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Isolation, Characterization, and Application of *Microbacterium paraoxydans* Bacteriophages in Milk Model

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By:

Samantha Proctor-Coplen

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Glossary

Additive – a substance added to something in small quantities to improve or preserve it

Antibiotic resistance – occurs when microorganisms such as bacteria and fungi develop the ability to defeat the drugs designed to kill them

Antibiotics – a medicine (such as penicillin or its derivatives) that inhibits the growth of or destroys microorganisms

Bacteriophage – a virus that parasitizes a bacterium by infecting it and reproducing inside it

Bacteriophage therapy – uses viruses to treat bacterial infections

Biopolymer –natural polymers produced by the cells of living organisms. Biopolymers consist of monomeric units that are covalently bonded to form larger molecules.

Electrospinning – a fiber production method that uses electric force to draw charged threads of polymer solutions or polymer melts up to fiber diameters in the order of some hundred nanometers

Endolysin – enzymes used by bacteriophages at the end of their replication cycle to degrade the peptidoglycan of the bacterial host from within, resulting in cell lysis and release of progeny virions

Fermentation – the chemical breakdown of a substance by bacteria, yeasts, or other microorganisms, typically involving effervescence and the giving off of the heat

Food safety – assessment, management, and communication of health risks associated with chemical and biological contaminants in food

Green Revolution –a great increase in the production of food grains (especially wheat and rice) that resulted in large part from the introduction into developing countries of new, high-yielding varieties, beginning in the mid-20th century

Human pathogen – an organism liable to cause human disease

Hydrophilicity – having an affinity for water, readily absorbing or dissolving in water

Hydrocolloid – a substance which forms a gel in the presence of water, examples of which are used in surgical dressings and in various industrial applications

Hydrogel – a gel in which the liquid component is water. A hydrogel is a crosslinked hydrophilic polymer that does not dissolve in water. They are highly absorbent yet maintain well defined structures.

Hydrophobicity – repelling tending not to combine with, or incapable of dissolving in water

Industrial Revolution – the process of change from an agrarian and handicraft economy to one dominated by industry and machine manufacturing

Lysogen – a bacterial cell or strain that has been infected with a temperate virus

Lytic phage – the phage takes over the machinery of the bacterial cell to make phage components; they destroy and lyse the cell, releasing new phage particles

McFarland – standards used in microbiology as a reference to adjust the turbidity of bacterial suspensions so that the number of bacteria will be within a given range to standardize microbial testing

Microbe – a microorganism, especially a bacterium causing disease or fermentation

Microliter – a unit of volume that represents one millionth of a liter

Milliliter – a unit of volume that represents one thousandth of a liter

Multiplicity of Infection – the ratio of agents (phages) to infection targets (bacterial cell)

Plaque – a clearing of bacterial growth formed by infection of one bacterium by a single virus, which then progresses in a circular pattern

Plaque forming unit – a measure used in virology to describe the number of virus particles capable of forming plaques per unit volume

Polycaprolactone – a biodegradable polyester with a low melting point of around 60°C

Polymer – a substance that has a molecular structure consisting chiefly or entirely of a large number of similar units bonded together

Pure Drug and Food Act and Meat Inspection Act of 1906 –a federal law that mandates for the inspection of meat products and forbids the sales, manufacturing or transportation of poisonous patent medicines and adulterated food

Purification – removal of contaminants

Temperate phage – a phage capable of replicating through the lysogenic cycle

Titer – the concentration of viruses expressed as infectious units per mL

Abbreviations

API 50CH (tests to study bacterial growth on different carbohydrates)- apiapi strips

API ZYM (detects bacterial enzymes)- api enzyme strips

ARG- Antibiotic resistant genes

CFU- Colony forming units

CRISPR- clustered regularly interspaced short palindromic repeats

DNA- Deoxyribonucleic acid

GRAS- Generally recognized as safe

PFU- Plaque forming units

RNA- Ribonucleic acid

WHO- World Health Organization

Abstract of Thesis

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TITLE OF THESIS: Bacteriophages and Food Safety

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ABSTRACT: Throughout history, humans have consistently implemented different strategies to prevent food-associated illnesses. Subsequent to our multiple technological advances, food safety is still a serious issue of concern. Overuse of antibiotics has diminished their efficacy for food preservation and safety, and there is a demand for natural (no preservatives) to less processed and naturally preserved food. Food bio-preservation includes the use of natural antimicrobials already present in the food. It has a long history of safe consumption and is seen as a plausible strategy to reduce the intense use of chemically synthesized food preservatives. Bacteriophages are antimicrobials that occur abundantly and naturally in foods, and can be isolated and purified from uncooked/raw/fresh products such as chicken and beef in conjunction with processed foods, seafood, and fermented products including yogurt and cheese.

To date, the combination use of several antimicrobial strategies, known as hurdle technology, has often been implemented as a means of improving the efficacy of food

bio-preservation. This research explores microbacteriophages and their possible ability to extend food safety and shelf life by killing *Microbacterium* spp. and reduce the risks associated with the presence of foodborne bacteria within the food chain. Bacteriophages have only started to be assessed as potential food bio-preservatives more recently and this research may support the implementation of bacteriophages for food safety. To test this potential, the phages lytic ability was tested in a milk model and in a sodium alginate hydrogel/PCL fiber matrix. Results showed that phages were successfully incorporated into the sodium alginate, as evidenced by their viability and lytic capability, and effectively reduced bacterial counts in a milk model. This constitutes a simple approach to antimicrobial control along the entire food chain in the food industry for the prevention of microbial spoilage.

CHAPTER 1: INTRODUCTION

1.1 FOOD SAFETY- Food safety encompasses the assessment, management, and communication of health risks associated with chemical and biological contaminants of food (Madison et al., 2022; Wu and Rodricks, 2020). Beginning in the late 19th century, with advances in the science of chemical synthesis, a wide variety of new chemical entities have emerged. Processes such as fermentation, cooking, and smoking introduce large numbers of diverse chemical entities, and plant and animal breeding create new varieties that alter the chemical composition of foods (Maske et al., 2021). There will always be opportunities for food contamination by microbes and chemicals naturally occurring in the environment and, subsequent to the industrial revolution, chemical products of industry (Zhang et al., 2022). A number of microbiological agents that are human pathogens may find their way into foods as well (Paczesny and Mierzejewski, 2021; Wu and Rodricks, 2020). Subsequent to our understanding of how to avoid these agents by proper handling and processing of susceptible foods, antibiotic resistance continues to emerge in foodborne bacteria, and contamination—often leading to illnesses and sometimes death—can result (Wu and Rodricks, 2020.)

The various federal laws under which food safety is regulated focus on contaminants and chemicals intentionally introduced (Venturini et al., 2022). Intentionally introduced substances are additives used to affect in some beneficial way the technical characteristics of the treated food (Lu et al., 2022). Federal law has safety requirements for a portion of the enormous numbers of chemicals that are natural

components of food: those that are known to cause adverse effects to human health (such as aflatoxin), by lowering maximum tolerable levels in food (Wu and Rodricks, 2020). Such components are, in the absence of specific safety related information, generally considered to be safe for consumption, although regulation can occur should evidence of undue risk arise for any specific member of this class of food substances (Storms et al., 2020).

1.2 HISTORY OF FOOD SAFETY IN UNITED STATES- Prior to the creation of the Pure Food and Drug Act and the Meat Inspection Act in 1906, concerns had arisen in the previous three decades about chemicals added to food without prior safety testing, and abysmal worker and food safety conditions in food processing facilities (Wu and Butz, 2004). In the first decades of regulating U.S. food safety, the FDA focused largely on evaluating the acceptability of chemical additives (Storms et al., 2020). A multitude of events led to dramatic increases in food production worldwide that is commonly termed the *Green Revolution*: the proliferation and global use of agricultural chemicals to fertilize soil and to control pests and weeds, post-WWII global security needs, and the rush to produce food more efficiently all over the world (Le et al., 2021; Wu and Butz, 2004). Irrigation technologies were improved for more reliable water availability to crops (Perkins, 1997). The U.S. government agencies in collaboration with private foundations, and later other international governing bodies, provided the funding that allowed these agricultural improvements to be disseminated worldwide (Perkins, 1997). While these, then new, agricultural chemicals effectively increased food production in many parts of the world and were a safety improvement over the arsenical pesticides

that preceded them, they carried their own set of safety concerns (Perkins, 1997). To continue food safety, the United States and other countries formed multiple international expert committees on food additives and microbial food risk assessment (Shang et al., 2021; Wu and Butz, 2004).

1.3 FOOD SAFETY RESEARCH IN RECENT YEARS- From a global perspective, how much does unsafe food contribute to human disease; specifically, the contribution of food contaminants? Beginning in 2006, WHO (World Health Organization) established the Foodborne Disease Burden Epidemiology Reference Group (FERG) to estimate the global burden of disease caused by food contaminants of multiple types: viruses, bacteria, parasites, and chemicals and toxins. (Dasgupta et al., 2022).

Among the findings are the following: Every year, roughly 1 in 10 people worldwide become ill from foodborne disease (Havelaar et al., 2015). In total, this means 33 million healthy life years lost (disability-adjusted life years: DALYs) and 420,000 deaths per year (Wu and Rodricks, 2020). One third of the foodborne disease-related deaths are in young children (Havelaar et al., 2015). Diarrheal diseases make up the largest cause of foodborne disease burden, and are caused primarily by nontyphoidal *Salmonella, Escherichia coli*, norovirus, and *Campylobacter* in food (Havelaar et al., 2015). Among parasites and chemicals/toxins, *Taenia solium* (pork tapeworm) and aflatoxin (mycotoxin produced by *Aspergillus* fungi in maize and nuts) caused the greatest disease burdens for their respective categories (Havelaar et al., 2015, WHO 2020a,b,c).

Interagency Food Safety Analytics Collaboration estimates that meat and poultry products are vectors for 30.9% of all foodborne illnesses (Scharff, 2020). This translates into 2.9 million annual illnesses, yielding economic costs of up to $10.9 billion (Scharff, 2020). The costliest food-pathogen pairs include *Campylobacter* spp. in poultry ($6.9 billion), *Salmonella* spp. in chicken and pork ($2.8 and $1.9 billion, respectively), and *Toxoplasma gondii* in pork ($1.9 billion) (Scharff, 2020). Results based on alternative attribution and economic model assumptions are also presented, generating meat and poultry attribution estimates ranging from 27.1 to 36.7% and economic costs of $8.1 to $22.5 billion (Scharff, 2020). In the United States, they have estimated that, annually, 31 foodborne pathogens cause about 9.4 million episodes of foodborne illness across the country (Scallan et al., 2011; Scharff, 2020). Milk spoilage accounts for over $6 billion in monetary losses per year, with spore-forming bacteria a major contributor (Scharff, 2020). Examining costs across a greater span of pathogens, Scharff (2020) estimates an annual loss in the United States of up to $20.3 billion.

1.4 HYDROGELS- The term gel refers to a semi-solid intermediate that is engineered to retain liquid-like (flowing) and solid-like (a finite elastic modulus) rheological behaviors when gel–sol transition takes place (Renard et al., 2006; Zha, 2021). Gels usually consist of three-dimensional polymeric networks of fibrous/chain or globular macromolecules, which tend to encapsulate considerable proportions of dispersing mediums such as water (hydrogels), gas (aerogels), or oil (oleogels) (Zha, 2021; Scharff, 2020). In the food industry, gelling is an effective technique facilitating food hydrogel design whereby the textural and sensorial properties of end-products, as

well as flavor release in the food matrix, can be amended (Renard et al., 2006; Zha, 2021). Numerous products are presented in the form of gels such as jam, jelly, confectionery products, desserts, quick-set gels, and so on. Gelling agents (gellants) are the basis of food hydrogels, most of which are natural biopolymers mainly deriving from dairy, fish, meat, and microorganisms. Structure variances of those hydrogels that existed in disparate sources conceivably prompt an enormous effect on designing hydrogel molecular structures with predictable functions (Renard et al., 2006; Zha, 2021). In the last decade, progress has been made in the design of food hydrogels using both animal-sourced protein (casein, gelatin, and egg white proteins) and microorganisms polysaccharides (agar, carrageenan, gellan gums, and xanthan) as gellants.

Currently, there is an urgent demand for the utilization of low cost, sustainable, non-toxic, and abundant natural gellants in the development of food hydrogel. Plant-based biopolymers, including plant proteins, starch, and polysaccharides (gums), are considered as credible as functional building blocks for various food systems (Zha, 2021).

Hydrogels are a class of materials with a three-dimensional hydrophilic network and are created by cross-linked polymer chains in an aqueous matrix (Khalesi, 2020). They can be sourced fundamentally from synthetic or natural polymers. Hydrogels have the potential to hold large amounts of water in their structure and still retain the properties of solid materials. (Khalesi, 2020; Zha, 2021). Hydrophilic functional groups such as amino, carboxyl, and hydroxyl groups in the backbone of polymer chains allow

water absorption in gel networks (Zha, 2021). Cross-links between polymer chains (physical or chemical links) assist in the maintenance of hydrogel networks (Khalesi, 2020). Therefore, structure, viscoelasticity, and water holding capacity of hydrogels depend on the type of polymers as well as the type and intensity of cross-links between polymer chains (Zha, 2021; Khalesi, 2020). The high-water content of hydrogels enables them to simulate the flexibility of natural tissues. The widespread application of hydrogels in various sectors including food, biochemical, pharmaceutical, cosmetic, and textile have encouraged researchers to study these materials (Khalesi, 2020; Zha, 2021).

1.5 SODIUM ALGINATE HYDROGEL- Sodium alginate is a cell wall component of marine brown algae and contains approximately 30 to 60% alginic acid (dos Santos, 2019). The conversion of alginic acid to sodium alginate allows its solubility in water, which assists its extraction (Li et al., 2007; dos Santos, 2019). The biggest advantage of alginates is its liquid–gel behavior in aqueous solutions (Sarheed et al., 2015; Navarro and Muniesa, 2017). When monovalent ions (sodium in sodium alginate) are exchanged for divalent ions (especially calcium), the reaction proceeds almost immediately, changing from a low viscosity solution to a gel structure (dos Santos, 2019; Navarro and Muniesa, 2017). The gelled mass is a copolymer composed of two kinds of monomer units (Navarro and Muniesa, 2017).

Antimicrobials can be applied on solid food surfaces using techniques such as spraying, dipping, or brushing. However, direct application of antimicrobials on food products faces important challenges, such as poor solubility in aqueous systems, limited

stability against chemical or physical degradation, uncontrolled release and possible adverse effects on food sensory qualities (Sezer et al., 2022; Yezhi et al., 2016). Protecting antimicrobials within a matrix is, therefore, an important strategy to address these issues (Cao et al., 2020; Mozaffari et al., 2022). This matrix can act as a delivery system, which can be classified into three major groups according to their structural and physicochemical properties: emulsion-based, micro or nanosized carrier-based, and film/coating-based (Cao et al., 2020; Yezhi et al., 2016).

In the last several years, several delivery systems have been developed using phages (Figure 1) but most of them are mainly directed to control the growth of pathogenic species in food products (Colom et al., 2015; Korehei and Kadla, 2014; Vonasek et al., 2014). Duyvejonck et al. (2019) completed an evaluation of the stability of bacteriophages in different solutions suitable for the production of magistral (for human use) preparations in Belgium. Vinner et al. (2019) completed the microencapsulation of *Salmonella*-specific bacteriophage Felix O1 using spray-drying in a pH-responsive formulation and direct compression tableting of powders into a solid oral dosage form.

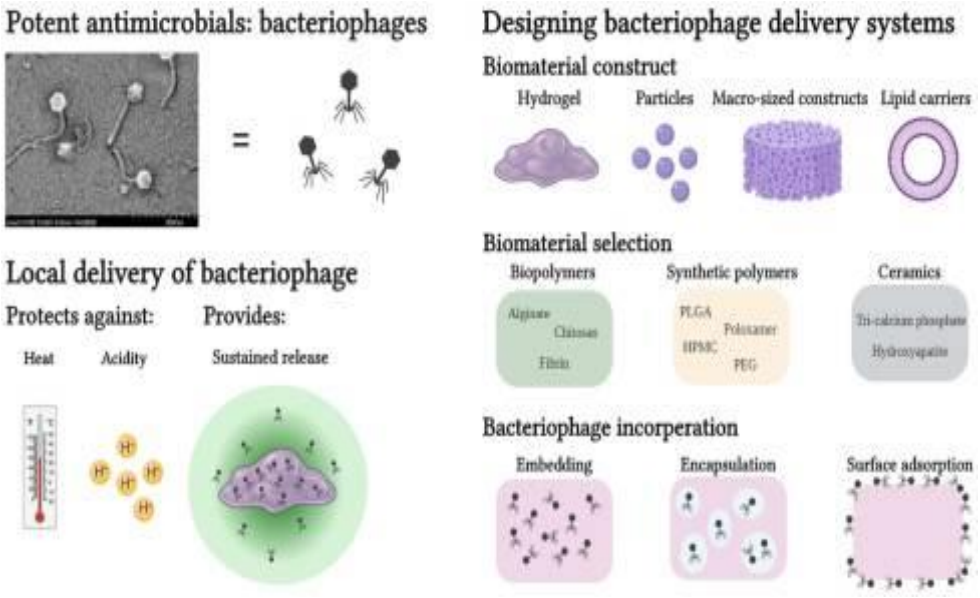

Figure 1: Various antimicrobial bacteriophage delivery systems. This figure was taken from Stoleru et al., 2021

1.6 ELECTROSPINNING- Charles Boys, in 1887, introduced the idea of electrospinning when he reported that fibers could be produced from a viscous, elastic liquid and an external electrical field (Woodings, 2003). Electrospinning uses electrostatic forces to generate synthetic fibers. A typical setup generates an electric field between the spinneret filled with polymer solution and a counter electrode at a distance by the drum collector. Though some researchers have shown minor success in using electrospinning methods for phage therapy applications, the success of the finished product is dependent on a variety of factors. First, the nanofibers should mimic the natural environment they will be used in, emphasizing the importance of choosing the type of polymer and materials for production (Nematie et al., 2017). Besides the solvent and type of polymer, ambient conditions and processing parameters must be considered (Xue et al., 2017); for example, a high molecular weight polymer with a

suitable solvent must be used for proper dissolution as low molecular weight polymers tend to produce beads instead of fibers. The evaporation rate and solidification rate of the jet is dependent on the volatility of the solvent. If the solvent is too volatile, the jet solidifies too quickly after exiting the spinneret; however, volatility that is too low produces wet fibers. Increasing the dielectric constant increases the voltage required to produce a stable jet. Since water has a high dielectric constant, it is not a favorable solvent for the dissolution of polymers through electrospinning (Sun et al., 2014), which can be problematic because most phages are stored long-term in phage buffer consisting mainly of water. A high concentration of polymer results in no jet formation, and low polymer concentration results in beaded droplets. A high viscosity leads to failed ejection of the solution from the spinneret, while a low viscosity produces very thin fibers. High electrical conductivity does not produce the Taylor cone, while perfectly insulated solutions cannot conduct charges (Xue et al., 2017). The flow rate, voltage, and distance between the collector and spinneret also affect the size and morphology of the fibers, as does temperature and humidity (Yang et al., 2017). All these factors must be considered and optimized when dealing with electrospinning nanofibers (Velasco-barazza et al., 2006.)

Packaging is used to protect foods from environmental influences and microbial contamination to maintain the quality and safety of commercial food products, to avoid their spoilage, and to extend their shelf life (Hultman et al., 2015; Munteanu and Vasile, 2021). Bioactive packaging is developing to additionally provide antibacterial and antioxidant activity with the same goals (extending the shelf life while ensuring safety of

the food products) (Munteanu and Vasile, 2021). Janani et al., (2020) successfully used antibacterial tragacanth gum-based nanocomposite films carrying ascorbic acid antioxidant for bioactive food packaging. Stoleru et al. (2021) completed a study on bioactive food packaging using Poly (lactic acid) surface functionalized by chitosan coating embedding clove and argan oil. Sganzerla et al. (2020) did a study involving bioactive food packaging based on starch, citric pectin and functionalized with *Acca sellowiana* waste by-product for apple conservation.

New solutions are designed using natural antimicrobial and antioxidant agents such as polysaccharides and natural inorganic nanoparticles (nano clays, oxides, metals such as silver) incorporated/encapsulated into appropriate carriers in order to be used in food packaging (Munteanu and Vasile, 2021). Electrospinning/electrospraying are receiving attention as encapsulation methods due to their cost-effectiveness, versatility, and scalability (Munteanu and Vasile, 2021). The electrospun nanofibers and electrosprayed nanoparticles can preserve the functionality and protect the encapsulated bioactive compounds (BC) (Munteanu and Vasile, 2021).

1.7 BACTERIOPHAGES- Bacteriologists presumably had encountered bacteriophages from the beginning of their discipline, but it was in 1915 that F. W. Twort reported a strange phenomenon he termed ‘‘glassy transformation’’ of cultures of micrococci (Abedon, 2005; Huang et al., 2018). He observed that the calf lymph, from which he was trying to grow vaccinia on cell-free medium, contained a serially transmissible agent that induced a dissolution of the bacteria, leaving only subcellular granules (Abedon, 2005). Independently, Felix d’Herelle in 1917, discovered a microbe

that was ‘‘antagonistic’’ to bacteria and that resulted in their lysis in liquid culture and killing in discrete patches (he called them plaques) on the surface of agar seeded with the bacteria (Abedon, 2005). Felix d’Herelle described these invisible microbes as ‘‘ultra-viruses’’ that invaded bacteria and multiplied at their expense, killing them, and he created the term bacteriophage, which literally means eater of bacteria (Abedon, 2005; Amano et al., 2019). He ascertained that the plaque count provided a way to enumerate these invisible agents, which he defined as particulate (Abedon, 2005; Amano et al., 2019). He was able to show that phage multiplied in ‘‘waves’’ or ‘‘steps’’ representing cycles of infection, multiplication, release, and reinfection (Abedon, 2005).

Obligate intracellular parasites, bacteriophages are viruses that infect and kill bacterial species and as such offer no risk to the health of humans, animals, or plants (Gencay and Brondsted, 2019). Microbacteriophages are bacteriophages that specifically infect *Microbacterium* spp. Bacteriophages, with a population of 10^31 residing in earths’ biosphere, outnumber bacteria by a factor of 10 (bacteria populations are 10^30) (Gencay and Brondsted, 2019; Hansen et al., 2019). This diversity and abundance of bacteriophages in our biosphere offers close to unlimited sources from which to isolate phages for application purposes in controlling foodborne pathogenic bacteria (Pujato et al., 2019; Gencay and Brondsted, 2019). With an estimated 10^31 bacteriophages in our biosphere, approximately 96% of those reside in the order *Caudovirales,* consisting of double-stranded DNA genomes encapsulated in a polyhedral capsid attached to a tail (Weng et al., 2020; Gencay and Brondsted, 2019). Within the order *Caudovirales* are three major families of tailed phages (*Siphoviradae, Myoviridae,*

and *Podoviridae*), presenting different tail morphologies (Huang et al., 2018; Gencay and Brondsted, 2019). *Podoviridae* bacteriophages consist of short, non-contractile tails; *Siphoviridae* phages employ long, flexible, non-contractile tails;, and *Myoviridae* are unique in carrying contractile tails (Barr et al., 2017; Gencay and Brondsted, 2019). Phages that are morphologically related and have been historically grouped together in families may have no similarity at the DNA level (Landers et al., 2012; Gencay and Brondsted, 2019). Advancing technology in DNA sequencing has increased the number of sequenced bacteriophage genomes, prompting a new genome- and proteome-based classification system (Huang et al., 2018; Gencay and Brondsted, 2019). This system allows further classification into species, genera, and sub-families by comparative genomic analysis (Mozafarri et al., 2022; Gencay and Brondsted, 2019). Phage taxonomy is bound to evolve. New phage families are likely to be discovered in unusual habitats, such as volcanic springs, hypersaline lagoons, or the mammalian rumen (Turner et al., 2021).

Phage structure consists of a head housing the genetic information enclosed in a protein capsid (Franz et al., 2018; Batinovic et al., 2019). A collar or neck region connects the head to the extended sheath (also known as the tail) (Madison et al., 2022; Batinovi et al., 2019). The sheath, which is enclosed by protective sheath proteins, produces a hollow cylindrical tube via which the viral DNA/RNA is injected or delivered into the host cell (Ishaq et al., 2020; Batinovic et al., 2019). The base plate, which is linked to the tail fibers, is located at the base of the sheath and enables attachment to the host cell (Gorski et al., 2018; Batinovic et al., 2019). The phage tail fibers connect to

receptors on the bacterium cell surface through adsorption, controlled by the base plate (Wu et al., 2020; Batinovic et al., 2019). The sheath punctures the bacterium cell membrane and injects genetic information (double or single-stranded DNA or RNA) into the cell cytoplasmic compartment (Batinovic et al., 2019).

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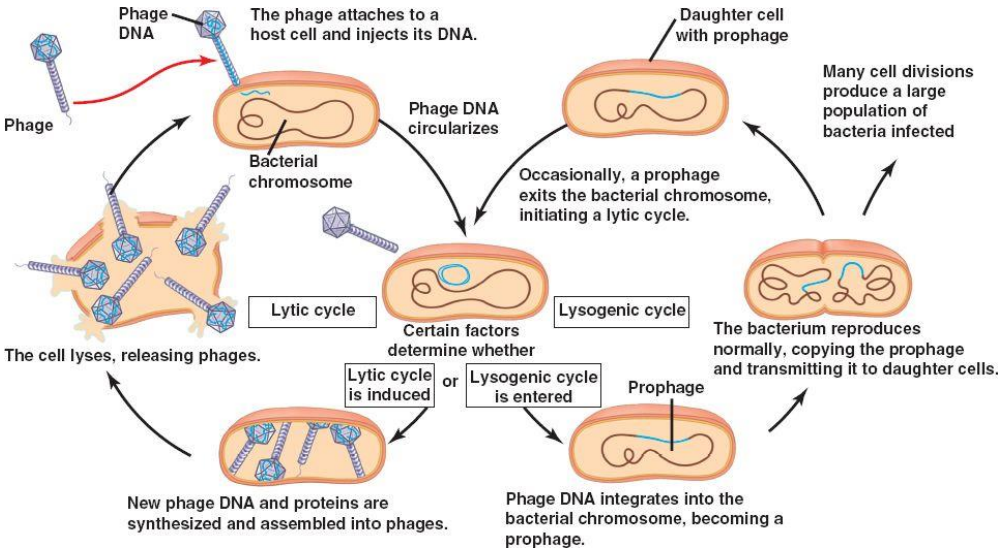


Figure 2: Lytic and lysogenic cycle of a bacteriophage. Figure taken from Venturini et al., 2022

Bacteriophages can replicate through a lytic cycle and/or a lysogenic cycle (Figure 2). Lytic phages hijack cell machinery to make phage components and lyse their host cells, whereas lysogenic or temperate phages incorporate their genetic material into the host bacterial genome (Venturini et al., 2022; Batinovic et al., 2019). Lysogenic infections can revert to lytic infection under unfavorable conditions, including changes in the immediate environment, lack of nutrients, or exposure to toxic material (Le et al.,

2021; Batinovic e al., 2019). Virulent or lytic phages exploit the lytic cycle to lyse their host cell and produce many progeny. The phage genome produces early proteins that break down the host DNA and hijack host cellular machinery. Phage proteins are synthesized using the host cell machinery. The heads and sheaths are assembled independently, and phage genetic materials are packed into the head to create daughter phage particles (Kitti et al., 2022; Batinovic et al., 2019). Phage enzymes rupture the host bacteria membrane, releasing new phage progeny into the environment (Batinovic et al., 2019).

Without a metabolism of their own, bacteriophages must hijack the replication machinery of bacterial cells in order to propagate, and this dependence on bacteria dictates bacteriophages must reside in an environment rich in bacterial species (De La Pena et al., 2022; Gencay and Brondsted, 2019). The bacteriophage DNA chromosome must retain the information to do three things: ensure its own replication to produce chromosomes to be encapsulated into progeny virions; commandeer the host cell metabolism and redirect it toward the production of progeny virions; and encode the structural proteins and enzymes required to assemble new virions (Huang et al., 2018).

1.8 BACTERIOPHAGES IN FOOD SAFETY- Bacteriophages are inherently low in toxicity, and as research recently revealed, the human gut microbiome consists of diverse and abundant bacterial species in conjunction with bacteriophages and other viruses (Bellasi et al., 2021Choi et al., 2022; Gencay and Brondsted, 2019;). Phongtang et al. (2019) showed bacteriophage control of *Salmonella* Typhimurium in milk, while

another study by Lee et al. (2017) examined *Listeria* phages for control of growth of *Listeria monocytogenes* in milk. Endersen et al. (2013) completed isolation and characterization of six novel mycobacteriophages and investigation of their antimicrobial potential in milk. McLean et al. (2013) researched phage inhibition of *Escherichia coli* in ultrahigh-temperature-treated and raw milk. High specificity of bacteriophages dictates other microbiota in the gut are untouched, leaving beneficial bacteria to propagate (Witte et al., 2022; Gencay and Brondsted, 2019; Han et al., 2022).

Bacteriophages' most enticing and exceptional trait is the specificity of their activity, or the ability to destroy just the bacterial pathogens of interest (Olszak et al., 2017; Lewis and Hill., 2020). Bacteriophages have a limited range of activity, overcoming the risk of impacting the entire microbiome by eradicating potentially helpful bacteria, causing secondary pathogen overgrowth, and causing the creation of resistant bacteria (Dasgupta et al., 2022; Yeh et al., 2018) . Data from Sarker et al. (2012) confirmed the specificity of bacteriophage action. Researchers administered 15 healthy adult volunteers an oral cocktail of 9 T4-like *E. coli* bacteriophages. After a 5-day period, the provided phages were found in the feces of nearly all treated patients, but no change was observed in the composition of their gut microbiota (Sarker et al., 2012).

Bacteriophages are less dangerous and tolerated since they only replicate in the target bacterium and do not infect mammalian cells (Barr et al., 2019; Yeh et al., 2018). Examinations in experimental animals and humans supports this conclusion (Huh et al.,

2019; Costa et al., 2019). People are continually exposed to bacteriophages because they are prevalent in the environment, implying that they are not xenobiotic to human bodies (Barr et al., 2019; Costa et al., 2019). A phagome (phage biome)-dominated virome (virus biome) exists in our healthy microbiome (Mills et al., 2017; Costa et al., 2019). Patients tolerate bacteriophages employed for therapeutic purposes as well (Yeh et al., 2018). Because bacteriophages can stay in the body of humans for lengthy time intervals, they do not require repeated administrations (Choi et al., 2016; Barr et al., 2019; Yeh et al., 2018). Only a few dosages are required due to the increase in bacteriophage concentration at the location after prior therapy (Skurnik et al., 2022; Costa et al., 2019).

1.9 ANTIBIOTICS AND CURRENT EFFICACY IN FOOD SAFETY- Antibiotic resistance in bacteria is a worldwide threat to human health. The overuse of antibiotics in human and veterinary medicine has led to the emergence of antibiotic resistance, and epidemiological studies verify that antibiotic consumption is directly connected with this emergence and the dissemination of antibiotic resistance bacteria (More, 2020; Ventola, 2019). Antibiotics are still widely used in livestock for prophylaxis and/or treatment of infectious diseases (Soto-Rodriguez et al., 2013; Rendueles et al., 2022). The continuous exposure of animals to antibiotics promotes the development of antimicrobial resistance (Rendueles et al., 2022; More, 2020.) Under persistent selective pressure, commensal bacteria in livestock become reservoirs of antibiotic resistance genes (ARGs), which can be acquired by pathogenic bacteria through horizontal transfers (Chan et al., 2013; Rendueles et al., 2022). Antibiotic resistant bacteria can be

transmitted through the consumption of meat and other livestock products (milk and eggs) and be disseminated in the environment by animal wastes (Gomes and Henriques, 2016; Manyi-Loh et al., 2018). Additionally, antibiotic residues have been detected in animal tissues and milk (Landers et al., 2012).

Decreasing the excess use of antibiotics in animal husbandry and in human medicine is especially important to decrease the occurrence and spread of antibiotic resistant bacteria (Canica e al., 2019; Fusco et al., 2018). Yet antibiotic use cannot be decreased to zero in the interest of human and animal health. It will be important, therefore, to define points of pathogen entry, trace transmission routes along the food chain, determine the evolution of transferable antibiotic genes, and, most importantly, to find control measures which prevent or diminish the entry and spread of resistant microorganisms or resistance genes (Mishra et al., 2021). Here, not only true foodborne pathogens are of importance, but also opportunistic pathogens such as *Microbacterium* spp., *Klebsiella* spp., *Enterobacter* spp., *Citrobacter* spp., and *Serratia* spp (Fusco et al., 2018). These are well known to occur in various foods (meats, milk) and to cause hospital infections (Fusco et al., 2018; Nordmann et al., 2012). Additionally, even non-pathogenic bacteria may become antibiotic resistant and can be relevant in spread (Fusco et al., 2018).

1.10 GENUS *MICROBACTERIUM-* *Microbacterium* spp. are high G+C% rod-shaped aerobes in the order Actinomycetales, which also contains the genera *Arthrobacter*, *Gordonia*, *Mycobacteria*, and *Streptomyces* (Buczolits, et al., 2008: Jacobs-

Sera et al., 2020). *Microbacterium* spp. are in the family, *Microbacteriaceae*, whereas the other four genera are in the families *Micrococcaceae*, *Gordoniaceae*, *Mycobacteriaceae,* and *Streptomycetaceae*, respectively (Mohd et al., 2017; Jacobs-Sera et al., 2020). Most *Microbacterium* strains do not carry CRISPR-Cas systems although various *Microbacterium* restriction-modification systems have been discovered. *Microbacterium* spp. do not contain mycolic acids in their cell walls (Jacobs-Sera et al., 2020).

Microbacterium spp. are prevalent throughout the environment, having been isolated from soil, plants, and food (Soto-Rodriguez et al., 2013: Jacobs-Sera et al., 2020). While some *Microbacterium* spp. have been shown to benefit plants by increasing drought resistance, others, typically uncommon species, have been associated with foodborne illness, bacteremia in patients, and cases of peritonitis (Amano et al., 2019; Adames et al., 2010), and some species have been isolated from a cystic fibrosis patient and cancer patient (Alonso et al., 2001; Amano et al., 2019; Jacobs-Sera et al., 2020).

Microbacterium species are common in dairy products and the mammalian intestinal tract (Buczolits et al., 2008). The microbiota that spoil long-life micro-filtered milk generally includes species of the genus *Microbacterium* (Bellassi et al. 2020). Long-life micro-filtered milk is milk that has been treated to a very fine filtration process (Buczolits et al., 2008; Jacobs-Sera et al., 2020) that removes more bacteria than pasteurization (Huang et al., 2018). It removes 99.5% of bacteria present in milk and

gives milk a longer shelf life, of up to 30 to 45 days (Huang et al., 2018; Bellasi et al., 2021). Properties of this microorganism's bacteriophage that could potentially modify the quality of micro-filtered milk are still unexplored when compared to better-known microorganisms, such as the spore-forming *Bacillus* and *Paenibacillus* spp., and Gram-negative contaminants, such as species *Pseudomonas* and *Acinetobacter* (Nedelkova et al., 2007; Bellassi et al., 2020). *Microbacterium* spp. are often isolated from psychrophilic microbiota of raw milk (Bellassi et al., 2020; Jacobs-Sera et al., 2020) and are characterized as highly proteolytic, lipolytic microorganisms (Bellassi et al., 2020; Huang et al., 2018).

Milk from a healthy animal under aseptic conditions is not sterile (Bozoglu et al., 2016). Bacteria in milk comes from the udder of the cow in the range of 100–1000 cells per ml. *Macrococcus caseolyticus, Mycobacterium freudenreichii,* and *Microbacterium liquefaciens* are the most frequent bacteria in aseptically drawn milk (Bozoglu et al., 2016). In raw milk, microorganisms come from inside the udder, animal surface area, feed, air, water, and equipment used for milking and storage (Bozoglu et al., 2016).

It has been shown that from the time of contamination until the end of the shelf life of micro-filtered milk, *Microbacterium*'s proteolytic and lipolytic activities are below the threshold of detection (Huang et al., 2018). Regarding dairy products, it is interesting to note that strains *Microbacterium* are often found on the rind of smear-ripened cheeses (Buczolits et al., 2008) and that the community of psychrophilic bacteria in raw milk, including *Microbacterium*, has the ability to form biofilms along the

whole milk chain (on the collection and storage equipment), which poses a risk to the stability of the milk (Huang et al., 2018).

1.11 *MICROBACTERIUM PARAOXYDANS-* Cells of *Microbacterium paraoxydans* are small, gram-positive, coryneform rods that grow aerobically at 20, 37, and 40°C (citation?). Colonies are bright yellow, smooth, and sometimes sticky, and reach a diameter of 2 mm after 48 h of incubation at 37°C on blood agar. Strains are motile by peritrichous flagella. They are catalase positive, and oxidase negative. Glucose, sucrose, maltose, galactose, fructose, mannose, and mannitol are acidified, but salicin is not. Esculin is hydrolyzed with some delay. Dnase, gelatin, and casein hydrolyses are positive and there is no decomposition of tyrosine (Laffineur et al., 2003).

In the API 50CH (tests to study bacterial growth on different carbohydrates) system, the following compounds are assimilated: glycerol, d-arabinose, ribose, glucose, galactose, fructose, mannose, rhamnose, mannitol, α-methyl-d-glucoside, *N*-acetylglucosamine, esculin, salicin, cellobiose, maltose, lactose, sucrose, trehalose, melezitose, gentiobiose, d-turanose, l-fucose, and gluconate. Sorbose, sorbitol, amygdalin, xylitol, and d-fucose are assimilated by some strains. When API ZYM (detects bacterial enzymes) strips are used, leucine arylamidase, phosphoamidase, and α-glucosidase are positive. Acid and alkaline phosphatases, esterase (C_4), β-galactosidase, *N*-acetylglucosaminidase, α-mannosidase, and α-fucosidase are variable. Esterase lipase (C_8), valine arylamidase, cystine arylamidase, trypsin, α-chymotrypsin, β-glucuronidase, and β-glucosidase are negative. D-ornithine is the diamino acid of the

peptidoglycan, and the main cellular fatty acids are anteiso-$C_{17:0}$, anteiso-$C_{15:0}$, and iso-$C_{16:0}$ (Laffineur et al., 2003).

CHAPTER 2- HYPOTHESIS AND OBJECTIVE-

The objectives of this research are to: 1) isolate a *Microbacterium paraoxydans* bacteriophage; 2) determine the One-step growth curve, pH, and temperature stability of the isolated phage; 3) determine the stability of the phage particle in the presence of sodium alginate and PCL nanofiber; 4) incorporate the *M. paraoxydans* bacteriophage into sodium alginate hydrogel, bound to polycaprolactone (PCL) nanofiber, and evaluate its' efficacy; and 5) study the efficacy of *M. paraoxydans* bacteriophage in controlling the host population in a milk model. My hypothesis states that the *M. paraoxydans* bacteriophage, incorporated into sodium alginate, will be stable and will successfully diffuse to control host bacteria. My hypothesis also states that placing *M. paraoxydans* phage in milk will successfully reduce the bacterial concentration.

To test this hypothesis, the *M. paraoxydans* bacteriophage was characterized by a) studying the phage stability in different pH's, b) studying the phage lytic ability at different multiplicity of infection (MOI), and c) performing a one-step growth curve assay. The hydrogel incorporated with the phage and attached to PCL nanofiber was characterized by a) testing the stability of the phage in the presence of the hydrogel and PCL nanofiber, and b) its' ability to lyse *M. paraoxydans* on a bacterial lawn.

CHAPTER 3-CHARACTERIZATION OF *MICROBACTERIUM PARAOXYDANS* BACTERIOPHAGE

Materials and Methods

Culture medium and bacterial culture preparation

Microbacterium paraoxydans provided by the SEAPHAGES program was grown in standard PYCa liquid medium (Peptone, 40% dextrose, 1M calcium chloride ($CaCl_2$), yeast extract (powder)) and incubated in a shaking incubator (Fisher Scientific #SHKE4450). On solid media, the bacteria were grown on standard PYCa plates (peptone, 40% dextrose, 1M $CaCl_2$, agar). Cultures grown were incubated at 37°C, and 50 µg/ml of cycloheximide was added to the liquid culture medium and PYCa agar plates to reduce contamination. Two-X PYCa top agar (peptone, yeast, agar, 40% dextrose, 1M $CaCl_2$) was diluted to a 1:1 ratio with PYCa liquid medium to make 1X PYCa top agar, which was used to plate the bacterial lawn. The phage lysate was diluted in phage buffer (pH 7.2. 10 mM Tris, 10 mM magnesium sulfate ($MgSO_4$), 70 mM sodium chloride (NaCl), and 1mM $CaCl_2$) to quantify the phage. For the agar-overlay method, 10 µL of each dilution was added to 250 µL of *M. paraoxydans* bacteria and incubated for 10 minutes. After incubation, 4 mL of 1X PYCa top agar was added to the bacteria and phage mixture and transferred to a PYCa agar plate. The plates were incubated at 37°C for 24 hours and assessed for plaques.

Microbacteriophage isolation and processing

The soil sample used for isolation of Microbacteriophages was collected from GPS location N 35.65499738 and W 97.469831454. The soil was mixed with PYCa liquid medium and incubated on a rotator for three hours at room temperature. Subsequent to this, the mixture was filtered using a 0.22µm filter. The filtrate was inoculated with *M. paraoxydans* and incubated overnight at 37°C in a shaking incubator for three consecutive days. After every 24 hours, the filtrate was removed from the shaker, centrifuged, filtered with a 0.22µm filter, serial diluted with phage buffer, and plated using the double agar overlay method. Plates were incubated at 37°C and assessed for plaques after 24 hours.

Stability of phages at different pH conditions

The stability of the Microbacteriophage, Samsparadox, was evaluated by incubating the phage in phage buffer adjusted to pH values (2,3,4,5,6,7,8,9,10,11,12) at room temperature for one hour following the protocol described in previous publications (Patton, C.J., & Kotturi, H. 2019). The pH of the phage buffer was adjusted using HCl or NaOH and was 0.22 µm filter-sterilized. The initial concentration of phage was $1x10^{10}$ PFU/mL, and it was incubated for one hour at room temperature. Following the one-hour incubation, the phage solution was diluted, plated using the agar-overlay method, and incubated at 37°C prior to assessment of plaques.

One-step growth curve of microbacteriophage Smedna

The one-step growth curve was done at a multiplicity of infection (MOI) of 1.0 (one bacterial cell to one bacteriophage) following protocol from previous work with Microbacteriophages (Huang et al., 2018). The host bacterium and phage were incubated at 37°C for one hour to allow for complete phage adsorption. Following incubation, 0.4% sulfuric acid (H_2SO_4) was added to inactivate unattached phage particles, and the solution was incubated again for five minutes at room temperature. The H_2SO_4 was neutralized by adding 0.4% sodium hydroxide (NaOH). The bacteriophage suspension was diluted in PYCa broth and incubated at 37°C. Every 30 minutes, for a duration of 8 hours, the sample solution was diluted and plated using the double agar overlay method. Following incubation, plaques were counted to determine the PFU/mL and plotted.

Activity of Microbacteriophages against host at different multiplicities of infection (MOI)

This assay was performed using two Microbacteriophages individually and in combination at different MOI. Phage activity was tested at MOI 100 (100 phages per bacterial cell), 10 (10 phages per bacterial cell), 1 (1 phage per bacterial cell), 0.1 (10 bacterial cells per phage), and 0.01 (100 bacterial cells per phage). Using a 96-well microtiter plate with six replicates for each MOI, 100 µl of host bacteria (*M. paraoxydans,* 10^5 CFU/mL) was dispensed in each well with 100 µl of diluted phage in PYCa liquid medium at different MOI's. The negative control used was *M. paraoxydans* in PYCa broth with no phage present. The 96-well microtiter plate was placed in the

spectrophotometer and incubated with shaking for 24 hours, with optical density (OD) 595nm taken every hour at various multiplicities of infection. Upon completion, data was assessed and plotted.

Stability of phage in Sodium Alginate and PCL fiber (SA-PCL Hydrogel system)

Phage lysate with a concentration of 1x10^10 PFU/ml was incubated at 37°C in the presence of sodium alginate hydrogel and PCL fiber for two weeks. Each day, lysate was serial diluted, plated on PYCa plates using double agar overlay method, and changes in the phage titer were determined.

Preparation of Sodium Alginate and PCL fiber (SA-PCL Hydrogel system)

To prepare the sodium alginate and PCL fiber hydrogel system, 0.5 grams of sodium alginate powder was added to a flask holding 18 ml sterile PBS (pH 7.4) on a hot plate with stirring capabilities. A sterile stir bar was added, and stirring commenced at 200 rpm at 90°C on the hot plate for three hours. Once a solution of sodium alginate was obtained, the temperature of the hot plate was reduced to 45°C, with the flask remaining on the hot plate for one hour to ensure mixture temperature reduction from 90°C to 45°C. Subsequent to this, 2 ml of phage lysate was added to the hydrogel mixture and stored at 37°C.

After mixing phage hydrogel solution, this mixture was then cast in 3D printed rings (4mm x 5mm). A phage hydrogel solution of 200 µl was pipetted into the casting ring and 100 µl of 1M $CaCl_2$ was pipetted over the hydrogel/casting ring matrix. Another

100 µl 1M $CaCl_2$ was also pipetted around the bottom of the ring to ensure the PCL fiber and gel adhere together. This was allowed to sit at room temperature for 10 minutes. Following this, the matrix was incubated at 4°C for 30 minutes. The molded phage-incorporated sodium alginate rings were pushed out of the casting ring and rinsed with approximately 100 µl of sterile PBS to remove any residual 1M $CaCl_2$. The rinsed disc was layered gently onto a PCL nanofiber disc of the same diameter to complete the SA-PCL hydrogel system.

Zone of inhibition

We tested the ability of bacteriophage incorporated into SA-PCL hydrogel matrix to lyse the host bacteria in a bacterial lawn using zone of inhibition assay. Using the double agar overlay method, PYCa plates were prepared with the host bacteria and the SA-PCL hydrogel system was placed on the solidified top agar in the plate and incubated at 37°C for 24 hours. Control SA-PCL hydrogel systems were prepared without phage lysate and plated with the experimental matrices. After incubation, the zone of inhibition around the SA-PCL hydrogel system was measured using a Vernier Caliper. Ten measurements were taken for each group.

DNA extraction

Genomic DNA was extracted from the bacteriophage implementing the PCI (phenol-chloroform-isoamly alcohol) technique as follows: heat block preheated to 55°C; DNAse I buffer was thawed out; 1ml of high titer lysate was transferred to a

microcentrifuge tube (aseptic technique employed) and 12.5µl Magnesium chloride ($MgCl_2$) added to tube; 0.5µl DNAse, 100µl DNAse I buffer, and 10µl RNAse were added in order to tube; tube was vortexed and incubated at room temperature for 30 minutes. After incubation, 40 µl 0.5M EDTA, 5 µl Proteinase K (12mg/ml), and 50 µl 10% SDS were added to the tube in order and vortexed. The tube was incubated at 55°C for 60 minutes with inversion every 20 minutes. The tube was removed and liquid was transferred to two new microcentrifuge tubes (500 µl each). In the fume hood, PCI was added to the sample tubes in 1:1 ratio, and tubes were inverted and centrifuged at 13,000 rpm for 5 minutes. The tube was removed from the centrifuge, and supernatant was removed and transferred to a new tube, while carefully not disturbing the bottom layer. PCI addition was repeated in a 1:1 ratio, with centrifugation until the cloudy middle layer in the sample tube was no longer visible. Supernatant was transferred to a new tube and 1 ml of 95% ethanol was added followed by the addition of 3M sodium acetate. Tubes were incubated on ice for 10 minutes. After incubation, tubes were centrifuged at 13,000 rpms for 10 minutes. A cotton-like pellet was observed after centrifugation and the supernatant was removed by decantation. Tubes were allowed to dry at room temperature (RT) for 10 minutes. Following this, 100 µl of nuclease free water was added to the tubes and the DNA pellet resuspended.

Transmission electron microscope (TEM) visualization of Microbacterium paraoxydans bacteriophage

TEM visualization of bacteriophage was accomplished by the following preparations for imaging. To a new microcentrifuge tube, 200 µl of phage lysate was

added. A copper-meshed grid was used and 5 µl of high titer lysate was pipetted onto the darker side of the grid. The grid was gently rinsed with 60 µl sterile DI water one drop at a time, then wicked off the edge of the grid with filter paper. The rinse step was repeated twice. Following this, 5 µl of 1% uranyl acetate was pipetted onto the grid and excess wicked off by applying filter paper to the edge of the grid. The grid was allowed to air dry for 5 minutes and then taken to OMRF (Oklahoma Medical Research Foundation) for TEM imaging.

Activity of Microbacteriphage in milk model

We followed the steps described in the protocol published by Huang et al. (2018). Briefly, powdered milk was prepared and sterilized according to manufacturer's directions. For the 4°C experimental group, 9 ml of skim milk was placed in a 50 ml tube. The skim milk was inoculated with 100µl of *M. paraoxydans* (10^5 CFU/ml) and vortexed; 1000 µl of bacteriophage lysate at MOI 100 was added to the skim milk and vortexed. The mixture was placed in a refrigerator (4°C) for 8 hours with a data point measured every 2 hours. Host bacterial cell count (CFU/ml) was determined by taking 200 µl of the milk mixture and performing 1:10 dilutions using PYCa liquid medium. Each serial dilution (100 µl) was spread onto PYCa plates and incubated for 24 hours. Plates were checked and CFU/ml calculated for every 2-hour data point. For the 4°C control group, 9 ml of skim milk was placed in a 50 ml tube. The skim milk was inoculated with 100µl of *M. paraoxydans* (10^5 CFU/ml) and vortexed; 1000µl of phage buffer was added to the milk mixture, vortexed, and stored at 4°C for 8 hours, with data gathered every 2 hours. Data for the CFU/ml was acquired by taking 200 µl of the milk

mixture and performing 1:10 dilutions using PYCa liquid medium. Each serial dilution (100 µl) was spread onto PYCa plates and incubated for 24 hours. Plates were checked and CFU/ml calculated for every 2-hour data point. For 28°C incubation temperature, the above steps were repeated with milk samples incubated at that specific temperature.

For determining the phage titer (PFU/ml) in milk sample incubated at 4°C in the experimental group, data points were taken every 2 hours by taking 200 µl of the milk mixture and placing it in a 0.22µm filter and filter sterilized. The filtrate was 1:10 serial diluted using phage buffer; 10µl of each dilution was added to 4 ml 1X PYCa top agar and plated by double agar overlay method. Plates were incubated for a 24-hour period and PFU/ml calculated for every 2-hour data point. The control group at 4°C PFU/ml data points were taken every 2 hours by taking 200 µl of the milk mixture and placing it in a 0.22µm filter and filter sterilized. The filtrate was 1:10 serial diluted using phage buffer; 10µl of each dilution was added to 4ml 1X PYCa top agar and plated by double agar overlay method. Plates were incubated for 24 hours, and PFU/ml calculated for every 2-hour data point. The same steps were followed for milk incubated at 28°C.

To summarize, *M. paraoxydans* host bacteria at 10^5 CFU/mL was inoculated into skim milk, bacteriophage Smedna subsequently inoculated at MOI 100 (10^7 PFU/mL) with bacteria into the skim milk, which was then incubated at 4°C and 28°C for 8 hours with a data point gathered every 2 hours.

Data analysis

All experiments were performed in triplicates, unless otherwise noted. GraphPad prism was used to calculate and establish multiple comparisons between samples. A p-value below 0.05 were considered statistically significant.

Results

Isolation of Bacteriophages

Two phages were isolated and given the names Samsparadox and Smedna. Samsparadox has a head approximately 50nm and a tail approximately 100nm and presents with clear plaques.

TEM analysis

To visualize Samsparadox, TEM images were obtained (Figure 3).

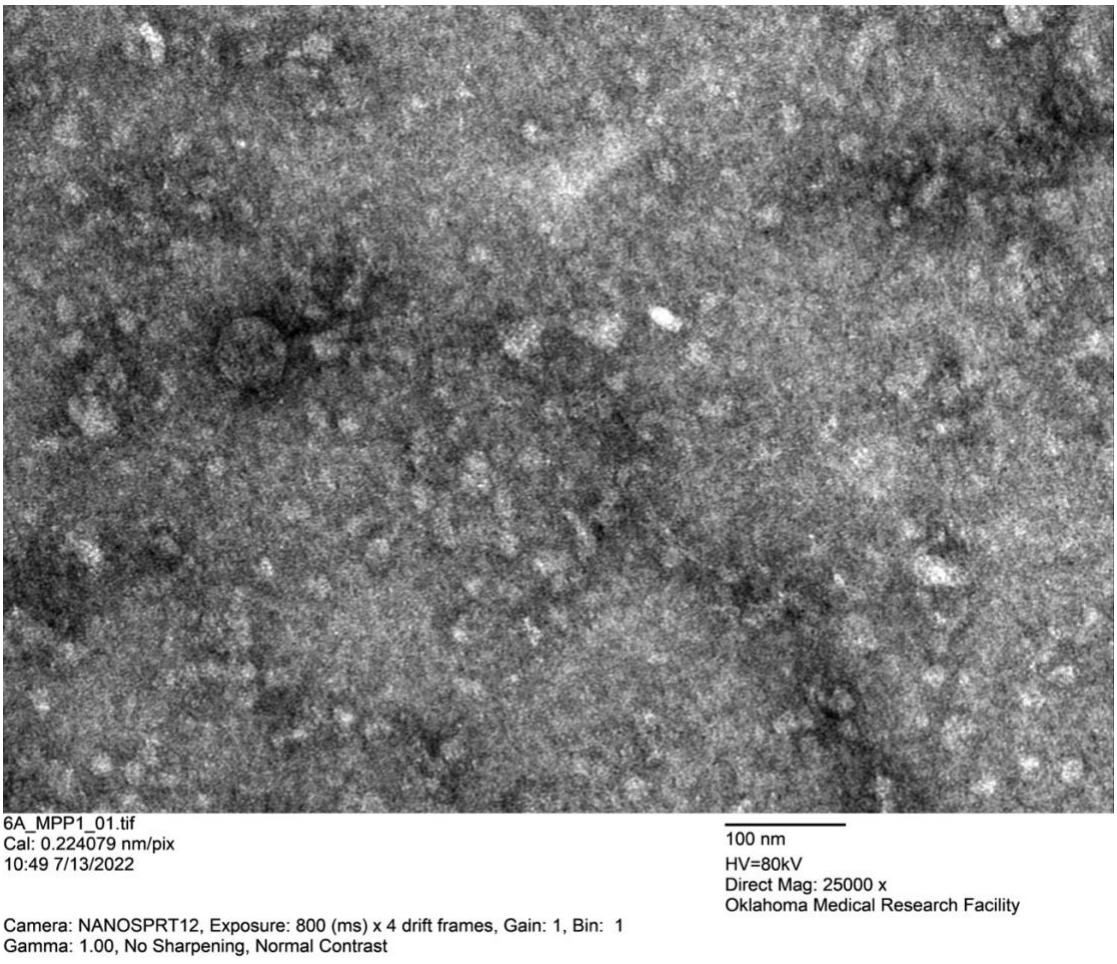


Figure 3: Phage Samsparadox TEM image with head approximately 50nm and tail 100nm.

pH stability assay

pH stability assay showed the Microbacteriphage, Smedna, to be stable at pH 2 through 12. There is a significant difference in infectivity between pH 3 and pH 4. Viable plaques were noted at all pH ranges tested (Figure 4). *Vibrio* phages studied by Yin et al. (2019) were found to retain activity at a broad range of pH 2-12 (Yin et al., 2019). Smedna is an excellent candidate for use because of its lytic ability at a wide range of pH's.

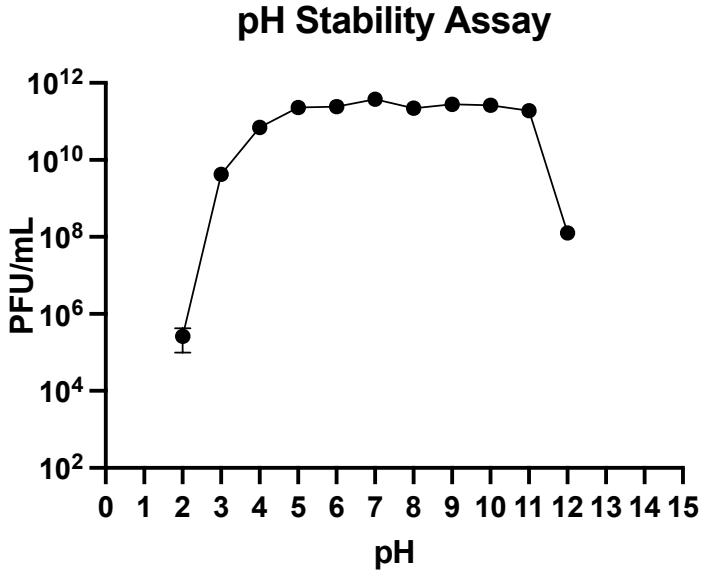


Figure 4: pH stability assay of Microbacteriophage Smedna

One step growth curve

A good understanding of the Microbacteriophage's latent period (time between injection or uptake of the viral genome into the host cell to release of new viral progeny by host cell lysis, hence the latent period begins with the viral attachment with the bacterial cell wall) is needed to determine its' potential use for food safety. I found that

the latent period of the phage was 3 hours, followed by a rise period of 2 hours (Figure 5).

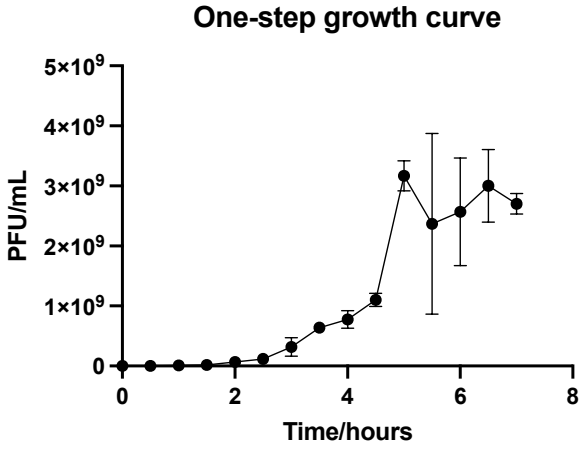


Figure 5: One-step growth curve of Microbacteriophage Smedna

Activity of Microbacteriophages at different MOI

The lytic ability of the phage against host bacteria at different MOI's was determined using a spectrophotometer with readings taken every hour for 24 hours. I tested both phages individually and together. My results indicate that phage Samsparadox at higher MOI (1, 10, and 100) lysed the host bacteria and was effective in preventing the emergence of phage-resistant strains. However, at low MOI (0.01 and 0.1), I could see some bacterial growth for 2–4 hours and subsequent lysis of host bacteria (Figure 6a). However, phage Smedna showed high lytic ability at higher MOI's (10 and 100). At lower MOI's (1, 0.1, and 0.01), the host bacteria continues to grow for approximately 7 hours with some decrease in growth (Figure 6b). Together, a mixture of phage Samsparadox and phage Smedna is highly effective at all MOI's over the 24-hour period and successfully inhibit emergence of phage-resistant strains (Figure 6c).

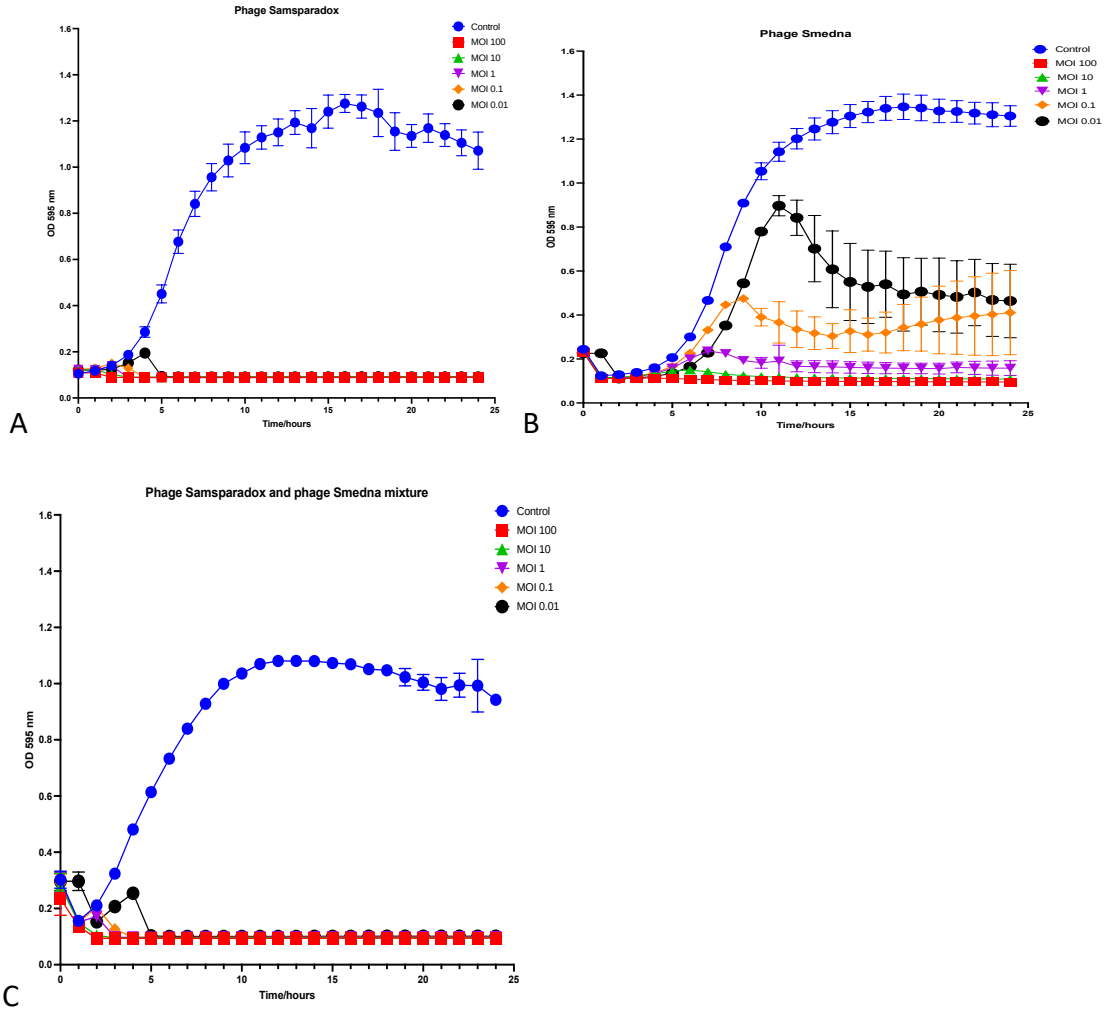


Figure 6a,b,c: Lytic ability of phage Samsparadox (a), Smedna (b), and phage mixture (c) at different MOI's over a 24-hour period.

Phage Smedna stability in presence of sodium alginate and PCL fiber

In order to examine the effect of sodium alginate and PCL fibers on the stability of phage particles, I incubated the phage lysate in the presence of these two compounds. My results show that at 37°C, there is no significant reduction in infectivity of phages particles over 15 days when compared with the control phage lysate incubated at the same temperature (Figure 7a,b).

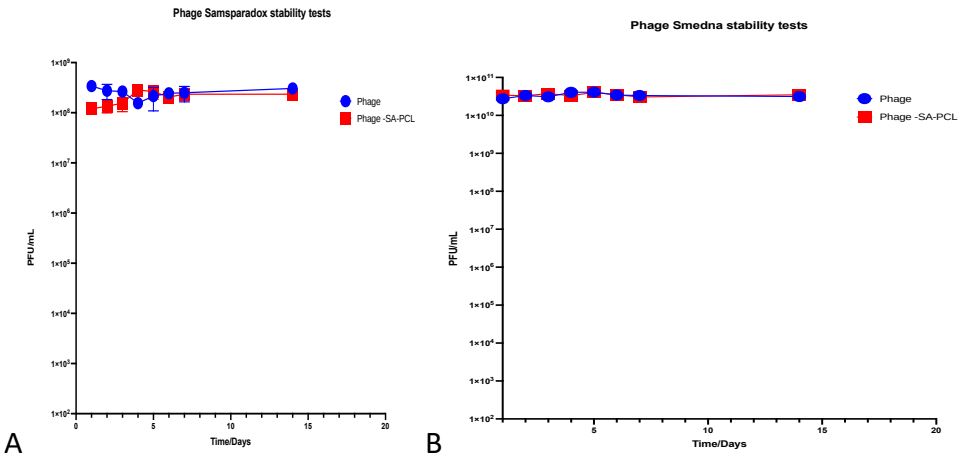


Figure 7a,b: Stability of phage Samsparadox (a) and phage Smedna (b) in the presence of sodium alginate hydrogel and PCL fibers.

Milk assay

No significant decrease (p=0.3) in bacterial count at 4°C was observed in milk following phage application at MOI 100, when compared with the control group at 4°C (Figure 8a). A decrease in the bacterial count was observed in the 28°C assay when compared with the control group at 28°C (Figure 8b).

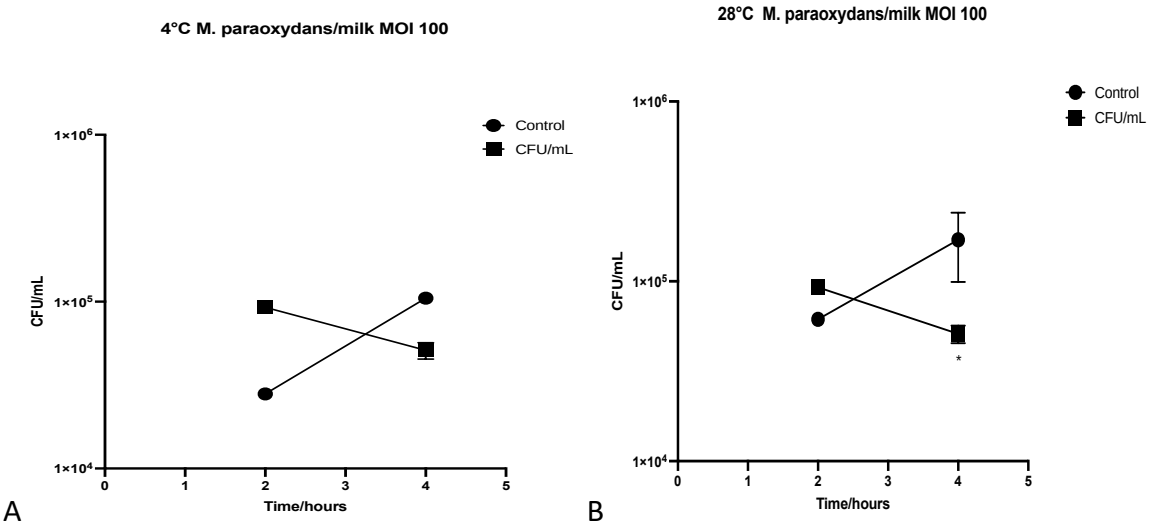
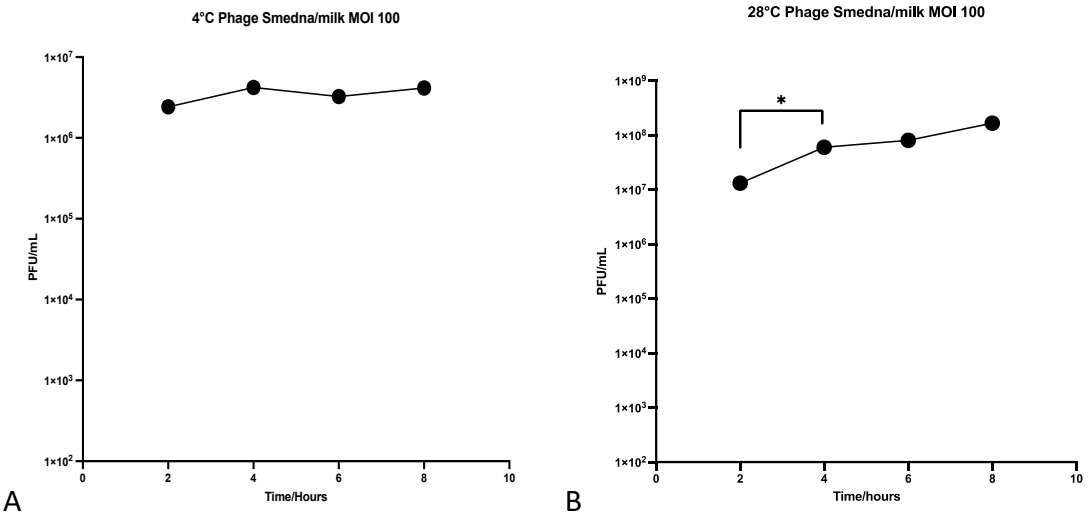


Figure 8a,b: Bacterial count at 4°C (a) and 28°C (b).

Results for the 4°C group PFU/ml showed an increase with no significance (p=0.25; with a slight decrease in bacteriophage activity between hour 4 and 5) over the 8-hour period (Figure 9a). Results for the 28°C group PFU/ml showed an increase much greater than the 4°C group (Figure 9b). Phage Smedna was effective in reducing bacterial counts in milk. At 28°C, phage Smedna was more activated, with a significant increase (p=*0.03).



A B

Figure 9a,b: Bacteriophage Smedna PFU/mL at 4°C (a) and 28°C (b).

Zone of Inhibition assay

My results indicate that the phage incorporated into SA-PCL hydrogel matrix is able to successfully diffuse into the top agar and inhibit the growth of host bacteria. This

can be visualized by a clear zone around the hydrogel matrix. Definite clearing and zones of inhibition averaging 20.01 mm (Figure 10) were visible on the bacterial lawns, while the control did not show any clearing or zones of inhibition. Analysis revealed a p value < 0.0001 for phage Smedna (Figure 11a), which indicated the difference in the control and phage Smedna was significant. Phage Samsparadox analysis results gave a p value < 0.0001 as well (Figure 11b), which indicated a significant difference between control and phage Smedna. Phage Smedna and phage Samsparadox mixture analysis also gave a p value < 0.0001 (Figure 11c), which subsequently indicated a significant difference between the control and the phage mixture. Bacteriophages show definite promise as a microbial control agent when incorporated into a sodium alginate hydrogel/PCL fiber matrix.

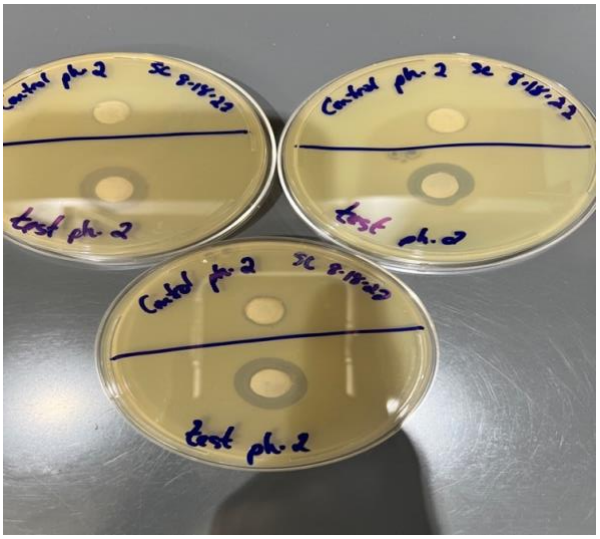
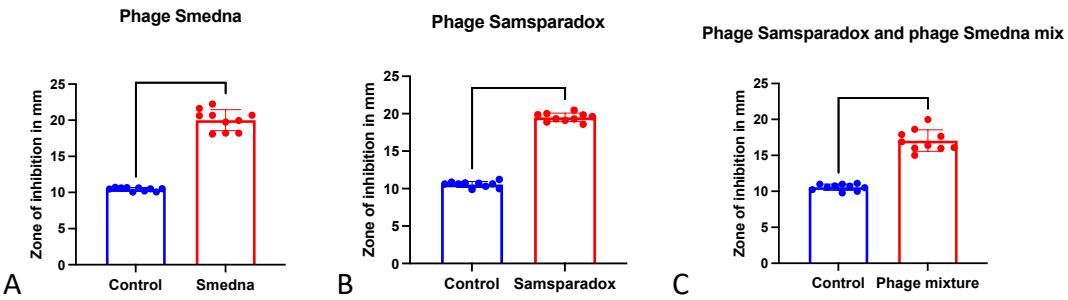


Figure 10: Top half of the plates shows control with no clearing. Bottom half of plates have clear zones of inhibition on *Microbacterium paraoxydans* bacterial lawn showing infectivity of *M. paraoxydans* bacteriophage Smedna in a hydrogel/PCL matrix.



Figures 11a,b,c: Phage Smedna (a), phage Samsparadox (b), and phage mixture zone of inhibition compared to control.

Discussion

Microbacterium spp. are coryneform bacteria that belong to the phylum *Actinobacteria*, the order *Actinomycetales*, and the family *Microbacteriaceae* (Bernard, 2015; Collins-Thompson et al., 1972). Several studies have identified strains belonging to the genus *Microbacterium* in milk after pasteurization and after the milk's shelf life at refrigeration temperatures (Noor et al., 2022). Heat-resistant and psychotropic strains *M. lacticum* and *M. flavum* were isolated from pasteurized milk after a prolonged storage period (14 days) at 7.2°C (Briggiler et al., 2016; Washam et al., 1977). Given the high thermal stability of *M. lacticum* strains isolated from pasteurized milk, they have also been used for thermal resistance experiments, in combination with an extrusion process, in which they were subjected to both mechanical and thermal energy (Bulut et al., 1999; Callaway et al., 2011).

Microbacterium spp. are often isolated from the psychrophilic microbiota of raw milk (von Neubeck et al., 2015; Machado et al., 2017; Yuan et al., 2019) and are characterized as highly proteolytic, lipolytic microorganisms (Baur et al., 2015; Hantsis-Zacharov and Halpern, 2007). However, it has been shown that from the time of contamination until the end of the shelf life of micro-filtered milk, *Microbacterium*'s proteolytic and lipolytic activities are below the threshold of detection (Li et al., 2022; Schmidt et al., 2012). Regarding dairy products, it is interesting to note that strains of the genus *Microbacterium* are often found on the rind of smear-ripened cheeses (Mounier et al., 2005, 2007) and that the community of psychrophilic bacteria in raw milk, including *Microbacterium*, has the ability to form biofilms along the whole milk chain, which poses a risk to the stability of the milk (Cabello et al., 2006; Medina et al., 2011;; Weber et al., 2019). However, no data are available on the genomic structures underlying the phenotypes of the genus *Microbacterium* that are involved in the preservation of extended shelf-life (ESL) milk (Garneau et al., 2011; Weber et al., 2019).

Bacteriophages have been studied as valuable antimicrobial alternatives to kill drug-resistant bacteria (Connerton et al., 2011; Hoang Minh et al., 2016). Phages are widespread and account for the largest number of organisms in nature (Weber et al., 2019). They are obligate intracellular bacterial parasites that target a range of bacterial strains within a species (Cooper, 2016). Phages self-replicate within bacterial cells during proliferation, self-limit as the host bacterium is reduced, and cause little damage to the surrounding microbiota that are not members of the target species (Xu, 2021). Moreover, phage composition is not toxic to eukaryotic cells (Wernicki et al., 2017).

Currently, there is a trend to utilize bacteriophages as biocontrol agents against pathogenic bacteria in the food industry to improve safety and avoid contamination (Cooper, 2016; Zinno et al., 2014). Additionally, some commercial phage products are approved by the Food and Drug Administration (FDA) and are currently marketed to target *Shigella* spp., *Salmonella* spp., *Escherichia coli*, and *Listeria monocytogenes* (Chang et al., 2022; Moye et al., 2018).

Bacteriophages have the potential for food safety uses due to their ability to lyse multi-drug resistant pathogens (Connerton et al., 2011; Feng et al. 2003). They are low cost, easy to manufacture, and have no documented side effects to date. My research lays a good foundation for continuing research and possible application of *M. paraoxydans* bacteriophages for food safety in the future. Foodborne illness acquired from the consumption of contaminated food remains a serious threat, with a great impact on human health and economics (Alves et al., 2020; Onyeaka et al., 2022a;). The World Health Organization (WHO), states that every year, approximately six hundred million cases of foodborne illnesses and 420,000 associated deaths occur worldwide (WHO 2015). Contamination of food products by pathogenic bacteria may take place at different stages along the food chain, from production, processing, and distribution, until their final preparation by consumers (Onyeaka et al., 2022b; Sillankorva et al., 2012).

The ability of these pathogens to grow on food matrices often leads to the establishment of microbial communities embedded on a self-produced extracellular matrix, known as biofilms, which confers them increased protection to antimicrobial

agents (Gouvea et al., 2016; Alves et al., 2018; Onyeaka et al., 2022c). In addition, the inappropriate overuse of antibiotics in both humans and animals (livestock industry) has led to an acceleration of microbial resistance (Jorge et al., 2019).

The most viable approach to deal with these challenges is to reduce the initial microbiological load and/or to prevent the growth of the remaining microorganisms on food products by the use of an active packaging (Yildirim et al., 2018). Antimicrobial active packaging has become the focus of great interest due to recent developments in materials science and engineering, the diversity in the methods of application, and the variety of food products that can be protected (Khaneghah et al., 2018).

Phages are natural predators of bacteria, and, like all other viruses, obligate intracellular parasites, which means their replication requires the host's machinery (Alves et al., 2020). Lytic phages, the most suitable for food applications, interact with the host's cell surface molecular receptor, causing the cell wall to be penetrable for the incorporation of the nucleic acid, whereas the capsid remains outside the cell (Alves et al., 2020). Inside the host, phages are reproduced quickly, forming new virus particles, and cause lysis of the bacterial cell (Lin et al., 2017). The increasing interest in phages for food application has been subsequent to the commercialization of phage-based products that have received regulatory approval from the Food and Drug Administration (FDA), such as EcoShieldTM and SalmoFreshTM (Moye et al., 2018; Ramos-Vivas et al., 2021;). Primary encapsulation strategies reported for phages were driven by the need to protect them from adverse conditions found in the digestive tract, such as low pH and activity of enzymes (Alves et al., 2020). In this context, phages have been encapsulated

mostly in alginate-based microspheres (Abdelsattar et al., 2019; Colom et al., 2017; Moghtader et al., 2017) and liposomes (Otero-Pazos et al., 2016).

Recently, studies have reported phage encapsulation to be further applied in food products (Alves et al., 2020). For example, the incorporation of phages targeting *E. coli* strains into matrices such as whey protein isolate (WPI) coatings/films (Tomat et al., 2019) or chitosan (Amarillas et al., 2018), has been shown to reduce the loss of phage activity during storage and to be a highly effective to prevent bacterial contamination of vegetable surfaces, meat, fish feed, and tomatoes (Gutierrez et al., 2016; Amarillas et al., 2018). A cocktail of phages targeting *Salmonella* has also been microencapsulated in WPI coatings and exhibited a high efficiency against serovars, but it was less efficient when applied on fresh foods (Petsong et al., 2019; Alves et al., 2018). Phage incorporation on alginate films has also been shown to prevent meat spoilage caused by *Pseudomonas fluorescens* (Alharaty et al., 2019; Alves et al., 2018).

CHAPTER 4: CONCLUSIONS

The food industry may be one of the most important industries in the world, as it provides a basic requirement of everyday life. Food products, however, are susceptible to microbial contamination, which may compromise their safety and quality (Chmielewski and Frank, 2003). Microbial contamination associated with foodborne diseases represents a great concern to public health, but also plays a fundamental role in the food industry in terms of food spoilage (Alves et al., 2018). Regardless of the recent advances achieved within the scope of preservation methods, manufacturing practices, and quality and hygiene control, between one-third to one-half of the food produced world-wide is not consumed (Batt, 2016; Endersen et al., 2014). The areas that contribute the most to food waste are households and processing plants (Stenmarck et al., 2016). Among the several reasons for this food loss, microbial spoilage plays a prominent role (Leyva Salas et al., 2017). A promising approach to deal with these challenges relies on the incorporation of antimicrobial compounds to prevent the growth of pathogenic and/or spoilage microorganisms that may be present on food surfaces (Alves et al., 2018). This strategy also contributes to maintain the quality and safety of food products, and to improve shelf-life (Lone et al., 2016; Valdés et al., 2017).

Among the antimicrobials used and incorporated along the food chain, bacteriophages have been recently recognized for their effectiveness in controlling bacterial pathogens in the agricultural and food industry (Sillankorva et al., 2012). Phages exhibit inherent features that make them promising antimicrobial candidates,

such as their ubiquity, high specificity against a target host or host range, self-replication capacity while their hosts are present, low inherent toxicity, easy and economical isolation and production, and a long shelf life (Abedon et al., 2017). From a food safety perspective, lytic phages are possibly one of the most harmless antibacterial approaches available (Alves et al., 2018). Lytic phages infect and multiply inside of their specific bacterial host, causing lysis and the release of newly formed virus particles, starting a new infection cycle in non-infected hosts that were not infected in the previous round (Pires et al., 2017). In recent years, use of phages has been a subject of great interest and research, prompting the FDA to assign some phage-based products the status of generally recognized as safe (GRAS) (FDA, 2012; Alves et al., 2018).

In conclusion, bacteriophages as an alternative to antibiotics in food, animals, and along the food chain is a promising approach for the control of microbial agents. Analyses of the biological characteristics (pH, lytic ability, stability, efficacy) and application of Smedna revealed that this phage is a promising candidate for use in a phage cocktail or other phage-based agents for food safety. *Vibrio* phages studied by Yin et al. (2019) were found to retain activity at a broad range of pH 2-12. Phage Smedna is an excellent candidate for use because of its lytic ability at a wide range of pH's (pH 2-12). The significant zone of inhibition created by my bacteriophages show definite promise as a microbial control agent when incorporated into a sodium alginate hydrogel/PCL fiber matrix. This outcome was supported by Alves et al. (2018), who successfully incorporated φIBB-PF7A bacteriophage onto sodium alginate-based films crosslinked with calcium chloride to prevent poultry spoilage caused by *Pseudomonas*

fluorescens. The overuse of antibiotics has diminished their effectiveness and many microbes are now antibiotic resistant, which indicates new methods of microbial control must be investigated. Bacteriophages are harmless to the biosphere and all things residing in it, with the exception of bacteria, so phages are a truly safe alternative for antimicrobial use along the entire food chain.

CHAPTER 5: BIBLIOGRAPHY

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