenum in substantial amounts. The oral phage has to transit through the duodenum with an elevated amount of bile acids after its passage through gastric acid. Our findings demonstrated that microencapsulation considerably increased the stability of phages in bile salts. Bile salts have considerably less impact on the action of free phages as compared to the effect of stomach acid. This may be because phages lack lipid elements and are therefore resistant to amphoteric substances like bile salts. These results were in conformity with the studies reported by Yin et al and Zhang et al (Yin et al., 2023) (Zhang et al., 2021).

In addition to keeping up with activity in bile salts and gastric acid, microencapsulated phages also need to be promptly released into the intestinal tract. Before examining the stability of the microencapsulated phage, we evaluated the stability of the free phages PAA, PAM, and PAR in SIF. When the microencapsulated phages were released into SIF during the course of the current investigation, the results revealed that the liberated phages had strong resistance to SIF, assuring their stability. According to our studies, the microsphere covering material must have completely reacted with intestinal fluid, causing a burst effect and the quick release of all phages. Such was also reported by Ramirez et al, where they determined the subsequent release of the encapsulated phages in pancreatin, in, in vitro studies (Ramirez et al, 2019).

Thus, our research suggests that phage microencapsulation can increase phage activity in vivo and increase their resistance in the gastrointestinal tract, which might enhance the effectiveness of phage therapy as an alternative to antibiotics, despite the fact that there are still many issues and challenges with phage therapy, particularly in gastrointestinal therapy.

Conclusion

In conclusion, the study's findings demonstrated that treating our potential phages with antibiotics simultaneously had a synergistic effect in *Pseudomonas Aeruginosa* growth. The PAS effect can therefore be utilised as a potential substitute to control antibiotic-resistant bacteria at decreased drug concentrations and to lessen the establishment of antibiotic and phage resistance, according to the research's most significant conclusion. The mechanism of the PAS effect needs to be better understood, though, in order to create new, successful antimicrobial approaches to combating antibiotic-resistant bacteria.

Future treatment plans for infections brought on by *P. aeruginosa* may be developed using phage-antibiotic synergism and related antibiotics that meet the requirements for therapeutic application. Additional benefits of this strategy include the quick onset of synergism, which may help lower the number of bacteria in vivo to levels that the immune system can handle, and the avoidance of antibiotic side effects associated with high doses of medication by using sub-inhibitory concentrations. Finally, it is important to consider the reported phenomenon's potential applicability in the management of *P. aeruginosa* in addition to further research aimed at better understanding the mechanisms underlying rapid synergism.

Furthermore, our findings suggested that encapsulation is an efficient way to deliver phages to gastrointestinal tract. Alginate-Agarose microencapsulated phage microspheres showed considerably higher survival rates than free phage under simulated gastrointestinal settings and more effective phage release in simulated intestinal fluid.Phages' efficacy in oral therapeutic applications can be enhanced by the present encapsulation technique. An effective substitute for antibiotics is revolutionary microencapsulated phage treatment.

Future Prospects

The future prospective of this research study constitutes of,

- To understand the underlying mechanism of PAS phenomenon, by determining the effect of antibiotic mode of action on phages viability and activity.
- Evaluating the molecular basis of mechanisms involved in developing a synergistic effect between phages and antibiotics.
- Further research is required to employ these phage cocktails as phage therapy before the commercialization of phage-based products based on the combination of bacteriophages with antibiotics.
- Exploration of other biocompatible material to carry out the enteric coating of bacteriophages.
- Thermal stability of phages while encapsulated in different bio-composite materials could be evaluated for efficient administration in human body.
- Storage and Shelf life of these encapsulated products is to be assessed for long term use and efficacy.

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